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α-Glucosidase Inhibitory Effect of Fermented Fruit Juice of *Morinda Citrifolia* L and Combination Effect with Acarbose

ADELINA SIMAMORA,[1]* ADIT WIDODO SANTOSO[2] and KRIS HERAWAN TIMOTIUS[1]

[1]Department of Biochemistry, Krida Wacana Christian University, Jakarta, 11510 Indonesia.
[2]Department of Herbal Medicine, Krida Wacana Christian University, Jakarta, 11510 Indonesia.

Abstract

Fermented fruit juice of *M. citrifolia* is supposed to be the future nutraceutical beverage due to its antidiabetic and antioxidant activities. The purposes of this study were to characterize the fermented juice microbiologically and chemically, and to evaluate its α-glucosidase inhibition and radical scavenging activities in vitro. The fruit of *M. citrifolia* was fermented and the fruit juice was obtained and evaluated for its radical scavenging activity based on a DPPH assay. Its in vitro antidiabetic activity on α-glucosidase inhibition was investigated, including its combined effect with acarbose by a Chou-Talalay method. The inhibition mode was evaluated by Lineweaver-Burk plots. The juice was identified for its microbiome with 16S sequencing method and pictured with SEM. The bioactive compounds were analysed with LC-MS. The main microbiome was yeast and tentatively identified as Candida. The yeast was not able to grow in the normal growth medium for yeast, such as sabouroud agar. The TPC of the juice was 1,193 µg GAE/ml. The main compounds identified by an LC-MS were short chain fatty acids (α-ketoglutaric acid and malic acid). The fermented fruit juice showed good α-glucosidase inhibitory and antioxidant activities with IC_{50} of 28.99 and 14.09 µg GAE/ml, respectively. The kinetic study showed a non-competitive inhibition on α-glucosidase. The combination of the juice with acarbose at higher concentrations produced an additive effect on α-glucosidase. However, at lower concentrations, an antagonistic effect was observed. The fermented fruit juice of *M. citrifolia* is a good beverage with strong antidiabetic and antioxidant effects.

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CONTACT Adelina Simamora adelina.simamora@ukrida.ac.id Department of Biochemistry, Krida Wacana Christian University, Jakarta, 11510 Indonesia.

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Introduction

Nutraceutical beverages are made primarily from plant components, such as fruit, seeds, rhizomes and vegetables, as well as animal products such as milk and dairy-based and alcoholic drinks. The taste, aroma, and flavor of such beverages should be first accepted, and thereafter other beneficial aspects can be gained for health promotion and disease prevention.[1] Currently, nutraceutical potential of plants has been explored for the treatment and prevention of diabetes mellitus.[2]

Morinda citrifolia L., commonly known as "mengkudu" or "pace" in Indonesia, was an evergreen shrub of about 3 to 6 m high. It is widely cultivated in many areas, including Asia, Australia, and Pacific islands. The fruit juice has been used to treat a broad range of illnesses such as bowel disorders, skin inflammation, liver diseases, urinary tract infection, cardiac diseases, and many more. The available literature indicates that the fruit juice of *M. citrifolia* lowers the level of the postprandial blood glucose, thus have antidiabetic activity.[3] However, to date, only a few studies have examined antidiabetic activities of the juice from fermented fruit of *M. citrifolia*. Nayak et al reported on anti-diabetic effects of the fermented fruit juice (fFJ) using animal (mice).[4] It was also reported that fFJ reduces body weights and improves glucose tolerance in mice.[5,6] Thus, it is interesting to study on various aspects of its *anti-diabetic effect of fFJ*, including the α-glucosidase inhibitory properties of this fermented fruit.

