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Original Article

Alpha-glucosidase inhibitory effect and inorganic constituents of *Phyllanthus amarus* Schum. & Thonn. ash

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Abstract

This study investigated the α-glucosidase inhibitory effect and determined the concentration of some inorganic constituents in *P. amarus* ash. Oral glucose and sucrose tolerance test were performed on normal mice. *In vitro* α-glucosidase inhibitory activity was evaluated by using yeast a-glucosidase. The element concentrations were measured by inductively coupled plasma (ICP) spectroscopy. Single oral administration of *P. amarus* ash did not show antihyperglycemic effect after glucose administration, but decreased blood glucose level after sucrose administration. The ash showed α-glucosidase inhibitory activity *in vitro* with IC_{50} of 982 mg/mL. The concentrations of K, Ca, Mg, Mn, Fe, Zn, Cu, Pb, Cr, Ni and Co in *P. amarus* ash were 35049.80±340.64, 3337.24±52.10, 1368.52±13.29, 90.81±1.34, 87.68±1.15, 18.28±0.22, 4.69±0.07, 1.07±0.15, 0.29±0.03, 0.20±0.04 and 0.10±0.02 mg/g, respectively. These results indicate that the antihyperglycemic effect of *P. amarus* ash might be partly due to the α-glucosidase inhibitory activity of the inorganic constituents.

Keywords: *Phyllanthus amarus*, inorganic constituent, α-glucosidase inhibitory activity, glucose tolerance, sucrose tolerance

1. Introduction

α-glucosidases are enzymes located in the brush-border surface membrane of intestinal cells involving in breaking down carbohydrates such as starch, glycogen and disaccharides to glucose by hydrolyzing terminal non-reducing 1-4 linked α-glucose residues to release a single α-glucose molecule (Chiba, 1997). Inhibition of α-glucosidases is important to control postprandial hyperglycemia in type 2 diabetes mellitus (American Diabetes Association, 2001). There is increasing evidence suggesting that postprandial hyperglycemia strongly correlates with diabetic complications especially those to the cardiovascular system (Tanaka, 2012). Acarbose, an α-glucosidase inhibitor, is the first line drug for reducing postprandial blood glucose in diabetic patients and was reported to reduce the relative risk of cardiovascular event in patients with impaired glucose tolerance and type 2 diabetes (Hanefeld, 2007; Breuer, 2003).

Despite the numerous modern medications to reduce blood glucose, the increasing use in diabetic patients of complementary and alternative medicine, especially herbs, dietary and mineral supplements, have been reported (Yeh *et al*., 2003). A number of herbs traditionally used for diabetes have been recorded for antihyperglycemic activity such as *Allium sativum, Aloe vera*, *Coccinia indica*, *Eugenia jambolana, Gymnema sylvestre*, *Ipomoea batatas, Momordica charantia*, *Ocimum sanctum, Silybum marianum, Trigonella foenum-graecum, Phyllanthus amarus, Piper sarmentosum*,

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Pterocarpus marsupium, *Tinospora cordifolia*, etc. (Suksomboon *et al*., 2011; Modak *et al*., 2007; Yeh *et al*., 2003). *Phyllanthus amarus* Schum. & Thonn. which belongs to the family Euphorbiaceae is a small herb well known for its medicinal properties. Various pharmacological activities of *P. amarus* including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective, and diuretic properties have been reported (Patel *et al*., 2011, Pramyothin *et al*., 2007, Wongnawa *et al*., 2006). The main active constituents of *P. amarus* are lignans (phyllanthin, hypophyllanthin, niranthin, etc.), flavonoids (quercetin, astragalin, rutin, kaempferol, etc.), ellagitannins (gallic acid, ellagic acid, etc.), alkaloids (securinine, dihydrosecurinine, etc.), triterpines (lupeol), sterol, and volatile oil (Patel *et al*., 2011). Although medicinal plants contain both organic and inorganic constituents, most of the studies done so far on hypoglycemic herbs were carried out with organic active principles (Modak *et al*., 2007). However, some inorganic elements such as potassium (K), calcium (Ca), zinc (Zn), magnesium (Mg), manganese (Mn), copper (Cu) and trace elements such as chromium (Cr), vanadium (V), cobalt (Co), molybdenum (Mo) and tungsten (W) have potential roles for glucose homeostasis (Pandey *et al*., 2012, Wiernsperger and Rapin, 2010). Most research works on hypoglycemic effect of *P. amarus* have also been carried out on the organic compounds (Patel *et al*., 2011), while little attention has been paid on the role of its inorganic constituents.

