

## Gas Chromatography-Mass Spectrometry Analysis and $\alpha$ -Glucosidase Inhibitory Activity of *n*-Hexane Extract of Bilajang Bulu (*Merremia Vitifolia*) Leaves

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Received: 12 October 2021; Accepted: 7 December 2021; Published: 15 December 2021

### Abstract

One of the most effective treatments for diabetes mellitus is to inhibit  $\alpha$ -glucosidase, which inhibits glucose absorption by the epithelium membrane of the small intestine. In South Sulawesi, Indonesia, *Merremia vitifolia* is used as a traditional medicine for the treatment of diabetes. The aims of this study are to (1) assess the extract's ability to inhibit  $\alpha$ -glucosidase and (2) identify volatile organic compounds in *n*-hexane of *Merremia vitifolia* extract. Extraction was conducted by maceration. The inhibitory activity of quercetin towards  $\alpha$ -glucosidase has the  $IC_{50} = 2.53 \pm 0.16 \mu\text{g/mL}$ , and by *n*-Hexane extract of *Merremia vitifolia* leaves has the  $IC_{50} = 14.4 \pm 1.52 \mu\text{g/mL}$ . Then, *n*-Hexane extract of *Merremia vitifolia* leaves has strong  $\alpha$ -glucosidase inhibitory activity. The compounds that have been identified based on Gas Chromatography-Mass Spectrometry (GC-MS) analysis with similarity index  $\geq 89\%$  which are caryophyllene (0.94%), (*E*)- $\beta$ -famesene (4.72%), neophytadiene (9.78%), phytol (65.94%), 9,12,15-octadecatrienoic acid (6.71%), 1,5-cyclodecadiene (6.76%), squalene (4.48%), stigmaterol (4.08%),  $\gamma$ -sitosterol (10.20%), Serratol (23.12%), vitamin E (2.78%) and lup-20(29)-en-3-one (21.04%). Based on a literature study, the presence of phytol, neophytadiene,  $\beta$ -caryophyllene, stigmaterol,  $\gamma$ -sitosterol, and lup-20(29)-en-3-one have contributed to the strong  $\alpha$ -glucosidase inhibitory activity of *n*-Hexane extract of *Merremia vitifolia* leaves.

Keywords: GC-MS; Inhibition of  $\alpha$ -glucosidase; *Merremia vitifolia*; Phytol

### Introduction

One of the chronic and serious human metabolic disorders is diabetes mellitus. The disease was characterized by a high concentration of plasma glucose, which led to major complications such as cardiovascular disease, diabetic neuropathy, and retinopathy. The number of patients increases every year all around the world,

especially in low-middle income countries, including Indonesia (Kumar et al., 2011; Munasaroh et al., 2018). One of the most effective treatments for diabetes mellitus is to inhibit  $\alpha$ -glucosidase, which inhibits glucose absorption by the epithelium membrane of the small intestine (Kumar et al., 2011). The enzyme catalyzes the hydrolytic cleavage of oligosaccharide into monosaccharide, i.e. glucose to be absorbed, so by the inhibition of

the enzyme, overall absorption of glucose by the small intestine could be delayed, lowering the postprandial blood glucose level, and assisting to avoid of late diabetic complications (Proença et al., 2017). One of the potentials candidates for diabetic drug development is plant secondary metabolites.

*Merremia vitifolia* (*M. vitifolia*) is one of the plants in Convolvulaceae family that is widely grown in Southeast Asian region, including Indonesia (GBIF Secretariat, 2019). This plant in Indonesia is called Akar Bulu. It is known as Bilajang Bulu in South Sulawesi, and its leaves are used as a traditional medicine to treat diabetics (Hasanah et al., 2019). The water extract of the plant has antibacterial activity against *Staphylococcus aureus* and contains flavonoid (Hasanah et al., 2020).

