

**BIOASSAY-GUIDED ANTIDIABETIC MECHANISMS OF
CINNAMOMUM INERS LEAVES EXTRACT**

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ABSTRACT

Background: *Cinnamomum iners* has been traditionally claimed to be consumed by diabetic patients as natural supplements. However, no scientific data is available to validate the folklore claim.. Hence, this study evaluated the antidiabetic mechanisms of active methanol extract, active chloroform fraction and active subfraction 1 of *C. iners* leaves which is the extension from our previous study. **Methodology:** The possible antidiabetic mechanism was validated using ex vivo model and immunochemistry procedure. In addition, the insulin stimulatory effect of active extract, active fraction and active subfraction of *C.iners* was determined using rat insulin ELISA kit.

Results: Our study demonstrated that the antihyperglycaemic effect of *C. iners* leaves is mediated both, at pancreatic level by direct stimulation of insulin and at peripheral level by increasing glucose uptake by muscle. **Conclusion:** Our study clearly justified the use of this plant in the indigenous system of medicine.

KEY WORDS: *Cinnamomum iners*, antidiabetic mechanism, antihyperglycaemic.

INTRODUCTION

Diabetes mellitus remains to be major global health problem in most countries with the number of people with diabetes multiply world-wide. It is estimated to become one of the world's main killers by 2030 (Nagappa et al. 2003). The conventional therapeutic measures are associated with undesirable side effect such as hypoglycaemic coma, liver problems and diarrhea (Suba et al. 2004). Hence, herbal remedies serve as alternative treatment for diabetes with lesser side effect and economically wise (Adam et al. 2009). *Cinnamomum iners*, also

known as *kayu manis hutan*, *medang kemangi* and *teja* is one of commonly used medicinal plants in Malaysia. It is native to the Malaysia, India, Myanmar, Indonesia, Thailand, Singapore, Brunei and Philippines. Based on ethnobotanical approaches, this plant has been traditionally claimed to be consumed by diabetic patients as natural supplements (Choi 2003). The plant leaves also have been promoted for significant therapeutic benefits; from fever, headache, for digestive system problem, rheumatism, wound healing and diabetes (Pengelly 2004). Despite the extensive use by local people, there have been only limited attempts to explore the pharmacological properties of this plant scientifically. An earlier hypoglycemic and anti-hyperglycemic screening of various extracts and fractions of *C. iners* by our team revealed that methanol extract, chloroform fraction and sub-fraction 1 exhibited the most potent glucose lowering activity. Our previous findings also clearly highlighted that cinnamic aldehyde is the active component of methanol extract, chloroform fraction and subfraction 1 (Mustaffa et al. 2014). Cinnamic aldehyde is a well established compound of *Cinnamomum* species for antihyperglycemic properties (Tandon et al. 2013; Qin et al. 2003; Alam et al. 2003; Kanappan et al. 2006). The present study which is the extension of the previous works was undertaken to elucidate the possible antidiabetic mechanism of the active methanol extract, active chloroform fraction and active subfraction 1 of *C. iners* leaves.

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, filtered and concentrated to obtain the respective extracts. Methanol extract (2 g) which was the active extract was fractionated by using liquid-liquid separation techniques to obtain chloroform, ethyl acetate and *n*-butanol fraction. Chloroform fraction which was the active fraction was further extracted in hexane-chloroform mixture (1:3) and chloroform to obtain subfraction 1 and subfraction 2. Active methanol extract, active chloroform fraction and active subfraction 1 was standardized using cinnamic aldehyde as marker compound.

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 transit room with 12 h-light-12 h dark cycle. Rats were fed with rat pellet and water *ad libitum*. The study was approved by the Animal Ethic Committee of USM (Reference number: USM /Animal Ethics Approval / 2012 / (78) (393). For experimental purpose, animals were fasted (12 h for diabetic rats and 6 h for normal rats) 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 the rats. Blood glucose level was measured after 72 h of STZ injection. Rats with fasting blood glucose concentrations within 12-22 mmol/L was considered as diabetic and used for the study [Brain et al. 1997; Kadnur et al. 2005].

Preparation of plasma insulin

Blood samples approximately 0.5 mL was collected from the tail vein using hematocrit-capillary tubes containing Na-heparin (15 units/mL of blood sample). All the samples were centrifuged at 12,000 rpm at 4 °C for 3 min. Then it was stored at –20 °C until used for insulin concentration measurement. Insulin concentrations in the plasma samples were assayed by enzyme-linked immunosorbant assay (ELISA) using a kit purchased from Crystal Chem, USA (Bank 1988; MacDonald & Gapinski 1989).