In the present work, the *in vivo* α-glucosidase inhibitory effect of the inorganic components in the ash of *P. amarus* was evaluated in normal fasted mice by oral glucose and sucrose tolerance tests. The *in vitro* α-glucosidase inhibitory effect was also tested by using yeast α-glucosidase. Moreover, the inorganic elemental analysis of *P. amarus* was carried out using inductively coupled plasma technique.

2. Materials and Methods

2.1 Chemicals

The drugs used in this study included tolbutamide (Ajax Finechem Pty Ltd), Glucobay® (acarbose 50 mg/tab), yeast α-glucosidase (Sigma), *p*-nitrophenyl α-D-glucopyranoside (Sigma). All other chemicals and solvents were of analytical reagent grade. Water was purified by Milli Q Water Purification System, Millipore, USA.

2.2 Preparation of *P. amarus* ash

Dried powder of *P. amarus* was purchased from Lampang Herb Conservation, Lampang, Thailand, and was identied by Assoc. Prof. Tanomjit Supavita, School of Pharmacy, Walailak University, Nakhon Si Thammarat. Twenty grams of *P. amarus* powder in a crucible was placed in an electric muffle furnace and maintained at 430-450°C overnight to destroy any organic compounds present in the powder (Sahrawat *et al*., 2002). After cooling, the ash was removed from the crucible and kept in a vacuum desiccator. The yield of the ash was 5.7 % w/w.

2.3 Animals

Male ICR mice, weighing 20-30 g, were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand. The animals were housed in the controlled room at temperature 25±2°C with a 12 hours light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory feed and water. The experimental protocol was approved by the Institutional Committee for Ethical Use of Animals, Prince of Songkla University, Thailand (Ref 19/2012).

2.4 Experimental protocol

The animals were divided into 4 groups, with 6 mice each and were treated as follows:

Group I (control group) was administered with 10 % gum acacia, 10 mL/kg

Group II was administered with *P. amarus* ash (90 mg/kg)

Group III was administered with tolbutamide (300 mg/kg)

Group IV was administered with acarbose (40 mg/kg)

The selected dose of *P. amaus* ash was 90 mg/kg to avoid toxic effect of some metals (Kar *et al*., 1999). The powder of tolbutamide, acarbose and the ash were suspended in 10 % gum acacia prior to the administration by the volume of 10 mL/kg.

2.5 Glucose/Sucrose tolerance test

After being fasted overnight, blood samples from all mice were obtained from the tail vein. Then the mice were orally given each drug through feeding tube. At 30 min after the drug administration, the substrate solution (glucose or sucrose, 2 g/kg body weight) was administered to the mice. Five more blood samples were collected at 15-, 30-, 60-, 120-, and 180-min intervals. The blood glucose levels were measured using a glucometer (GlucoDr , All Medicus Co., Ltd).

