

Antidiabetic Effects and *in vitro* Antioxidant Activity of *Pseuderanthemum palatiferum* (Nees) Radlk. ex Lindau Leaf Aqueous Extract

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Abstract

Fresh leaves of *Pseuderanthemum palatiferum* (Nees) Radlk. ex Lindau which have therapeutic effects for various diseases including diabetes mellitus, have been widely used by Thai people who are seeking an alternative drug or complementary medicine. However, few studies of *P. palatiferum* biological activities have been reported. This study investigated the hypoglycemic effect of *P. palatiferum* leaf aqueous extract using an oral glucose tolerance test (OGTT) in normal rats and streptozotocin-nicotinamide-induced diabetic rats. A single oral administration of the aqueous extract at doses of 0.25 and 0.50 g/kg significantly decreased the blood glucose level in diabetic rats but not in normal rats. Both doses of the extract produced blood glucose lowering effect in diabetic rats after 30 minutes as well as glibenclamide, a hypoglycemic drug. Evaluation of the *in vitro* antioxidant activity demonstrated that the extract exhibited DPPH radical scavenging activity ($IC_{50}=221.14$ μ g/ml), reducing activity, lipid peroxidation inhibitory effects, and protective effects against AAPH-induced hemolysis ($HT_5=181.19$ min). The total flavonoid content of the extract was 227.81 ± 10.70 mg/100g extract equivalent to kaempferol. The present investigation on antidiabetic and *in vitro* antioxidant effects of *P. palatiferum* leaf aqueous extract supports the use of *P. palatiferum* leaves as a complementary medicine for diabetes.

Key words: *Pseuderanthemum palatiferum*, Hoan-ngoc, Antidiabetic effects, Streptozotocin-nicotinamide-induced diabetic rats, Antioxidant activity

INTRODUCTION

Diabetes continues to be a major health problem worldwide. The number of diabetic patients has rapidly increased in most parts of the world, including Thailand (1). The prevalence of diabetes mellitus in Thailand has been predicted to increase from 2.4% in 1995 to 3.7% in 2025 (2). The use of medicinal plants as an alternative treatment for diabetes has become increasingly popular because synthetic antidiabetic agents produce some serious side effects and are expensive (3, 4).

Pseuderanthemum palatiferum (Nees) Radlk. ex Lindau, a 1–2 m high shrub, belongs to the Acanthaceae family. Its vernacular names are *xuan-hoa* or *hoan-ngoc* in Vietnam and *phaya wanon* in Thailand. Recently, *P. palatiferum* has been of particular interest to diabetic patients in Thailand. Its fresh leaves have been claimed to cure various diseases including diabetes (5). In the Mekong Delta in the southern region of Vietnam, fresh leaves of *P. palatiferum* are used for treatment and prevention of various types of human diseases such as hypertension, inflammation, cancer, gastrointestinal disorders, and kidney and liver diseases. The leaves are also used to treat animal diseases such as diarrhea in pigs and dogs, and cholera in chickens and ducks (6). Recently, a hypoglycemic effect from 80% ethanol leaf extract of *P. palatiferum* was reported in streptozotocin-induced diabetic rats. The extract also improved the lipid profile of the streptozotocin-induced diabetic rats, i.e., a reduction of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL), and an increase of high-density lipoprotein (HDL) levels (7).

In a recent phytochemical study, flavonoid glycosides –kaempferol-3-methyl ether-7-O- β -glucoside, and apigenin-7-O- β -glucoside were isolated from *P. palatiferum* leaf extract (8). Interestingly, kaempferol and apigenin glycosides have been shown to possess hypoglycemic activity. For example, kaempferitrin (kaempferol-3,7-O-(α)-L-dirhamnoside) increased glucose uptake in

rat soleus muscle (9) and activated the insulin signaling pathway, resulting in reduction of blood glucose level (10). Apigenin-6-C-(β)-L-fucopyranoside also showed a significant hypoglycemic effect in diabetic rats, and stimulated insulin secretion and glycogen synthesis (11). Since glycosides are high-polarity compounds, they can be extracted with a high-polarity solvent. As *P. palatiferum* is generally recommended to be used in the form of a decoction, this study therefore followed the traditional method of preparation by using water to extract the bioactive compounds from *P. palatiferum* leaves.