Measurement of glucose uptake by isolated rat abdominal muscle

Glucose uptake by isolated rat abdominal muscle was measured according to Gray & Flatt 1998; Perez et al. 2000. The sacrificed rats abdominal muscle were cut into small squares (0.09-0.15 g) and transferred in Kreb's-Ringer bicarbonate buffer (KRB; 118 mM NaCl, 5 mM KCl, 1.28 mM CaCl₂, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃) in the presence of 95% O₂ and 5% CO₂ for 10 min to acclimatize the muscles. After acclimatization, the KRB solution was then replaced with KRB containing 11.1 mM of glucose, and samples were collected from the KRB solution as a baseline reading. The KRB solution was treated with 1 mg/mL of extract and 1 mg/mL of metformin in the presence or absence of 1 IU/mL insulin. The KRB solution was aerated for 5 min followed by incubation for 30 min in a shaking water bath. Samples were then collected after 30 min, and the muscle

weight was measured. The concentration of glucose in the KRB solution was determined before and after the incubation period to determine the amount of glucose uptake by muscle by using a Stat Fax Analyzer (LabCommerce Inc, USA).

Studies on regeneration of β -cells in streptozotocin-induced rats

This test was carried out as per described (Hassan et al. 2010). At the end of the 12th day of treatment, the rats were sacrificed, and the pancreas was removed and fixed in 10% buffered formalin for two days. The pancreatic tissue was embedded in paraffin after dehydrated with a series of alcohols (70%, 80%, 90% and 100% ethanol) and xylene for 30 min. Then, the tissues were embedded with an embedding ring. The paraffin-embedded tissues sections were serially cut at 5 μ m in thickness and placed on poly-L-Lysine-treated glass slides (Sigma, USA). After that, the slides were placed in a 45 °C oven for 1 h. Then, the tissues were deparaffinized followed by incubation for 15 min in 3% H₂O₂ in methanol to quench the endogenous peroxides. The sections were then washed in phosphate buffer saline (PBS) for 5 min. After that, the sections were blocked by incubating with diluted normal serum for 20 min. Then, the sections were incubated with primary antibody (Guinea-pig polyclonal anti-insulin body from Zymed laboratories, San Francisco, CA, USA) diluted to 1:100 in PBS for 30 min for detection of insulin. Next, the sections were washed with PBS for 5 min followed by incubation with diluted biotinylated secondary antibody solution for 30 min. Vectastain ABC (Vector Laboratories, USA) reagent was incubated with the tissues for 30 min and then the slides were washed again for 5 min. Sections were then incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB) (DAKO, Japan) for 3 to 5 min at room temperature. Next, slides were washed three times in distilled water followed by addition of two to four drops of DAB enhancer to cover the tissues on the slides and incubated for 1 to 3 min at room temperature. Then, the nuclei were counterstained with Harris Hematoxylin. The tissues were then dehydrated in a graded series of alcohol which consisted of three rinses in 95% ethanol, two rinses in absolute alcohol and three clearings in xylene for 3 min each. Finally, the tissues were mounted with DPX (BDH, UK). The assessment was done by direct microscopic examination of 10 islets for each group of rats using a Leica MZ6 optical microscope (Leica Mikroskopie und Systeme, Germany) equipped with a Leica Qwin (Leica Imaging Systems, Cambridge, England).

Statistical analysis

Data were expressed as mean \pm standard error of mean (SEM). Statistical analysis was made using one-way analysis of variance (ANOVA) followed by Dunnett's t-test for post-hoc analysis. p -values < 0.05 and < 0.001 were considered to be statistically significance.

