Anti-lipolytic, α-amylase inhibitory and antioxidant activities of *Pseuderanthemum palatiferum* (Nees) Radlk. leaf ethanolic extract

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*Pseuderanthemum palatiferum* (Nees) Radlk. has been used extensively as a medicinal plant for the treatment of diabetes mellitus in various parts of Southeast Asia. However, the mechanism of its anti-diabetic action has not been fully characterized yet. The objectives of this study were to investigate the in vitro effects of *P. palatiferum* leaf ethanolic extract (PPE extract) on adipocyte lipolysis, α-amylase enzyme activity and antioxidant activity. The PPE extract at every concentration tested (10, 25, 50, 100, 250, 500 µg/ml) caused a significant inhibition on basal lipolysis in adipocytes of the high fat diet-fed rats. The PPE extract also produced a concentration-dependent anti-lipolytic action against isoprenaline (0.1 µM)-induced lipolysis in the normal pellet diet-fed rat derived adipocytes. Inhibition of α-amylase was detected using the 3,5-dinitrosalicylic acid assay, the PPE extract having an IC₅₀ of 4.99 mg/ml. An antioxidant activity, as evaluated by the DPPH assay (IC₅₀), was 58.13 µg/ml. In the ferric reducing power assay, the extract exerted antioxidant activity with a vitamin C equivalent antioxidant capacity (VCEAC) and a trolox equivalent antioxidant capacity (TEAC) of 2.33 and 2.04 g/100 g extract, respectively. The anti-lipolytic, α-amylase inhibitory and antioxidant activities of PPE extract detected here help justify the traditional use of this plant in the treatment of diabetes mellitus.

**Key words:** *Pseuderanthemum palatiferum*, diabetes mellitus, adipocyte lipolysis, α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing power assay.

**INTRODUCTION**

Diabetes mellitus (DM) is a chronic metabolic disorder caused by impairment in insulin secretion and/or insulin...
action. The incidence of DM, especially type 2 DM has increased substantially in modern society in which people live with a sedentary lifestyle. Treatment of type 2 DM includes both drug therapy and lifestyle modifications (ADA, 2014). Although several groups of anti-diabetic agents with various mechanisms of action have been developed, certain treatment problems, including unresponsiveness and adverse drug reactions, still arise regularly. Additionally, the high cost of some novel anti-diabetic agents is another main obstacle in diabetic treatment, especially in developing countries. Therefore, other treatment options, particularly medicinal plants, have been used widely as alternative anti-diabetic therapies.

*Pseuderanthemum palatiferum* (Nees) Radlk. has been used traditionally as a medicinal plant for the treatment of DM in several Southeast Asian countries, especially in Vietnam and Thailand. The leaf ethanolic extract of the plant produced a significant plasma glucose-lowering action in streptozotocin-induced diabetic rats without causing any acute toxicity (Padee et al., 2010). However, the mechanisms of its anti-diabetic action have not been clearly established yet. Chronic elevations of plasma free fatty acid (FFAs) and postprandial plasma glucose contribute to the pathogenesis of insulin resistance and type 2 diabetes (Shulman, 2000; Bensellam et al., 2012; Rachek, 2014). Additionally, the increased plasma levels of both FFAs and postprandial glucose are linked to the production of reactive oxygen species which play a key role in pancreatic β-cell destruction and diabetic complications (Bakker et al., 2000; Karunakaran and Park, 2013). The plasma level of FFAs is primarily controlled by adipocyte lipolysis (Zhai et al., 2010). Meanwhile, the level of postprandial plasma glucose is regulated chiefly by the activity of intestinal carbohydrate-digesting enzymes, including α-amylase (Nolte Kennedy, 2012).

Since adipocyte lipolysis, α-amylase enzyme activity and oxidative stress are associated with the development of diabetes and its complications, proper regulation of these biological functions may impede pathogenesis of the disease. The objectives of this study were to investigate the *in vitro* effects of *P. palatiferum* leaf ethanolic extract (PPE extract) on three diabetes-related biological activities, adipocyte lipolysis, α-amylase enzyme activity and antioxidant activity.