*M. vitifolia* leaves had been evaluated for its antioxidant, thrombolytic, anti-arthritic and anti-nociceptive potential (Akter et al., 2021), but in this research, *n*-Hexane was used to extract the non-polar compound from *M. vitifolia* leaves, and to identify the volatile organic compound by using Gas Chromatography-Mass Spectrometry (GC-MS) and to evaluate the extract activity to inhibit  $\alpha$ -glucosidase.

## Research Methods

### Materials and Tools

Identification of *M. vitifolia* (Burm. f.) Hallier f. (family Convolvulaceae) plant was conducted by the Research Center of Biology, Indonesian Institute of Sciences (LIPI) based on the morphology characteristic of the plant. Fresh leaves of *M. vitifolia* were collected from the forest around Pelita Harapan University. Around 500 g of leaves were collected, cleansed with water, let dry at room temperature, and cut into small pieces. Then it was dried for 36 hours at 80 °C in the oven. The dried pieces of leaves were refined into powder with a blender. As much as 83.69 grams of dried powder were collected. The solvent that was used for maseration was *n*-Hexane (Analytical Grade, Merck). For column chromatography, sea sands, cotton,

ethyl acetate (Analytical Grade, Merck), TLC 60 aluminium plat F254 (Merck), and Silica gel 60 (0.2-0.5 mm) were used for column chromatography.

The phosphate buffer was made by dissolving 3.59 g of  $\text{Na}_2\text{HPO}_4$  in 100 mL of distilled water (solution A) and 1.39 g of  $\text{NaH}_2\text{PO}_4$  in 100 mL of distilled water (solution B). Solution A is added with solution B until it reaches pH 7.0 then stirs it with distilled water so that the volume becomes 200 mL. Enzyme for inhibition test was  $\alpha$ -glucosidase Type I (EC 3.2.1.20) of *Saccharomyces cerevisiae* and its synthetic substrate, *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (*p*-NPG) were purchased from Wako. A maximum of 1.0 mg of  $\alpha$ -glucosidase (62 units / mg) was dissolved in 100 ml of phosphate buffer pH 7.0 containing 200 mg of bovine serum albumin. For testing enzyme stock 10x diluted with phosphate buffer pH 7.0 (equivalent to 0.062 units). *p*-Nitrophenyl- $\alpha$ -D-glucopyranoside 150.65 mg was dissolved in 25 mL of pH 7,0 phosphate buffer. The substrate solution was diluted to the  $\text{Na}_2\text{CO}_3$  0.2 M solution is used to stop the enzymatic reactions.

UV light was used to detect spot in TLC. Rotary evaporator (Heidolph) was used for solvent evaporation, GC-MS (Agilent 7890B with MSD 5977 A) was used to identify the volatile organic compounds in the extract based on NIST17 library.

### Procedure

#### Maseration

Around 83.69 g of dried powder of *M. vitifolia* leaves were separated into two (41.0 g on each) and put them into two Erlenmeyer flasks 1000 mL and added 410 mL of *n*-Hexane into each flask, cover the top of flask with plastic shield and aluminium foil, and stirred with magnetic stirrer for 6 hours at room temperature. Filtered the dissolved extract with filter paper and evaporated the solvent with rotavapour. Collected and weighted the extract, and stored it in dark at room temperature.

### *Inhibition of $\alpha$ -glucosidase assay*

Inhibition of  $\alpha$ -glucosidase assay was conducted based on the report of Munasaroh, Tamat, & Dewi (2018). As much as 4,0 mg of sample was dissolved in 500  $\mu$ L of DMSO as a main solution, then diluted with DMSO in order to obtain a varied concentration of the sample at 50; 25; 12.5; 6.25  $\mu$ g/mL. Quercetin 4,0 mg as a reference substance was dissolved into 500  $\mu$ L of DMSO to obtain a varied concentration of the quercetin at 20; 10; 5; 2.5  $\mu$ g/mL.

