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Alpha-Glucosidase Inhibitory Effect of Methanolic Extracts from Indonesian Plants

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ABSTRACT

Alpha-glucosidase inhibitors (AGIs) are oral anti-diabetic drugs used for type-2 diabetes treatment by retarding the carbohydrate digestion. There are numerous natural products with AGI action, however, only limited number of Indonesian plants have been studied for their AGIs potency. In this research, 57 samples of plants naturally grown in Indonesia were tested in vitro for their AGIs activities. Some of them are traditionally utilized for diabetes treatment. The study showed that the methanolic extracts of gambir fruit (Uncaria gambir), banana leaf (Musa paradisiaca), lemongrass stem (Cymbopogon citratus), bitter gourd fruit (Momordica charantia), and onion bulb (Allium cepa L.) exhibited the highest AGIs activities at 0.02 g/ml concentration. The gradient extraction using n-hexane, ethyl acetate, and butanol resulted in ethyl acetate and butanol fractions showing high AGIs activity, indicating that the AGIs activities in these five samples were influenced by the existence of semi-polar to polar compounds. Antioxidant activity, total phenolic and flavonoid contents were also evaluated. Gambir fruit exhibited the highest antioxidant activity followed by onion bulb, banana leaf, bitter gourd fruit, and lemongrass stem with IC_{50} of 101.75 ppm, 1,963.57 ppm, 2,377.64 ppm, 5,859.14 ppm and 5,910.14 ppm, respectively. Total phenolic and flavonoid analysis showed that the activity of gambir fruit was supported by the high content of phenolic (77.56 mg GAE/g), while the onion bulb exhibited the highest flavonoid content (8.94 mg QE/g). Positive correlation of antioxidant activity and total phenolic content with AGIs was found, however, there was no correlation between AGIs and total flavonoid content.

Keywords : alpha-glucosidase inhibitors, Indonesian plants, diabetes, antioxidant

Introduction

Diabetes mellitus (DM) is a metabolic, chronic disease that has become a significant public health problem and is one of four priority non-communicable diseases targeted for action by world leaders. Over the past few decades both the number of cases and the prevalence of diabetes have been steadily increasing and is growing most rapidly in low- and middle-income countries (World Health Organization, 2016). The estimated number of adult suffering from diabetes worldwide in 2015 is 415 million and expected to increase to 642 million in 2040, if the rise is not halted (International Diabetes Federation, 2015). Indonesia is ranked one of the world's top five worst affected nations for people living with diabetes, together with China, India, Brazil and the United States (World Health Organization, 2016). One of well-known oral treatments for type 2 diabetes is the use of □-glucosidase inhibitors (AGIs) drugs. □-Glucosidase (EC 3.2.1.20), an enzyme located in the brush-border surface membrane of intestinal cells, is responsible for the hydrolysis of polysaccharide or disaccharides into monosaccharide in the small intestine. Studies showed that glucose absorption was retarded by inhibiting the catalytic activity of a-glucosidase and thus, lowered the effect on postprandial blood glucose and insulin levels (Robinson *et al*., 1991; Braun *et al*., 1995; Dwek *et al*., 2002). Consumption of AGIs that reversibly inhibit □-glucosidases, such as maltase and sucrase in the intestine, delayed carbohydrate digestion and hence, sugar absorption from the gut (Campbell *et al*., 1996; Kumar *et al*., 2011). The effects of monotherapy with AGIs for patients with type 2 diabetes were reviewed (Laar *et al*., 2005). No evidence for an effect on mortality or morbidity was found. Furthermore, AGIs exhibited significant beneficial effects on glycemic control and postload insulin levels, but not on plasma lipids. These indicate the possible use of AGIs as a first-line agent or in combination with other antihyperglycemic drugs.