It is known that oxidative stress plays an important role in the pathogenesis of diabetes. High levels of oxidative stress markers, such as thiobarbituric acid reactive substances (TBARS) and oxidized low-density lipoprotein (LDL), have been reported in diabetic patients (12, 13). Oxidative stress could impair the insulin-induced GLUT4 translocation, which is an essential step in blood glucose regulation (14). Antioxidants particularly flavonoids which are electron-donating compounds are known to possess potent scavenging activity towards free radicals. Therefore, it would be interesting to determine the antioxidant activity and total flavonoid content of *P. palatiferum* extract.

The aims of this study were to investigate the hypoglycemic effect of *P. palatiferum* leaf extract in normal and streptozotocin-nicotinamide-induced diabetic rats, and to evaluate its *in vitro* antioxidant activity. The total flavonoid content of this plant extract was also determined.

MATERIALS AND METHODS

Chemicals and biological products

Nicotinamide, o-dianisidine, PGO enzyme and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St Louis, MO, USA); 2,2'-azobis (2-amidinopropane hydrochloride) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), 99% linoleic acid, ascorbic acid and Trolox were also obtained from Sigma Chemical Co.

Glibenclamide tablets (Daonil) were purchased from Aventis Pharma Ltd (Bangkok, Thailand). All other chemicals and reagents used were of analytical grade. Defibrinated sheep blood was obtained from the National Laboratory Animal Centre of Thailand, Salaya district, Nakhon Pathom province, Thailand.

Plant material

Pseuderanthemum palatiferum (Nees) Radlk. ex Lindau. fresh mature leaves were collected from Hoan Ngoc Garden, Khlong Luang district, Pathum Thani province, Thailand, from May to December 2010. The leaves of the node at 15 cm or more below the shoot were defined as mature leaves. The plant samples were identified by Prof. Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. Voucher specimens (PBM 04831) were then deposited at the herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University.

Preparation of plant extract

Fresh leaves of *P. palatiferum* were washed and dried in a hot-air oven at 50°C and ground with an electric grinder. The dried materials were soaked in distilled water for 10 min before heating, and then boiled at 100°C for 5 min. The decoction was filtered and lyophilized; the dried aqueous extract (yield, 19.18% dry wt., 2.57% fresh wt.) was then collected and kept in a closed container.

Animals

Male Wistar rats, each weighing 120–150 g, were obtained from the National Laboratory Animal Centre of Thailand, Salaya district, Nakhon Pathom province, Thailand. They were housed with free access to food and water in an air-conditioned room (25°C) under a 12 h light/dark cycle for at least one week prior to the experiments. The animal protocol was approved by the Animal Ethics Committee, Faculty of Pharmacy, Mahidol University (No.7/2552).

Hypoglycemic activity determination

Induction of diabetes in rats

Non-insulin-dependent diabetes mellitus (NIDDM) was induced in the overnight-fasted rats using the method of Masiello *et al.* (1998) (15) with slight modification. Nicotinamide (75 mg/kg) was subcutaneously injected 15 min before an intraperitoneal injection of 75 mg/kg streptozotocin (STZ). The urine glucose concentration of each rat was checked daily using a urine glucose strip. Four weeks after NIDDM induction, fasting plasma glucose (FPG) levels were measured; rats with an FPG level higher than 160 mg/dl were considered diabetic and were used in the experiment.

Oral glucose tolerance test (OGTT) in normal and diabetic rats

Normal rats were fasted overnight and then randomly divided into four experimental groups with 5–6 rats in each group. The aqueous extract and glibenclamide were dissolved in distilled water and orally administered to the rats as follows:

Group 1: distilled water (control)

Group 2: glibenclamide, 5 mg/kg body wt. (positive control)

Group 3: *P. palatiferum* aqueous extract, 0.25 g/kg body wt

Group 4: *P. palatiferum* aqueous extract, 0.50 g/kg body wt

All animals were orally fed with glucose (1.25 g/kg body wt.) 30 min after receiving the test solution. Blood samples were drawn from the tail vein just prior to administration of the extract or drug (defined as time -30), before glucose administration (defined as time 0), and every 30 min until 150 min after glucose administration (16).

