Antidiabetic, Antibacterial and Antioxidant Activities of Different Extracts from *Bracea javanica* (L.) Merr Seeds

Adelina Simamora¹*, Kris Herawan Timotius¹, Adit Widodo Santos⁴

**ABSTRACT**

Introduction: The seeds of *B. javanica* are known as herbal material for anticancer, antimalaria and antiamaeoba. Limited report is available on their antidiabetic and antibacterial properties. Methods: Methanol (ME) and Ethyl acetate extracts (EAE) were studied for their inhibition activities on α-glucosidase in vitro, including combination of ME with acarbose and inhibition mechanism. Antibacterial activity was tested by well diffusion and microdilution methods. The extracts were evaluated for their in vitro antioxidant property by DPPH assay, as well as their phenolic and flavonoid contents. Results: ME exhibited a strong α-glucosidase inhibition activity (*IC₅₀* of 2.938 mg/ml) compared to EAE and acarbose (*IC₅₀* of 1745.05 and 823.99 µg/ml, respectively). A low dose of ME gave an additive inhibition on α-glucosidase when combined with acarbose. By a kinetic analysis, ME was found to inhibit α-glucosidase in a mixed-type inhibition. Both ME and EAE showed strong antibacterial activities against gram negative and positive bacteria. The strongest inhibition was observed against *C. violaceum* and *S. mutans* for ME (MIC of both 0.387 mg/ml) and *P. aeruginosa* for EAE (MIC 2.938 mg/ml). Both extracts showed weaker antioxidant activities than standards; IC₅₀ of 164.73 and 4225.40 µg/ml, respectively. ME was rich in phenolics (271.97 µg/ml) compared to EAE and acarbose (IC₅₀ of 273.917 µg/ml). Conclusion: This study can recommend *B. javanica* seeds as a source for mixed type inhibition with acarbose may have important role for the treatment of diabetes mellitus. Key words: Additive inhibition, Antibacterial, *Bracea javanica*, α-glucosidase inhibitor, Mixed type inhibition.

**INTRODUCTION**

Diabetes Mellitus (DM) is one of the most common metabolic disorders, which is characterized by high level of postprandial blood glucose (hyperglycemia). This condition may be caused by defect in insulin secretion as a result of impaired pancreatic β-cells, as in type 1 DM. In type 2 DM, this condition may be due to a combination of insulin resistance and insufficient insulin secretion.¹ Around the world, diabetes has affected 346 million people and the number is estimated to increase by twofold in 2030.² Glycemic control is considered an effective therapy in the management of T2DM. Blood glucose is predominantly sourced from enzymatic hydrolysis of dietary polysaccharides by α-amylase and α-glucosidase in the epithelium of small intestine. Accordingly, inhibition of these enzymes can delay the conversion of dietary carbohydrates into glucose, thus lowering postprandial plasma glucose. Therefore, inhibitors that target carbohydrate hydrolyzing enzymes become an important approach in the treatment of hyperglycemia. α-Glucosidase inhibitor drugs such as viglibose, acarbose and miglitol are currently used for treating T2DM. Nevertheless, they are reported to cause unfavorable side effects associated with gastrointestinal complications such as loose stools, flatulence and abdominal discomfort.³ In view of this, finding new α-glucosidase inhibitors with minimal adverse side effects is of great importance.

Plant materials are good source for drugs, in particular α-glucosidase inhibitors.⁴ *Bracea javanica* (L.) Merr which belongs to Bracea genus and Sima-r出版社 family, is an evergreen shrub of about 3 m high. It is widely distributed in Asia Pacific regions such as Indonesia, Thailand and Malaysia. The seeds from *B. javanica* have been used traditionally for treating diabetes mellitus.⁵ *B. javanica* has been previously studied for their biological activities, such as antimalarial,⁶ anti-protozoal,⁷ anti-inflammatory,⁸ antileukemic⁹ and anthelminthic.¹⁰ Some chemical compounds have been reported to be isolated from this plant,¹¹ including quassinoid which is known to have various pharmacological properties such as antitumor and anticanacer.¹² To date, only a few studies reported the antidiabetic activities of this plant, such as inhibition activity of the seed extracts against glycogen phosphorylase¹³.
and in vivo studies using animal models. Nonetheless, there is no report available on in vitro inhibitory properties of the seed extract against α-glucosidase. In addition, the mechanism underlying the inhibition and their interaction with the prescribed synthetic inhibitor acarbose is still unknown. Therefore, the study aimed to investigate α-glucosidase inhibition properties of *B. javanica* seeds extracts by in vitro enzymatic experiments, including their combination effect with acarbose. The present study extends the investigation by examining the antibacterial and antioxidant potentials of the extracts.