Acarbose, a complex oligosaccharide, is an effective α-glucosidase inhibitor and has been clinically proven in maintaining glycemic control. Together with other synthetic α-glucosidase inhibitors such as viglibose and miglitole, acarbose has been reported to reduce the progression of diabetes as well as its complications, such as neuropathy, nephropathy, and retinopathy.[7] Therefore, it has become one of the first line drugs of choice in the management of type 2 diabetes mellitus (T2DM). T2DM patients are recommended to take 100 – 200 mg three times daily for a maintenance dose. Nonetheless, long term use of acarbose is known to cause adverse side effects associated with gastrointestinal and hepatic adverse effects.[8] In an effort to reduce undesirable side effects caused by large doses of single medication, combined use of drugs has gained more attention. Herbal extracts from fruit and teas have been reported to possess inhibitory activities against α-glucosidase and have few side effects.[9] Many studies have reported combined inhibition of herbal extracts or compounds with acarbose.[10,11] However, until recently, little information is available on the possible interaction between acarbose and fFJ of *M. citrifolia*.

Therefore, the present study aimed to investigate α-glucosidase inhibitory activities of fFJ of *M. citrifolia*, including enzyme inhibition mechanism and its effect on α-glucosidase when combined with acarbose. The chemical and microbial profiles of the fFJ was also evaluated.

Materials and Methods

Chemicals and Reagents

3,5-di-tert-butyl-4- hydoxytoluene (BHT), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Folin & Ciocalteu's phenol reagent, α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), and *p*-nitrophenyl-α-D glucopyranoside (*p*NPG) were purchased from Sigma-Aldrich (St. Louis, USA). Gallic acid (GA) was purchased from Santa Cruz Biotechnology (Dallas, USA). Acarbose was purchased from United States Pharmacopeia. All solvents and other chemicals were of analytical grade

Preparation of fFJ

FFJ was obtained directly from the local producer in Tasikmalaya, West Java, Indonesia (product name: Lentera Morinda). According to the producer, the fermentation process was performed as follows: the fully ripe fruit was washed. The fruit was then kept in airtight plastic bags and left to ferment spontaneously for 100 days at room temperature. The airtight bags were opened to end of the fermentation process and the liquid was filtered to obtain a clear liquid which was ready for consumption. The liquid was stored at -20 ^{o}C before used for bioactivity analysis.

Total Phenolic Content (TPC) Assay

TPC was determined by a modified Folin-Ciocalteu method[12] and estimated by a gallic acid standard curve. Results were expressed as µg gallic acid equivalent (µg GAE)/ml.

Liquid Chromatography-Mass Spectrophotometry (LC-MS) Analysis

The fFJ was analyzed on a Mariner Biospectrometry system equipped with a Hitachi L 6200 binary pump. The column used was a Shimp-pack C8, 150 × 6 mm i.d. Sample was prepared in methanol with 0.3% formic acid, injected at a flow rate of 1 ml/min. The HPLC was fitted with a Q-tof mass spectrometer interface, with an ESI (Electrospray Ionisation) source in a positive ion mode. Nitrogen was used as the nebulizing gas at a flow rate of 10 (arbitrary units). Voltages were optimized over time and after instrument maintenance for each segment; capillary and tube lens voltages were in the range of 17-46 and 65-115 V, respectively. The spray voltage and capillary temperature for all samples were set at 4 kV and 275 °C. In each segment, three scans were recorded: (1) full scan with ranges, (2) selected ion monitoring (SIM) scan for isolating the precursor ions, and (3) selected reaction monitoring (SRM) mode for isolating the fragment ions of samples for quantifications. The sample molecular weights, precursor ions, collision energies and fragment ions used for quantifications were reported.

Scanning Electron Microscopy (SEM)

Pellet of fFJ was obtained by centrifugation at 2000 g for 10 mins at 4 °C. The supernatant was discarded, and the pellet was washed in phosphate buffer saline (PBS) twice. The washed pellet was resuspended in glutaraldehyde (3%) in a 1:1 ratio and incubated at room temperature for 12 h. After washing in PBS three times for 15 mins, pellet underwent successive dehydration steps in a graded ethanol solution (30, 50, 70, 80, 90, and 100 %) each for 5 mins. Cells were air dried and coated with gold (Tioe Q150RS) for 15 s (20 nm). They were observed using scanning electron microscopy (SEM) (Hitachi TM3000). The S16 sequencing technique was then carried out to identify the microbiome in fFJ.