Diabetic rats similarly divided into four groups and fasted for 4–8 h prior to the experiments were subjected to the same protocol as normal rats.

Blood glucose analysis

Blood samples were centrifuged for 2 min to obtain the plasma, which was determined for glucose level by the PGO

enzyme method (17). Absorbance was read at a wavelength of 450 nm using a spectrophotometer (Pharmacia, USA). Plasma glucose concentrations were expressed as mean \pm SEM.

In vitro antioxidant activity determination

DPPH radical scavenging assay

The free radical scavenging capacity was determined based on the electron-donating ability of antioxidant compounds according to Brand-Williams *et al.* (1995) (18). Two ml of 0.2 mM DPPH solution was added to 1 ml of extract (50-500 μ g/ml of methanol). A mixture of 2 ml of DPPH solution and 1 ml of methanol was used as a control. After 30 min incubation in the dark at room temperature, the absorbance of each sample was measured at 515 nm. The scavenging capacity of each sample was expressed as percentage of inhibition as calculated by the following equation:

$$\% \text{ inhibition} = [(A_c - A_s) / A_c] \times 100$$

where A_c = absorbance of the control, and A_s = absorbance of the sample solutions.

The 50% inhibition concentration (IC_{50} value) was calculated from the regression equation of the curve of % inhibition versus extract concentration. The experiment was done in triplicate and the average IC_{50} value was calculated. The DPPH radical scavenging capacity of the extract was expressed as mean $IC_{50} \pm SD$. Trolox was used as a reference standard.

Reducing power assay

Based on the $Fe^{3+} \rightarrow Fe^{2+}$ transformation in the presence of antioxidants, reducing power was determined according to the methods of Oktay *et al.* (2003) (19), Gülçin *et al.* (2006) (20) and Ferreira *et al.* (2007) (21). A 500 μ l sample of *P. palatiferum* aqueous extract (25-500 μ g/ml of methanol) was mixed with 500 μ l of 0.2 M sodium phosphate buffer and 500 μ l of 1% w/v potassium ferricyanide. After incubating the mixtures at 50°C for 20 min, 2 ml of 10% w/v trichloroacetic acid was added to each; the samples were then centrifuged at 650 rpm for 10 min. The supernatant (500 μ l)

was drawn and mixed with 500 μ l of deionized water and 100 μ l of 0.1% w/v ferric chloride. The absorbance of the mixtures was spectrophotometrically measured at 700 nm. Trolox was used as a reference standard. The Fe^{2+} content was evaluated using a standard curve of $FeSO_4$ (0.025-0.25 mM). The mean of three readings was expressed as mM $FeSO_4$ equivalent \pm SD.

Thiobarbituric reactive substance (TBARS) assay

Lipid peroxidation was induced by Fenton-like reaction according to the methods of Choi *et al.* (2002) (22) and Matkowski and Piotrowska (2006) (23) with slight modification. The reaction mixture comprised 150 μ l of *P. palatiferum* aqueous extract (1-10 mg/ml of methanol), 500 μ l of 0.6% v/v linoleic acid in 5% sodium dodecyl sulfate (SDS), 450 μ l of tris-HCl buffer (pH 7.5) and 100 μ l of 4 mM $FeSO_4 \cdot 7H_2O$ solution. Linoleic acid peroxidation was initiated by the addition of 200 μ l of 2 mM ascorbic acid to the reaction mixtures. After incubating for 30 min at 37°C, 5.5% w/v of trichloroacetic acid was added to terminate the peroxidation reaction. Then 1 ml of 1% w/v thiobarbituric acid was added to the reaction mixtures, followed by boiling for 15 min. The mixtures were centrifuged at 3500 rpm for 10 min, after which the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was read at 532 nm. The malondialdehyde (MDA) content was evaluated using a standard curve of malondialdehyde bis (dimethyl acetal) (0.006-3 mM). The percentage of linoleic acid peroxidation inhibition was calculated using the following equation:

$$\% \text{ linoleic acid peroxidation inhibition} = [(M_c - M_s) / M_c] \times 100$$

where M_c = MDA equivalent of the control, and M_s = MDA equivalent of the sample solution. IC_{50} value was determined from the regression equation of the curve of % linoleic acid peroxidation inhibition versus extract concentration. The experiment was done in triplicate and the average IC_{50} value was then calculated. The lipid peroxidation inhibitory activity of the

extract was expressed as mean $IC_{50} \pm SD$. Trolox was used as a reference standard.

AAPH-induced hemolysis assay

The inhibitory activity of free radical-induced red blood cell hemolysis was determined by the method described by Deng *et al.* (2006) (24). Briefly, defibrinated sheep blood (10 ml) was centrifuged at 2500 rpm for 5 min, and the plasma was carefully removed. Erythrocytes were washed three times with 10 mM phosphate buffered saline (PBS) before adding 4 ml of PBS to obtain a 20% v/v suspension. Two concentrations (0.5 and 1 mg/ml) of *P. palatiferum* aqueous extract were prepared using PBS as a solvent. One hundred μ l of each concentration of the extract was added to a mixture of 100 μ l of 20% v/v erythrocyte suspension and 200 μ l of 200 mM AAPH solution. Trolox (50 mg), a reference standard, was dissolved in 0.5 ml of DMSO. Then, PBS (100 ml) was added and the Trolox solution adjusted to a final concentration of 500 μ g/ml. A mixture of 20% v/v erythrocyte suspension (100 μ l) and 3.6 ml of distilled water was used as a negative control for complete hemolysis. The reaction mixtures were shaken gently at 37 °C. Every 30 min for 3 h, 400 μ l of each sample was drawn, diluted with 4 ml of 0.15 M NaCl, and centrifuged at 3000 rpm for 10 min. The supernatant was then removed and the absorbance determined at 540 nm. The percentage of hemolysis was calculated by the following equation:

$$\% \text{ hemolysis} = (A_s/A_c) \times 100$$

where A_c =absorbance of the negative control, and A_s = absorbance of the sample solution. Antioxidant activity was expressed as the time required to reach 50% hemolysis (HT_{50}).

Total flavonoid content

The total flavonoid content was evaluated using the method of Meda *et al.* (2005) (25). A volume of 500 μ l of 2% w/v $AlCl_3$ in methanol was mixed with 500 μ l of the sample solution. After 10 min, the absorbance of the mixture was measured at 415 nm against methanol. The

total flavonoid content of the extract was calculated using a regression equation of the standard curve of kaempferol (0.5–12.5 μ g/ml). The mean of three readings was used and was expressed as mg of kaempferol equivalent (KE)/100 g of extract.

Statistical analysis

Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test. Statistical comparisons between two groups were performed by Student's unpaired *t*-test. The level of significance was $P < 0.05$. Data were analyzed by SPSS (version 15.0 for Windows).

RESULTS

Oral glucose tolerance test in normal and diabetic rats

The effects of *P. palatiferum* leaf aqueous extract on oral glucose tolerance of normal rats are shown in Fig. 1a. Changes in the plasma glucose level in extract-treated normal rats (0.25 g/kg and 0.50 g/kg) were not different from that of the control group, whereas glibenclamide-treated normal rats showed a significant decrease in plasma glucose levels 60–150 min after glucose administration. This result indicated that the extract did not have a hypoglycemic effect in normal rats. In contrast, both doses of *P. palatiferum* extract produced an antidiabetic effect at 30 min and 90–150 min in diabetic rats similar to the effect of glibenclamide, showing a significant reduction in plasma glucose level after 30 min (Fig. 1b).

DPPH radical scavenging activity

The DPPH scavenging activities of *P. palatiferum* leaf aqueous extract and a reference standard (Trolox) exhibited dose-dependent response. The IC_{50} value of Trolox was 28.08 ± 0.64 μ g/ml. The extract with IC_{50} of 221.14 ± 6.33 μ g/ml had a lower DPPH scavenging activity when compared to Trolox.