RESULTS AND DISCUSSION

As depicted in Figure 1, only subfraction 1 and cinnamic aldehyde showed an increase in insulin concentration as compared to diabetic non-treated group. This indicated that subfraction 1 possessed direct insulinotropic effect in which might be due to the presence of cinnamic aldehyde as its bioactive compound. Cinnamic aldehyde is a known antidiabetic compound that exerts the antidiabetic effect by promoting insulin secretion (Adisakwattana et al. 2011). However, methanol extract and chloroform fraction failed to stimulate an increase in insulin concentration. As expected, metformin did not showed increase in insulin level after 14 day treatment as metformin is known not to stimulate the release of insulin from β cells of pancreas. Streptozotocin induced diabetes has been shown to cause selective destruction of β cells of islets of Langerhans (Junod et al. 1967; Zhang & Tan, 2000). This lead to decrease in insulin production and eventually will cause hyperglycaemia. Risbud & Bhonde (2002) reported that pancreatic endocrine cells have the potential to proliferate after the induction with STZ. Hence it is possible that, one of the mechanism for antidiabetic action of *C. iners* via initiating cell regeneration. At present, no drug is able to arrest the progressive loss of pancreatic β -cell function (Kan et al . 2001). Hence, medicinal plants which possess insulin secretagogue property remains as a key tool to control the increasing figures of diabetic patients (Hansotia & Drucker, 2005). The beta cell regenerating potential of *C. iners* active extract/active fraction and active subfraction was evaluated using immunohistochemistry procedure. This procedure employs the principle of distinguishing normal insulin producing cell (brown colour) from the destroyed cell. The results of this study demonstrated marked changes in the distribution pattern of insulin-positive cells in pancreatic tissue of diabetic rats compared with that of normal rats [Figure 2(A) and 2(B)]. Figure 2(A) shows the islets of normal rats with a relatively large area of insulin producing cells (80.46% of islets size) (Table 1). As indicated in Figure 2(B), there was a small number of insulin-positive cells (7.69% of islets size) in the diabetic control group after 14 days. In contrast, treatment of streptozotocin-induced diabetic rats with cinnamic aldehyde and subfraction 1 of *C. iners* resulted in a larger area (11.3% and 9.43% of islets size) of insulin positive immunoreactivity for the presence of insulin, however there is no significant changes

as compared to diabetic control group [Figure 2(C) and 2(D)]. This suggests that cinnamic aldehyde and subfraction 1 promote insulin secretion by the existing β cell islet which is partially destructed by STZ. However, the pancreas of diabetic rats treated with methanol extract (Figure 2E) and chloroform fraction (Figure 2F) of *C. iners* failed to exhibit significant increase of insulin positive cells as compared to diabetic control group with the percentage of insulin positive cells of 5.16% and 6.45% respectively. It seems that the antihyperglycaemic effect of active methanol extract and active chloroform fraction is brought by an extrapancreatic mechanism such as glucose uptake by muscle and glucose inhibition by intestine as reported earlier in our previous studies (Mustaffa et al., 2014). The results obtained is in line with the data collected for the measurement of insulin level in the blood circulation which showed only cinnamic aldehyde and subfraction 1 is capable of promoting insulin secretion. The results obtained suggest that the antihyperglycaemic effect of subfraction 1 might be due to the presence of cinnamic aldehyde in which mimic the antidiabetic mechanism of cinnamic aldehyde in promoting insulin secretion by pancreatic β -cell (Hansotia & Drucker, 2005).

Skeletal muscle serves as the important target tissues for the uptake of glucose at peripheral level (DeFronzo et al. 1981). Figure 3 showed glucose uptake (mg/dL per g tissue weight) by isolated rat abdominal muscle in the absence and presence of insulin. Experimental group treated with methanol extract and subfraction 1 resulted in significant increase ($p < 0.05$) in glucose uptake when compared with the control group. Cinnamic aldehyde also showed significant glucose uptake in isolated rat abdominal muscle. Besides, metformin also indicated significant increase in glucose uptake as compared to the control. It is reported by DeFronzo and team (1991) that the antihyperglycaemic effect of metformin is presumably as a result of physiological response of several tissues, including the uptake of glucose by skeletal muscle and adipose tissue. The glucose uptake by methanol extract treated group is higher as compared to subfraction 1 and cinnamic aldehyde alone. The clearing rate of glucose from the perfusion medium by methanol extract is almost similar to metformin (Figure 3). This might be due to synergistic activity of various compound present in the methanol extract that enhance the insulin-stimulated glucose transport across membrane of skeletal muscle. On the other hand, the glucose uptake property of subfraction 1 might be due to the presence of cinnamic aldehyde, in which glucose uptake property of cinnamic aldehyde had been reported (Tandon et al. 2013). However, *C. iners* chloroform fraction was not capable to increase the glucose uptake by rat abdominal muscle. The antihyperglycaemic

effect of active chloroform fraction might be brought by other extrapancreatic mechanism such as glucose inhibition by intestine as claimed previously (Mustaffa et al., 2014). The presence of insulin in the KRB (Kreb's-Ringer bicarbonate buffer) significantly increased the glucose uptake by isolated rat abdominal muscle in metformin and *C. iners* leaves extract/fraction/subfraction treated group when compared with control. Hence, metformin and the extract/fraction/subfraction participates directly in the uptake of glucose at the peripheral level or as an insulin coadjuvant in which insulin act as a fat and glucose storage promoter, thus enhance metabolic function in targeted cell (Hassan et al. 2010).

