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In vitro evaluation of the effect of *Pluchea indica* extracts in promoting glucose consumption activity on a liver cell line

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Abstract

Background: Type 2 diabetes mellitus is a chronic metabolic disorder that is associated with mortality and morbidity. Recently, several plant-based agents have been used in the management of diabetes. *Pluchea indica* has been traditionally consumed as a medicinal plant in Southeast Asia, and its leaves have demonstrated induction of hypoglycemic effect in normal rats. This *in vitro* study aimed to evaluate the potency of *P. indica* extracts in stimulating glucose consumption in human liver CCL-13 cell line model. **Methods**: *P. indica* leaves were dried and extracted using a series of organic solvents and water. The effect of the extracts on cell viability was determined by MTT assay. The glucose consumption was analyzed using glucose oxidase method. **Results**: Our results revealed that the methanol extract of *P. indica* could significantly increase glucose consumption of cells in a concentration-dependent manner, which suggests the usefulness of the extract as an antidiabetic candidate via stimulation of glucose uptake into the liver cells. **Conclusion**: Our study suggests that *P. indica* is a potential natural candidate for diabetes mellitus management.

Keywords: diabetes mellitus type 2, liver, cell line, glucose oxidase, Pluchea indica

Introduction

Diabetes mellitus is a chronic disease that is characterized by persistent hyperglycemia with disturbances in carbohydrate, fat, and protein metabolisms resulting from the defects in insulin secretion or insulin action or a combination of both the factors.[1] The worldwide figure of people with diabetes in the year 2015 indicated that 415 million people had the disease and the number was estimated rise to 642 million by 2040.[2] Over 90% of the global cases account for type 2 diabetes. Modernized and sedentary lifestyles have led to over-nutrition and lack of physical exercises, thereby increasing the prevalence of obesity as a known risk factor for the development of type 2 diabetes.[3,4] Type 2 diabetes is primarily managed by controlling blood glucose levels and practicing a healthy lifestyle.[5] A number of medications with varying mechanisms of action are commonly prescribed to control the blood glucose levels i.e. α-glucosidase inhibitors, insulin secretagogues, biguanides, thiazolidinedione, and insulin mimetic agents. However, these drugs may cause various adverse effects, such as gastro-intestinal complaints and hypoglycemia.[6] These reasons highlight the importance of identifying novel antidiabetic drugs with minimum side effects.

Plant-derived natural products have been playing an important role in improving the human health.[7] *Pluchea indica* (L.) Less (Compositae) is found abundantly in the tropical coasts of the South and Southeast Asian regions. The roots and leaves are reported to possess anti-inflammatory[8], antimicrobial[7], hepatoprotective, antiulcer[9], and wound healing properties.[10] Two independent *in vivo* studies using rat models demonstrated the anti-hyperglycemic effect of *P. indica* in streptozotocin-induced diabetic rats. Moreover, *P. indica* also significantly induced hypoglycemic effect in normal rats.[11,12] A study on 3T3-L1 adipocyte cells revealed that *P. indica* may play a role in the control adipogenesis of 3T3-L1 cells by decreasing lipid accumulation and inhibiting adipogenesis.[13] However, no attempt has yet been made to explore the antidiabetic property of *P. indica* using liver cell model. Investigating the effect of new drug candidates on hepatic glucose metabolism is essential because the liver is the main organ for controlling blood glucose.[14] This *in vitro* research was

designed to explore the effect of *P. indica* extracts on glucose consumption in human liver CCL-13 cell line.