Reducing power

The reducing power assay is based on the $Fe^{3+} \rightarrow Fe^{2+}$ transformation in the

presence of antioxidants, in which the yellow-colored Fe^{3+} /ferricyanide complex, is reduced blue-colored ferrous form Fe^{2+} (19-21). The reducing powers of *P. palatiferum* leaf aqueous extract and Trolox are shown in Fig. 2. Trolox showed a dose-dependent reducing power at concentrations of 50–500 $\mu\text{g/ml}$, while the

reducing power of 1000 $\mu\text{g/ml}$ Trolox was not significantly different from that of 500 $\mu\text{g/ml}$ Trolox. The extract showed a dose-dependent reducing power at concentrations of 100–1,000 $\mu\text{g/ml}$. However, the extract exhibited less potent reducing power when compared to Trolox at the same concentration.

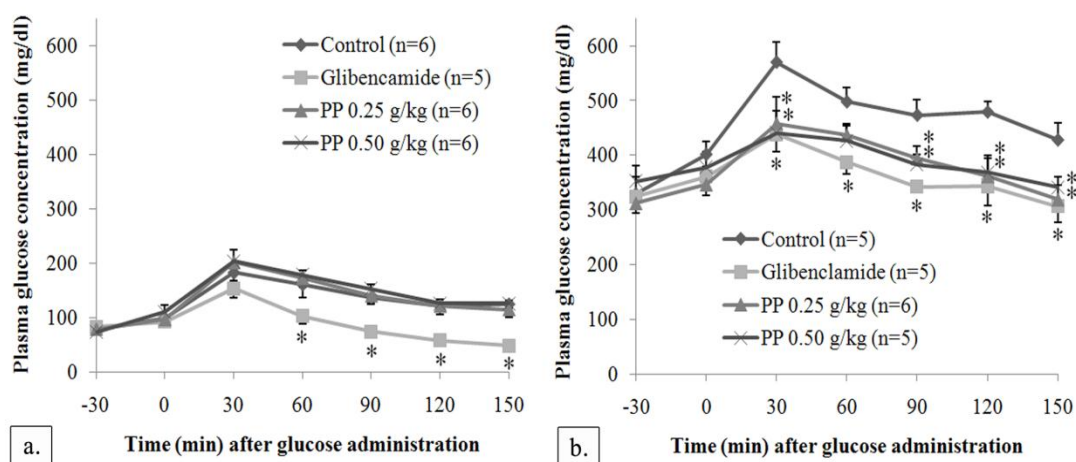


Figure 1. Effects of *P. palatiferum* leaf aqueous extract (PP), 0.25 and 0.50 g/kg, on oral glucose tolerance of (a) normal rats and (b) diabetic rats. * $P < 0.05$ compared with the respective control group at each time point.

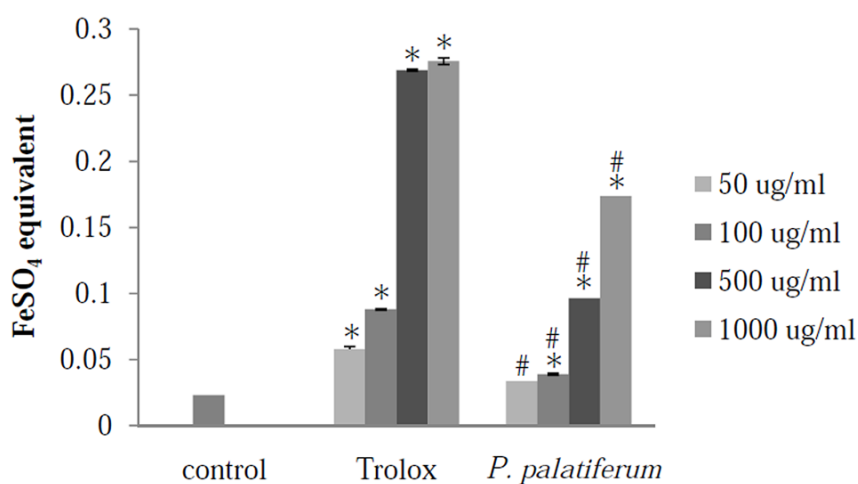


Figure 2. FeSO_4 equivalents of *P. palatiferum* leaf aqueous extract and Trolox compared with the control. * $P < 0.05$ compared with the control. # $P < 0.05$ compared with the same concentration of Trolox.