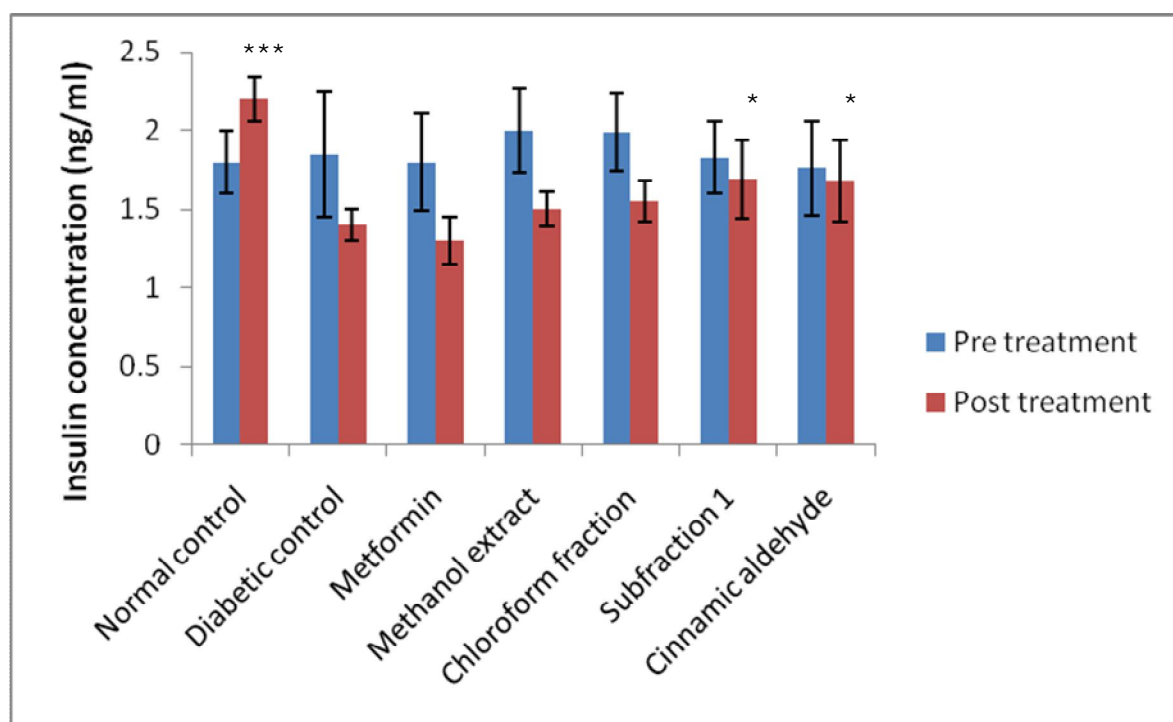
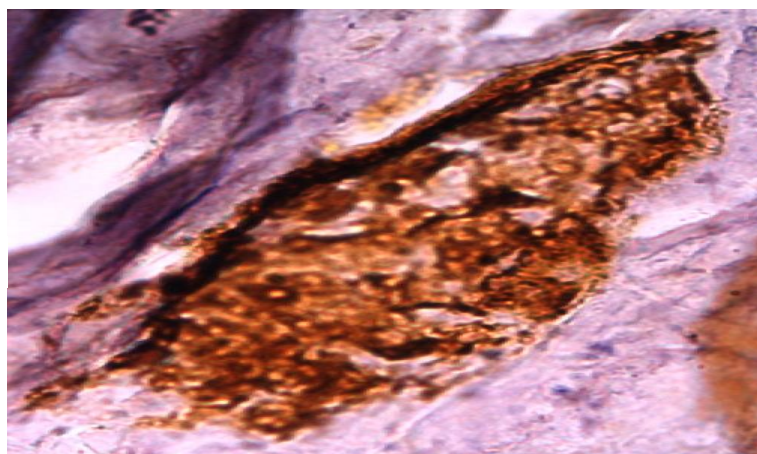
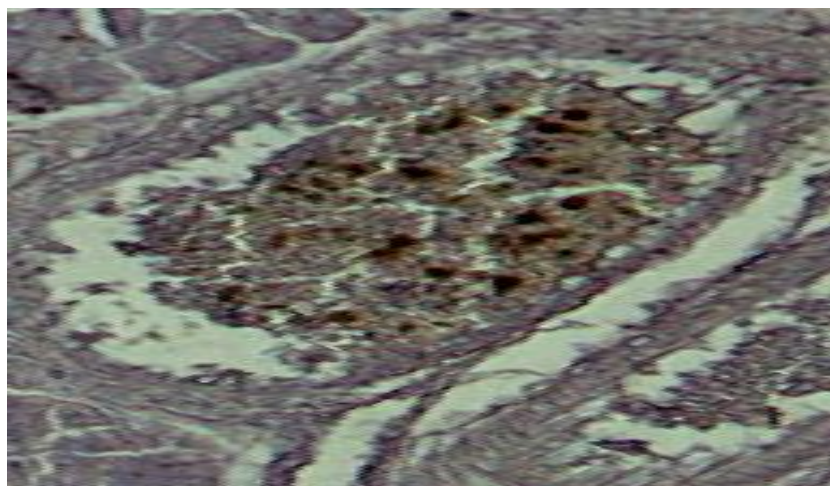