Methods

Plant material and extract preparation. The leaves of *P. indica* were collected in August 2010 from the coastal areas in Kuantan, Pahang state, Malaysia. The plant was identified by Dr. Shamsul Khamis from the University Putra Malaysia. A voucher specimen of *P. indica* (no. MT-11-12) was deposited at the Herbarium, Kulliyyah of Pharmacy, International Islamic University Malaysia. The *P. indica* leaves were dried and ground into powder (294 g) before subjecting them to a serial extraction of organic solvents in the sequence of *n*-hexane, dichloromethane, ethyl acetate, and methanol. The extraction was performed using a Soxhlet apparatus at room temperature and concentrated in a rotary evaporator at 55 °C. A different sample of *P. indica* powdered leaves (25 g) was extracted using distilled water and freeze-dried to yield a dried extract. The total yield (w/w) of the extracts included *n*-hexane (4.16%), dichloromethane (1.45%), ethyl acetate (4.96%), methanol (14.13%), and water (15.01%). The extracts were stored in airtight glass containers at 4 °C.

Cell growth and treatment. The liver cell line was purchased from American Type Culture Collection No. CCL-13 (USA). All the reagents for cell culture and maintenance were from Invitrogen (USA). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C under an atmosphere of 95% air and 5% CO_2.

Cell viability assay. Different concentrations of extracts or 0.2% ethanol (as a control vehicle) were prepared as working solutions in cell growth medium DMEM for 48h of treatment. The cells (2×10^5 cells/mL) were plated in 100 mL of medium/well of a 96-well plate. Cell viability was determined by adding 20 µL of 5 mg/mL MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Invitrogen, USA) and further incubated at 37 °C for 3 h. The supernatant was removed, and 100 µL DMSO was added to each well for 1 h. The absorbance was measured in a spectrophotometer at 570 nm (reference: 630 nm). All experiments were repeated three times in triplicates.

Glucose oxidase assay. The glucose concentration was determined by both the activities of glucose oxidase and horseradish peroxidase. Glucose (GO) Assay Kit (Code GAGO-20) was purchased from Sigma (USA). The cells were seeded in a 96-well plate at a density of 2×10^5 cells/mL. Glucose consumption was measured after a 24 h incubation with samples at concentrations of 0.2, 0.1, and 0.05 mg/mL, respectively. Cellular glucose uptake was assayed in 10 µL of medium using glucose oxidase method as prescribed in the manufacture protocols prior to absorbance measurement in a spectrophotometer. The color intensity of the reaction product at 540 nm was found to be proportional to the original glucose concentration in the sample. The standard glucose curve was prepared according to the previous study.[15] The amount of glucose in the wells (i.e., the quantity of glucose consumed by the cells) was calculated by subtracting the glucose concentration in cell-plated wells from those of blank wells.[16]

Statistical analysis. The data were representative of at least triplicate experiments and analyzed by comparing experimental samples of the same treatment conditions as a group with negative control (untreated) samples using one-way ANOVA, followed by evaluation by Dunnett's post-hoc test. The results were reported as the mean (standard error of means). The statistic significant considered as $p < 0.05$.

Results

The phytochemical screening of the crude extracts (i.e., *n*-hexane, dichloromethane, ethyl acetate, and methanol) revealed the presence of carbohydrates, saponins, sterol/ terpenes, phenol, and flavonoids, as shown in Table 1.

Next, various concentrations of the *P. indica* extracts were exposed on the cells for 48 h and then measured by MTT assay to determine the maximum concentration of the extracts that could be tolerated without causing toxicity (Figure 1). The results demonstrated that the concentrations selected for the study (0.05, 0.1, and 0.2 mg/mL) did not impair cell viability during the period of incubation.

Finally, the glucose oxidase assay was performed to examine the glucose uptake activity of the extracts. As shown in Figure 2, the methanol extracts significantly elevated glucose consumption in a concentration-dependent manner, whereas the level of glucose uptake induced by other extracts remained unchanged.

