Trad. Med. J., May - August 2016
Vol. 21(2), p 63-68
ISSN : 1410-5918

Submitted : 18-03-2016
Revised : 27-07-2016
Accepted : 05-08-2016

COMBINATION OF ETHANOLIC EXTRACT OF α-GLUCOSIDASE INHIBITORY ACTIVITY OF *Phaleria macrocarpa* (SCHEFF.) BOERL. FRUITS AND *Annona muricata* LINN. LEAVES

AKTIVITAS PENGHAMBATAN α-GLUCOSIDASE OLEH KOMBINASI EKSTRAK ETANOLIK BUAH *Phaleria macrocarpa* (SCHEFF.) BOERL. DAN DAUN *Annona muricata* LINN.

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ABSTRACT

Antidiabetic activities of Phaleria macrocarpa fruits and Annona muricata leaves through α-glucosidase inhibition have been reported. Each extract of the two medicinal plants showed weak α-glucosidase inhibitory activities. The effect of synergies is expected from combining the two extracts. The purpose of this research was to investigate α-glucosidase inhibitory activity of the combination of ethanolic extract of P. macrocarpa fruits and A. muricata leaves and to confirm the presence of different chemical constituents of the most active individual and extracts combination by thin layer chromatography (TLC). Extracts were obtained by maceration method using 96% ethanol. Various concentration of extracts combination was analyzed based on IC_{50} of the enzyme inhibition of each individual extract. The result showed that α-glucosidase inhibitory activity of each extracts was concentration dependent, with IC_{50} values of individual extracts of P. macrocarpa fruits and A. muricata leaves were 261.34 and 428.79 µg/mL, respectively. However, the extracts combination with the highest inhibition activity (84.51±0.79%) was obtained at its IC_{50} of P. macrocarpa fruits and two-third IC_{50} concentration of A. muricata leaves. The TLC profile of the most active extract combination showed a phytochemical constituent that is also detected in the profile of each individual extract. This can be assumed as a synergistic effect due to increased phytochemical contents of the combined extract.
***Keywords**: α-glucosidase inhibitory, medicinal plants, combination extract, phytochemical profiling*

ABSTRAK

Buah Phaleria macrocarpa dan daun Annona muricata telah dilaporkan memiliki aktivitas antidiabetes dengan menginhibisi aktivitas enzim α-glukosidase. Masing-masing tanaman herbal tersebut menunjukkan aktivitas inhibisi yang rendah terhadap enzim α-glukosidase. Kombinasi dua tanaman ini diharapkan dapat memberikan efek sinergis. Penelitian ini dilakukan untuk mengevaluasi aktivitas inhibisi enzim α-glukosidase gabungan ekstrak etanol buah P. macrocarpa dan daun A. muricata dan mengidentifikasi profil kromatografi lapis tipis (KLT) masing-masing buah P. macrocarpa, daun A. Muricata dan gabungan ekstrak yang memiliki aktivitas terbaik. Ekstraksi dilakukan dengan teknik maserasi menggunakan etanol 96%. Kombinasi ekstrak gabungan diformulasi berdasarkan nilai IC_{50} dari masing-masing ekstrak. Hasil analisis menunjukkan bahwa aktivitas inhibisi bergantung pada konsentrasi, dengan nilai IC_{50} masing-masing ekstrak buah P. macrocarpa dan daun A. Muricata secara berturut-turut adalah 261.343 dan 428.790 µg/mL. Gabungan ekstrak yang memiliki aktivitas inhibisi tertinggi (84.52±0.79%) terdiri dari gabungan ekstrak buah mahkota dewa pada konsentrasi IC_{50} dan ekstrak daun sirsak pada konsentrasi 2/3 IC_{50}. Berdasarkan profil KLT, terdeteksi adanya senyawa bioaktif pada ekstrak gabungan yang memiliki aktivitas paling baik, yang mana senyawa tersebut juga terdeteksi pada masing-masing ekstrak. Hal ini dapat diasumsikan bahwa dengan meningkatnya kandungan senyawa fitokimia yang terkandung dalam gabungan ekstrak dapat dihasilkan suatu efek sinergis.
***Kata kunci**: inhibitor α-glukosidase, tanaman obat, ekstrak gabungan, profil fitokimia*

