Antioxidant and α-Glucosidase Inhibitory Compounds of *Centella asiatica*

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**Abstract**

*Centella asiatica*, as known as Pegagan was previously reported to have anti-hyperglycemic effects in animal diabetic model rats. However, its α-glucosidase activity *in vitro* assay not yet reported. Our goal in this study is to isolate and identify active compounds as α-glucosidase inhibitor and antioxidant from aqueous ethanol 70% (v/v) extract of *C. asiatica*. The extract was partitioned by *n*-hexane, EtOAc, and *n*-butanol sequentially. Among the fractions tested, EtOAc fraction was showed the highest antioxidant and α-glucosidase inhibitory activities with an IC \(_{50}\) values of 45.42 and 73.17 μg/mL, respectively. The antioxidant activity was conducted by determination of DPPH radical scavenging activity, whereas α-glucosidase inhibitory activity was determined against yeast α-glucosidase. Furthermore, isolation of the ethyl acetate extract yielded two active compounds, which were identified as kaempferol (1) and quercetin (2). Both of the compounds showed good yeast α-glucosidase inhibitory activity with IC\(_{50}\) values of 16.50 and 21.61 μg/mL, respectively. In addition those compounds also could scavenge DPPH radical activity with IC\(_{50}\) values of 9.64 and 11.97 μg/mL, respectively. Due to its ability in reducing α-glucosidase activity and scavenging free radical activity, the *C. asiatica* appears to be a potential as a good resource for future development of antioxidant and antidiabetic drug.

**Keywords:** *Centella asiatica*, α-glucosidase inhibitory activity, kaempferol, quercetin

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1. **Introduction**

Diabetes mellitus is a complex metabolic disordered affected by insulin secretion, insulin action or both. Diabetes mellitus is associated with an increased risk of morbidity, mortality and overall health care worldwide. There are two major categories of diabetes mellitus, such as type 1 diabetes occurs because of the absolute deficiency of insulin and type 2 diabetes (more prevalent category) occurs mostly due to combination of insulin resistance and inadequate compensatory insulin secretary response. A primary risk factor in development of type 2 diabetes mellitus (DM) is postprandial hyperglycemia\(^1\). Furthermore, there are considerable evidences of possible role for reactive oxygen species, generated as a result of hyperglycemia, in causing many of the secondary complications of diabetes, such as nephropathy, retinopathy and neuropathy\(^2\).

One therapeutic approach to treat diabetes is to control postprandial hyperglycemia based on α-glucosidase inhibitor. In digestive tract, α-glucosidase can change oligosaccharide and disaccharide become monosaccharide. α-Glucoside inhibitor can inhibits this enzyme so that it helps in reducing the digestion and absorption of glucose. In other words, postprandial hyperglycemia can be decreased. Nevertheless, using synthetic agents (including acarbose, miglitol and emiglicate) continuously to inhibit α-glucosidase activity should be limited due to can cause many side
effects (such as flatulence, abdominal cramp, vomiting and diarrhea). Several studies have been accomplished to screen natural agents to inhibit α-glucosidase activity without or with fewer side effects. On the other hand, some researchers suggest that natural antioxidants have capability to reduce oxidative damage and decrease the fact of diabetic complications. Other report informed that the combination of α-glucosidase inhibitor and antioxidants will become more effective for the prophylaxis of type 2 diabetes with the use of dietary supplements. Consequently, screening natural drugs using postprandial hyperglycemia and reactive oxidative stress as dual target can be a potential strategy to prevent the multiple disorders of type 2 DM.

For many years, *C. asiatica*, as known as Pegagan in Indonesia, is widely used in folk medicine to treat a wide range of illness. The ethanolic extract of *C. asiatica* showed significant anti-diabetes activity in streptozotocin diabetic rats as judged from body weight, serum glucose, lipids, cholesterol and urea, and liver glycogen levels. In addition, ethanolic extract of *C. asiatica* exhibited significantly higher antioxidative activity than water extract. However, there are no reports that inform possibility of *C. asiatica* in decreasing hyperglycemic by using α-glucosidase inhibitor (*in vitro*). Therefore, the aim of this present study is to identify the active fraction from *C. asiatica* as α-glucosidase inhibitor in decreasing hyperglycemic and reducing scavenging free radical activity.