Discussion

The liver plays a vital role in regulating glucose homeostasis.[17] In response to ingestion of glucose or a mixed meal, glucose enters hepatocytes via the glucose transporter GLUT2 and is used to synthesize glycogen. In the fasting state, the liver sustained the level of blood glucose through glycogenolysis and gluconeogenesis, which subsequently released the glucose into the blood circulation.[18,19] Controlling hepatic glucose metabolism has been considered as a strategy in the development of antidiabetic drugs. For instance, metformin has been widely used to reduce the hepatic glucose output in

treating type 2 diabetes mellitus.[20] Other liver-targeted antidiabetic candidates, such as activators of glucokinase, inhibitors of fructose 1,6-bisphosphatase, inhibitors of glycogen phosphorylase, and antagonists of the glucagon receptor are currently being studied in different phases of clinical trials.[21] However, so far, none of them have received approval. Hence, the potential to specifically target hepatic glucose homeostasis still remains to be exploited.

Table 1. Qualitative analysis of the phytochemicals of *P. indica* extracts

Detection	Extracts				
	***n*-hexane**	**dichloromethane**	**ethyl acetate**	**methanol**	**water**
Alkaloids	−	−	−	−	−
Carbohydrate	N.T.	N.T.	N.T.	+	+
Glycosides	N.T.	N.T.	N.T.	+	+
Saponin	N.T.	N.T.	N.T.	+	+
Amino acids	N.T.	N.T.	N.T.	−	−
Terpene	+	−	−	−	−
Steroids	+	+	+	−	−
Phenolics	−	−	+	+	+
Flavonoids	−	−	−	+	+

Key: + presences of constituent; − absence of constituents; N.T. not tested

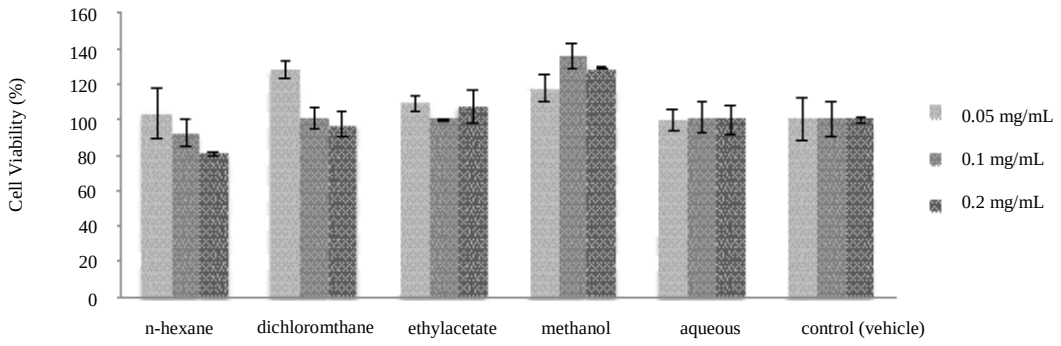

Figure 1. The CCL-13 cell viability after 48 h of exposure using different concentrations of extracts (0.05, 0.1, and 0.2 mg/mL) were analyzed by MTT assay. Data were presented as means ± SE of three replicates and expressed as percentages of the control value.