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INTRODUCTION

Medicinal plants were found in various places throughout the world, especially in tropical countries such as Indonesia. Currently, the use of medicinal plants (herbal medicine) is increasing, both in Indonesia and in developed countries in America and Europe. Herbal medicines have been effectively used to treat many diseases and it can be an alternative for synthetic or modern medicine (Kumar *et al*, 2011). The research of medicinal plant as a source of therapy for various diseases still needs to be explored. Therapy for diseases such as diabetes is increasing due to the change of way of life. Drug to control hyperglycemic condition can be achieved by controlling the absorption of dietary blood glucose through the inhibition of α-glucosidase mechanism. Currently, many researches explore not only single plants but also combined medicinal plants. This combined formula is expected to exert interaction specifically a synergistic effect.

Phaleria macrocarpa (Scheff.) Boerl (*P. macrocarpa*) is a member of *Thymelaeaceae* family, extensively used as a traditional medicine to treat various diseases including diabetes. Its fruit has been reported to have antidiabetic activity. Methanol and boiled water extracts of *P. macrocarpa* fruits showed inhibition of α-glucosidase of 40.26% and 26.53%, respectively, but still had low activities (Sugiwati *et al*, 2006). Inhibitory activities of ethanol crude extract from *P. macrocarpa* fruits was also low (29.22%) (Suparto *et al*, 2008). Methanol extract showed lowered blood glucose by 56.25% and 58.33% in diabetic rats after 12-day treatment (Ali *et al*, 2012). Other medicinal plants, *Annona muricata linn* (*A. muricata*) leaves had showed antidiabetic activities. Adewole and Caxton-Martins, (2009) reported that the water extract of *A. muricata* leaves had beneficial effect to decrease blood glucose levels and increase insulin concentration in rat induced streptozotosin. In addition to that, Adeyemi *et al*, (2009) had proven that the methanol extract of *A. muricata* leaves can also decrease blood glucose levels of rat induced streptozotosin. However, both plants individually still had weak activities.

No reports were found yet on the effect of *P. macrocarpa* fruits-*A. muricata* leaves ethanolic extract combination act as α-glucosidase enzyme inhibition. The combination of both extracts would be expected to provide synergistic effect by increasing inhibition of α-glucosidase activity. IC_{50} values, namely the concentration of extract that can inhibit 50% of α-glucosidase activity, were used as a point to produce ethanolic extracts combination. The synergistic effect is the combined effect of bioactive compounds that showed higher activity which cannot be found in individual compound (Rasoanaivo *et al*, 2011). These bioactive compound further detected by thin layer chromatography (TLC) which commonly used as tool for standardization, authentication and quality control of medicinal plants. Therefore, this research was to investigate α-glucosidase inhibitory activity of *P. macrocarpa* fruit and *A. muricata* leaves ethanolic extracts combination, and TLC profile of the most active of individual and extracts combination.

METHODOLOGY

Materials and instrumentation

P. macrocarpa fruits and *A. muricata* leaves were collected from Biopharmaca Research Center, Bogor Agricultural University (Indonesia) garden. Ethanol 96%, dimethyl sulfoxide (DMSO) the enzyme α-glucosidase (Sigma G 3651-250UN), ρ-nitrophenyl α-D-glucopiranoside (PNG) (Sigma N 1377-5G), *Bovine Serum Albumin* (BSA), acarbose tablets (Bayer, Jakarta-Indonesia), n-butanol, and chloroform. The instruments used in this study were rotary evaporator (Heidolph®), oven (memmert) microplate reader (epoch™), CAMAG Linomat 5, and CAMAG Reprostar 3.

