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Combination of FTIR-based Fingerprinting and Chemometrics Analysis for Discrimination of *Tithonia diversifolia* Leaves Extracts and Correlation with α-Glucosidase Inhibitory Activity

Mohamad Rafi[1,2*], Zuhelmi Aziz[3], Davita Purmasari[3] , Alfi Hudatul Karomah[1]
[1] Tropical Biopharmaca Research Center, International Research Institute of Food, Nutrition, and Health, IPB University, IPB Taman Kencana Campus, Jalan Taman Kencana No. 3, Bogor 16128, Indonesia
[2] Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jalan Tanjung Dramaga IPB Campus, Bogor 16680, Indonesia
[3] Faculty of Pharmacy, University of Pancasila, Srengseng Sawah, Jagakarsa, South Jakarta 12640, Indonesia

ABSTRACT

Tithonia diversifolia, known as Mexican sunflower, has been widely used as an herbal medicine to treat diabetes. This study used FTIR fingerprint spectra combined with chemometrics to differentiate *T. diversifolia* leaves extracts with different extracting solvents and their correlation with the inhibition of α-glucosidase activity. *T. diversifolia* collected from two growing locations (West Bandung and Sleman, Indonesia) was extracted with absolute ethanol, 50% ethanol, and water using ultrasonication. The ethanol absolute extract yielded a higher IC_{50} than the 50% ethanol and water extract. The FTIR spectra of each extract had a different profile, implying that the composition and the concentration of the metabolite extracted were relatively distinct. Absorbance data from the FTIR spectra in the 4000–400 cm−1 range were used to group all extracts according to the extracting solvent using principal component analysis (PCA). Before PCA, the FTIR spectra were subjected to signal preprocessing using a standard normal variate. We found that all of the extracts could be distinguished based on the extracting solvents using principal components (PC) 1 and 2 with a cumulative percentage of approximately 87%. Partial least square regression (PLSR) was used to correlate the FTIR spectra and the inhibition of the α-glucosidase activity to obtain a functional group of a metabolite that contributed to inhibiting the α-glucosidase activity. From the PLSR, peaks from the wavenumbers at ~3300 cm^{-1}, ~3000 cm^{-1}, ~1650 cm^{-1}, ~1350 cm^{-1}, and ~1100 cm^{-1} corresponded to the O-H, CH_3, CH_2, C=C, and C-O, which were thought to be responsible for inhibiting the α-glucosidase. Therefore, these functional groups were owned by the metabolites in the *T. diversifolia* leaves extracts that contributed to the inhibition of α-glucosidase.
Keywords: chemometrics; FTIR spectra; inhibition of α-glucosidase; *Tithonia diversifolia*

INTRODUCTION

Tithonia diversifolia, or Mexican sunflower, belongs to the family Asteraceae, a plant native to Mexico and Central America, but is now widely grown in many regions, such as Indonesia (Amanatie & Sulistyowati, 2015). Empirically, *T. diversifolia* has been used by people in Central America, South Asia, and Africa to treat diseases such as bruises, flatulence pain drugs, leprosy, and liver disease (Utami *et al*., 2012). *T. diversifolia* leaves have some biologically beneficial properties such as antidiabetic, antiviral, antimalarial, and anticancer properties (Darmawi et al., 2015; Sari et al., 2018). The secondary metabolite contained in *T. diversifolia* is responsible for these biological properties.

Essential oils, alkaloids, flavonoids, saponins, triterpenoids, and polyphenol metabolites are present in *T. diversifolia*. In order to ensure consistency in the quality, safety, and efficacy of *T. diversifolia*, an evaluation of its bioactive metabolites' composition and concentration levels is required. Raw materials must be standardized because many variations in the composition and concentration levels of the bioactive metabolites could occur. Several factors, such as the environment of the growing location, harvesting time, post-harvest processes, and plant parts, can influence fluctuation in the levels of the bioactive metabolites (Simmler et al., 2018). In addition, the type and concentration of the extracting solvent affect the compounds extracted from a plant. This is because the compounds contained in plants vary in terms of their chemical characteristics and polarity (Thouri et al., 2017).