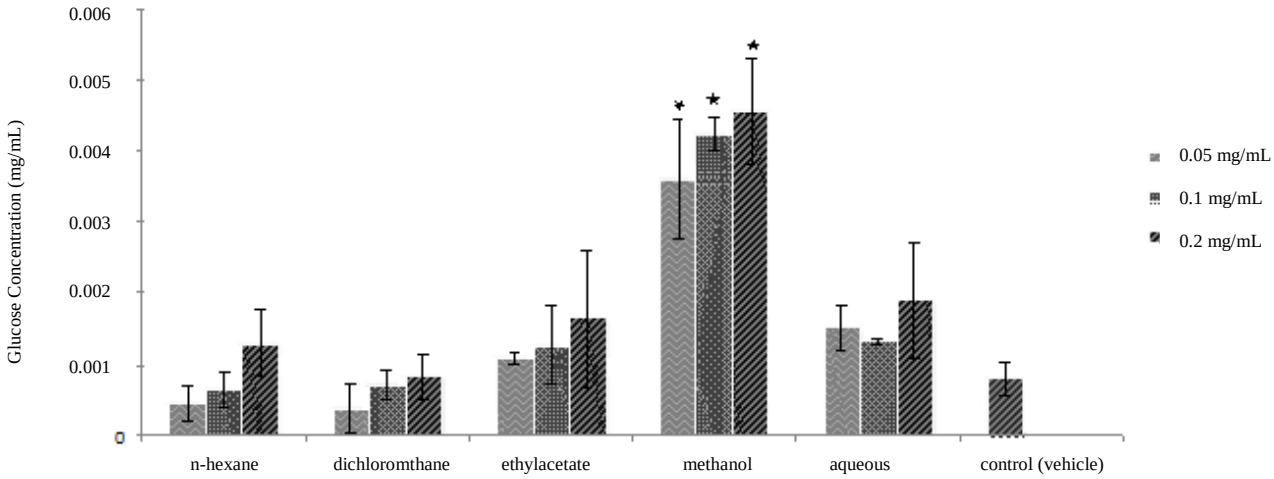

Figure 2. The methanol extracts of *P. indica* enhanced the glucose consumption in CCL-13 cells in a concentration-dependent manner. The glucose consumption of methanol extracts at the concentrations of 0.05, 0.1, and 0.2 mg/mL displayed significant difference at $p < 0.05$ for all samples groups. The mean score for the methanol-treated cells (0.05, 0.1, and 0.2 mg/mL) was significantly different from that of the control group.

Clinical trial of new drugs can be only started after multiple rounds of pre-clinical experiments, involving tests on animal and cell culture, have been conducted. Hepatic glucose metabolism has been extensively studied both *in vivo* and *in vitro*. Primary human liver cells (hepatocytes) have been reported as the best *in vitro* model; however, their supplies are quite limited.[22] Hence, several other cell lines derived from the liver such as IHH, HepaRG, HepG2, and Huh-7 have been utilized as a model. In the present study, we utilized the human CCL-13 liver cell line as an *in vitro* model to demonstrate the glucose uptake induced by plant medicine *P. indica*. The glucose uptake activity was analyzed using a chromogenic glucose oxidase method, which was chosen due to its reliability, convenient, and economical for bulk screening of new drug candidates.[15]

The crude extracts of *P. indica* were prepared by sequential extraction methods. We identified that the polar compounds were more abundant than non-polar ones in which both methanolic and aqueous extracts exhibited highest yields. The qualitative screening revealed that the polar compounds contain phenolic and flavonoid substances. Similar studies reported that the methanolic extract[23] and ethanol extract[24] of *P. indica* leaves had the highest phenolic compound than the other parts of plants. The potential of phenolic compounds of plant origin have also been considered in the management of oxidative stress-linked chronic diseases, such as diabetes and hepatic glucose homeostasis.[25] In agreement with this, our findings revealed that methanol extracts of *P. indica* induced the uptake of glucose by the CCL-13 cells. We postulate that the phenolic compound in the methanolic extract of *P. indica* may be responsible for promoting glucose consumption in the CCL-13 cell line. The result is supported by previous *in vitro* study using glucose oxidase method in HepG2 cell line, which demonstrated that phenolic compounds had significant glucose lowering activity.[26] We also detected the presence of phenolic substances in the aqueous and ethyl acetate extracts; however, no significant increase of glucose uptake was observed. We speculate that the proportion and the type of phenolic compounds in the extracts might influence the results. Further studies are warranted to identify the bioactive compound(s) responsible for the induction of glucose uptake and elucidate the antidiabetic mechanisms of *P. indica* on different cell lines as well as in an *in vivo* model.

Conclusions

The *P. indica* methanol extract was found to increase the glucose uptake in human liver cell line. Further study will be focused on determining the active ingredient(s) of *P. indica* which play a role as anti-diabetic activity on different *in vitro* and *in vivo* models.

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Conflict of Interest Statement

There are no conflicts of interest to declare.

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