Extraction

Samples of *P. macrocarpa* fruits and *A. muricata* leaves were dried until the percentage of water was below of 10%, the dried results were then grounded and sieved using 40-mesh of sieve size. Each sample was extracted with maceration technique with ethanol 96% as solvent, and dried using rotary evaporator.

Phytochemical Analysis

Phytochemical screening was analyzed to determine the presence of bioactive compounds; such as flavonoids, phenolics, terpenoids, steroids, alkaloids, saponnins, and tannins compounds. The analysis performed according to standard methods that were described by Harborne (1984).

Alpha-Glucosidase Inhibitory Assay

Inhibition of α-glucosidase assay was using previous method described (Sugiwati *et al*, 2009; Sancheti *et al*, 2009). Enzyme solution was prepared by dissolving 1.0 mg of α-glucosidase in 0.01 M phosphate buffer (pH 7.0). The reaction mixture was contained of 50 µL of 0.01 M phosphate buffer (pH 7.0), 10 µL of test sample, 25 µL of 10 mM *ρ*-nitrophenyl α-D-glucopyranoside, and 25 µL of 0.04 units/mL α-glucosidase. This reaction mixture was incubated at 37 °C for 30 minutes. Enzyme reaction was terminated by

adding 100 mL of 0.2 M sodium carbonate. The absorbance of *p*-nitrophenol released was measured using microplate reader at 410 nm. Acarbose was used as positive control. All experiments were carried out in triplicate. The percentage of inhibitory activity was calculated by the following formula:

Inhibitory activity % = $(A_{control} - A_{sample})/A_{control}$ X 100

The IC_{50} value was calculated by plots log of concentration versus the percentage of inhibition curves. The combined extracts were made by IC_{50} values of each extract using numerical methods in order to minimize the variation of the extracts combined, and then activity of α-glucosidase inhibitory was assay by the same method.

Metabolite Profiling Using Thin Layer Chromatography (TLC)

P. macrocarpa fruits, *A. muricata* leaves and combined ethanol extracts were diluted in ethanol. The extracts were filtered and applied on TLC plate using CAMAG Linomat V semi-automatic sample spotter. The plate was developed in CAMAG glass twin chamber that was containing n-butanol:chloroform (6:4 v/v) as a mobile phase, After development, the plates were dried in air and scanned under UV 254 nm and 366 nm using CAMAG Reprostar 3 and documented with WinCATS Software.

RESULTS AND DISCUSSION

Phytochemical Analysis

Qualitative phytochemical screening was analyzed to determine secondary metabolites which contained in the ethanol extract of *P. macrocarpa* fruit and *A. muricata* leaves. The result showed ethanolic extracts of both *P. macrocarpa* fruits and *A. muricata* leaves contained flavonoids, phenolics, terpenoids, steroids, alkaloids, saponnins, and tannins. This result was in accordance with previous reports (Lay *et al*, 2014; Gavamukulya *et al*, 2014). Secondary metabolite of each extracts predicted to be contributed for antidiabetic activity. This is preliminary information about composition of each ethanol extract, and these phytochemicals were suspected to be contributing for antidiabetic activity (Qi *et al*, 2010).

α-Glucosidase Inhibition Activities of *P. macrocarpa* fruits and *A. muricata* leaves ethanolic extracts

α-Glucosidase inhibitory activity of *P. macrocarpa* fruits and *A. muricata* leaves ethanolic extracts were analyzed at range of concentration from 125 to 1000 µg/mL. The result of α-glucosidase enzyme inhibition of both extract showed a trend of concentration dependent (Figure 1). Figures 1 illustrate the percentage of α-glucosidase inhibitory activities of *P. macrocarpa* fruits and *A. muricata* leaves extracts.

