M calabura TLC

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Chromatographic (TLC) Fingerprinting, Antioxidant, α-Glucosidase, and Lipase Inhibitory Activities of Ethanol Extract of *Muntingia calabura* L. Fruits

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ABSTRACT : *Muntingia calabura* fruit is traditionally used for the treatment of many ailments including diabetes mellitus. The medicinal properties might be related to its antidiabetic and antioxidant properties of its constituents. This study aimed to determine phytochemical profiles of ethanol extract of *M. calabura* fruit by thin layer chromatography and to evaluate its antioxidant and inhibitory activities on glucosidase and pancreatic lipase. Extract was subjected to a separation using a silica gel 60 F254 TLC plate, using chloroform: methanol: formic acid 8:1.5:0.5 (v/v/v) mobile phase system. The spots were observed under UV light at 254 and 366 nm, and with vanillin-sulfuric acid spraying for post-derivatization. Phytochemical profiles were obtained using phenolic assay. Antioxidant and enzyme inhibitory activities were determined using *in vitro* methods. The presence of phenolic compounds was confirmed in the seven spots obtained. All spots showed strong DPPH radical scavenging activity and reducing capacity by phosphomolybdenum assay with activities between 0.61 to 7.69 mmol TE/gram dried weights. All spots were able to inhibit α-glucosidase and lipase, with activities ranging from 0.25 to 3.88 ACAEs/g dried weight and 3.22 to 40.01 OE/g dried weight, respectively. The study concludes *M. calabura* fruit extract contained a variety of antioxidant, antidiabetic, and anti-obesity compounds, thus supporting its traditional use.

KEYWORDS: Acarbose equivalents, α-glycosidase inhibition, kersen, lipase inhibition, total antioxidant activity.

I. INTRODUCTION

Muntingia calabura L. which belongs to the family Elaeocarpaceae is widely distributed in tropical and subtropical areas. The trees can grow up to 12 m with horizontally spreading leaves. *M calabura* is known as Jamaican cherry, and in Indonesia, it is locally known as kersen or talok. The fruit is round (1 – 1.25 cm) and sweet with tiny seeds inside. Traditionally, *M calabura* is used for the treatment of gastric ulcer, swelling, headache, measles, stomachache, mouth pimples, and cold [1]. Previously, studies have been done to scientifically prove the medicinal properties of *M calabura*. The leaves were reported to have anti-nociceptive [2], gastro-protective [3], and anti-bacterial [4] activities. The fruit was shown to have antioxidant [5] and anti-inflammatory [6] activities. Only recently, we have reported enzyme (α-glucosidase, lipase, and xanthine oxidase) inhibition activities of *M calabura* fruit [7]. In the previous study, we reported the presence of flavonoid and phenolic compounds in the ethanol extract of *M calabura* fruit. These classes of secondary metabolites have been known for their various medicinal properties [8]. Identification of phytochemical constituents in plant extracts is important to allow further research regarding these compounds. TLC is a rapid and economical technique for preliminary identifications of phytochemical constituents in plant extracts. Thin layer chromatogram of a crude sample presents a distinct profile or pattern that reflects phytochemical constituents in the sample. This fingerprinting technique has been widely used for the evaluation of crude drugs to identify any active principals and possible contaminants [9]. Further, fingerprint profile allows for future standardization of medicinal substances of plant origin. The present study determined the fingerprint profile of *M calabura* fruit extract by TLC developed under two eluent systems. In addition, the separated constituents were analyzed for their antioxidant and enzyme (α-glucosidase and lipase) inhibition activities.

II. MATERIALS AND METHODS

Materials: All reagents were purchased from Sigma-Aldrich (St Louis, USA), *i.e.* acarbose, α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside, Folin-Ciocalteu reagent, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), porcine pancreatic lipase (EC 3.1.1.3), *p*-nitrophenylpalmitate, and orlistat. Solvents used were of analytical grades.

Extract preparation : *M. calabura* fruits were obtained from the Botanical Garden of Jember University, East Java, Indonesia, in August 2018. After washing thoroughly under running water, the fruits were dried using food dehydrator and pulverised. The coarse powder (50 g) was macerated in ethanol (1:5 ratio, w/v) for 72 h at room temperature. After filtering through a Whatman no 1 filter paper, the solvent was removed under reduced pressure using a rotary evaporator (Buchi R3, Flawil, Switzerland). The crude extract was stored at 4 °C until analysis.