Around 10  $\mu$ L of a varied concentration of sample dissolved in DMSO were prepared and 5 min pre-incubated with 250  $\mu$ L of enzyme solution, and 490  $\mu$ L of phosphate buffer 0.1 M (pH 7.0) at 37 °C. And roughly 5  $\mu$ L of various concentration of quercetin dissolved in DMSO were prepared and 5 min pre-incubated with 250  $\mu$ L of enzyme solution, and 495  $\mu$ L of phosphate buffer 0.1 M (pH 7.0) at 37 °C. The addition of 250  $\mu$ L p-NPG 5 mM was to initiate the reaction. After 15 minutes of reaction incubation, 1 mL of Na<sub>2</sub>CO<sub>3</sub> 0.1 M was added to stop the reaction.  $\alpha$ -Glucosidase activity was measured by GC-MS at 400 nm as the concentration of p-nitrophenol (pNP) released. The blank was made with phosphate buffer instead of enzyme and had the right background absorbance. Equation 1 was used to compute the inhibitory activity:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control reaction}}{\text{Absorbance in the presence of sample}} \times 100\%$$

The IC<sub>50</sub> is calculated by using linear regression eq. Where x axis is sample concentration and y axis is % inhibition.

### *Column chromatography*

The eluent used for column chromatography was determined based on the separation of spots with TLC silica gel. The best separation was observed by using 4:1 ratio of *n*-Hexane and ethyl acetate. Column tools set were prepared. About 25 g of silica gel 60 (0.2-0.5

mm) was put into column with eluent of 4:1 ratio of *n*-Hexane : ethyl acetate, respectively. About 0.1 g of *M. vitifolia* leaves extract was dissolved into eluent and put into column for separation.

Separation was conducted with 8 times adding of 20 mL eluent 4:1 ratio of *n*-Hexane: ethyl acetate, and 3 times of adding 20 mL eluent of 8:1 ratio of *n*-Hexane : ethyl acetate. There are 20 fractions that has been collected, for further TLC silica gel evaluation. Based on the TLC spots, fraction 1 and 2 with no clear spot observed. Fraction 3 – 9 have more than one spot in TLC, and fraction 9 – 18 also have more than one spot and shows similar TLC pattern, fraction 19 – 20, no clear spots. Because of the pattern similarity of the spots in the TLC layer, researchers decided to mix together fraction 1 – 9 into fraction of 'F1 Merremia', and fraction 9 – 20 into fraction 'F2 Merremia' for further GC-MS analysis after removal of solvents.

Operation conditions for gas chromatography was automatic sample injection with 1  $\mu$ L injection volume at 250 °C, and helium as carrier gas, CH<sub>2</sub>Cl<sub>2</sub> was used as solvent. The initial temperature 40 °C hold for 1 min, ramp at 10 °C/min to 300 °C for 4 min. Single Quadrupole MS method was used with scan time segment start from mass 30.00 to 600.00. Scan speed 781 [N=3] u/s, scan frequency 1.3/sec, cycle time 754 ms and step size 0.1 m/z.

## **Result and Discussion**

### ***The n-Hexane extract of M. vitifolia leaves***

The weight of *n*-Hexane extract resulted from the maceration of 83.69 g *M. vitifolia* was 0.182 g (0.203% yield). The yield is smaller than ethanol extract (2.61%) reported by Hasanah, et al (Hasanah et al., 2019). The color of the extract was chartreuse with the strong unique odor. The fraction of 'F1 Merremia' has yellow-orange color, while the 'F2 Merremia' fraction has green color.

### ***Inhibition of $\alpha$ -glucosidase results***

The inhibition of  $\alpha$ -glucosidase assay by *n*-hexane extract of *M. vitifolia* leaf was conducted with quercetin as positive control. Table 3 shows  $\alpha$ -Glucosidase inhibitory activity by quercetin has  $IC_{50} = 2.53 \pm 0.16$   $\mu\text{g/mL}$ , and Table 4 shows  $\alpha$ -glucosidase inhibitory activity by *n*-hexane extract has  $IC_{50} = 14.4 \pm 1.52$   $\mu\text{g/mL}$ . Quercetin shows stronger inhibition activity than *n*-Hexane extract. But still, *n*-Hexane extract of *M. vitifolia* leaves has strong  $\alpha$ -glucosidase inhibitory activity.