### MATERIALS AND METHODS

#### Preparation of *P. palatiferum* leaf ethanolic extract (PPE extract)

*P. palatiferum* (Nees) Radlk. was planted and harvested in Roi Et province, Thailand. The specimens were authenticated by the Plant Varieties Protection Division, Department of Agriculture, Ministry of Agriculture and Cooperatives. A voucher is deposited at the Herbarium of Pharmaceutical Chemistry and Natural Product Research Unit, the Faculty of Pharmacy, Mahasarakham University, Thailand (code: MSU.PH-ACA-P1). The leaves were dried by hot-air oven at a temperature of 50°C for 45 h. The dried leaves (100 g) were macerated with 80% ethanol (1,000 ml) for 7 days. The extract was then dried using a rotary evaporator (Heidolph Laborota 4000, Germany) followed by a freeze dryer (Christ Alpha 1-4, Germany). The leaf ethanolic extract of *P. palatiferum* (PPE extract) was then obtained with % yield of 12.7% w/w. The PPE extract was stored at -20°C until use.

#### Study on lipolysis in rat adipocytes

##### Preparation of adipocytes

Male Wistar rats (150 to 170 g), obtained from the National Laboratory Animal Center Mahidol University, Thailand were kept at a constant temperature (25 ± 1°C) with a 12 h dark-light cycle. The rats were fed *ad libitum* with free access to water. After 1 week acclimatization, the rats (n = 16) were randomly assigned into two groups (8 rats per group) and fed with two different diet types, normal pellet diet (NPD) and high fat diet (HFD). NPD used was CP mice feed (food no.082, Bangkok, Thailand), whilst HFD was prepared following the formulation of Srinivasan et al. (2005). The amount of fat in the NPD and HFD are approximately 12 and 56%, respectively. After 3 weeks, the rats were sacrificed and the epididymal fat pads were collected for preparation of adipocyte suspension. All procedures with the animals were approved by the animal research ethics committee, Mahasarakham University, Thailand (no.0127/2555). Adipocyte suspensions were prepared by collagenase digestion (collagenase type II, Sigma, 0.25 mg/ml), according to the method of Rodbell (1964), with slight modification. Hanks buffer containing 5 mM glucose and 0.5% bovine serum albumin (BSA, with less than 0.005% (w/v) free fatty acid) (Sigma) was used in the preparation of adipocyte suspension. The adipocytes were resuspended with the buffer into 1:1 (v/v) proportion and stored at 37°C before performing experiments.

##### Adipocyte lipolysis assay

Adipocytes were incubated for 60 min in microfuge tubes at 37°C with Hanks buffer, pH 7.4, containing 5 mM glucose, 0.5% BSA and various concentrations of the PPE extract at 10, 25, 50, 100, 250 and 500 µg/ml. Adipocyte lipolysis was examined both in the absence (basal lipolysis) and the presence of 0.1 µM M isoprenaline (isoprenaline-induced lipolysis). After 1 h incubation, the cell free incubation media were collected and the concentrations of glycerol were measured using a glycerol assay kit from Sigma.

##### In vitro study on α-amylase enzyme activity

The α-amylase enzyme activity assay was performed according to the method of Ali et al. (2006). Freshly prepared porcine pancreatic α-amylase enzyme (200 µl, 4 unit/ml) was pre-incubated at 25°C for 5 min with 40 µl of the PPE extract, DMSO (a negative control) or α-amylase inhibitor from wheat seed (*Triticum aestivum*) (a positive control). A 400 µl volume of potato starch solution (0.5% w/v) and 160 µl of distilled water were subsequently added and incubated at 25°C for further 3 min. A 200 µl volume of the mixture was taken into a new microfuge tube containing 100 µl of 3,5-dinitrosalicylic acid (DNS) color reagent solution (96 mM DNS and 5.31 M sodium...
RESULTS