α-Glucosidase Assay

α-Glucosidase inhibitory activities of fFJ of *M.citrifolia* was determined based on our reported procedure.[13] Different concentrations of fFJ were prepared by serial dilutions. The reaction mixture was prepared consisting of the sample (50 µl), 50 µl of phosphate buffer (50 mM, pH 6.8) and 50 µl of α-glucosidase (0.5 U/ml). This mixture was incubated for 5 mins at 37 °C, thereafter, 100 µl of substrate p-nitrophenyl-α-D-glucoside (1 mM) was added to start the reaction. The reaction was further incubated for 20 mins at 37 °C and stopped by the addition of 750 µl Na_2CO_3 (100 mM). The absorbance was recorded at 405 nm in a spectrophotometer. Control solution was prepared by replacing the sample with buffer

The inhibition percentage was calculated using the following equation:

$$\% \text{ inhibition of } \alpha\text{–Glucosidase} = \frac{\text{A control - A sample}}{\text{A control}} \times 100\%$$

Where A control is the absorbance of control; A sample is the absorbance of sample. The α-glucosidase inhibitory activity was expressed as IC_{50} and determined from the graph plotted against the percentage of inhibition. The value was compared with the positive control acarbose, a standard prescribed medicine for DM.

Table 1: α-Glucosidase inhibitory activities of fFJ of *M. citrifolia* and acarbose.

Concentration (ug GAE/ml)	Inhibition (%)	IC50 (ug GAE/ml)
11.93	9.69 ± 2.60	
17.9	24.17 ± 8.03	
23.86	37.78 ± 5.40	28.99 ± 4.31
29.83	52.57 ± 4.75	
35.8	65.53 ± 0.75	
Acarbose		823.99 ± 0.06

Table 2: The CI (combination index) values of the combined inhibitory activities of FJ of *M. citrifolia* and acarbose on α-glucosidase.

Acarbose IC_{50}	fFJ M. citrifolia value ratio IC_{50}				
	0.25	0.5	1	2	2.7
0.25	3.62	5.42	4.91	1.76	1.11
0.5	2.68	2.67	3.2	1.65	0.85
1	2.3	3.17	3.35	1.54	0.97
2	2.6	2.4	2.98	2.97	1.11
2.7	2.34	2.3	2.65	1.93	0.97

Evaluation of Combined Inhibitory Effect Against α-Glucosidase

The combined effect of fFJ of *M. citrifolia* and acarbose, a prescribed α-glucosidase medicine, was evaluated using a drug combination method developed by Chou-Talalay.[14,15] Firstly, IC_{50} of individual inhibitor (fFJ of *M. citrifolia* or acarbose) were determined using dose-response curves. Then, combined fFJ of *M. citrifolia* and acarbose at different IC_{50} combinations were examined for their inhibitory effects on α-glucosidase. Data were calculated using Compusyn software® to obtain combination indexes (CI) for each individual or combined inhibitors.

Based on the CI values, drug combinations were considered of having synergistic effects when CI values < 0.9, additive effects CI = 0.9 – 1.1, or antagonistic effects CI > 1.1.

Determination of Enzyme Inhibition Mechanism

The inhibition type of fFJ of *M. citrifolia* on α-glucosidase activity was determined using Lineweaver-Burk plot analysis. In this method, a kinetic assay was carried out using *p*-nitrophenyl-α-D glucopyranoside as a substrate at different concentrations (0.15-1 mM).[16] The substrate was incubated with α-glucosidase (0.5 U/ml) in the absence and presence of fFJ of *M. citrifolia* at different concentrations (35.79; 71.59; 89.49 µg GAE/ml). Double reciprocal plots (1/[S] and 1/[V]) were constructed to point out the type of inhibition.