2. Materials and methods

2.1. General

$^1$H and $^{13}$C NMR spectra were recorded with JEOL-NMR operating at 500 MHz ($^1$H) and 125 MHz ($^{13}$C), using CD$_3$OD as solvent. UV spectra were measured with UV/VIS Hitachi 200, FT-IR Shimadzu. MS spectra were obtained with a Mariner Liquid Chromatography-Mass Spectrometer. Chromatography column was carried out using Merck Si-gel 60 and TLC analysis on pre-coated Si-gel plates (Merck Kieselgel 60 F$_{254}$).

α-Glucosidase from yeast *Saccharomyces cerevisiae* (EC 3.2.1.20), bovine serum albumin and $p$-nitrofenil-$\alpha$-D-glucopyranoside as synthetic substrate of $\alpha$-glucosidase (Wako Pure Chemical Industries, Ltd). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Follin-Ciocalteu 2N were purchased from Sigma. Gallic acid, quercetin, vanillin, Na$_2$CO$_3$, AlCl$_3$, NaNO$_2$, NaOH, H$_2$SO$_4$, were obtained from Merck. Other commercially available chemical were used as received and solvents were distilled before using.

2.2. Plant Materials

*C. asiatica* was collected as whole plant from Bandung. The whole plant was cleaned off of dirt and other debris. The plant was dried at 45°C in an oven and milled to make fine powder.

2.3. Extraction and isolation

The dried entire plant (1.1 kg) was extracted twice with 70% aqueous ethanol at room temperature for 8 h. The filtrate was then evaporated to dryness with a rotary evaporator, under reduced pressure at 40°C to obtain crude extract (260 g). Some of the dried extract (130 g) was suspended in water (1.0 L) and partitioned with $n$-hexane, ethyl acetate (EtOAc), and $n$-butanol, continuously. Each solute fraction was concentrated under vacuum to give $n$-hexane (12 g), EtOAc (7.6 g), $n$-butanol (5 g), and water (98.8 g) fractions. The EtOAc fraction (5 g) was separated by column chromatography (CC) on silica gel using a stepwise gradient from 100% $n$-hexane, to 100% EtOAC, to 50% EtO:MeOH. Eluted fractions were combined on their TLC pattern to yield nine fractions (1-9). Recrystallization of fractions 2 and 3 with CHCl$_3$ and MeOH afforded compound 1 (15 mg) and 2 (15 mg).

Compound 1: yellow powder, IR (KBR) $\nu_{\text{max}}$: 3421, 1662, 1608, 1498, 1381, 1012 cm$^{-1}$. $^1$H-NMR (500 MHz, acetone-$d_6$) $\delta$H: 8.17 (2H, d, $J$= 9.0 Hz, H-2', H-6'), 7.03 (2H, d, $J$= 9.0 Hz, H-3', H-5'), 6.54 (1H, d, $J$= 1.9 Hz, H-6), and 6.27 (1H, d, $J$= 1.9 Hz, H-8). $^{13}$C-NMR (125 MHz, acetone-$d_6$) $\delta$C: 176.7 (C-1), 165.1 (C-7), 162.5 (C-5), 160.2 (C-4'), 157.9 (C-9), 147.1 (C-3) 136.7 (C-2), 130.5 (C-2', C-6'), 123.4 (C-1'), 116.4 (C-3', C-5'), 104.2 (C-4), 99.2 (C-6), 94.6 (C-8). The yielded a squasi-molecular ion peak [M+H]$^+$ at $m/z$ 287.2214 for C$_{15}$H$_{10}$O$_6$. The MS and NMR data were fully agreement with literatures of kaempferol.
3. Compound 2: yellow powder, IR (KBR) $\nu_{\text{max}}$: 3271, 1664, 1610, 1357, 1097 cm$^{-1}$. $^1$H-NMR (500 MHz, acetone-$d_6$) $\delta_H$: 7.82 (1H, d, $J = 2.6$ Hz, H-2'), 7.71 (1H, dd, $J = 1.9$ Hz, 8.5 Hz, H-6'), 6.99 (1H, d, $J = 8.5$ Hz, H-3'), 6.52 (1H, d, $J = 2.6$ Hz, H-8), 6.26 (1H, d, $J = 1.9$ Hz, H-6)). $^{13}$C-NMR (125 MHz, acetone-$d_6$) $\delta_C$: 176.6 (C-1), 165.1 (C-7), 157.8 (C-9), 148.4 (C-5), 145.9 (C-2), 148.4 (C-4'), 146.9 (C-3), 136.8 (C-2'), 123.8 (C-1'), 121.5 (C-2'), 116.2 (C-3'), 115.8 (C-6'), 104.1 (C-4), 99.2 (C-6), 94.5 (C-8). The yielded a squasi-molecular ion peak [M+H]$^+$ at $m/z$ 303.2372 for C$_{15}$H$_{10}$O$_7$. The MS and NMR data were compatible with literatures of quercetin.$^7,8$