Figure 1 *M. calabura* fruit

TLC analysis: The crude extract was subjected to a thin layer chromatographic separation. An F_{254} TLC plate 2 x 7.5 cm (Merck, Darmstadt, Germany) was used as a stationary phase. Before use, methanol was run on the plate, which was then followed by drying off the solvent at 100 °C to ensure dryness. The TLC chambers were conditioned in mobile vapor for about 10 mins. Two solvent systems were used separately as eluents for separation, *i.e.* (A) chloroform: methanol: formic acid (8:1.5:0.5, v/v/v) and (B) chloroform: ethyl acetate (6:4, v/v). Developed chromatograms were observed under visible light, UV light at 254 and 366 nm, and after spraying with vanillin-sulfuric acid for post-derivatization. UV visualization was carried out using a CAMAG UV visualizer. R_f values for each observed spot was calculated using the following formula, and ImageJ was used to quantify the intensity of each spot.

R_f = (Distance travelled by each spot)/(Distance travelled by solvent front)

Preparative TLC: The crude extract was chromatographed by a preparative TLC which was performed on an F_{254} TLC plate 5 x 10 cm. Chloroform: methanol: formic acid (8:1.5:0.5, v/v/v) was used as an eluent system. The separated bands were visualized by visible and UV (at 254 and 366 nm) light. The bands were scraped from the plate and extracted with ethanol. Each filtrate was separated from the silica powder by centrifugation (100,000 rpm for 10 mins) to obtained clear fraction. In this way, seven main fractions were obtained from the preparative TLC, which were then analyzed for their bioactivities.

Bioautography assays

Bioautography by Folin-Ciocalteu reagent: To identify phenolic compounds in the spots, the developed chromatogram using solvent system A was sprayed by Folin-Ciocalteu reagent (10%, methanol). The plate was directly observed. The presence of phenolic compounds was observed as dark blue spots.

Bioautography by DPPH reagent: The developed chromatogram was sprayed with DPPH solution in ethanol (0.6 mM). The chromatogram was observed after waiting for 5 mins in daylight. Spots containing free radical scavengers appeared as cream or yellow spots against purple background.

Bioactivity assays

Total antioxidant activity/phosphomolybdenum method: Each fraction obtained from the preparative TLC was tested for their total antioxidant activity by phosphomolybdenum method as reported previously [10]. Phosphomolybdenum reagent consisted of sulfuric acid (0.6 M), trisodium phosphate (28 mM), and ammonium molybdate (4 mM). Reaction mixture was prepared by adding phosphomolybdenum reagent (3 mL) into each fraction (0.3 mL). The mixture was incubated in hot water bath (95 °C) for 90 mins. After cooling at room temperature, the absorbance was read at 695 nm. Trolox (0.08 – 2.00 mmol/mL) was used to generate a calibration curve. Results were expressed as trolox equivalent/gram dried weight (mmol TE/g DW).

α-Glycosidase inhibitory activity : Fractions were also analyzed for their α-glucosidase inhibitory activities using the already published methods [11]. An aliquot of each fraction (50 μL) was added with phosphate buffer

(50 µL, 50 mM, pH 6.8). α-Glucosidase (0.5 U/mL in phosphate buffer) was added to the solution and the mixture was incubated at 37 °C for 5 mins. The substate *p*-nitrophenyl-α-D-glucopyranoside (1 mM, 100 μL) was added to start the reaction and the reaction mixture was further incubated at 37 °C for 20 mins. The reaction was stopped by the addition of Na_2CO_3 (100 mM, 750 μL) and the absorbance was measured at 405 nm on a spectrophotometer. Acarbose (0.02 – 0.16 mmol/mL) was used to generate a standard curve. Activity of each fraction was presented as mol acarbose equivalent/gram dried weight (molACE/g DW) [12].

Pancreatic lipase inhibitory activity: Pancreatic lipase inhibition activity of each fraction was determined as reported [7]. Each fraction (100 μL in DMSO) was added with pancreatic lipase (300 μL, 200 U/mL in tris buffer, 50 mM pH 8 with NaCl 30 mM). The mixture was incubated at 37 °C for 5 mins. A solution of *p*-nitrophenyl palmitate (10 mM in DMSO) was added to start the reaction, and the mixture was further incubated at 37 °C for 20 mins. Ethanol (500 μL) was added to stop the reaction, and absorbance was read at 405 nm. Orlistat (0.20 – 0.80 mol/L was used to generate a standard curve and results were presented as mol orlistat equivalent/g dried weight (molOE/g DW).

III. RESULTS

TLC analysis: The TLC chromatograms of *M. calabura* fruit extract in both solvent systems were presented in Fig. 2. In general, both solvent systems were able to separate the secondary metabolites in the extract into separated components. Following illuminations under UV light (at 254 and 366 nm) and derivatization with vanillin, solvent system A (chloroform: methanol: formic acid) yielded more observable spots (7 spots) with R_f values in the range of 0.07 – 0.89. In comparison, solvent system B (chloroform: ethyl acetate) obtained only 4 spots with R_f in the range of 0.04 – 0.37. ImageJ analysis (Table 1) showed that the separated spots in both solvent systems had various quantities.