Effects of the PPE extract on adipocyte lipolysis

NPD-fed rat derived adipocyte lipolysis

In basal lipolysis, the glycerol concentration released from adipocytes of the NPD-fed rats was 86.17 ± 2.10 µg ml⁻¹/ml packed cell volume (PCV)/h (n = 4). The PPE extract at any concentration tested (10, 25, 50, 100, 250 and 500 µg/ml) did not cause any significant change in the basal lipolysis as shown in Figure 1A. Isoprenaline at a concentration of 0.1 µM significantly increased adipocyte lipolysis with a glycerol level of 177.27 ± 28.21 µg ml⁻¹/ml PCV/h (p < 0.05; n = 4), calculated as 205.72 ± 32.74% of the basal lipolysis. The PPE extract at concentrations of 25, 50, 100, 250 and 500 µg/ml had a significant inhibitory action on the isoprenaline-induced lipolysis (p < 0.05; n = 4) (Figure 1B). The median concentration of the PPE extract which inhibited the isoprenaline-induced lipolysis (IC₅₀) was 67.19 µg/ml (pIC₅₀ = 4.17 ± 0.31).

HFD-fed rat derived adipocyte lipolysis

The glycerol level in the basal lipolytic condition of the HFD-fed rat derived adipocytes was 66.12 ± 9.15 µg ml⁻¹/ml PCV/h (n = 4). The PPE extract at every concentration tested significantly decreased the basal lipolysis (p < 0.05; n = 4) (Figure 1C). Isoprenaline at a concentration of 0.1 µM only slightly increased lipolysis in adipocytes derived from the HFD-fed rats, with the glycerol level of 71.47 ± 2.72 µg ml⁻¹/ml PCV/h (n = 5). This level was not statistically different from that of the basal lipolysis. The PPE extract at any concentration used in the experiment did not produce any significant change in the isoprenaline-induced lipolysis (Figure 1D).

Effects of the PPE extract on in vitro α-amylase enzyme activity

DMSO (negative control) did not inhibit α-amylase enzyme (% inhibition = 0.00 ± 1.98%). The α-amylase inhibitor from wheat seed (positive control) significantly inhibited α-amylase enzyme activity with the % inhibition of 70.38 ± 7.40%. The PPE extract at concentrations of 5, 10, 20 and 50 mg/ml produced a significant inhibition against α-amylase enzyme activity (p < 0.05; n = 3) as shown in Table 1. The maximal α-amylase inhibition of 59.86 ± 4.46% was found at the PPE extract concentration of 20 mg/ml. The PPE extract caused a concentration-dependent inhibitory action on α-amylase enzyme activity.
Table 1. *In vitro* effects of the PPE extract on $\alpha$-amylase enzyme activity.

<table>
<thead>
<tr>
<th>PPE extract concentration (mg/ml)</th>
<th>% inhibition (mean ± SD)</th>
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<tbody>
<tr>
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<tr>
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<td>5</td>
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<tr>
<td>10</td>
<td>51.72±9.50*</td>
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<tr>
<td>20</td>
<td>59.86±4.46*</td>
</tr>
<tr>
<td>50</td>
<td>59.04±2.34*</td>
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* $p$<0.05; One-way ANOVA, Bonferroni post-hoc test; $n = 3$

Figure 1: Effects of the PPE extract on adipocyte lipolysis. (A) Basal lipolysis in adipocytes derived from the NPD-fed rats. (B) Isoprenaline (0.1 $\mu$M)-induced lipolysis in adipocytes derived from the NPD-fed rats. (C) Basal lipolysis in adipocytes derived from the HFD-fed rats. (D) Isoprenaline (0.1 $\mu$M)-induced lipolysis in adipocytes derived from the HFD-fed rats. The data are expressed as the mean ± SEM ($n=4$). * $p$<0.05 when compared with the control (One-way ANOVA followed by Bonferroni post hoc test).
enzyme activity with an IC_{50} of 4.99 mg/ml (pIC_{50} of 2.30 ± 0.12) (Figure 2).

**In vitro antioxidant activity of the PPE extract**

**DPPH assay**

The PPE extract produced scavenging activity against DPPH free radical with an IC_{50} of 58.13 µg/ml (pIC_{50} of 4.24 ± 0.08) (n = 5). Ascorbic acid, positive control, exerted antioxidant activity with an IC_{50} of 4.16 µg/ml (pIC_{50} of 5.38 ± 0.05) (n = 5).