The  $IC_{50}$  value of  $\alpha$ -glucosidase inhibitory activity of *M. vitifolia n*-Hexane extract is stronger compared to several other plants extract such as *n*-Hexane extracts of *Garcinia fruticose Lauterb* ( $IC_{50} = 643.20$   $\mu\text{g/mL}$ ) (Zahratunnisa et al., 2017), the extract of *Quercus gilva* Blume ( $IC_{50} = 110.0$   $\mu\text{g/mL}$ ), *Xylosoma congestum* Merr. ( $IC_{50} = 182.3$   $\mu\text{g/mL}$ ), *Quercus dentata* Thunb ( $IC_{50} = 42.2$   $\mu\text{g/mL}$ ), *Quercus glauca* Thunb ( $IC_{50} = 44.7$   $\mu\text{g/mL}$ ) and *Podocarpus macrophyllus var maki* ( $IC_{50} = 45.2$   $\mu\text{g/mL}$ ) (Indrianingsih et al., 2015). The tropical plants that had  $\alpha$ -glucosidase inhibitor activity within an  $IC_{50} > 200$   $\mu\text{g/mL}$ , mean weak inhibitory activity (Indrianingsih et al., 2015).

### ***GC-MS analysis***

The GC-MS analysis was conducted to the fraction of 'F1 Merremia' and 'F2 Merremia'. Separation of the *n*-Hexane extract into both fractions before the GC-MS analysis was meant to avoid peaks overlapping and baseline noise in gas chromatography. There are 10 volatile organic compounds (Table 1) that have been identified by GC-MS in fraction 'F1 Merremia' (Figure 1) based on the NIST17 library with a similarity index (SI) higher than 89%. Similarity index less than 89% was not considered. Phytol (48.79%) was the most abundance compound in 'F1 Merremia' fraction. Meanwhile, in the fraction 'F2 Merremia', there 4 volatile organic

compounds (Table 2) that have been identified by GC-MS based on NIST17 library with SI is more than 91% (Figure 2). The most abundance compound in 'F2 Merremia' fraction was Serratol (23.12%).

The GC-MS analysis reveals various compounds in *n*-hexane extract of *M. vitifolia* which several of them have biological activities. Neophytadiene, phytol and Lup-20(29)-en-3-one were presence in both F1 and F2 Merremia fractions. Total of percentage abundance neophytadiene, phytol and lup-20(29)-en-3-one in both fractions were 9.78%, 65.94% and 21.04%, respectively. Phytol is the most abundance chemical, and  $\beta$ -Caryophyllene is the least abundance chemicals that were detected by GC-MS in *n*-Hexane extract of *M. vitifolia* (Figure 3). The high quantity of phytol in *M. vitifolia* was originally documented here, to the best of the researcher's knowledge. Phytol has been found in *Rhaphidophora pinnata* by several different researchers (27.64%) (Tualeka et al., 2018), *Lantana camara* (4.0%) and *L. radula* (29.2%) (Passos et al., 2012), *Argemone mexicana* (8.03%) (Gawade & Farooqui, 2017), *Ipomoea Caprae* and *Merremia Umbellata* (Ganjir et al., 2013), and *Justicia gendarussa* (Phatangare et al., 2017).

Phytol, an acyclic diterpene alcohol, is a precursor for the biosynthesis of chlorophyll, vitamin E, and vitamin K, as well as having antioxidant and antinociceptive properties (Santos et al., 2013). Phytol was shown to be cytotoxic agent against seven tumor cells using the MTT assay in vitro with highest activity to the breast adenocarcinoma MCF-7 (Pejin et al., 2014). Phytol have demonstrated anti-inflammatory, antioxidant, antinociceptive, anxiolytic, metabolism-modulatin, immune-modulating, and antimicrobial effects (Islam et al., 2018). Phytol and its derivative compounds are known as potent immune adjuvant (Chowdhury & Ghosh, 2012).