**MATERIALS AND METHODS**

**Chemicals and plant material**

All solvents and chemicals were analytical grade. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), Folin-Ciocalteu’s phenol reagent, *p*-nitrophenyl-α-D glucopyranoside and α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae* were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rutin and Gallic Acid (GA) were purchased from Santa Cruz Biotechnology (Dallas, USA). Acarbose was purchased from United States Pharmacopeia (Rockville, USA).

The seeds of *B. javanica* were collected from Padang, West Sumatera, Indonesia in March 2016. The plant was identified by one of the authors (KHT). Thoroughly washed seeds were air-dried in the shade and ground to a coarse powder. It was then kept in an air-tight container at 4°C until further used.

**Seed extraction**

The powdered seeds were macerated at ambient temperature overnight successively with n-hexane, ethyl acetate and methanol. Each extract was filtered and dried by vacuum evaporator. The Ethyl Acetate (EAE) and Methanol Extracts (ME) were kept at 4°C in darkness until further use.

**Determination of total phenolic content**

A colorimetric method using Folin-Ciocalteu reagents was used to estimate the total phenolics of the seed extract, based on previously described procedure. A gallic acid (12.5 – 200 µg/mL) standard curve was obtained with a linear coefficient value of 0.9976 and was used for the estimation of phenolic content of the seed extracts. Data were presented as mg gallic acid equivalent (mg GAE)/100 g dry weight of biomass.

**Determination of total flavonoid content**

The total flavonoid content of the seed extracts was estimated based on aluminum chloride method with slight modification. A rutin (3.2 – 500 µg/mL) standard curve was obtained with a linear coefficient value of 0.9999. This was then used for the estimation of total flavonoid content of the extracts. Data were presented as mg rutin equivalent (mg RE)/100 g of dry weight of biomass.

**Determination of α-glucosidase inhibitory activity**

Evaluation of inhibitory activity of the seed extracts against α-glucosidase was performed based on our procedure. Each extract was diluted to obtain an appropriate dilution series of different concentrations. Into each sample (50 µL) was added DMSO (5 µL), phosphate buffer (45 µL, 50 mM, pH 6.8) and α-glucosidase (50 µL, 0.5 unit/mL). The solution was kept at 37°C for 5 min, after which substrate (100 µL, 1 mM *p*-nitrophenyl-α-D glucopyranoside) was added to start the reaction. The reaction mixture was then incubated at 37°C for 20 min and stopped by the addition of 750 µL Na₂CO₃ (100 mM). Absorbance was read at 405 nm on a spectrophotometer (Biochrom Libra S-22, Germany). The absorbance of each sample was corrected with blank sample. Acarbose was used as a positive control of α-glucosidase inhibitor. The percentage (%) of inhibition was calculated as follows:

\[
\alpha - \text{Glucosidase inhibition} (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%
\]

Where: A control is absorbance of control and A sample is absorbance of sample. The IC₅₀ value was described as the extract concentration needed to inhibit 50% of α-glucosidase activity under the assay condition. All measurements were performed in triplicates.