**Ferric reducing power assay**

The PPE extract exerted ferric reducing power with a vitamin C equivalent antioxidant capacity (VCEAC) and a trolox equivalent antioxidant capacity (TEAC) of 2.33 and 2.04 g/100 g extract, respectively.

**DISCUSSION**

**Effects of the PPE extract on adipocyte lipolysis**

Both basal lipolysis and isoprenaline (0.1 µM)-stimulated lipolysis were investigated in adipocytes derived from the NPD- or HFD-fed rats. HFD feeding in rodents have been known to induce obesity and insulin resistance, which is a crucial pathogenesis of type 2 DM (Buettner et al., 2006). The basal lipolysis in adipocytes derived from the NPD-fed rats was not affected by the PPE extract. On the contrary, the PPE extract caused a significant inhibition of basal lipolysis in the HFD-fed rat adipocytes in a concentration-independent manner (Figure 1C). The elevation of basal lipolytic level was reported in adipocytes of the rats fed with HFD for 7 to 8 weeks (Portillo et al., 1999; Gaidhu et al., 2010). However, the difference in the basal lipolytic levels between the NPD- and the HFD-fed rat adipocytes was not observed in this study. This may be due to the shorter duration of HFD-feeding (3 weeks). The changes in several basal lipolytic-related proteins have been described in adipocytes of the HFD-fed rats (Portillo et al., 1999; Gaidhu et al., 2010). The expressions of adipose triglyceride lipase (ATGL) and comparative gene identification-58 (CGI-58) were increased in the HFD-fed rat adipocytes, whilst the level of perilipin expression was decreased (Gaidhu et al., 2010). A target site of the PPE extract may be over-expressed in HFD-fed rat adipocytes. Thus, the anti-lipolytic action of the PPE extract can only be found obviously in these adipocytes, but not in adipocytes isolated from the NPD-fed group. Nonetheless, further experiments are needed to investigate the exact anti-lipolytic site of action of the PPE extract. The PPE extract caused a concentration-dependent inhibition of isoprenaline-induced lipolysis in adipocytes isolated from the NPD-fed rats (IC_{50} = 67.19 µg/ml), but it did not cause any significant change in the HFD-fed rat adipocytes (Figure 1B and D). In this study, the lipolytic response to isoprenaline in adipocytes of the HFD group was dramatically lower than that of adipocytes isolated from the NPD-fed rats. This is in agreement with the previous studies in which adipocytes of the HFD-fed rats had a lower degree of β-agonist-induced lipolysis (Matsuo et al., 1995; Portillo et al., 1999; Gaidhu et al., 2010). The reduction in lipolytic response to β-agonists in adipocytes of the HFD-fed rats was found to be associated with an alteration in β-adrenoceptor binding affinity (Matsuo et al., 1995). The expressions of some essential proteins involved in the β-agonist-stimulated lipolytic pathway, such as perilipin and adipose phospholipase A2, were modified in the HFD-fed rat adipocytes as well (Gaidhu et al., 2010). Thus, the difference in isoprenaline-induced lipolytic response to the PPE extract between the NPD- and HFD-fed rat adipocytes is likely to be explained by the alteration in isoprenaline-induced lipolytic pathway.