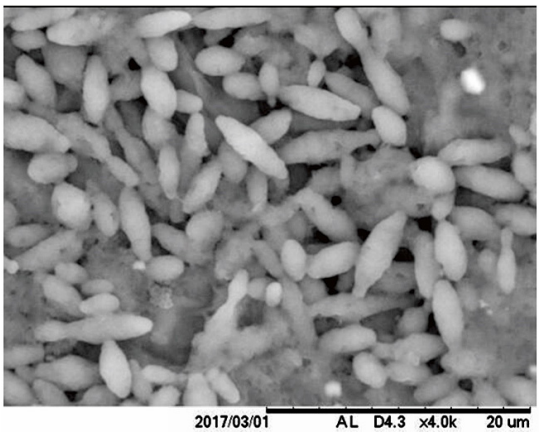

Fig. 1: The SEM Image of Yeast in fFJ of *M. Citrifolia*

DPPH Assay

The free radical scavenging activity of fFJ of *M.citrifolia* was measured based on DPPH assay according to the previous method[17] with minor modification. The assay is based on the ability of a substrate to donate a hydrogen atom in order to scavenge the DPPH radical. DPPH solution (0.6 mM in ethanol) was prepared and 1 ml of this solution was added to 3 ml of sample in various concentration. The mixture was immediately vortexed and incubated for 30 minutes in darkness at room temperature. The decrease in absorbance was measured at 517 nm using a spectrophotometer. BHT (1.67 – 53.00 µg/ml) and ascorbic acid (10 – 80 µg/ml) were used as reference solutions and ethanol was used as a blank solution. The percentage of inhibition activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_{\text{Control}} - A_{\text{sample}})}{A_{\text{Control}}} \times 100$$

Where A control is the absorbance of control; A sample is the absorbance of sample. The concentration of the sample and the references required to scavenge 50% of the DPPH radical was defined as IC_{50} and was determined by the graph plotted against the percentage of inhibition. The value expressed as µg GAE/ml and was compared with the reference solutions.

Tabel 3: The DPPH inhibitory activity (IC_{50}) of fFJ of *M.citrifolia* and standards

Concentration (ug GAE/ml)	Inhibition (%)	IC_{50} (prediction) (ug GAE/ml)
1.49	10.98 ± 2.45	
2.98	19.41 ± 2.46	
5.97	24.4 ± 2.32	14.09 ± 2.16
8.95	36.19 ± 2.01	
11.93	42.82 ± 1.6	
Ascorbic acid		53.24 ± 0.82
BHT		21.36 ± 0.80

Statistical Analysis

All assays were carried out in triplicate. The results were expressed as mean value and standard deviation (SD). Regression method was used to calculate IC_{50}.

Results and Discussion

Characteristic of fFJ of *M. Citrifolia*

Due to fermentation process, the juice was dark in color and had low pH (pH 4).

The main microbe found in fFJ is yeast (Figure 1). The SEM image showed cylindrical yeast cells with budding on the apical site. Based on the 16S sequencing method, the yeast was identified as Candida. Interestingly, the yeast was not able to grow in the normal growth medium for yeast, such as Sabouroud agar.

The plant phenolics are an important class of secondary metabolites that have been associated with the plants' biological activities, such as antidiabetic and antioxidant activities.[18,19] Our study found that fFJ was rich in phenolics as shown in its TPC, 1193 ± 4.72 µg GAE/ml. When the juice extract was further evaluated for its chemical compositions, the LC-MS analysis showed two main peaks (Figure 2), with 146 m/z (100%) and 132 m/z (100%). These are designated for two organic compounds, namely α-ketoglutaric and malic acids, which were in agreement with previous report.[20]

Inhibitory effect of fFJ on the Activity of α-glucosidase

The fruit of *M. citrifolia* has been widely used in the Asia Pacific region for the remedy of various diseases such as diabetes mellitus, urinary tract infection, liver and cardiovascular diseases.21 In the present study, fermented fruit juice (fFJ) of *M. citrifolia* was used for the investigation of antidiabetic properties.

citrifolia inhibited α-glucosidase in a concentration-dependent manner giving rise to IC_{50} of 28.99 µg GAE/ml (Table 1). The IC_{50} of acarbose was 823.99 ± 0.06 µg/ml, which is close to previous report.[22] These results indicated fFJ of *M. citrifolia* is a more effective inhibitor when compared to acarbose under the same assay condition.