2.4. Determination of Total Phenol Content (TPC)

The total phenol content was determined by using the Folin-Ciocalteau (FC) method according to Singleton (Singleton et al., 1999)$^9$ with some modifications. For each sample, 500 $\mu$L of the samples (100 $\mu$g/mL) was added to 3 mL distilled water and 500 $\mu$L of 2N FC reagent. The mixture was allowed to stand at room temperature for 8 min, and then 1.5 mL of 20% sodium carbonate was added to the mixture. The reaction mixture was incubated at room temperature for 2 hours. The resulting blue complex was measured at 765 nm. Gallic acid was taken as standard for the calibrating curve. The total polyphenol content was calibrated using the calibration curve based linear equation. The total polyphenol content was expressed as mg gallic acid equivalent/g dry extract.

2.5. Determination of Total Flavonoid Content (TFC)

The TFC were assessed following a previously reported spectrophotometric method with minor modification.$^{10}$ Briefly, for each samples, an aliquot of 500 $\mu$L extract were diluted with 3.2 mL of water. Initially, 150 $\mu$L of 5% NaNO$_2$ solution was added to each sample. After 5 minutes, 150 $\mu$L of 10% AlCl$_3$ was added. Then, 6 minutes later, 1 mL of 1.0M NaOH was then added and mixed well. Absorbance was read at 510 nm. Quercetin was taken as standard for the calibrating curve. The total flavonoid content was calibrated using the calibration curve based linear equation. The total flavonoid content was expressed as mg quercetin equivalent/g dry extract.

2.6. Determination of $\alpha$-glucosidase activity

The enzyme inhibition activity for $\alpha$-glucosidase was assessed according to the methods reported by Kim et al. (2004), with minor modifications. The assay uses pNPG ($p$-nitrophenyl-$\alpha$-D-glucopyranoside) as the substrate, which is hydrolyzed by $\alpha$-glucosidase to release $p$-nitrophenol, a color agent that can be monitored at 405 nm. Briefly, 5 $\mu$L of a sample solution dissolved in DMSO at various concentrations (5 to 50 $\mu$g/mL) was mixed with 495 $\mu$L of 100 mM phosphate buffer (pH 7.0) and 250 $\mu$L of 3 mM pNPG solution. The reaction mixture was pre-incubated for 5 min at 37$^\circ$C, the reaction was started by adding 250 $\mu$L of the enzyme solution (0.065 unit/mL). Then, incubation was continued for 15 min. The reaction stopped by adding 1 mL of 0.1 M Na$_2$CO$_3$. The released $p$-nitrophenol was monitored at 400 nm.

2.7. Determination of DPPH radical scavenging activity

The scavenging effect of the extracts on the DPPH radicals was estimated according to the method of Hamid et al., 2002,$^6$ 0.5 mL each sample (10-200 $\mu$g/mL) in methanol was mixed with 0.5 mL of daily prepared methanol 1mM DPPH solution. Then, methanol were added until the total volume of mixture get 2.5 mL. The reaction was shaken well and incubated in dark at 37$^\circ$C for 30 minutes. Then, the absorbance was measured at 517 nm. A blank was used to remove the influence of the color from samples.

The percent inhibition of $\alpha$-glucosidase inhibitory and DPPH radical scavenger activities were assessed using the following formula: $\%$ Inhibition $= [1 - (A_s/A_0)] \times 100$, where $A_0$ was the absorbance of the control reaction and $A_s$ was the absorbance in the presence of the sample. The $IC_{50}$ values were calculated from the mean inhibitory values by applying logarithmic a regression.
3. Result and discussion