Therefore, an evaluation of the composition and concentration of metabolites that do not fluctuate is necessary to obtain consistent biological activity. A metabolomics approach can determine the effect of the type and concentration

***Corresponding author: Mohamad Rafi**
Email: mra@apps.ipb.ac.id

of the extracting solvent on the plant metabolites and evaluate its correlation with the α-glucosidase inhibitory activity of *T. diversifolia*. Metabolomics is a comprehensive quantitative and qualitative analysis of plant metabolites that can produce high-quality data (Salem et al., 2020). This metabolomics approach can display fingerprint profiles of all plant metabolite components and can be used for evaluating the changes in metabolite profiles under various conditions (Xiao et al., 2022).

A fingerprinting analysis could be used to determine the changes in the metabolite composition and concentration of the *T. diversifolia* extract due to the type and concentration of the extracting solvent by using a Fourier transform infrared spectrophotometer (FTIR). FTIR was used in this study because the method is fast and straightforward, does not damage the sample, and is easy to prepare. The FTIR spectrum produces complex and challenging data to distinguish the different extraction solvents. Therefore, it was necessary to use chemometric analysis such as principal component analysis (PCA) and partial least squares regression (PLSR) to discriminate the extracting solvent and form a correlation with the α-glucosidase inhibitory activity in the *T. diversifolia* extract, respectively.

FTIR combined with chemometrics analysis has been reported to determine the changes in metabolite profiles and their correlation with plant bioactivity (antioxidant, inhibition of α-glucosidase, etc.). This combination has been used to correlate metabolites and bioactivity to identify the functional group associated with the antioxidant activity and/or the inhibition of the α-glucosidase of *Orthosiphon aristatus* (Juliani et al., 2016; Ahda et al., 2023), *Phaleria macrocarpa* (Easmin et al., 2017), *Momordica charantia* (Khatib et al., 2017), *Salacca zalacca* (Saleh et al., 2018), *Sonchus arvensis* (Rohaeti et al., 2021), *Syzygium polyanthum* (Rafi et al., 2021), and *Sesbania grandiflora* (Noviany et al., 2023). However, no reported paper has distinguished the *T. diversifolia* extracts according to the different extracting solvents using FTIR and its correlation with the α-glucosidase inhibitory activity. Therefore, this study evaluated the differences in the FTIR spectrum fingerprints and the α-glucosidase inhibition of *T. diversifolia* extracts with different extraction solvents. Furthermore, it combined the above with PCA and PLSR to predict the active functional groups.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol absolute was purchased from Merck (Darmstadt, Germany). The α-glucosidase enzymes from *Saccharomyces cerevisiae* recombinant and substrate p-nitrophenyl-α-D-glucopyranoside (PNPG) were obtained from Sigma Aldrich (St. Louis, USA). Acarbose, sodium carbonate, potassium phosphate monobasic, potassium phosphate dibasic, water (free CO_2), and potassium bromide (spectroscopy grade) were obtained from Sigma Aldrich (St. Louis, USA).

Plant materials

T. diversifolia leaves were obtained from two locations, i.e., the Manoko experimental garden, Lembang, Bandung, West Java, and PT. Natura Alam Persada, Kaliurang, Yogyakarta. The plant was identified in Herbarium Bogoriense, Cibinong, Bogor, Indonesia. All of the samples were cut into small pieces, dried at room temperature, and pulverized.

Sample extraction

The sample from the two locations was extracted using three different solvents, namely ethanol absolute, 50% ethanol, and water, with a ratio of powdered sample to solvent of about 1:10. Extraction was carried out using an ultrasonicator (Ultrasonic LC 30 H, Elma) for 30 min. The filtrate obtained was concentrated with a rotary evaporator (Heidolph, Germany) at 40°C. The extract was dried using a freeze dryer (Eyela FDU-1200, Japan).