Lipid peroxidation inhibition

The lipid peroxidation inhibitory effects of *P. palatiferum* leaf aqueous extract and Trolox occurred in a dose-dependent manner at concentration 50–500 µg/ml as shown in Fig. 3. For both Trolox and the extract, the percentages of lipid peroxidation inhibition at concentrations of 500 and 1,000 µg/ml were not significantly different. Therefore, the maximum inhibitory concentration for both appeared to be 500 µg/ml. The IC₅₀ of Trolox was 249.65 µg/ml. The IC₅₀ of *P. palatiferum* extract, however, could not be demonstrated because the percentage of inhibition of *P. palatiferum* extract did not reach 50% at concentrations of 500 and 1,000 µg/ml. Nevertheless, the extract showed a less potent lipid

peroxidation inhibitory effect than Trolox at the same concentration.

Inhibition of AAPH-induced hemolysis

The inhibitory effects against AAPH-induced hemolysis are expressed as incubation time used to produce 50% hemolysis (HT₅₀). *P. palatiferum* leaf aqueous extract at concentrations of 500 and 1,000 µg/ml significantly prolonged HT₅₀ from 62 min in the control to 103.71 and 130.77 min, respectively, as shown in Fig. 4; whereas 500 µg/ml of Trolox showed HT₅₀ of 181.19 min.

Total flavonoid content

The content of flavonoids equivalent to kaempferol in *P. palatiferum* leaf aqueous extract, as evaluated by UV spectrophotometry was found to be 227.81 ± 10.70 mg/100g extract.

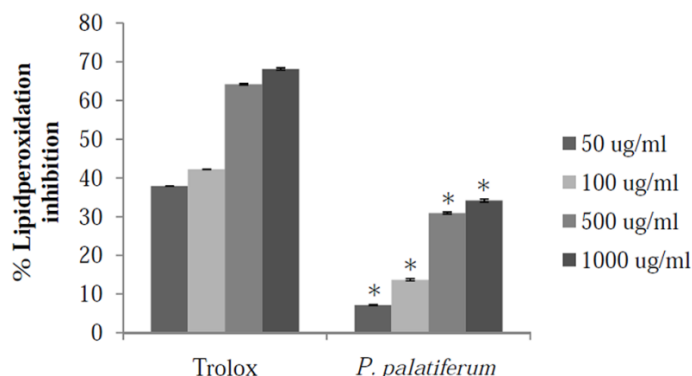


Figure 3. Percentage of inhibition of lipid peroxidation by Trolox and *P. palatiferum* leaf aqueous extract. **P* < 0.05 compared with the same concentration of Trolox.

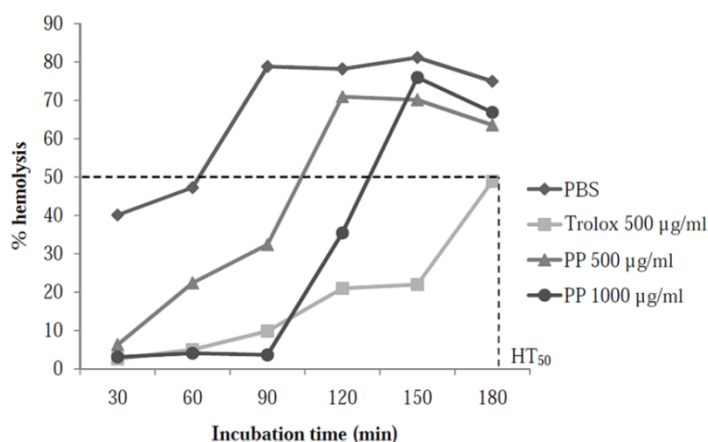


Figure 4. Inhibitory effects against AAPH-induced hemolysis of *P. palatiferum* leaf aqueous extract (PP) at 500 and 1,000 µg/ml, compared to the control (PBS) and 500 µg/ml of Trolox.