Phytol and neophytadiene also has been reported to have the  $\alpha$ -glucosidase inhibition activity (Gawade & Farooqui, 2017). The molecular docking analysis depicted that phytol had the best docking binding energy for inhibition of enzyme  $\alpha$ -

glucosidase, compare to neophytadiene and  $\beta$ -Caryophyllene (Oso & Olaoye, 2020).

Table 1. Chemical composition of 'F1 Merremia' fraction

<b>Compound</b>	<b>Retention Time (minute)</b>	<b>% Abundance</b>	<b>Similarity Index</b>
Caryophyllene	12.697	0.94	99
(E)- $\beta$ -Famesene	13.079	4.72	95
Neophytadiene	17.366	2.10	99
Phytol	20.035	48.79	99
9,12,15-Octadecatrienoic acid	20.307	6.71	99
1,5-Cyclodecadiene	20.433	6.76	89
Squalene	25.674	4.48	99
Stigmasterol	28.940	4.08	99
$\gamma$ -Sitosterol	29.482	10.20	99
Lup-20(29)-en-3-one	30.112	3.01	99

Table 2. Chemical composition of 'F2 Merremia' fraction

<b>Compound</b>	<b>Retention Time (minute)</b>	<b>% Abundance</b>	<b>Similarity Index</b>
Neophytadiene	17.346	7.68	99
Phytol	20.068	17.15	91
Serratol	20.383	23.12	99
Vitamin E	27.768	2.78	99
Lup-20(29)-en-3-one	30.024	18.03	99