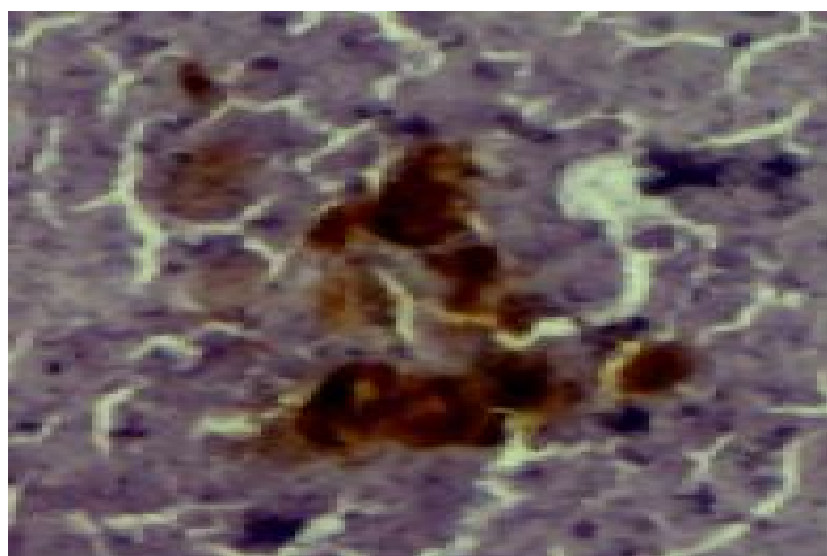
Figure 1. The effect of before and after 12 day of oral treatment with methanol extract, chloroform fraction and subfraction 1 of *C. iners*, metformin and cinnamic aldehyde on insulin levels of diabetic rats. The values are given as mean \pm SEM (n=6). * and *** indicates significant different at $p < 0.05$ and $p < 0.001$ compared with diabetic control.