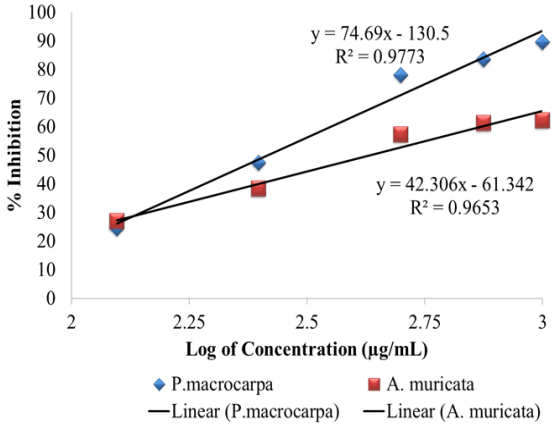

Figure 1. Inhibition of α-glucosidase activity of *P. macrocarpa* fruits and *A. muricata* leaves ethanolic extract

Its inhibitory activity was varied at 24.53±4.94 – 89.68±0.55% and 26.92±4.43 – 62.30±2.97% for *P. macrocarpa* fruits and *A. muricata* leaves extract, respectively. The 96% ethanolic extract of *P. macrocarpa* showed higher activity than the 30% ethanolic extract of *P. macrocarpa* fruits that previously reported by Suparto *et al*, (2008). However, Sugiwati *et al*, (2006) reported that the methanol (40.26%) and water (26.30%) extract at 50µg/mL had higher inhibition activities compared to this study. The difference in activity can be due to the source of samples and type of extraction solvent frequently affects the biological activities of plant extracts. Kumar *et al*, (2011) reported that the inhibition activity of *A. muricata* had higher activities using water, methanol, and ethyl acetate extract compared to our study with ethanolic extract. The IC_{50} was calculated by plotting log of concentration versus the percentage of inhibition of each extracts and also for acarbose. Inhibitory activities of *P. macrocarpa* fruit showed higher activity compared to *A. muricata* leaves, as indicated by IC_{50} values of 261.34 and 428.79 µg/mL, respectively. However, both of these extracts showed lower activity compared to acarbose as positive control. The IC_{50} of acarbose was 0.62 µg/mL.

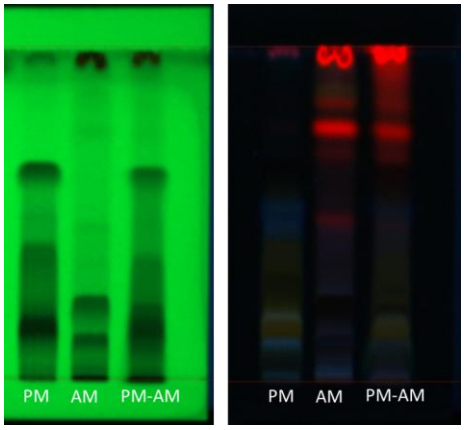

Figure 2. TLC profile of *P. macrocarpa* fruits (PM), *A. muricata* leaves (AM) and combined of ethanol extract (PM-AM) under UV 254 nm (left) and 366 nm (right).

Table I. α-Glucosidase inhibitory activity of *P. macrocarpa* fruits, *A. muricata* leaves, and combination of both extracts.

Composition Based on IC_{50} values	*P. Macrocarpa* Fruits (μg mL^{-1})	*A. Muricata* Leaves (μg mL^{-1})	Inhibition (%)[a]
1: 1	261.34	428.79	82.79±0.94
1: 1/3	261.34	142.93	81.62±0.86
1: 2/3	261.34	285.86	84.51±0.79
1/3:1	87.114	428.79	63.89±0.94
2/3: 1	174.23	428.79	84.01±0.71

[a]The results expressed ± relative standard deviation.

Table II. *Rf* values and colors of each bands from ethanolic extract of *P. macrocarpa* fruits, *A. muricata* leaves, and combination of both ethanolic extract.