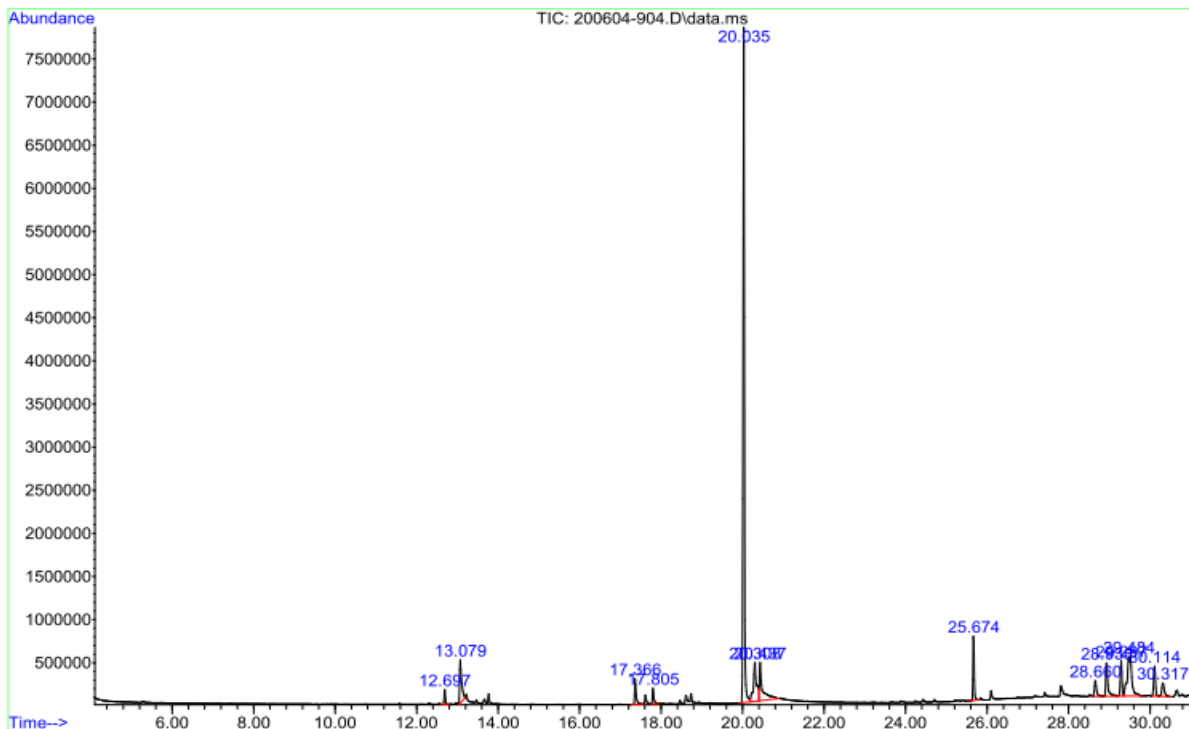


Figure 1. GC-MS Chromatogram of 'F1 Merremia' fraction

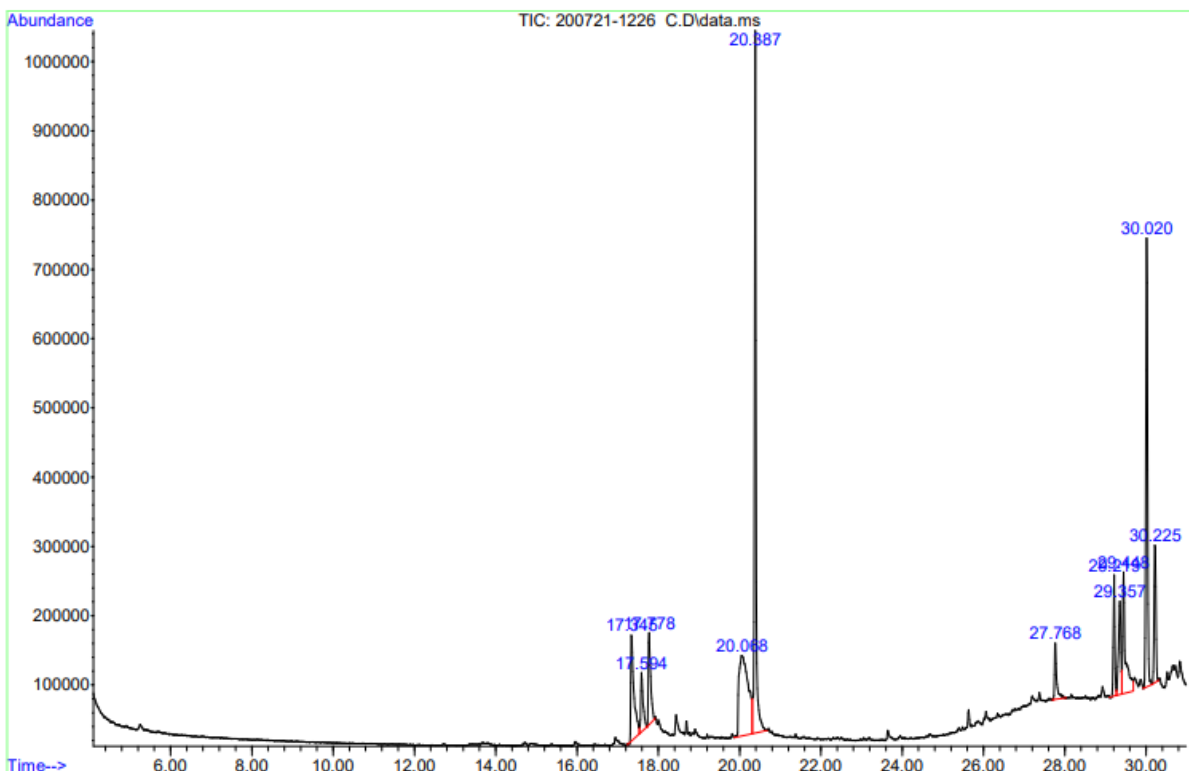


Figure 2. GC-MS Chromatogram of 'F2 Merremia' fraction

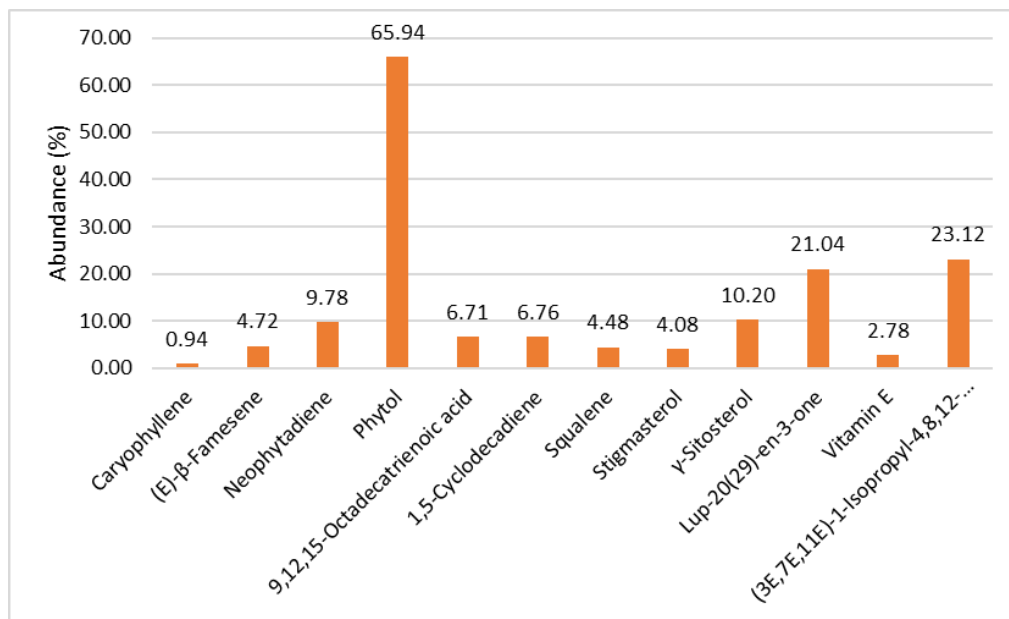


Figure 3. Chemical abundance (%) of *n*-Hexane extract of *M. vitifolia* leaves based on GC-MS.

Table 3. α-Glucosidase inhibition by quercetin

Sample name	% Inhibition in μg/mL				IC <sub>50</sub> (μg/mL)
	2.5	5	10	20	
Quercetin	49.93 ± 1.54	66.54 ± 3.84	73.64 ± 3.26	99.48 ± 0.61	2.53 ± 0.16

Table 4. α-Glucosidase inhibition by *n*-hexane extract of *M. vitifolia* leaves

Sample name	% Inhibition in μg/mL				IC <sub>50</sub> (μg/mL)
	6.25	12.5	25	50	
<i>n</i> -hexane extract	12.583 ± 6.5	46.81 ± 7.07	77.12 ± 0.03	96.69 ± 0.78	14.4 ± 1.52

Docking binding energy of phytol, neophytadiene, β-Caryophyllene, and acarbose toward α-glucosidase were -6.8, -5.3, -6.2 and -9.8 kcal/mol, respectively (Oso & Olaoye, 2020). Similar to the report of Nokhala et al.,(2020) based on molecular docking results showed that phytol, vitamin E, stigmasterol, and quercetin have α-glucosidase inhibitory effect, and stigmasterol has lowest bonding energy.

Phytol and stigmasterol also were reported to responsible for α-glucosidase inhibitory activity (Murugesu et al., 2018).

Stigmasterol has shown α-glucosidase inhibitory effect and significant antidiabetic activity in streptozotocin-nicotinamide induced diabetic mice (Kumar et al., 2013). Another sterol molecule, γ-sitosterol is an epimer of β-sitosterol, has antihyperglycemic activity by increasing insulin secretion in response to glucose (Sirikhansaeng et al., 2017). High concentration of stigmasterol dan γ-sitosterol were observed in *Parinarium glaberimum* Hassk (Tahya et al., 2020) which is known traditionally in Moluccas to treat diabetic patients.