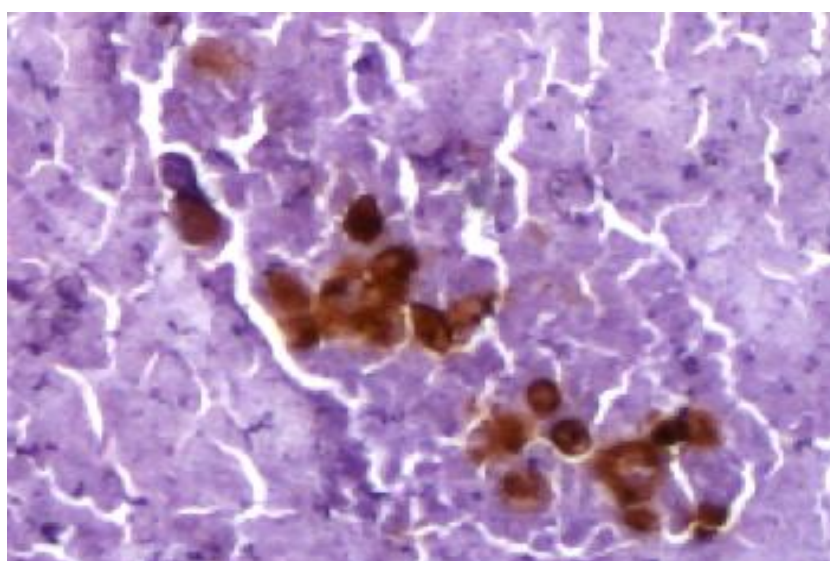
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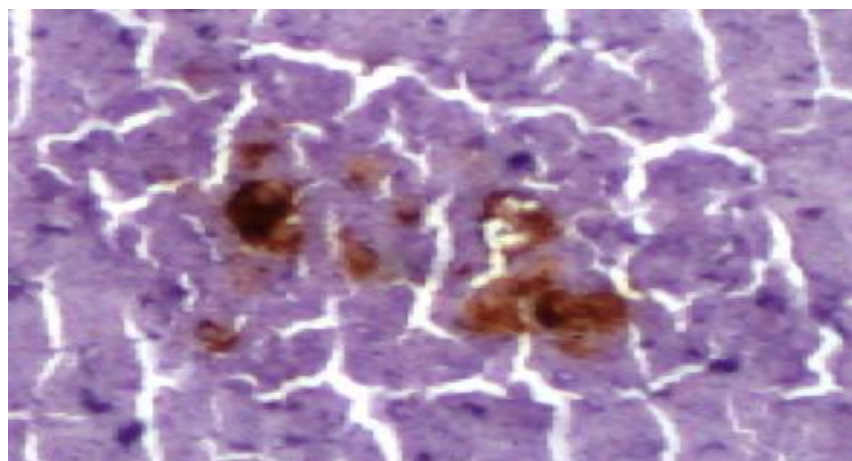
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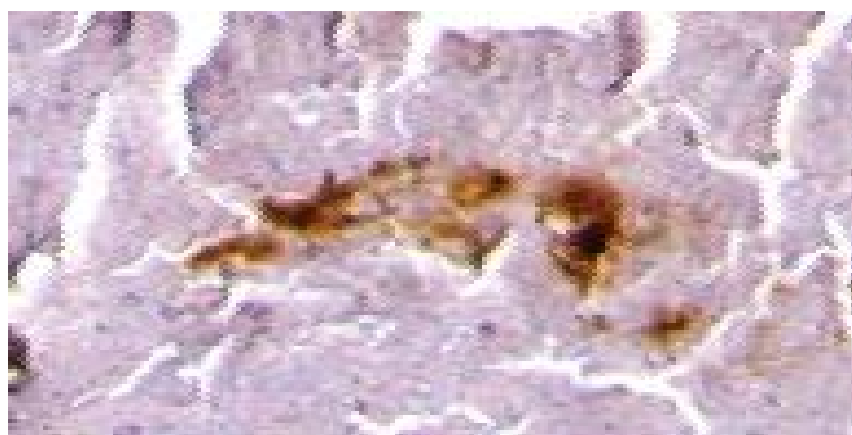
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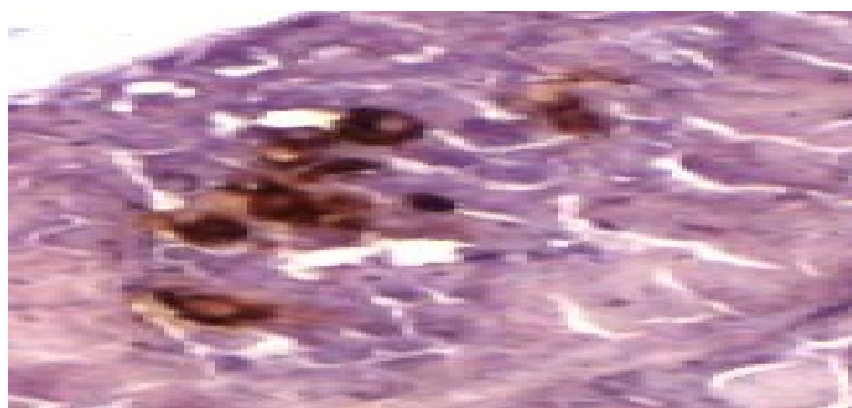
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Figure 2. Light micrograph of rat pancreas showing insulin immuno-staining of β -cells (brown colour) in islet of Langerhans A) normal rat, B) control diabetic rat, C) diabetic rat treated with cinnamic aldehyde D) diabetic rat treated with subfraction 1 of *C. iners*, E) diabetic rat treated methanol extract of *C.iners*, F) diabetic rat treated with chloroform fraction of *C. iners*, G) diabetic rats treated with metformin ($\times 400$).