2.6 *In vitro* α-glucosidase inhibitory assay

α-glucosidase inhibitory activity was determined according to Kumar *et al*. (2010) and Gowri *et al*. (2007) with some modification. In brief, 50 μL of test sample (50-1250 μg/mL in phosphate buffer, pH 6.8) was reconstituted in 100 μL of 100 mM phosphate buffer, pH 6.8 and incubated with 50 μL yeast α-glucosidase (0.25 U/mL in phosphate buffer) for 15 min at 37°C before 50 μL of substrate (5 mM *p*-nitrophenyl α-D-glucopyranoside, in phosphate buffer) was added

and then incubated for 15 min at 37°C. The reaction was stopped by adding 1 mL of Na_2CO_3 (0.1 M). Release of *p*-nitrophenol was measured at 405 nm by spectrophotometer (Spectronic Genesys 20). Individual blank for test sample was prepared by replacing substrate with 50 µL of the buffer. The control sample was prepared in similar manner but using 50 µL of buffer in place of test sample. All the tests were run in triplicate. The percentage of enzyme inhibition was calculated as (1-B/A)x100, where A represents the absorbance of control sample, B represents the absorbance of test sample. The IC_{50} values were determined by regression analysis of the data for at least five concentrations of sample.

2.7 Assay of inorganic elements in *P. amarus*

Two hundred milligrams of *P. amarus* dry powder or 100 mg of the ash was digested with 2 mL of conc. nitric acid and incubated at 90°C for 1 h, then adjusted with deionized water to the volume of 10 mL. The digested sample was used for the assay of inorganic elements using the ICP spectrometer (PerkinElmer®, Optima 4300DV, USA).

2.8 Statistical analysis

All the results were expressed as mean ± standard deviation. Statistical analysis was performed using one way analysis of variance (ANOVA), followed by least significant difference (LSD) test. Differences were considered to be statistically different when p value was < 0.05.

3. Results

3.1 Oral glucose and sucrose tolerance tests

In oral glucose tolerance test, there was no significant difference in the blood glucose level and the area under curve (AUC) between *P. amarus* ash-treated, acarbose-treated and the control group, whereas those of the tolbutamide-treated group were lower when compared with the control group (Figures 1, 2). In contrast, in the oral sucrose tolerance test, the postprandial blood glucose level at each time measured from T30-T180 minute, as well as the AUC, in the acarbose-, tolbutamide- and *P. amarus* ash-treated group were significantly lower than the control group (Figures 3, 4).

3.2 *In vitro* a-glucosidase inhibitory activity

The ash of *P. amarus* showed inhibitory activity against yeast α-glucosidase with the maximum inhibition of 50.04±3.55 % at the concentration of 1 mg/mL, compared to 59.23±4.12 % of acarbose at the concentration of 1.25 µg/mL. The IC_{50} of *P. amarus* ash was 982.13±162.69 mg/mL, whereas that of acarbose was 816.87±99.65 µg/mL (Table 1, Figure 5).

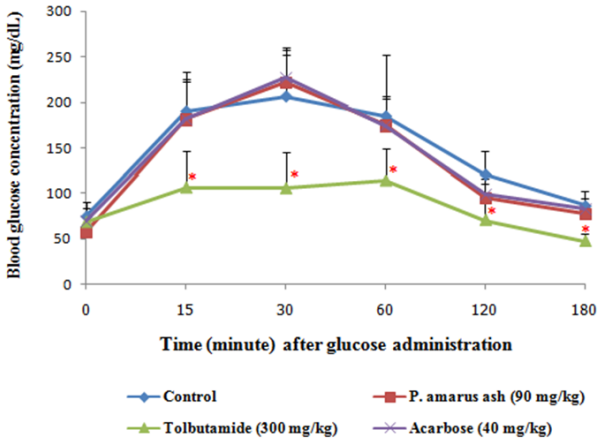

Figure 1. Effects of *P. amarus* ash, tolbutamide and acarbose on oral glucose tolerance in normal mice (X±SD, N=6, * significantly different from control, $p<0.05$)