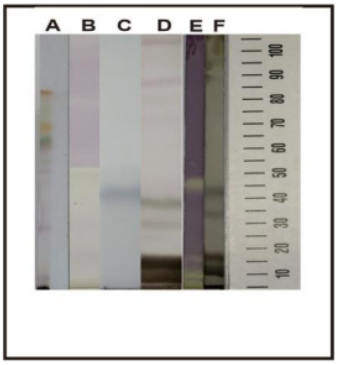

Figure 2 Chromatograms from fruit extract of *M. calabura* in solvent system A (chloroform: methanol: formic acid). The following are visualizations of the separated spots after (A) post-derivatization with vanillin-sulfuric acid (B) DPPH spraying (C) Folin-Ciocalteu spraying (D) sulfuric acid (in methanol) spraying. (ii) solvent system B (chloroform: ethyl acetate). The following are visualization after (E) DPPH spraying and (F) sulfuric acid (in methanol) spraying.

Phenolic profile: The chromatogram displayed the presence of phenolic compounds in all the separated components (Fig. 2B), as can be seen from the dark blue spots on the TLC plate, following Folin-Ciocalteu spraying.

Table 1 ImageJ analysis from the separated components of fruit extract of *M. calabura* in solvent system A (chloroform: methanol: formic acid) and B (chloroform: ethyl acetate).

Solvent	Number of Spot	Resolution Factor (RF)	Density/Intensity (%)
A	7	0.07	27.36
		0.22	13.02
		0.42	13.09

		0.61	8.10
		0.67	7.01
		0.79	11.28
		0.89	14.01
B	4	0.04	47.16
		0.09	20.93
		0.33	10.59
		0.37	21.32

DPPH radical scavenging activity : TLC-DPPH assay was used to evaluate radical scavenging activity of the separated components of *M. calabura* fruit extract. Fig. 2B and 2E show developed chromatograms of both solvent systems which were sprayed by DPPH solution. Similar results were observed for both chromatograms. In both cases, every spot in the chromatograms was visible as cream spot.

Table 2 Antioxidant activity by phosphomolybdenum method and enzyme inhibitory activities of TLC fractions of *M. calabura* fruit.

Samples	Phosphomolybdenum (mol TE/gram DW)[a]	α-Glucosidase (mol ACAEs/g DW)[b]	Lipase (mol OE/g DW)[c]
Fraction 1	5.13 ± 0.07	1.09 ± 0.02	26.00 ± 7.24
Fraction 2	7.34 ± 0.05	3.88 ± 0.15	40.01 ± 2.48
Fraction 3	2.16 ± 0.03	0.52 ± 0.02	10.12 ± 6.32
Fraction 4	0.78 ± 0.05	0.96 ± 0.06	4.13 ± 4.41
Fraction 5	0.61 ± 0.05	0.25 ± 0.12	3.22 ± 5.41
Fraction 6	6.64 ± 0.03	1.80 ± 0.09	34.10 ± 1.51
Fraction 7	7.69 ± 0.09	1.18 ± 0.11	36.28 ± 3.47

[a]mol Trolox Equivalents/gram Dried Weight of TLC fraction
[b]mol Acarbose equivalents/gram Dried Weight of TLC fraction
[c]mol Orlistat equivalents/gram Dried Weight of TLC fraction

Total antioxidant activity: Phosphomolybdenum reaction was used to determine reducing activity of the separated components of *M calabura* fruit. The method quantifies the reducing potential based on the reduction of Mo(VI) into its lower oxidation state Mo(V) by antioxidant compounds. Results are presented in Table 2. Various reducing capacities were observed among all isolated components (F1 – F7) obtained by scraping the TLC preparative bands. Activities were in the range of 0.61 to 7.64 molTE/gr DW, with F7 and F5 showed the highest and the lowest activities, respectively.

α-Glucosidase inhibition activity: Antidiabetic activity of TLC fractions of *M calabura* fruit were tested based on their inhibition on α-glucosidase. This enzyme is central in digestion of polysaccharide to monosaccharide. Thus, inhibition of α-glucosidase may affect post prandial blood glucose level. All fractions inhibited α-glucosidase in various activities (Table 2) ranging from 0.25 – 3.88 molACE/g DW. Fraction 5 had the strongest inhibition, whereas F2 showed the weakest inhibition on α-glucosidase.

Lipase inhibition activity: All components (F1 – F7) showed pancreatic lipase inhibition activity, in the range of 3.22 – 40.01 molOE/g DW (Table 2). Fraction 2 showed the strongest inhibition activity, whereas F5 was the weakest inhibitor.