Inhibition of α-glucosidase activity

In all, 50 µL of the extract solution was added to 450 µL of the phosphate buffer (pH 7.0) and 250 µL of the substrate solution, p-nitrophenyl-α-D-glucopyranoside, 10 mM. The mixture solution was incubated for 5 min at 37°C. We added 250 µL of the enzyme solution (0.025 U/mL) and incubated it for 20 min at 37°C. After the incubation, 1000 µL of sodium carbonate 0.2 M was added. Then, as much as 200 µL of the solution was inserted into the microplate reader ELX 800 (BioTek, Winooski, USA) using micropipettes, and its absorbance was measured at 405 nm. The positive control used was acarbose. The percentage of inhibition activity was calculated using the equation below (Mechchate et al., 2021):

$$\% \text{ inhibition} = \frac{C - (S1 - S0)}{C} \times 100\%$$

With C: absorbance of enzyme activity in the absence of inhibitors; S1: absorbance of p-nitrophenol as enzyme activity with the addition of test substances; S0: absorbance of p-nitrophenol with the addition of test substances without the addition of enzymes. The IC_{50} value was calculated using a linear regression equation, the sample concentration on the X-axis, and the percentage of inhibition on the Y-axis.

Measurement of the FTIR spectrum

The dry extract of *T. diversifolia* was mixed and homogenized with KBr in the ratio of 1:100. Then, the mixture was made into pellets by using a hydraulic pump and pre-vacuumed for 3 min after it was pressed at a pressure of 80 KN for 5 min. The obtained pellets were placed in a sample holder. Measurement of the FTIR spectrum was performed using FTIR spectrophotometer Tensor 37 (Bruker Optik GmbH, Karlsruhe, Germany) with a deuterated triglycine sulfate (DTGS) detector in the mid-infrared region (4000–400 cm^{-1}) at a resolution of 4 cm^{-1} operated with OPUS software version 4.2 (Bruker Optik GmbH, Karlsruhe, Germany).

Chemometric analysis

Absorbance data at wavenumbers 4000–400 cm^{-1} from the FTIR spectra of the *T. diversifolia* extracts were used as the variable for the chemometric analysis by using Unscrambler X version 10.1 (Camo, Oslo, Norway). Before being subjected to a chemometric analysis, we carried out preprocessing, namely, a standard normal variate, to reduce scatter and improve the clarity of the signal. Principal component analysis (PCA) was used to group the extracts with different extracting solvents. The functional groups from the *T. diversifolia* compound that exhibited the inhibition of α-glucosidase compounds were identified using PLSR by correlating the FTIR spectra and the IC_{50} value of the inhibition of the α-glucosidase activity (Umar et al., 2021).

RESULTS

Extraction of *T. diversifolia* leaves

T. diversifolia leaves originating from West Bandung and Sleman, Indonesia, were extracted using an ultrasonicator. Ethanol absolute, water, and their mixture (50% ethanol) were used as the extracting solvents. The extract obtained was expressed in percentage yield (Table I). In this study, we found that the extraction yield of the *T. diversifolia* leaves from West Bandung ranged from 3.57% ± 0.53% to 14.13% ± 0.76%, while that from Sleman ranged from 1.69% ± 0.31% to 6.14% ± 1.08%.

Metabolites from the *T. diversifolia* leaves from West Bandung were extracted more in water solvents than in absolute and 50% ethanol, as indicated by the highest extraction yield. Furthermore, the ANOVA results showed that the extraction yields of the *T. diversifolia* leaves for each solvent differed significantly at the 5% confidence level (p-value < 0.05). This showed that the extraction solvent significantly affected the extracted metabolites. In addition, the *T. diversifolia* leaves from West Bandung had a higher extraction yield than those from Sleman for all solvents used in this work.

Inhibition of α-glucosidase activity

Preliminary tests were conducted before determining the α-glucosidase inhibition activity to obtain optimum conditions for the enzyme to work optimally. This study's optimization included enzyme concentration, substrate concentration, pH, and incubation time. This optimization needed to be performed because these factors significantly influenced the enzyme's work. The principle of the α-glucosidase enzyme inhibitory activity test is that the α-glucosidase enzyme hydrolyzes *p*-nitrophenyl-α-D-glucopyranoside into yellow *p*-nitrophenol and glucose. The test was carried out *in vitro*, with the enzyme activity measured based on the absorbance results of the yellow color of p-nitrophenol. The results of the optimization of the α-glucosidase enzyme are presented in Table II.

The α-glucosidase inhibitory activity was determined on the ethanol absolute, 50% ethanol, and water extracts of the *T. diversifolia* leaves using acarbose as a positive control. The IC_{50} value shows the extract concentration (ppm) that can inhibit 50% of the activity of the α-glucosidase enzyme. The presence of an extract that could provide inhibitory activity against the α-glucosidase enzyme was determined from the absorption of the p-nitrophenol formed. The results of the α-glucosidase enzyme inhibitory activity from the samples are presented in Table III.