The anti-lipolytic action of the PPE extract is reported for the first time in this study. The anti-lipolytic action of the PPE extract demonstrated in this study is likely to be associated with its plasma glucose-lowering action reported previously by Padde et al. (2010). A reduction in adipocyte lipolysis leads to a lower amount of released glycerol, which is a major precursor in hepatic gluconeogenesis. Additionally, FFAs, another product of adipocyte lipolysis, also play a key role in the regulation of plasma glucose level and glucose uptake by interfering
with glucose transporter 4 (GLUT4) functions (Shulman, 2000). FFAs are also precursors of hepatic very low density lipoprotein (VLDL) synthesis (Malloy and Kane, 2012). The plasma levels of VLDL and high-density lipoprotein (HDL-C) are linked, since triglyceride in VLDL can be transferred into HDL-C. Subsequently, HDL-C becomes triglyceride-rich HDL-C, which can be easily degraded (Taskinen, 2003). Thus, the anti-lipolytic effects of the PPE extract is likely to be implicated in its plasma triglyceride-lowering and HDL-C-increasing actions reported previously as well (Padee et al., 2010). Moreover, FFAs released during adipocyte lipolysis are also involved in pancreatic β-cell apoptosis and insulin resistance (Shulman, 2000; Cernea and Dobreanu, 2013). The negative regulation of basal adipocyte lipolysis by the PPE extract thus potentially provides beneficial impacts especially in persons having a high amount of fat deposition or taking a high-fat diet, who are at high risk of type 2 DM. Nonetheless, further experiments are required to prove whether the anti-lipolytic action of the PPE extract can still be found in vivo where several factors implicated in the regulation of adipocyte lipolysis exist.

Effects of the PPE extract on in vitro α-amylase enzyme activity

The PPE extract produced a significant α-amylase enzyme inhibition in a concentration-dependent manner with an IC$_{50}$ of 4.99 mg/ml. A maximal α-amylase enzyme inhibition of approximately 60% was found at a concentration of 20 mg/ml. A similar level of inhibition was observed at the PPE extract concentration of 50 mg/ml, the highest concentration tested. The achievement of the plateau inhibition was thus suggested at these concentrations. To our knowledge, this is the first report of α-amylase inhibitory activity from a PPE extract. It was reported that the methanolic extract of P. palatiferum dry leaves (1 mg/ml) exerted an in vitro inhibitory action against α-glucosidase enzyme, with 7% inhibition (Rungprom et al., 2010). This is somewhat similar to our results in which 1 mg/ml of the PPE extract produced approximately 10% inhibition on α-amylase enzyme activity (Table 1). However, the IC$_{50}$ of the PPE extract against α-glucosidase enzyme activity has not been reported yet. Thus, the potency of the extract against these two enzymes cannot be compared. Both α-amylase and α-glucosidase enzymes are essential for intestinal carbohydrate digestion. The inhibition of both enzymes would hence produce an advantage over the inhibition of each enzyme alone. Further research thus should be performed to investigate the effects of the PPE extract on α-glucosidase enzyme activity.

It is still not known which phytochemical components of the PPE extract the active compounds are acting against the α-amylase enzyme. Several phytochemicals, including β-sitosterol, stigmasterol and kaempferol, were isolated from the PPE extract (Giang et al., 2003). The in vitro inhibitory activity of these phytochemicals against α-amylase was demonstrated previously (Tadera et al., 2006; Nkobole et al., 2011; Takahama and Hirota, 2013). Additionally, it was reported that β-sitosterol, stigmasterol and kaempferol significantly decreased the plasma glucose levels in streptozotocin- or alloxan-induced diabetic rats (de Sousa et al., 2004; Kumar et al., 2013). These phytochemicals thus possibly play a role in the plasma glucose-lowering action of the plant extract via the inhibition of carbohydrate-digesting enzymes. Nonetheless, additional experiments will be required to identify the specific phytochemicals in the PPE extract inhibiting α-amylase.

The α-amylase inhibitory action of the PPE extract potentially acts as one of its mechanisms of action against DM. However, the postprandial plasma glucose level, an indicator of carbohydrate digestion and absorption, after the treatment with the PPE extract has not been investigated yet. In vivo carbohydrate tolerance tests are therefore needed to determine whether the extract controls plasma glucose level mainly via its action against carbohydrate-digesting enzymes.

Postprandial hyperglycemia is implicated in the destruction of pancreatic β-cells via induction of glucotoxicity (Dubois et al., 2007; Bensellam et al., 2012). The high level of plasma glucose after meals is also linked to oxidative stress, inflammation, and endothelial dysfunction (Ceriello et al., 2005). These derangements are a crucial pathophysiology of atherosclerosis, which leads to the development of coronary artery disease and myocardial infarction, the major cause of death in diabetic patients. Several epidemiological studies indicated postprandial hyperglycemia as the direct and independent risk factor of cardiovascular diseases in diabetic patients (Cariello et al., 2005). Control of postprandial plasma glucose using inhibitors of carbohydrate-digesting enzymes is thus likely to reduce the risk of cardiovascular complications.