In the prevention and treatment of diabetes and obesity, plant-based medicines and functional foods affecting beneficial physiological effects have gained high interest in the last decades. There were many studies conducted with the aim to search for effective and safe AGIs from natural sources (Matsui *et al*., 2001; Tundis *et al*., 2010; Gunawan-Puteri *et al*., 2012; Yonemoto *et al*., 2014). Many results showed significant antihyperglycaemic effect with slight or no side effects. Therefore, natural AGIs from plant sources offers a potential strategy for the control of hyperglycaemia. Indonesia is the second largest biodiversity in the world after Brazil, with around 40,000 endemic plant species including 6,000 medicinal plants. Unfortunately, numerous Indonesian medicinal plants potency still remain unknown until now. According to Aditama, until October 2014 only 41 standardized herbal medicines and 6 phytopharmaca were listed in the National Agency of Drug and Food Control (Aditama, 2015). As continuation of the screening of Indonesian plants for their AGIs potency, in this research 57 natural plant samples were investigated. The determination of prospective plants were assessed *in vitro* for their inhibitory effect using rat intestinal glucosidase. Furthermore, the antioxidant activity, total phenolic and total flavonoid content were also examined, and their correlation to AGIs activity assessed.

Materials and method

Materials

The fresh or dried leaves, barks, fruits, seeds, or bulbs of 57 samples were obtained from CV Sekar Utami, Jakarta, CV Karya Tama, Lampung and Pasar Modern BSD, Tangerang. Rat

intestinal acetone powder and DPPH (2,2-diphenyl-1-picrylhydrazyl) were commercially available from Sigma Aldrich, Germany. K.tartaric acid, quercetin, gallic acid, NaOH, folin-ciocaltau phenol reagent, glucose and sucrose were supplied from Merck, Germany. All other chemicals used were purchased from PT. Bratachem, Indonesia, unless otherwise stated. The samples used are shown in Table 1.

Table 1. Samples for AGIs evaluation

Scientific name	Parts	Scientific name	Parts	Scientific name	Parts
Abelmochus esculentus	Fruit	*Coriandrum sativum L.*	Fruit	*Phaleria macrocarpa*	Fruit
Aleurites moluccana	Fruit	*Cuminum Cyminum L*	Seed	*Phyllanthus niruri*	Leaf
Allium Cepa	Bulb	*Cymbopogon citrates*	Stem	*Physalis angulata L.*	Leaf
Allium cepa L.(var Aggregatum)	Bulb	*Durio zibethinus*	Skin	*Piper betle L.*	Leaf
Aloe vera	Skin	*Eichhornia crassipes*	Leaf	*Piper cocatum*	Leaf
Amomum compactum	Fruit	*Elettaria cardamomum*	Fruit	*Plantago major L*	Leaf
Ananas comocus	Leaf	*Euphoria Longana*	Skin	*Quisqualis indica L*	Leaf
Andrographis paniculata	Leaf	*Garcinia mangostana*	Skin	*Ruellia tuberosa L.*	Leaf
Annona muricata L	Leaf	*Guazuma ulmifolia*	Leaf	*Ruellia tuberosa L.*	Leaf
Artocarpus altilis	Leaf	*Gynura procumbens*	Leaf	*Senna Alata*	Leaf
Azadirachta indica	Leaf	*Illicium Verum*	Flower	*Senna Alexandrina*	Leaf
Brassica oleracea	Stem	*Luffa acutangula*	Fruit	*Stachytarpheta mutabilis*	Leaf
Catharanthus roseus L.	Leaf	*Momordica charantia*	Fruit	*Swietenia macrophylla*	Leaf
Centella asiatica	Leaf	*Morus alba L.*	Leaf	*Tinospora crispa L.*	Leaf
Cinnamomum verum	Bark	*Musa paradisiacal*	Skin	*Trigonella foenum-graecum*	Seed
Citrullus lanatus	Skin	*Nicotiana tabacum*	Leaf	*Typhonium flagelliforme*	Leaf
Citrus nobilis (var. Microcarpa)	Seed	*Ocimum sanetum*	Leaf	*Uncaria gambir*	Fruit
Clinacanthus nutans L.	Leaf	*Persea americana*	Skin	*Zingiber zerumbet*	Leaf
Clitoria ternatea	Stem	*Persea Americana*	Seed	*Ziziphus mauritiana*	Leaf

Sample preparation

Fresh samples were firstly cut and dried at 50 °C for 24h. Five grams (dry weight) of each sample was subjected to 100 mL of 50% (v/v) aqueous methanolic extraction for 24h at room temperature. The crude extract was obtained by vacuum filtration through filter paper

(Whatman No. 5C, 70 mm) and was evaporated to dryness using rotary evaporated under reduced pressure at 50 °C and redissolved with 50% (v/v) aqueous dimethyl sulfoxide. Samples were kept in a dark place at 4 ± 2 °C prior to the glucosidase inhibitory activity assay.