Earlier studies reported a positive relationship between the total phenolic content and the ability to inhibit α-glucosidase.[23] Indeed, some plant derived- phenolics have been reported to exhibit more effective α-glucosidase inhibition activities than acarbose.[24,25] In this direction, the observed inhibition activity of fFJ may be related to the action of polyphenol compounds on the enzyme.

Combined Inhibitory Effect of Ffj of *M. Citrifolia* with Acarbose.

The use of the antidiabetic drug acarbose is reported to be related to gastrointestinal adverse effects. Strong inhibition on intestinal α-glucosidase resulted in undigested polysaccharide in the large intestine. This leads to bacterial fermentation which produces gases such as methane and carbon dioxide, causing complications, such as abdominal cramping, flatulence, and diarrhea. Therefore, reducing the dosage of acarbose may potentially reduce the side effects. Previous studies reported that plant materials have inhibitory activities on α-glucosidase[26] indicating that they may be effective therapeutic agents for controlling postprandial hyperglycemia with fewer side effects. Thus, experiments were then performed to evaluate whether fFJ in combination with acarbose has a synergistic, additive, or antagonistic effect.

Combined effects of fFJ of *M. citrifolia* and acarbose on α-glucosidase activities were analysed by

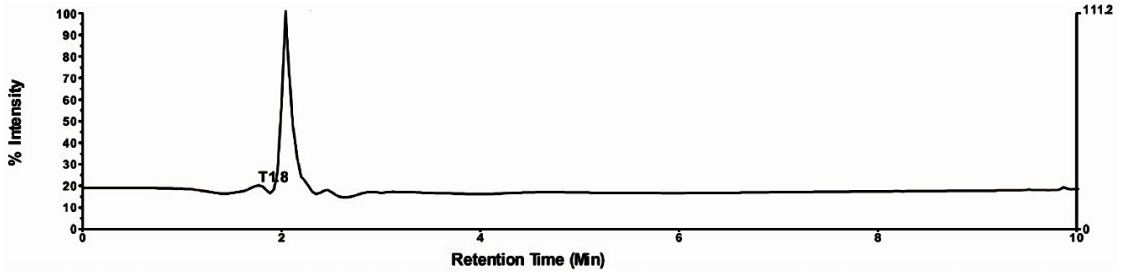

Fig. 2: The LC-MS Profile of fFJ of *M. citrifolia*.

combination index (CI) values. The CI values were in the range of 0.85 – 5.42 (Table 2). Based on the CI values, there were four CI values calculated between 0.85 – 1.11, suggesting an additive effect. These were obtained when acarbose was combined with fFJ at 2.7 folds of its IC_{50}. The additive effect explains the increased inhibitions observed at these combinations. In contrast, an antagonistic effect was observed when acarbose was combined with fFJ at 0.25 – 2 folds of its IC_{50} values.

Our findings revealed that fFJ of *M. citrifolia* showed an additive effect when fFJ at various concentrations combined with acarbose at high concentration. The additive combination between plant polyphenols and acarbose has been reported previously by other researchers.[27] This effect suggests that polyphenols may be used in conjunction with acaborse in maintaining glycemic control, thus may be beneficial in the treatment of T2DM. To the best of our knowledge, this is the first study to investigate the combined effect of fFJ of *M. citrifolia* and acarbose.