$\beta$ -Caryophyllene has been reported to be directly beneficial to treat diabetes mellitus (Kumawat & Kaur, 2019) and inflammation of colon (Colitis) (Bento et al., 2011).  $\beta$ -Caryophyllene was found to be therapeutic in the following diseases: Alzheimer (Hu et al., 2017), epilepsy (Oliveira et al., 2016), osteoblast dysfunction (Shan et al., 2017), and cancer (Tabana et al., 2015). Lup-20(29)-en-3-one, belongs to pentacyclic lupane-type triterpenes is the oxidized form of lupeol. Lupeol itself is well known to have many beneficial pharmacological activities including anti-inflammatory, anti-hyperglycemic, antioxidant, anti-dyslipidemic and anti-mutagenic effects (Tsai et al., 2016). Because of high structural similarity to Lupeol, the Lup-20(29)-en-3-one could be a multi-target compound to explore various pharmacological potency with many potential targeting proteins including  $\alpha$ -glucosidase. Several other pentacyclic lupane-type triterpenes have been reported to show  $\alpha$ -glucosidase inhibitory activity stronger than acarbose (Yin et al., 2014). Another activities of lup-20(29)-en-3-one are to increase the melanin content of the tested cell without cytotoxicity (Yin et al., 2014) and an active compound against trypanomastigotes of *Trypanosoma cruzi* (Polanco-Hernández et al., 2012).

The second major component of *n*-Hexane extract of *M. vitifolia* leaves is (3E,7E,11E)-1-isopropyl-4,8,12-trimethylcyclotetradeca-3,7,11-trienol also called as serratol or cembrenol. This organic compound belongs to the class of cembrane diterpenoids. Serratol compound has been reported as antiprotozoal compound (Schmidt et al., 2011). Serratol was tested for in vitro activity against 4 protozoan pathogens in human, which are *Trypanosoma brucei rhodesiense* (East African Human Trypanosomiasis, sleeping sickness), *T. cruzi* (Chagas' disease), *Leishmania donovani* (Kala-Azar), and *Plasmodium falciparum* (Tropical Malaria). They found that this compound is active against *T. brucei* and *P. falciparum*. These activities were 10- to 15-fold higher than its cytotoxicity against rat skeletal

myoblasts (Schmidt et al., 2011). In researcher best knowledge, this report is the first to identify serratol in *M. vitifolia*. These open the other potential to isolate serratol for further research in Malaria drugs development in Indonesia.

Based on the literatures reported, the presence of phytol, neophytadiene,  $\beta$ -caryophyllene, stigmaterol,  $\gamma$ -sitosterol, and lup-20(29)-en-3-one were responsible to the strong  $\alpha$ -glucosidase inhibitory activity of *n*-Hexane extract of *M. vitifolia* leaf.

### Conclusion

The amount of  $\alpha$ -Glucosidase inhibitory activity by quercetin has the  $IC_{50} = 2.53 \pm 0.16$   $\mu$ g/mL, and by *n*-Hexane extract of *M. vitifolia* leaves has the  $IC_{50} = 14.4 \pm 1.52$   $\mu$ g/mL. *n*-Hexane extract of *M. vitifolia* leaves has strong  $\alpha$ -glucosidase inhibitory activity. *M. vitifolia* leaves could be good potential target for antidiabetic drugs development. There are 12 compounds that have been indentified based on GC-MS analysis of *n*-Hexane extract of *M. vitifolia* leaves with similarity index more than 89%. Caryophyllene, (E)- $\beta$ -famesene, neophytadiene, phytol, 9,12,15-octadecatrienoic acid, 1,5-cyclodecadiene, squalene, stigmaterol,  $\gamma$ -sitosterol, Serratol, vitamin E and lup-20(29)-en-3-one. Phytol is the most abundance chemical, and  $\beta$ -Caryophyllene is the least abundance chemicals that were detected.

### Acknowledgement

This work was financially supported by LPPM Universitas Pelita Harapan (UPH), with registration number P-036/FIP/I/2020 and P-005-FIP/II/2021. Thanks to LPPM UPH and Fakultas Ilmu Pendidikan UPH.

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