In vitro antioxidant activity of the PPE extract

The PPE extract scavenged DPPH free radicals in a concentration-dependent manner with an IC$_{50}$ of 58.13 µg/ml. The DPPH scavenging potency of the extract was lower than that of ascorbic acid (IC$_{50}$ of 4.16 µg/ml). The aqueous extract of P. palatiferum also showed in vitro antioxidant activity, with an IC$_{50}$ of DPPH radical scavenging activity of 221.14 µg/ml (Chayarop et al., 2012).
Thus, it suggests that the active antioxidative agents are more efficiently extracted with a less polar solvent (ethanol). Various ethanolic plant extracts also possessed higher DPPH scavenging potency than that of their aqueous counterparts (Nguyen and Eun, 2011). The phenolic compounds, especially flavonoids, a group of well known antioxidants, have been identified in both ethanolic and aqueous extracts of *P. palatiferum* (Nguyen and Eun, 2011; Chayarop et al., 2012). It was reported that the level of total phenolic contents in the ethanolic extract of *P. palatiferum* was higher than in the aqueous extract (Nguyen and Eun, 2011). This is in agreement with the higher DPPH scavenging potency of the ethanolic extract. Several groups of flavonoids, such as flavon-3-ols, flavanones, anthocyanidins, flavonols, flavones and isoflavones, exhibit anti-diabetic actions substantially via their antioxidant activity (Babu et al., 2013). It was reported that flavonoids and flavonoids metabolites were protected against oxidative stress-induced pancreatic β-cell dysfunction and cell death and also restored insulin secretion (Fernandez-Millan et al., 2014; Martin et al., 2014). Additionally, the anti-inflammatory action of flavonoids also contributed to its protective effect against the development of diabetes in an animal model (Fu et al., 2011).

Following the ferric reducing power assay, the PPE extract produced an antioxidant activity with VCEAC and TEAC values of 2.33 and 2.04 g/100 g extract, respectively. The ethanolic extract of *P. palatiferum* was reported to have low reducing power which was not associated with its DPPH scavenging activity (Nguyen and Eun, 2011). The aqueous extract of *P. palatiferum* also exhibited less potent reducing power when compared to trolox (Chayarop et al., 2012). Although the PPE extract exhibited a less potent antioxidative activity when compared to the reference antioxidants in both DPPH radical scavenging assay and ferric reducing power assay, its antioxidant activity was confirmed by two antioxidant assays, which specify different mechanisms of antioxidative action. Treatment with antioxidants has been found to reduce oxidative stress and protect pancreatic β-cells (Kaneto et al., 1999). The antioxidative activity was described in various extracts of medicinal plants traditionally used for the treatment of diabetes, such as *Tinospora crispa*, *Momordica charantia*, and *Pandanus amaryllifolius* (Cavin et al., 1998; Ghasemzadeh and Jaafar, 2013; Sulaiman et al., 2013). Antioxidative activity of the PPE extract is thus likely to partially explain its mechanism against diabetes.

**Conclusion**

The PPE extract exerted anti-lipolytic action within a concentration range similar to that which produced DPPH radical scavenging activity. However, its α-amylase inhibition was observed only at higher concentrations. Further *in vivo* pharmacodynamics and pharmacokinetics studies are still required to determine the exact mechanism of anti-diabetic action of the PPE extract. Over function of adipocyte lipolysis, postprandial hyperglycemia and oxidative stress are closely associated with the pathogenesis of diabetes and its complications (Bakker et al., 2000; Sakuraba et al., 2002; Karunakaran and Park, 2013). Therefore, anti-lipolytic, α-amylase inhibitory and anti-oxidative activities of the PPE extract demonstrated here potentially contribute to its anti-diabetic effects.

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**Conflict of interest**

Authors declare no conflict of interest.

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