Glucosidase inhibitory activity assay

The rat intestinal glucosidase inhibitory activity was determined using the method described previously with slight revision (Jong-Anurakkun *et al*., 2007). Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, and centrifuged at 10,000 rpm (4 °C, 60 min). The crude enzyme solution obtained from the supernatant was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0). The final crude enzyme solution showed specific activities (0.137 unit/mg protein), which was measured by using sucrose as a substrate. The inhibitory activity against sucrose hydrolysis was measured by the following procedures. Two test tubes, as sample and control, containing 200 µl sucrose solution (56 mM) in potassium phosphate buffer (0.1 M, pH 7) and two test tubes, containing 400 µl potassium phosphate buffer (0.1 M, pH 7) as each blank were pre-incubated at 37 °C for 5 min. The control and control blank defined as 100 % and 0 % enzyme activity, respectively. The working samples diluted in 50 % DMSO (100 µl) were added to the sample and sample blank test tubes while 50 % DMSO (100 µl) was added to the control and control blank test tubes. And then crude rat intestinal glucosidase (200 µl) was added only to the test tubes containing sucrose solution (sample and control). The reaction was carried out at 37 °C for 15 min and stopped by adding Tris-HCl buffer (2 M, pH 6.3, 750 µl). Procedures for inhibitory activity assay against maltose hydrolysis were basically the same as above except for replacing sucrose solution (56 mM, 200 µl) with maltase solution (3.5 mM, 350 µl) and for reducing the amount of enzyme solution from 200 µl to 50 µl. The reaction mixtures were then passed through a short column of aluminium oxide 60 (5 x 500 mm, 1.5 g) for removing phenolics which may interfere with the following glucose quantification. Each mixture was taken 0.5 ml to other test tubes and was added with 0.5 ml DNS solution. All mixtures were incubated for 5 minutes in boiling water. The absorbance was measured using UV-Vis spectrophotometer at 540 nm wavelength. Inhibitory activity was calculated by the following equation:

$$Inhibitory\ activity\ (\%) = \frac{(Abs\ control - Abs\ control\ blank) - (Abs\ sample - Abs\ sample\ blank)}{(Abs\ control - Abs\ control\ blank)} \times 100\%$$

The experiments were done in duplicate, and the results were presented as % inhibition, which is defined as the AGIs activity at certain concentration under the assay conditions.

Analysis of □-glucosidase inhibiting principle in relation to its antioxidant activity, total flavonoid content and total phenolic content

Five samples with the highest inhibitory activity were further extracted in gradient extraction system and were analyzed for its antioxidant activity, total phenolic content, and total flavonoid content. Dried samples (100 g) was macerated for 24 hours in n-hexane. The crude extract was filtered using vacuum filtration and the filtrate was referred as hexane extract. The remaining solid materials were re-extracted again with ethyl acetate, and the remaining solid from ethyl acetate extract was then filtrated with n-butanol. The evaluation of AGIs activity was done using rat intestinal glucosidase inhibitory assay with the same procedure in the screening of AGIs. Antioxidant activity was analyzed using DPPH radical scavenging

activity, total phenolic content was measured using Folin-Ciocalteau assay, while total flavonoid was measured using aluminium chloride assay.

Results and Discussion

Screening of AGIs

In this study, 57 samples from 56 species and 43 families were examined. Among them, 8 samples showed high inhibitory activity at concentration of 0.10 g/ml and five samples have more than 50% AGIs activity (Figure 1.(a)). The five samples, namely UG, MP, CC, MC, and AC were further observed in gradient eluent systems. The gradient eluent using solvents with different polarity (hexane, ethyl acetate and butanol) allows rough separation of the samples based on its general polarity. AGIs activity in each extract allowed prediction of the nature of active compounds and suitable fractionation system. The extraction yield of the samples in different solvent ranged from 0.9 to 12.5 mg/g (Table 2). The amount of extracted yield indicates that many compounds inside the samples were semi polar and polar compounds since ethyl acetate and butanol showed higher yield compared to hexane.