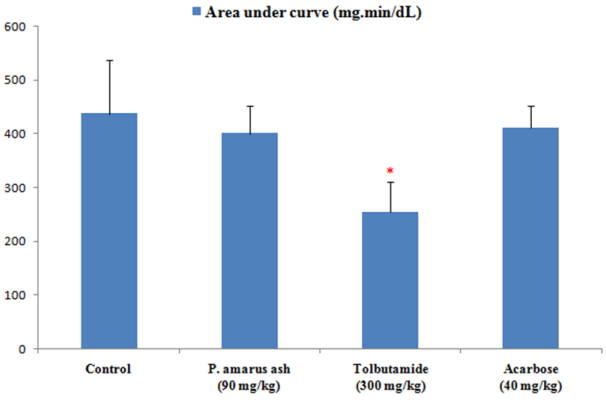

Figure 2. The area under the curves in oral glucose tolerance test after administration of *P. amarus* ash, tolbutamide and acarbose in normal mice (X±SD, N=6, * significantly different from control, $p<0.05$)

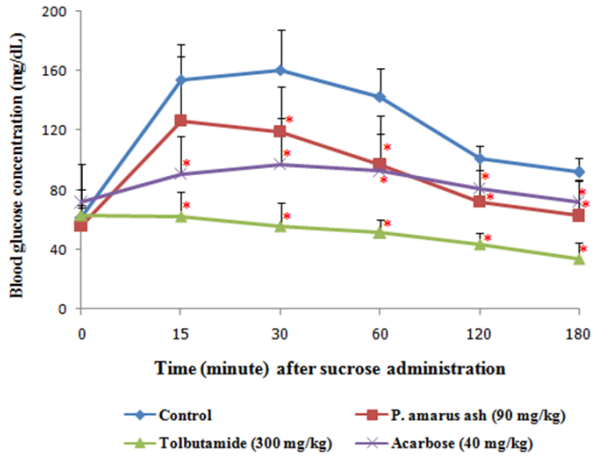

Figure 3. Effects of *P. amarus* ash, tolbutamide and acarbose on oral sucrose tolerance in normal mice (X±SD, N=6, * significantly different from control, $p<0.05$).

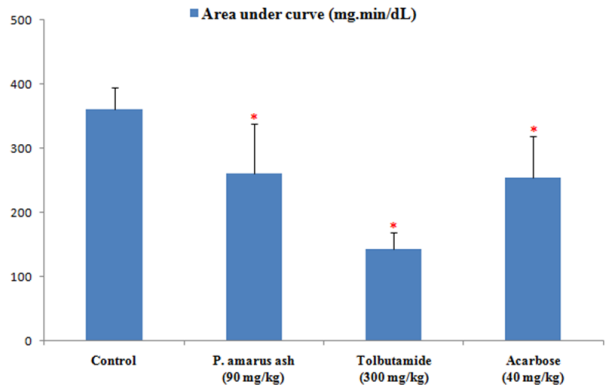

Figure 4. The area under the curves in oral sucrose tolerance test after administration of *P. amarus* ash, tolbutamide and acarbose in normal mice (X±SD, N=6, * significantly different from control, $p<0.05$).

3.3 Concentration of some elements in *P. amarus*

Table 2 shows some inorganic constituents found in the dry powder and ash of *P. amarus*. The concentrations of K, Ca, Mg, Mn, Fe, Zn, Cu, Pb, Ni, Cr, Se, V, As, Co and Cd in *P. amarus* dry powder were 2334.17±28.82, 1596.50±37.40, 519.00±12.70, 36.00±0.42, 27.30±1.32, 20.85±0.03, 1.80±0.05, 0.70±0.08, 0.25±0.01, 0.20±0.01, 0.20±0.10, 0.15±0.05, 0.15±0.04, 0.05±0.01 and 0.05±0.01 µg/g, respectively. Those of K, Ca, Mg, Mn, Fe, Zn, Cu, Pb, Ni, Cr and Co in the ash were 35049.80±340.64, 3337.24±52.10, 1368.52±13.29, 90.81±1.34, 87.68±1.15, 18.28±0.22, 4.69±0.07, 1.07±0.15, 0.20±0.04, 0.29±0.03, and 0.10±0.02 µg/g, respectively, whereas As, Cd, Se, and V were not detectable at the detection limit of 2, 0.1, 5, and 0.05 µg/g ash, respectively.