P. macrocarpa		*A. muricata*		Combined extract *of P. macrocarpa-A. muricata*	
Rf values	UV 366 nm	*Rf* values	UV 366 nm	*Rf* values	UV 366 nm
0.36	Pale yellow	0.26	Blue	0.25	Pale yellow
0.45	Blue	0.50	Red	0.46	Red
0.52	Light blue	0.67	Red	0.67	Red
0.67	Blue	0.75	Red	0.75	Red
0.76	Red	0.82	Red	0.84	Red
-	-	0.88	Pale yellow	-	-

Activity of α-Glucosidase Inhibition of Combined Extract

α-Glucosidase inhibition activity of *P. macrocarpa* fruits–*A. muricata* leaves extract combination was performed to investigate the synergistic effect of both extracts. As far as we have acknowledged, this is the first report of crude extract combination (in ethanol 96%) from *P. macrocarpa* fruits and *A. muricata* leaves as antidiabetic activity through the inhibition of α-glucosidase enzyme mechanism (Table I).

Percentage of inhibition of ethanolic extracts combination showed increasing activity compared to individual extract (63.89±0.94% until 84.51±0.79%). It means all combination of these extracts act synergistically in increasing the α-glucosidase inhibitory activity (Table I). The highest inhibitory activity (84.51±0.79%) was acted by 1:2/3 of extracts combination. Based on this result, it can be speculated that the α-glucosidase inhibitory activity of combined extracts was affected by the constituents

and synergistically effect of combination of the extracts.

This effect cannot be found in the individual extract. Generally, the metabolite secondary of medicinal plant extracts is responsible to give synergistic action (Kumar *et al*, 2011). Compounds found in extract with low or no activity can help other compounds to achieve higher activities to the target. This condition is referred to synergistic concept of herb extracts combination. However, not all composition of extracts combination can be said to act synergistically; in vivo studies need to be done to determine and confirm the effect in metabolism system which cannot be detected if there is antagonistic in vitro study (Rasoanaivo *et al*, 2011).

TLC profile of *P. macrocarpa* fruits, *A. muricata* leaves and combination of both ethanolic extract

The quality of medicinal herbs is affected by their chemical constituents, especially the presence of secondary metabolite. TLC profiling is one of qualitative method commonly used to analyze the chemical constituents of medicinal herbal extracts. TLC profile was analyzed using silica gel 60 F_{254} as a stationary phase and n-butanol:kloroform (6:4 v/v) as a mobile phase. The colors and *Rf* values of each bands from *P. macrocarpa* fruits, *A. muricata* leaves extract and extracts combination (Table II and Figure 2).

TLC profile confirmed the presence of different chemical constituents with *Rf* values in range of 0.2 to 0.8. Bands of chemical constituents were separated with proper resolution. The resolution value was in range of 1.5 to 4.6. Chemical constituents in each individual extract were also monitored in TLC profile of extract combination. TLC profile indicated that extract combination contained similar or related chemical constituents as detected in each individual extracts. Under UV 366 nm light blue, pale yellow and red bands were detected (Figure 2), it signified the presence of phenolic compounds with aromatic structure. The bands also detected under UV 254 with a common *Rf* values. According to Harborne (1984), phenolic compound with aromatic structure showed intense absorption under UV 366 nm with colored bands such as yellow, red, blue, grey, and brown. According to literature, *P. macrocarpa* fruits contained a class of phenolic compounds, and these compounds showed antidiabetic activity (Sugiwati *et al*, 2006; Ali *et al*, 2012). *A. muricata leaves* also contained higher phenolic compounds (Vijayameena *et al*, 2013).

CONCLUSION

Combination of ethanolic extract of *P. macrocarpa* fruits and *A. muricata* leaves showed higher antidiabetic activity through α-glucosidase inhibitor. Both extracts predicted to act synergistically as α-glucosidase inhibitor. TLC profile showed that the ethanolic extract combination contained related bioactive compounds with each individual extract of *P. macrocarpa* fruits and *A. muricata* leaves.

ACKNOWLEDGEMENTS

One of the authors (ER) is thankful to the Directorate General of Higher Education for the Scholarship (BPPDN) of Lecturer Candidate in 2013 and the authors give high appreciation to Biopharmaca Research Center Bogor Agricultural University for the use of facilities in performing the enzyme analysis.

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