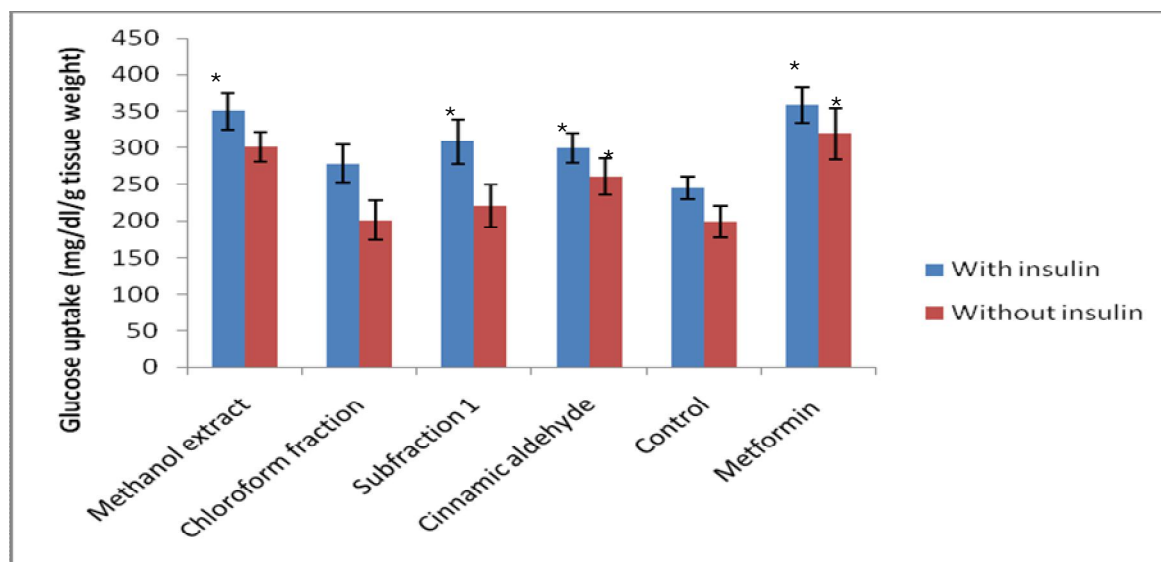


Figure 3. Effect of methanol extract, chloroform fraction and subfraction 1 of *C. iners* and metformin and cinnamic aldehyde on glucose uptake in isolated rat abdominal muscle (in the present or absence of insulin). Data represent means \pm SEM of $n=6$. * $p<0.05$ compared with negative control.

Table 1. Effect of *C. iners* methanol extract, chloroform fraction, subfraction 1 and metformin on islet cell in streptozotocin-induced diabetic rats.

Experimental Group	Percentage of insulin-positive cells per islet
Normal control	$80.46 \pm 3.21^{***}$
Diabetic control	7.69 ± 0.58
Diabetic rats treated with metformin	12.46 ± 2.15
Diabetic rats treated with cinnamic aldehyde	11.30 ± 2.18
Diabetic rats treated with methanol extract of <i>C. iners</i>	5.16 ± 0.85
Diabetic rats treated with chloroform fraction of <i>C. iners</i>	6.45 ± 1.02
Diabetic rats treated with subfraction 1 of <i>C. iners</i>	9.43 ± 1.95

Each value represents the mean \pm S.E.M ($n = 10$); *** indicates significant difference as compared to the diabetic control group at $p < 0.001$.

CONCLUSION

The antihyperglycaemic effect of *C. iners* leaves is linked to direct pancreatic and extrapancreatic mode of action by promoting insulin stimulative effect and increasing glucose uptake by muscle. Future therapeutic approach may apply *C. iners* as natural antihyperglycaemic agent which is relatively low in cost with minimal side effect.