(a) (b)

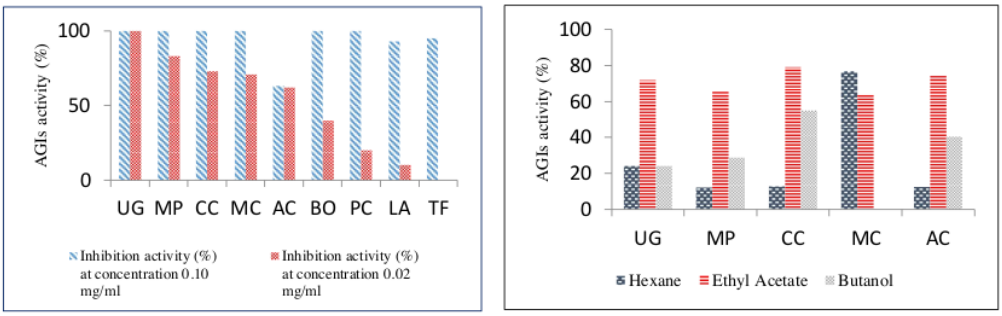

Figure 1. (a) AGIs activity of *Uncaria gambir* (UG), *Musa paradisiaca* (MP), *Cymbopogon citratus* (CC), *Momordica charantia* (MC), *Allium cepa L.* (AC), *Brassica oleracea* (BO), *Piper cocatum* (PC), *Luffa acutangula* (LA), and *Typhonium flagelliform* (TF). (b) AGIs activity of gradient-extracted fractions of UG, MP, CC, MC, and AC

AGIs activity assay for each extract in the gradient extraction of the five samples resulted in the finding of ethyl acetate and butanol extracts with higher AGIs activity compared to hexane extracts (p-value < 0.05, Figure 1.(b)). The result was in line with previous research in isolating the active compound for AGIs from several plants, which showed that ethyl acetate fraction showed the higher AGIs activity among other fractions (Laar, 2005; Dewoto, 2007). The finding indicates that the compound(s) responsible for AGIs was probably semi-polar or polar compound(s), since it has higher AGIs activities in ethyl acetate and butanol extracts. However, the hexane extract of MC showed the highest AGIs activity, followed by ethyl acetate extract and butanol extract of MC with AGIs activity value of 76.70, 63.65 and 0 % respectively. Matsuur *et al.* found that the active compound from methanolic extract of the bitter melon seeds that effectively inhibited alpha-glucosidase was D-(+)-Trehalose (Matsuur *et al.*, 2002). This indicates that there might be other non-polar compound responsible for AGIs activity in hexane fraction.

Table 2.Yield gradient extraction of UG, MP, CC, MC and AC

Name	Yield (mg/g)

	Hexane	Ethyl Acetate	Butanol
UG	1.30±0.10	7.60±0.20	9.77±0.01
MP	2.40±0.20	12.77±0.20	29.50±0.20
CC	8.00±0.20	12.00±0.20	16.70±0.50
MC	7.10±0.20	12.40±0.20	5.60±0.10
AC	0.90±0.20	15.10±0.40	11.60±0.04

Antioxidant activity, total flavonoid content and total phenolic content

Compound(s) that is responsible for AGIs activity might also contribute to antioxidant activity (Apea-Bah *et al*., 2009). Therefore, DPPH radical scavening activity assay was used to determine the antioxidant activity of the samples. IC_{50} value indicates the concentration of extracts that is needed to reach 50% inhibition to the DPPH radical. The smaller IC_{50} value means less amount of concentration needed to reach 50% inhibition or in other words the antioxidant activity is stronger. Statistical analysis was used to determine the samples with the highest antioxidant activity and the result showed that UG has the higest antioxidant activity followed by AC and MP, afterwards CC and MC (p-value < 0.05). The result was in line with Apea-Bah *et al*. that catechin, which is a powerful antioxidant in neutralizing free radicals (Braichu *et al*., 2013), was the major bioactive compound present in UG (Apea-Bah *et al*., 2009). In addition, there was a strong correlation between AGIs and antioxidant activity (r = 0.973). This indicates that the increase in the overall AGIs activity of the samples can be attributed to the increase of antioxidant activity. This also indicates that the chemical compound(s) contributing to AGIs activity also exhibits antioxidant activity. This was in agreement with previous research that there was a strong correlation of AGIs activity and antioxidant activity of some selected medicinal plants in Malaysia (Sugiwati *et al*, 2009). Compared to UG, MC, CC and MP, AC has the lowest AGIs activity. However, the antioxidant activity is moderately high compared to other extracts. Therefore, AC was not included in the correlation of AGIs and antioxidant activity. It could be assumed that in AC, the compound(s) responsible for AGIs activity was not the same with the compound(s) responsible for antioxidant activity.