4. Discussion and Conclusions

To investigate the *in vivo* α-glucosidase inhibitory activity, we compared the effects of *P. amarus* ash on oral glucose and sucrose tolerance focusing on postprandial blood glucose rather than fasting blood glucose. The postprandial blood glucose profile is determined by carbohydrate absorption, insulin and glucagon secretion, and their coordinated effects on glucose metabolism in the liver and peripheral tissues (American Diabetes Association, 2001). Sucrose is a disaccharide of glucose and fructose with an α-1,2 glycosidic linkage. It is hydrolyzed to glucose and fructose by sucrase, a kind of a-glucosidase, and is absorbed by the small intestine (Drozdowski and Thomson, 2006). Drugs with α-glucosidase inhibitory activity such as acarbose decreased oral absorption of sucrose resulting in inhibition of the increase in blood glucose but did not inhibit absorption of glucose which is a monosaccharide (Hayakawa *et al*., 1984). The present study showed that *P. amarus* ash and acarbose did not suppress hyperglycemia on oral glucose tolerance test, whereas tolbutamide, a sulfonylurea antidiabetic drug which stimulate insulin secretion, decreased blood glucose level. This suggests that the inorganic constituents of *P. amarus* ash at the dose of 90 mg/kg may not be involved in the reduction of glucose absorption. In

Table 1. Inhibitory effect of *P. amarus* ash and acarbose on yeast α-glucosidase (X± SD, N=3)

	Maximum % inhibition (concentration, mg/mL)	IC_{50} (µg/mL)
P. amarus ash	50.04±3.55 (1.0)	982.13±162.69
Acarbose	59.23±4.12 (1.25)	816.87±99.65

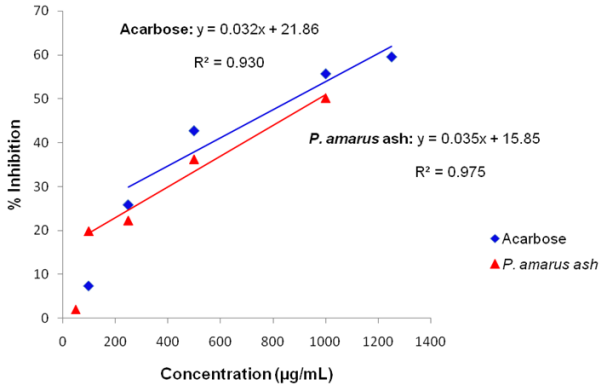

Figure 5. Inhibitory effect of *P. amarus* ash and acarbose on yeast α-glucosidase.

Table 2. Concentrations of some inorganic constituents in *P. amarus* (X±SD, N=3)

Elements	Concentration (µg/g)	
	Dry powder	Ash
K	2334.17±28.82	35049.80±340.64
Ca	1596.50±37.40	3337.24±52.10
Mg	519.00±12.70	1368.52±13.29
Mn	36.00±0.42	90.81±1.34
Fe	27.30±1.32	87.68±1.15
Zn	20.85±0.03	18.28±0.22
Cu	1.80±0.05	4.69±0.07
Pb	0.70±0.08	1.07±0.15
Cr	0.20±0.01	0.29±0.03
Ni	0.25±0.01	0.20±0.04
Co	0.05±0.01	0.10±0.02
As	0.15±0.04	ND
Cd	0.05±0.01	ND
Se	0.20±0.10	ND
V	0.15±0.05	ND