*C. asiatica* is being used as a natural source of medicine for long time. The main active compound of *C. asiatica* are pentacyclic triterpenes which showed wide range of defensive and therapeutic effect, most prominently influencing of collagen production and deposition in wound healing\textsuperscript{11}. Recently study of ethanol extract of *C. asiatica* showed highly promising hypoglycemic activity which capable inhibited absorption of glucose both by inhibition of intestinal disaccharides enzyme and $\alpha$-amylase\textsuperscript{12}. However, there are no reported studies of $\alpha$-glucosidase inhibitor compounds from *C. asiatica* extract. In the present study, the EtOAc extract of *C. asiatica* showed the highest activity against $\alpha$-glucosidase and free radical DPPH. In particular the EtOAc extract has the highest content of total phenol and flavonoid compound. Total phenol and flavonoid were presented in Table 1. The total phenol contents were expressed as mg/g gallic acid equivalent using the standard curve equation: $y=0.0901x+0.0548$, $R^2=0.9959$, where $y$ is absorbance at 765 nm and $x$ is total phenolic content in the different samples of *C. asiatica* in mg/g. The result showed total phenol of each sample has various ranged from 24.1 to 187.3 mg/g and decreased in the following order: EtOAc fraction > 70% aqueous ethanol extract > n-hexane fraction. In addition, total flavonoid determined as mg/g. Quercetin used as standard with curve equation $y=0.0108x-0.004$, $R^2=0.9975$. Samples have total flavonoid content ranged from 51.8 to 438.0 mg/g and decrease in the following order: EtOAc fraction > n-hexane fraction > ethanol 70% extract > n-butanol fraction. Based on the results, EtOAc fraction showed the highest phenol and flavonoid content compared to ethanol 70% extract, n-hexane fraction, and n-butanol fraction.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Phenol (mg/g)</th>
<th>Total Flavonoid (mg/g)</th>
<th>Antioxidant Inhibitory (%)$^1$</th>
<th>$\alpha$-glucosidase Inhibitory (%)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol 70% extract</td>
<td>24.1 ± 0.27</td>
<td>51.8 ± 0.96</td>
<td>31.21</td>
<td>5.39</td>
</tr>
<tr>
<td>n-hexane fraction</td>
<td>3.5 ± 0.07</td>
<td>92.3 ± 0.19</td>
<td>7.06</td>
<td>5.86</td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>187.3 ±0.84</td>
<td>438.0 ±1.34</td>
<td>92.99</td>
<td>37.95</td>
</tr>
<tr>
<td>Butanol fraction</td>
<td>8.4 ± 0.31</td>
<td>85.8 ± 0.60</td>
<td>11.81</td>
<td>13.30</td>
</tr>
</tbody>
</table>

$^1$% inhibitory activity at 200 $\mu$g/mL.  
$^2$% inhibitory activity at 50 $\mu$g/mL.

The antioxidant activity of *C. asiatica* was evaluated by its ability to scavenge DPPH free radicals. The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H\textsuperscript{4}. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The EtOAc showed highest inhibitory DPPH free radical up to 92.99% at 200 µg/mL (Table 1), with an IC\textsubscript{50} value of 45.42 µg/mL. The IC\textsubscript{50} values were calculated from the mean inhibitory values by applying logarithmic a regression analysis.

Based on previous report, presence of substances with free hydroxyls plays important role in powerful antioxidant activity of polar extract\textsuperscript{13}. Siriwardhana and Shahidi (2002)\textsuperscript{13} informed that there was a strong positive correlation between total polyphenol content and DPPH free radical scavenging activity\textsuperscript{14,15}. In this case, phenol and flavonoid contribute to confer scavenging ability in *C. asiatica*. However, some extracts of *C. asiatica* did not have the positive correlation between total flavonoid and antioxidant activity. For example, the results exhibited that total flavonoid in ethanol 70% extract was smaller than n-butanol fraction, but the antioxidant inhibitory activity of ethanol 70% extract was bigger than n-butanol fraction. A n-butanol extract of *C. asiatica* assumed contain flavonoid-glycosides. Flavonoid would give antioxidant activity which has OH in ortho C-3’, C-4’, OH in C-3, oxo function in C-4, double bond at C-2 and C-3. The OH with ortho position in C-3’-C-4’ had the highest influence to antioxidant activity of flavonoid\textsuperscript{16}. The flavonoid aglycones would give higher antioxidant activity than flavonoid glycosides\textsuperscript{16}. Presence of these glycosides can disturb the double bond between C-2 and C-3 and a free hydroxyl in
C-3 position of flavonoid that can control the ability of flavonoid to inhibit oxidation processes\(^\text{17}\). This condition can be a possibility in reducing antioxidant activity.