**Determination of combined inhibition effect of acarbose and extract on α-glucosidase**

Drug combination method developed by Chou-Talalay was used to evaluate combined inhibition effect of acarbose and the most active extract on α-glucosidase. It required for the inhibitors to be used individually and in combination at different ratios of IC₅₀ obtained from the α-glucosidase assay. α-Glucosidase (0.5 U/mL) and PNPG (1 mM) were used for the assay. The % of inhibition data were then treated using Compusyn® software to calculate Combination Index (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 α-glucosidase inhibition mode**

The inhibition mode of the most active extract was determined by performing an enzyme kinetic assay based on previous method. In this assay, substrate (*p*-nitrophenyl α-D-glucopyranoside) was prepared at increasing concentrations (0.25 – 1.25 mM). The solution was incubated with α-glucosidase (0.5 U/mL) in the absence and presence of extract (inhibitor) at different concentrations in phosphate buffer (50 mM pH 6.8). Double reciprocal plots of 1/[S] and 1/[V] were generated based on the Lineweaver-Burk method to describe the inhibition mode of the extract.

The Lineweaver-Burk graph was further processed to obtain the values of Kᵢ and Kᵢ’. The Kᵢ’ value which was the dissociation constant of inhibitor binding to α-glucosidase was obtained from the secondary replot of the slope vs [I] of the Lineweaver-Burk graph. The Kᵢ’ value which is the dissociation constant for inhibitor binding to enzyme-substrate (ES) complex was obtained from secondary replot of intercept vs [I].

**DPPH radical scavenging activity**

*B. javanica* seed extracts was evaluated for their antioxidant activities by measuring the effect of ME and EAE on DPPH radicals, according to a method previously described. Into 1 ml of DPPH solution (0.6 mM in ethanol) was added 3 mL of extract or standard solutions (Ascorbic acid and BHT) at different concentrations (10 – 100 µg/mL). The reaction was incubated at ambient temperature in darkness for 30 min. The absorbance was measured at 517 nm with spectrophotometer (Bio Chrom Libra S22). Ethanol (3 mL) in place of extract is used as control. The percentage inhibition activity was determined using the following equation:

\[
\text{DPPH inhibition} (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%
\]

Where: A control is absorbance of control solution and A sample is absorbance of sample. The IC₅₀ value was concentration of extract needed to scavenge 50% of DPPH radicals under the assay condition. All measurements were performed in triplicates.
Antibacterial activity: Agar well diffusion

Evaluation of antibacterial activity of ME and EAE was conducted by an agar well diffusion method on eight bacterial strains i.e Staphylococcus aureus ATCC 25923, Staphylococcus aureus COWAN I, Staphylococcus epidermidis ATCC 12228, Chromobacterium violauncium WT ATCC 12472, Escherichia coli ATCC 25922, Escherichia coli ATCC 35218, Pseudomonas aeruginosa PAO I and Streptococcus mutans ATCC 14721. Mueller-Hinton Agar (OXOID CM0337) was used as growth medium. Bacterial suspension (100 μL, McFarland 0.5) was spread on the agar plate. The well diameter was 0.5 cm and filled with 20 μL of the extracts. The incubation was carried out at 37°C for 24 h. The inhibition zone was observed and recorded.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of ME and EAE were determined based on a microwell dilution method in a 96-well plate against the selected bacterial strains. The initial solution was prepared by dissolving each extract in DMSO. This was then followed by preparing serial dilution to give different concentrations. Negative controls were prepared containing bacteria without antimicrobial solution. The bacterial toxicity of the solvent was also evaluated. MIC was determined as a concentration to inhibit the bacterial growth after 24 h. MIC was observed as the last dilution showing no apparent growth (turbidity). For the determination of MBC, a 10 μL of the culture medium was aspirated from each tube (in the MIC test) that showed no noticeable growth and sub-culturing it on fresh Mueller-Hinton Agar. This was then incubated at 37°C for 24 h. MBC was determined as the lowest concentration of the extracts required to kill the bacterium. MBC was identified as the least concentration showing no noticeable growth on the agar subculture.

Statistical analysis

Experiments in this study were conducted in three replicates. Results were expressed as mean ± Standard Deviation (SD). IC\textsubscript{50} values and the kinetics of enzymatic reactions were determined using regression method.