DISCUSSIONS

STZ-nicotinamide-induced diabetes in adult rats was used in this study as a model for non-insulin-dependent diabetes mellitus (NIDDM). The administration of suitable dosages of nicotinamide partially protects β -cells from the cytotoxic effect of STZ. Thus, the diabetes syndrome in this experiment appears closer to human NIDDM, in that it demonstrated a significant response to glucose and sensitivity to sulfonylureas, a class of hypoglycemic drug (15). These drugs, including glibenclamide, stimulate insulin secretion from pancreatic β -cells via the closing of the ATP-dependent potassium channel (K_{ATP}) (26). In the present experiment, *P. palatiferum* leaf aqueous extract at doses of 0.25 g/kg and 0.50 g/kg showed hypoglycemic effects in STZ-nicotinamide-induced diabetic rats but not in normal rats. The results were consistent with those of Padee *et al.* (2010) (7), who reported that the ethanol extract of *P. palatiferum* leaves (0.25 g/kg) significantly decreased fasting plasma glucose (FPG) levels in STZ-induced diabetic rats but not in normal rats. They also found an increase in serum insulin level in the extract-treated diabetic group as well as in the glibenclamide-treated group. Therefore, the hypoglycemic effect of *P. palatiferum* extract might be due to its stimulatory effect on insulin secretion. However, glibenclamide exhibited plasma glucose-lowering effects in both normal and diabetic rats, while the extract showed this effect only in diabetic rats. This suggested that the insulin secretion stimulation pathway of the extract might be different from that of glibenclamide. Nevertheless, other mechanisms such as stimulation of glucose uptake, reduction of glucose absorption, and increase in insulin sensitivity might also be involved. Further studies on hypoglycemic mechanisms are required in order to clarify these issues.

The present investigation indicated that the extract had the ability to scavenge DPPH, reduce Fe^{3+} , and inhibit linoleic acid peroxidation. The presence of flavonoids in the extract might contribute to the free

radical scavenging activity and help terminate the chain reaction due to their hydrogen donating ability (21). In the hemolysis study, *P. palatiferum* extract significantly prolonged HT_{50} compared to the control. This suggested that the extract could protect against free radical-induced biomembrane damage, but the effect was not as potent as that of Trolox. The protective effects of the extract against AAPH-induced hemolysis might be due to its lipid peroxidation inhibitory activity which corresponded with its linoleic acid inhibitory effects as determined by TBARS assay. However, the content of flavonoids equivalent to kaempferol in *P. palatiferum* leaf aqueous extract was 227.81 ± 10.70 mg/100g extract which accounted for only 0.2% of the extract.

Although the active compounds which exhibit antidiabetic activity in *P. palatiferum* were not identified, a previous phytochemical study of this plant reported the presence of flavonoids, including apigenin and kaempferol glycosides (8). These compounds might be responsible for antioxidant activity which helps prevent oxidative stress, an important part of the pathogenesis of diabetes.

CONCLUSION

An oral glucose tolerance test (OGTT) in normal and streptozotocin-nicotinamide-induced diabetic rats demonstrated that *P. palatiferum* leaf aqueous extract at doses of 0.25 g/kg and 0.50 g/kg showed hypoglycemic effects in diabetic rats but not in normal rats. The antioxidant activity of *P. palatiferum* leaf aqueous extract, investigated by four methods showed that the extract exhibited DPPH radical scavenging activity, reducing power, lipid peroxidation inhibitory effects, and some protective effects against AAPH-induced hemolysis. The observed antioxidant activity of the extract corresponded with the small amount of total flavonoid content present in the extract. The results support the use of *P. palatiferum* leaves as a complementary medicine for diabetes. Further investigations of the mechanisms of antidiabetic action and the compounds responsible for this activity are necessary.

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