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REFERENCES

1. Adam Z, Hamid M, Ismail A, Khamis S. Effect of *Ficus deltoidea* extracts on hepatic basal and insulin stimulated glucose uptake. *Journal of Biological Sciences* 2009; 9(8):796-803.
2. Adisakwattana S, Hsu WH, Yibchok-anun S. Mechanisms of p-methoxycinnamic acid-induced increase in insulin secretion. *Hormone and Metabolic Research* 2011; 43(11):766-773.
3. Alam K, Mahpara S, Mohammad MAK, Khan NK, Richard A. Cinnamon improves glucose and lipids of people with type 2 diabetes, *Diabetes Care* 2003; 26: 3215–3218.
4. Bank HL. A quantitative enzyme-linked immunosorbent assay for rat insulin. *Journal of Immunoassay* 1988; 9: 135–158.
5. Brain R, Margaret CC, Jian K, Ramesh KG, John HM. Strain differences in susceptibility of streptozotocin-induced diabetes: Effect on hypertriglyceridemia and cardiomyopathy. *Cardiovascular Research* 1997; 34:199-205.
6. Choi OH. *Tumbuhan Liar, Khasiat Ubatan dan Kegunaan Lain*. 1st ed. Kuala Lumpur, Malaysia. Utusan Publications and Distributors Sdn Bhd: 2003; pp. 132-33.
7. Defronzo RA, Barzilai N, Simonson DC. Mechanism of metformin action in obese and lean non insulin dependent diabetic subjects. *Journal of Clinical Endocrinology & Metabolism* 1991; 73:1294-1301.
8. Gray AM, Flatt PR. Antihyperglycemic Actions of *Eucalyptus globulus* (eucalyptus) are associated with pancreatic and extra-pancreatic effects in mice. *Journal of Nutrition* 1998; 128: 2319-2323.
9. Hansotia T, Drucker DJ. GIP and GLP-1 as incretin hormones: lessons from single and double incretin receptor knockout mice. *Regulatory Peptides* 2005; 128: 125-134.
10. Hassan Z, Yam MF, Ahmad M, Yusof APM. Antidiabetic properties and mechanism of action of *Gynura procumbens* water extract in streptozotocin-induced diabetic rats. *Molecules* 2010; 15: 9008-9023.

11. Junod A, Lambert AE, Orci L, Pictet R, Gonet AE, Renold AE. Studies of the diabetogenic action of streptozotocin. Proceedings of the Society for Experimental Biology and Medicine 1967; 126:201-205.
12. Kadnur SV, Goyal RK. Comparative antidiabetic activity of methanolic extract and ethyl acetate extract of *Zingiber officinale* Roscoe. Indian Journal of Pharmaceutical Sciences 2005; 67:453-457.
13. Kan EE. The importance of β -cell failure in the development and progression of type 2 diabetes. The Journal of Clinical Endocrinology and Molecular Metabolism 2001; 86: 4047-4058.
14. Kannappan S, Jayaraman T, Rajasekar P, Ravichandran MK, Anuradha CV. Cinnamon bark extract improves glucose metabolism and lipid profile in the fructose-fed rat. Singapore Medical Journal 2006; 47: 858-863.
15. MacDonald MJ, Gapinski JP. A rapid ELISA for measuring insulin in a large number of research samples. Metabolism 1989; 38: 450-452.
16. Nagappa AN, Thakurdesai PA, Rao NV, Singh, J. Antidiabetic Activity of *Terminalia catappa* Linn Fruits. Journal of Ethnopharmacology 2003; 88:45-50.
17. Pengelly A. Constituents of Medicinal Plants. Cambridge, USA. CABI Publishing: 2004, p. 66.
18. Perez C, Dominguez E, Canal JR, Campillo JE, Torres M.D. Hypoglycaemic activity of an aqueous extract from *Ficus Carica* (Fig Tree) leaves in streptozotocin diabetic rats. Pharmaceutical Biology 2000; 38: 181-186.
19. Qin B, Nagasaki M, Ren M, Bajotto G, Oshida Y, Sato Y. Cinnamon extract (traditional herb) potentiates *in vivo* insulin regulated glucose utilization via enhancing insulin signaling in rats. Diabetes Research and Clinical Practice 2003; 62:139-148.
20. Risbud MV, Bhonde RR. Models of pancreatic regeneration in diabetes. Diabetes Research and Clinical Practise 2002; 58:155-165.
21. Suba V, Murugesan T, Arunachalam G, Mandal SC, Sahu BP. Antidiabetic potential of *Barleria lupulina* extract in rats. Phytomedicine 2004; 11: 202-205.
22. Tandon R, Gupta A, Ray A. Mechanism of action of anti-diabetic property of cinnamic acid. A principal active ingredient from the bark of *Cinnamomum cassia*. International Journal of Therapeutic Applications 2013; 9:39-45.
23. Zhang XF, Tan BKH. Effects of an ethanolic extract of *Gynura procumbens* on serum glucose level, cholesterol and triglyceride levels in normal and streptozotocin-induced diabetic rats. Singapore Medical Journal 2000; 41: 9-13.