ND = not detectable (detection limit of As, Cd, Se, V = 2, 0.1, 5, and 0.05 µg/g ash, respectively)

contrast, in the oral sucrose tolerance test, *P. amarus* ash suppressed hyperglycemia after administration of sucrose, a disaccharide, as did acarbose, an α-glucosidase inhibitor, suggesting that the inorganic substances in *P. amarus* ash may play a role in decreasing glucose absorption which may be due to the inhibition of α-glucosidase activity in the intestine. This result was consistent with the previously reported antidiabetic effect of *P. amarus* aqueous extract (Patel *et al*., 2011) which might possibly be exerted in part through some dissolved inorganic constituents via α-glucosidase inhibitory activity. To confirm this activity, *in vitro* α-glucosidase inhibitory activity was carried out by using yeast a-glucosidase. It was found that *P. amarus* ash showed α-glucosidase inhibitory activity with nearly the same potency as acarbose when considered by the maximum percent inhibition and IC_{50} (Table 1, Figure 5). However, the maximum percent inhibition of *P. amarus* ash was limited by its solubility. The ethanol and hexane extract of *P. amarus* have also been reported to possess α-amylase inhibitory activity *in vitro* (Tamil *et al*., 2010, Ali *et al*., 2006).

Some recent reports have shown that inorganic compounds such as $CuSO_4$, $ZnSO_4$, $VOSO_4$, $NiSO_4$ and $FeSO_4$ possess α-glucosidase inhibitory activity *in vitro* (Zeng *et al*., 2012, Yoshikawa *et al*., 2010, Yoshikawa *et al*., 2009), and the synergistic inhibition of Cu, Zn, V and genistein (flavonoids) on α-glucosidase was also demonstrated (Wang *et al*., 2004). Therefore, in the present work we determined some inorganic constituents in *P. amarus* which may be involved in glucose homeostasis using the ICP technique. Table 2 shows that the major inorganic constituents in *P. amarus* dry powder are K, Ca and Mg (2334-519 μg/g), whereas Mn, Fe, Zn and Cu were found in moderate amount (36-2 μg/g), Ni, Cr, Se, V and Co were at trace level (0.25-0.05 μg/g). When compared to the amount reported elsewhere, K, Ca, Mg, Mn, Fe and Cu found in the present study were lower (<10 folds) than those reported previously (*Adedapo et al*., 2004). This variation might be due to the different amount of these metals in different area where the plant grows. It is noted that some elements such as Se and V, while existing in dry plant, were not detectable in the ash. It is possible that they might be lost due to volatilization during ashing (Welna *et al*., 2011).

Some elements found in *P. amarus* have been reported to have an effect on glucose homeostasis. For example, K, Ca, Mg, Cr, Mn, Cu, V and Zn are responsible for the secretion of insulin from beta cells of the islets of Langerhans, are involved in insulin receptor binding and signaling pathway, and are cofactors of many enzymes in glycolysis (Pandey *et al*., 2012, Wiernsperger and Rapin, 2010). There is accumulating evidence that the metabolism of several trace elements is altered in diabetes mellitus. Blood level of Zn, Mn, Cr and Mg were found to be lower in diabetic patients than in aged-match healthy controls (Kazi *et al*., 2008, Campbell and Nadler, 2004; Salgueiro *et al*., 2001). Supplementation of such elements is beneficial for improving insulin resistance, glucose tolerance and oxidative stress in some experiments (Wiernsperger and Rapin, 2010). The results from this study and previous reports suggest that the beneficial effect of *P. amarus* in controlling blood glucose level involves both organic and inorganic compounds.

Concerning the safety from toxic metals in herbs, we also determined the concentrations of Pb, As and Cd in *P. amarus*. Pb was found at 0.70 μg/g, whereas As and Cd were 0.15 and 0.05 μg/g, respectively. However, the amount of these toxic metals were far less than the allowance limit of heavy metals in herb products (Pb=10 μg/g , As=20 μg/g, Cd=0.3 mg/g, WHO, 2004).

In conclusion, the present study demonstrates that *P. amarus* contains some inorganic constituents which have beneficial effect on glucose homeostasis in part via α-glucosidase inhibitory activity, whereas some toxic metals such as Pb, As and Cd were found in negligible amount.

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