RESULTS

Extract yields, total phenolic and flavonoid contents

The yields from methanol and ethyl acetate extractions were 27.33% and 1.90% (w/w), respectively. The total phenolic contents in ME and EAE were 277.54 and 1.86 mg GAE/100 g DW (dried weight of biomass), respectively. The total flavonoid contents in ME and EAE were 124.90 and 0.08 mg RE/100 g DW.

The yields are in agreement to those obtained from the previous study by Ablat et al.\textsuperscript{15} who reported the lowest yield for ethyl acetate seed extract compared to more polar solvents such as water which obtained the highest yield. However, the same author obtained high contents in phenolics and flavonoids in Ethyl Acetate Extract (EAE) compared to Methanol Extract (ME). This can be attributed to the difference in extraction methods used.

\textit{a} Glucosidase inhibition activity

To evaluate the antidiabetic potential of \textit{B. javanica} seeds, the extracts were assessed for their inhibition activity on a-glucosidase. Acarbose, a prescribed synthetic inhibitor, was selected as a positive control to compare the inhibitory activities of the samples. Firstly, ME and EAE were tested for their inhibition activity against a-glucosidase in dose-dependent experiments. Both ME and EAE inhibited a-glucosidase in a concentration dependent manner (Table 1). When the concentration of the extracts increased, the enzyme activity was decreased rapidly. The

| Table 1: a-Glucosidase inhibitory activities of \textit{B. javanica} seed extracts and acarbose (mean ± SD). |
|---------------------------------|---------------|------------------|-------------------|
| **Concentration** (μg/ml) | **Inhibition (%)** | **IC\textsubscript{50} (μg/ml)** |
| ME\textsuperscript{a} | 98.95 | 20.08 ± 8.09 | 271.97 ± 24.99 |
| | 197.90 | 35.49 ± 4.05 |
| | 296.85 | 63.54 ± 7.18 |
| | 395.80 | 69.11 ± 9.91 |
| EAE\textsuperscript{a} | 376.12 | 3.34 ± 1.58 | 1745.05 ± 30.34 |
| | 752.24 | 19.55 ± 8.22 |
| | 1504.48 | 39.29 ± 8.89 |
| | 1880.60 | 56.07 ± 3.72 |
| Acarbose | | 823.99 ± 0.06 |

\textsuperscript{a}ME: methanol extract.

\textsuperscript{b}EAE: ethyl acetate extract.

Experiments were conducted at different ratio of their IC\textsubscript{50} values. Values of 0.25, 0.5, 1 and 1.5 represents 0.25, 0.5, 1 and 1.5-fold of their IC\textsubscript{50} values, respectively.

IC\textsubscript{50} values for ME and EAE were 271.97±24.99 and 1745.05±30.35 μg/mL, respectively. The IC\textsubscript{50} values for acarbose was 823.99±0.06 μg/mL, which is close to previous reports.\textsuperscript{25}

\textit{Type of a-glucosidase inhibition}

Further to investigate the mechanism underlying the additive inhibition, the inhibition type of ME on a-glucosidase was examined based on the Lineweaver-Burk double reciprocal plots. Figure 1a exhibited that the inhibition of ME on a-glucosidase produced data lines which intersected at one point at the second quadrant of the graph. This result points to a mixed competitive and noncompetitive mode. Our previous work and
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Secondary plots were generated for the calculation of $K_i$ and $K_i'$. The $K_i$ value which was the dissociation constant of ME binding to α-glucosidase was obtained from the secondary replot of slope vs ME of the Lineweaver-Burk graph. A straight line of $R^2 = 0.9989$ (Figure 1b) and $K_i$ value of $650 \mu$g/mL were obtained. The secondary replots of intercept vs ME obtained a straight line with $R^2 = 0.9051$ (Figure 1c), which is the dissociation constant for ME binding to Enzyme-substrate (ES) complex.