Table 3. AGIs activity (0.02 g/ml), phenolic content, flavonoid content, and antioxidant activity of UG,MP, CC, MC, and AC

Name	AGIs (%)	Phenolic Content mg GAE/g	Flavonoid Content mg QE/g	Antioxidant Activity IC_{50} (ppm)
UG	100.00	67.57^{a}	1.57^{c}	101.75^{a}
MP	83.21	14.95^{c}	2.40^{b}	2377.64^{b}
CC	73.11	6.14^{d}	1.00^{d}	5910.14^{c}
MC	71.00	15.20^{c}	0.99^{d}	5859.14^{c}
AC	62.34	19.90^{b}	8.94^{a}	1963.57^{b}

Phyoconstituents of plants such as phenolic and flavonoid compound also contribute to AGIs activity (Kumar *et al*., 2011). The total phenolic content of the samples extracts ranged between 6.14 to 67.57 mg GAE/g (Table 3). ANOVA single factor showed that the total phenolic content between the crude extracts has highly significant difference with p-value <0.05. The plant extracts which has the highest phenolic content was UG fruit extract,

followed by AC, MC and MP, and the least was CC extract (p-value <0.05). The total phenolic content obtained by Kassim *et al*. from methanolic extract of UG leaves was higher (99.25 mg GAE/g) than that of UG fruit obtained in this study (67.60 mg GAE/g). This confirms the fact that different part of plant contains different amount of phenolic compounds. The correlation between AGIs activity and total phenolic content showed moderate positive correlation with r = 0.798. The positive correlations indicate that the increase in AGIs activity can be attributed with the amount of phenolic compounds. This was in agreement with previous research that there was a significant correlation between AGIs and total phenolic content in selected medicinal plants in Nigeria (Manaharan, *et al*., 2012).

The flavonoid content of the extracts ranged between 1.00 to 8.94 mg QE/g (Table 3). The statistical analysis indicated that the amount of flavonoid content in AC, MP, UG were significantly different to MC and CC (p-value < 0.05). Among the samples, AC bulb extract had the highest flavonoid content. The correlation between AGIs activity and total flavonoid content value showed a moderate negative correlation (r = -0.538). However, the statistical analysis revealed that the correlation was not significant (p-value > 0.05). Therefore, no correlation between AGIs activity and total flavonoid content was found. This indicates flavonoid compound do not contribute to AGIs activity. This was in agreement with previous research that there was no correlation between total flavonoid content and AGIs activity (Adefegha *et al*., 2012).

Conclusions

Among those plants, the methanolic extract of *Uncaria gambir, Musa paradisiaca, Cymbopogon citratus, Momordica charantia* and *Allium cepa L.* showed highest AGIs activities 100%, 83%, 73%, 71%, and 62% respectively at concentration 0.02 g/ml. The gradient extraction of the five samples with the highest AGIs activity showed that the ethyl acetate fraction extract had the highest AGIs. This indicates that the compound(s) responsible for the inhibitory effect is most likely to be semi-polar or polar. UG also had the strongest antioxidant activity (DPPH IC_{50} = 101.75 ppm) and highest total phenolic content (67.57 mg GAE/g). This confirmed the positive correlation between AGIs activity with antioxidant activity and total phenolic content, which indicates that there might be the same compound(s) responsible for both activities. On the other hand, no correlation was found between AGIs activity and total flavonoid content. The AGIs activity of *Allium cepa L.* was indicated to be supported by the high content of flavonoid (8.94 mg QE/g).

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