One of the therapeutic approaches for preventing diabetes mellitus is to retard absorption of glucose via inhibition of \(\alpha\)-glucosidase and \(\alpha\)-amylase\(^\text{18}\). In this study, we assessed the antidiabetic activity of fraction and isolated compounds against yeast \(\alpha\)-glucosidase. A yeast \(\alpha\)-glucosidase enzyme from \(S.\ cerevisiae\) which categorized as \(\alpha\)-glucosidase type was selected for general screening of \(\alpha\)-glucosidase inhibitors\(^\text{18}\). The result of \(\alpha\)-glucosidase assay in this experiment reported that EtOAc fraction has the highest \(\alpha\)-glucosidase inhibitory activity (Table 1). The optimal concentration of EtOAc fraction for the 50\% inhibition (IC\(_{50}\)) against \(\alpha\)-glucosidase from yeast was 73.12 \(\mu\)g/mL. To clarify the active constituents in the EtOAc fraction of \(C.\ asiatica\), we further separation and isolation of active compound by column chromatography to yielded two pure active compounds. Identification of active compounds was conduct by spectroscopic methods (FTIR, LCMS, and NMR).

Compound 1 was isolated as yellow powder. FT-IR spectrum of 1 in KBr pellet exhibits peaks at 342 cm\(^{-1}\) (OH), 1662 cm\(^{-1}\) (>C=O), 1608 cm\(^{-1}\) (C-C aromatic stretch), and 1381cm\(^{-1}\) (O-H bending). The ESI-MS yielded a quasi-molecular ion peak [M+H]\(^+\) at \(m/z\) 287.2 revealed the molecular formula to be \(C_{15}H_{10}O_{6}\) indicating seven degrees of unsaturation. The \(^1\)H-NMR spectrum showed two peaks at \(\delta\) 6.27 (1H, d, \(J = 1.9\) Hz) and 6.54 ppm (1H, d, \(J = 1.8\) Hz) consistent with the meta protons of flavonoid H-6 and H-8 on A-ring and an AA'BB' system at \(\delta\) 8.17 (2H, d, \(J = 9\) Hz, H-2', 6') and 7.03 (2H, d, \(J = 9\) Hz, H-3', 5') corresponding to the protons on B-ring. Based on FT-IR, MS, \(^1\)H NMR and \(^13\)C NMR data, 1 was identified as 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4\(H\)-chromen-4-one (kaempferol)\(^\text{1}\).

Compound 2 was obtained as yellow powder. \(^1\)H and \(^13\)C NMR spectra of 1 and 2 were similar. The \(^1\)H-NMR spectrum showed two peaks at \(\delta\) 6.26 (1H, d, \(J = 2.6\) Hz) and 6.52 ppm (1H, d, \(J = 2.6\) Hz) consistent with the meta protons of flavonoid H-6 and H-8 on A-ring. However, significant difference was found around an ABX system at \(\delta\) 7.82 (1H, d, \(J = 2.6\) Hz, H-2'), 7.71 (1H, dd, \(J = 1.9\) Hz, 8.5 Hz, H-6') and 6.99 (1H, d, \(J = 8.5\) Hz, H-5'). The EI-MS of compound 2 yielded a quasi-molecular ion peak [M-H]\(^-\) at \(m/z\) 303.2372 for \(C_{15}H_{10}O_{7}\). These results clearly indicated the hydrogen atom (H) of 1 to be replaced by a hydroxyl group in 2. The MS and \(^1\)H-NMR data were compatible withthose literatures of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4\(H\)-chromen-4-one (quercetin)\(^\text{1,11}\).

Based on the spectra data and compared with previous literature, the compounds were identified as kaempferol (1) and quercetin (2) as showed in Figure 1.

![Fig. 1. Structure of kaempferol (1) and quercetin (2)](image)

Both of the compounds showed good yeast \(\alpha\)-glucosidase inhibitory activity with IC\(_{50}\) values of 16.50 and 21.61 \(\mu\)g/mL, respectively. In addition those compounds also could scavenge DPPH radical activity with IC\(_{50}\) values of 9.64 and 11.97 \(\mu\)g/mL, respectively. Since these active compounds are flavonol compounds, their inhibitory activities are not significant different. In this study, kaempferol more active than quercetin, due to different number of hydroxyl group in ring B. These result was supported by the other studies which reported that kaempferol and quercetin as multi-targeting compounds have a potential for Type 2 Diabetes Mellitus (T2DM) treatment\(^\text{8}\).

**Conclusion**

The obtained result in this study clearly demonstrated that kaempferol and quercetin from ethyl acetate fraction of \(C.\ asiatica\) was contributed in its \(\alpha\)-glucosidase inhibitory and antioxidant activity. The findings indicated that \(C.\ asiatica\) could be considered as potential source of natural antidiabetic and antioxidant agents.
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