**Antioxidant activity**

DPPH assay assesses the ability of antioxidant compounds to scavenge free radical by donating a hydrogen radical or transferring an electron to the stable DPPH radical, to become its reduced form DPPH-H. Table 3 presents $IC_{50}$ of *B. javanica* seed extracts and standards. Scavenging activity of both ME and EAE on DPPH radical were evident, with ME showed a stronger activity of more than 6 times stronger than EAE. However, compared to the standards BHT and ascorbic acid, the activities were much weaker.

**Antibacterial activity**

Antibacterial activity of seed extracts from *B. javanica* were examined against gram positive bacteria (*S. aureus, S. epidermidis* and *S. mutans*) and gram-negative bacteria (*E. coli, P. aeruginosa* and *C. violacium*) by determining the DIZ, MIC and MBC. The results are presented on Table 4. ME and EAE exhibited notable antibacterial activity against all tested bacteria. In general, ME exhibited stronger activity than EAE with MICs in the range of 0.387 to 3.092 mg/mL and MBC in the range of 0.774 to 6.184 mg/mL. Among different bacteria, gram negative strain *E. coli* and *S. aureus* were found to be the most sensitive to ME (DIZ of 20.75 and 20.20 mm, respectively), whereas *S. aureus* was the most sensitive to EAE (DIZ of 18.90 mm).

**DISCUSSION**

Natural products from plants are still valuable sources to develop alternative compounds with low side effect but having desirable α-glucosidase inhibition property. Ethnobotanical information indicates that around 400 plant medicines have been used to control hyperglycemia. In another report, more than 400 compounds exhibiting inhibitory activity on α-glucosidase have been identified from plant medicine, suggesting the potential of medicinal plants to be employed in management of DM. In this direction, we set to investigate the α-glucosidase inhibition properties of *B. javanica*.

In this work, it was found that the seeds of *B. javanica* extracted in methanol was a more effective α-glucosidase inhibitor, three times stronger than the standard acarbose. On the other hand, EAE showed a weaker inhibition activity when compared with acarbose. Association between polyphenolics and inhibition of key enzymes associated with carbohydrate hydrolyzes has been reported previously. Further, it has been reported that these secondary metabolites modulate enzymatic breakdown of dietary carbohydrates through binding with α-glucosidase. In view of this, the difference in inhibition activities between ME and EAE may be associated with the level of phenolic and flavonoid in the extracts. To the best of our knowledge, the present study is the first time α-glucosidase inhibition activity was reported for *B. javanica*, which may be a new source of inhibitor for T2DM treatment.

Table 3: DPPH radical scavenging activity of *B. javanica* seed extracts and standards (Mean ± SD).

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Inhibition (%)</th>
<th>$IC_{50}$ (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>ME</td>
<td>123.69</td>
<td>9.04 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>247.38</td>
<td>17.01 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>494.75</td>
<td>36.26 ± 0.37</td>
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<tr>
<td></td>
<td>618.44</td>
<td>46.42 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>742.13</td>
<td>56.82 ± 0.43</td>
</tr>
<tr>
<td>EAE</td>
<td>1062.50</td>
<td>25.09 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>2125.00</td>
<td>42.93 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>4250.00</td>
<td>54.60 ± 1.03</td>
</tr>
<tr>
<td></td>
<td>8500.00</td>
<td>72.35 ± 1.03</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>53.24 ± 0.82</td>
<td></td>
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<tr>
<td>BHT</td>
<td>21.36 ± 0.80</td>
<td></td>
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</table>