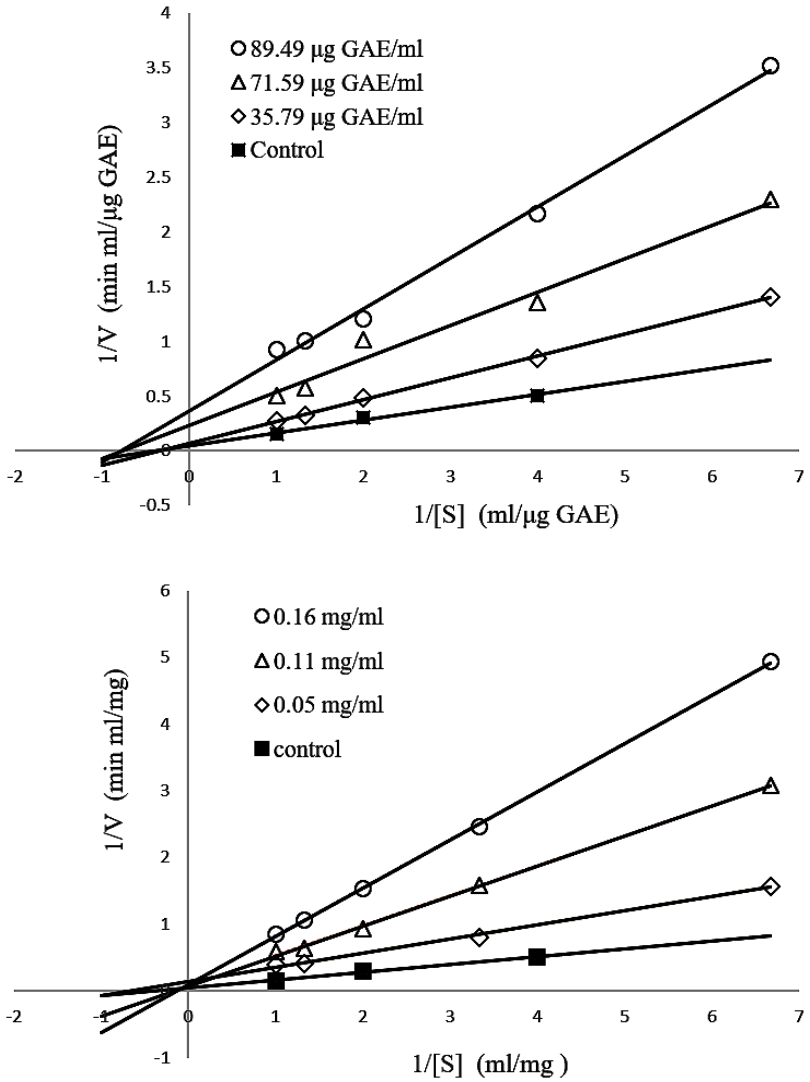

Fig. 3: Lineweaver-Burk plots for the inhibition of α-glucosidase by (a) fFJ *M. citrifolia* and (b) acarbose with *p*NPG as a substrate

Enzyme Inhibition Mechanism

Kinetic assays were carried out to determine the mode of inhibition of fFJ of *M. citrifolia* and acarbose on α-glucosidase. The mode of inhibitions was evaluated based on Lineweaver-Burk plots. For fFJ of *M. citrifolia*, the plots generated straight lines with different intersections on the Y-axis (Figure 3), suggesting a non-competitive inhibition. In contrast, the plots for acarbose gave straight lines with a point of intersection on the Y axis. This observation indicates that acarbose inhibited α-glucosidase in a competitive mode, as also found by others.[10]

The molecular interactions of the plant polyphenols on the specific binding sites on α-glucosidase are still unclear. Modeling studies proposed that the hydroxyl groups in polyphenolics structure may interact with the polar groups (amide, guanidine, carboxyl groups) in the active sites of the enzyme by the formation of hydrogen bonds. These interactions may change the molecular conformation of the enzyme, resulting in a decrease in enzyme activity.[28,29]

Antioxidant Activity of fFJ of *M. Citrifolia*

In the DPPH assay, fFJ exhibited scavenging activities in a concentration-dependent manner, in the range of 1.49 to 11.93 µg GAE /mL (Table 3), obtaining IC_{50} of 14.09 µg GAE/mL. The IC_{50} values of standard compounds BHT and ascorbic acid were 21.36 and 53.24 µg/ml. These results suggest that fFJ had stronger antioxidant activity than standards.

Conclusion

In conclusion, fFJ of *M. citrifolia* was demonstrated to inhibit α-glucosidase in vitro more effectively than acarbose. Its combination with acarbose is possible to be used in the management of type 2 diabetes mellitus. This study can recommend fFJ as a candidate to be developed as a nutraceutical beverage. However, further in vivo studies were necessary to elucidate the combination effect of fFJ and acarbose on α-glucosidase activity.

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Conflict of Interest

We declare we have no conflict of interest.

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