IV. DISCUSSION

Pharmacological properties of plants have been associated with the phytochemicals they contain, including phenolic and flavonoid compounds. A positive correlation between phenolic content and antioxidant activity of various plants have been well documented [13, 14]. In addition, enzyme inhibitory activities of plants have been attributed to the phenolic and flavonoid content [15, 16]. Phytochemical profiles obtained from thin layer chromatograms demonstrated the presence of various components in the ethanol extract of *M. calabura*. Following visualisation under visible and UV (254 and 366 nm) light and post derivatisation with vanillin-sulfuric acid, the solvent system A proved to have higher resolution when compared to system B, obtaining higher number of separated components and longer range of R_f values.

In this study, the presence of phenolic compounds was confirmed for all the separated spots, in both solvent systems. Recently, several phenolics and flavonoids have been identified in *M. calabura* fruit by LC-MS/MS analysis, including gallic acid, cyaniding-3-O-glucoside, genticic acid, and gallochatechin [17].Antioxidant activity of the developed chromatograms was determined by a TLC-DPPH method. This method offers a fast and convenient method to identify radical scavenger compounds in a developed chromatogram. In the presence of antioxidant compounds, the stable DPPH radicals were reduced to their corresponding DPPH-H reduced form, by donation of electrons or protons. This leads to decolorization of the purple reagent into yellow. In this study, all spots in the developed chromatograms appeared as light yellow and cream colour, indicating radical scavenging activity. Similar finding was also reported by others [5, 17]. Antioxidant activity of the separated components was also evaluated by their reducing potential. Compounds with reducing capacity suggest that they are electron donors, thus can reduce any oxidized species into their reduced states. The present study evidenced the reducing capacity of all the spots, supporting antioxidant activity observed in the DPPH assay. Previously, we reported the reducing capacity of the ethanolic fruit extract [7]. Findings in the present study clearly demonstrates that some components showed higher activity than others. It is possible that the observed activity in the crude extract originated from the additive effect of all components. The reductive capacity of the *M calabura* fruit was also reported by others, using different metal ions (Fe3+) as an oxidant [5]. Phenolic compounds contain hydroxyl groups attached to the benzene rings. Donation of the hydrogen atom and the resulting stability of the phenolic or flavonoid radicals contribute to the antioxidant activity of plant extract [18].

α-Glucosidase inhibition serves as a key strategy in controlling post prandial hyperglycemia. Acarbose, a synthetic inhibitor, was reportedly to cause adverse side effects that may affect its clinical use [19]. Natural inhibitors of plant origin may serve as a safer alternative. The present study found that all fractions were α-glucosidase inhibitors. This activity may be related to the phenolic compounds in the fractions. Studies reported that phenolic derivatives inhibit α-glucosidase [20]. Findings in this study support results of our previous study in which ethanol crude extract of *M. calabura* fruit was demonstrated to inhibit α-glucosidase [7]. This might be explained by additive inhibition of components in the extract. Obesity is a metabolic disorder which contribute to the onset and progression of diabetes mellitus and cardiovascular diseases [21]. Pancreatic lipase plays important role in lipolytic hydrolysis of dietary fats into fatty acids. Inhibition on lipase would reduce energy absorption and intake, thus may potentially be used for the treatment of obesity. Lipase inhibition can be an effective strategy for the treatment of obesity. Currently, orlistat is one of the first line drugs prescribed for the treatment of obesity. However, orlistat was reported to cause adverse side effects such as oily stools, diarrhea, and the loss of fat-soluble vitamins [22]. The catalytic role lipase plays in the digestion of triglycerides ~~into~~ makes this enzyme a clinical target for the treatment of obesity. This study found that every fraction isolated from the chromatogram was able to inhibit pancreatic lipase with various activity. This finding supports result from our previous study, in which the crude extract of *M. calabura* was evident to inhibit pancreatic lipase. Secondary metabolites namely phenolic and flavonoid compounds have been associated with pancreatic lipase inhibition [23].

V. CONCLUSION

The developed thin layer chromatograms of *M. calabura* fruit extract identified a variety of secondary metabolites. The separated components were demonstrated to have antioxidant activities and anti-enzyme (α-glucosidase and lipase) activities. These suggest that the observed activities may be associated with the secondary metabolites. Findings suggest possible application of the fruit extract as antidiabetic and anti-obesity agents. Future works must be directed in addressing the underlying mechanism of these enzyme inhibitions, probably by a computational study.

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BIOGRAPHIES

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Prof Dr Kris Herawan Timotius is a lecturer in microbiology and biochemistry at the Faculty of Medicine and Health Sciences, Krida Wacana Christian University. His research focus on herbal medicine including natural antibiotics, antiquorum sensing, antibiofilm, enzyme therapy, and anti-enzyme activities such as alpha glucosidase and xanthine oxidase.

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