![Figure 1: (a) Lineweaver-Burk plot for the inhibition of α-glucosidase by *B. javanica* ME. (b) Secondary replot of slope vs ME from the Lineweaver-Burk plot for the determination of $K_i$ ($R^2 = 0.9989$) (c) Secondary replot of Y-intercept vs ME from the Lineweaver-Burk plot for the determination of $K_i'$ ($R^2 = 0.9051$).](image)
In this study it was found that the combination of acarbose at various concentrations and ME at low concentrations exerted an additive effect. This effect indicates that ME may substitute for or be used in conjunction with acarbose in maintaining glycemic control in the management of T2DM. Previous studies have reported combined inhibition of plant extracts and acarbose. For instance, Adisakwattana et al. reported additive inhibition for combination of cinnamon bark aqueous extracts and acarbose on intestinal α-glucosidase. However, no information available on the range of acarbose dose and the amount of cinnamon extracts concentrations required to produce the effect. In the present study ME and acarbose exhibited additive inhibition at low concentrations and turned into moderate antagonistic effect at higher concentrations. Long term use of acarbose leads to accumulation of undigested carbohydrates in the colon, which serves as substrates for bacteria fermentation. In consequence of this is troublesome gastrointestinal symptoms such as diarrhea and flatulence. In some cases, long term use of acarbose was reported to cause acute severe hepatotoxicity. These side effects tend to increase in higher dose, as in the case of chronic T2DM patients where progressive increase dosages are prescribed. Therefore, reduction of the required dose of acarbose may potentially minimize the multiple adverse effects of this medicine. Furthermore, this may ultimately reduce the cost associated with pharmaceutical DM management. Our study suggests that combinations of acarbose and ME at low dose may serve as a possible combination therapy in preference of only acarbose treatment. The study found that B javanica seed extract gave a mixed type inhibition of non-competitive and competitive, whereas acarbose inhibited α-glucosidase in a competitive mode. When compared, the $K_i$ value of ME was almost three times more than $K_i$ value, suggesting that binding of ME to the ES was stronger than ME to enzyme alone. This finding indicates that the inhibition of ME was non-competitive, predominant over competitive. The additive effect exerted from the combination of ME and acarbose is possibly come from the different inhibition binding sites of the two inhibitors at α-glucosidase.

This study also found that the seed extracts showed antioxidant activities. Many studies demonstrated that chronic hyperglycemia as in T2DM induced generation of free radicals through glucolipotoxicity and mitochondrial pathways. This condition may cause oxidative damages in cell membrane, leading to the development of various complications in diabetes, including retinopathy, cardiomyopathy, nephropathy and nephropathy. In particular, destruction in pancreatic β cells which are known to be vulnerable to free radicals ultimately results in impaired insulin secretion. Therefore, antioxidant intake such as from diets may help protect cells from damages, thus may prevent diabetes and diabetic complications.

The present study also found that ME and EAE exhibited good antibacterial activity. Good antibacterial activity observed for EAE indicates that phenolics and flavonoids may not play major role in inhibiting the tested bacteria. Previously, many bioactive compounds have been identified from different parts of B. javanica, such as derivates of quassinoids that had anticancer and antiprotozoal activities. However, to the extent of our knowledge, only a few studies reported antibacterial activities of B. javanica. Previous reports included anti-tuberculosis activity of quassinoids isolated from B. javanica seeds and an antibacterial peptide, named Brucin isolated from the fruit of B. javanica which had specificity for Streptococcus pyogenes. Thus, the current study evidences the potential of the seeds from B javanica to be explored as antibacterial agent.

### CONCLUSION

The present study concludes that methanol seed extract of B. javanica has a therapeutic potential better than ethyl acetate extract, thus can be a new potential source for antidiabetic and antibacterial agent. Combination of B. javanica seed extract with acarbose is likely to be employed in diabetes therapy, with a possibility of reducing acarbose dosage.

### ACKNOWLEDGEMENT

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ABBREVIATIONS

- **ME**: Methanol Extract; **EAE**: Ethyl Acetate Extract; **GAE**: Gallic Acid Equivalence; **DPPH**: 2,2-diphenyl-1-picryl-hydrazyl.

### REFERENCES

ABOUT AUTHORS

Adelina Simamora, MSc (Pharm) is a researcher in the centre for herbal medicine studies and a lecturer in Biochemistry at the Faculty of Medicine Krida Wacana Christian University. At present, her research focus on the bioactive potential of medicinal plants, with special interest in various enzyme inhibition properties. Currently, her work involves various Indonesian medicinal plants and inhibition properties on α-glucosidase, xanthine oxidase, and lipase. Her interest also spans to transition and rare earth metals and their application as sensor in biological fluid.

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