According to the results, the IC_{50} value of the absolute ethanol extract of the *T. diversifolia* leaves from the two growth locations had greater α-glucosidase inhibitory activity than the 50% ethanol and water extracts. However, all the extracts had a higher IC_{50} value than acarbose. In contrast, there were no significant differences between the extracts of the *T. diversifolia* leaves from West Bandung and Sleman.

Table I. Yield of *T. diversifolia* leaf extracts

Location of growth	Yield (%)		
	Absolute ethanol	50% Ethanol	Water
West Bandung	3.57 ± 0.53[c]	11.18 ± 1.48[b]	14.13 ± 0.76[a]
Sleman	1.69 ± 0.31[b]	6.14 ± 1.08[a]	5.82 ± 1.11[a]

Mean values (n = 5) followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$)

Table II. Optimized condition for determination of α-glucosidase inhibition activity

Parameter	Optimum Results
Enzyme concentration	0.025 U/mL
pH	7.0
Incubation time	20 min
Substrate concentration	10 mM

Table III. Inhibitory activity of the α-glucosidase from *T. diversifolia* leaf extracts

Extract	IC_{50} (ppm)	
	West Bandung	Sleman
Ethanol absolute	55.78 ± 2.05[c]	53.02 ± 1.71[c]
Ethanol 50%	64.76 ± 2.07[b]	64.92 ± 3.49[b]
Water	85.96 ± 2.71[a]	84.80 ± 2.63[a]
Acarbose	44.90	

Mean values (n = 10 from 5 replications of each extract with duplicate analysis of each replication) followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$)

FTIR spectrum of *T. diversifolia* leaf extracts

The FTIR spectra of each sample extract were obtained from the FTIR spectrophotometer in the mid-infrared region at a wave number of 4000–400 cm^{-1}. Figure 1 shows the FTIR spectra of the *T. diversifolia* leaf extracts based on different geographical origins and solvent extractions and we can see that the pattern of the FTIR spectra of all extracts gave a distinctive spectrum profile. The FTIR spectra of all the sample extracts showed a broad peak at 3400 cm^{-1}, indicating the presence of a hydroxy group (O-H). A peak at wavenumber 2930–2850 cm^{-1} indicated saturated alkanes or C-H stretching vibrations. The absorption peak from the C=O functional group was detected at the wavenumber of 1738–1728 cm^{-1}. The broad absorption at the wavenumber 1650–1600 cm^{-1} indicated C=C stretching vibrations. A peak at the 1400–1382 cm−1 wavenumber showed an absorption from C-C. Absorption at wavenumber 1156–1060 cm^{-1} indicated an absorption from C-O.

Clustering of *T. diversifolia* leaf extracts using PCA

The absorbance data from the FTIR spectra of the absolute ethanol, 50% ethanol, and water extracts of the *T. diversifolia* leaves were used as the variables for clustering the extracts using PCA. The PCA method obtained unique information from the very complex absorbance of data by reducing the data and extracting information. Therefore, the differences among the three extracts could be identified based on the variations in the solvents and the geographical origins. This study's absorbance data were 60 × 1866 variables from wavenumbers 4000–400 cm^{-1}. The PCA score plot obtained is presented in Figure 2. The PCA results from wavenumber 4000–400 cm^{-1}, preprocessed using a standard normal variate, yielded a plot with a cumulative percentage of the two PCs of 87% of the total variance (PC-1 = 70%, PC-2 = 17%). The *T. diversifolia* leaf extracts could be grouped based on the extracting solvent but not on geographical origin, as shown in Figure 2.

Correlation of FTIR spectra and inhibition of α-glucosidase of *T. diversifolia* leaf extracts using PLSR

In this study, we used partial least squares regression (PLSR) to determine variables (absorbance) that contributed significantly to the α-glucosidase inhibitory activity. Thus, we could predict the significant functional groups from the metabolites responsible for inhibiting the α-glucosidase activity. The PLSR method correlates the x-variable (absorbance from the FTIR spectrum of the samples) as the predictor with the

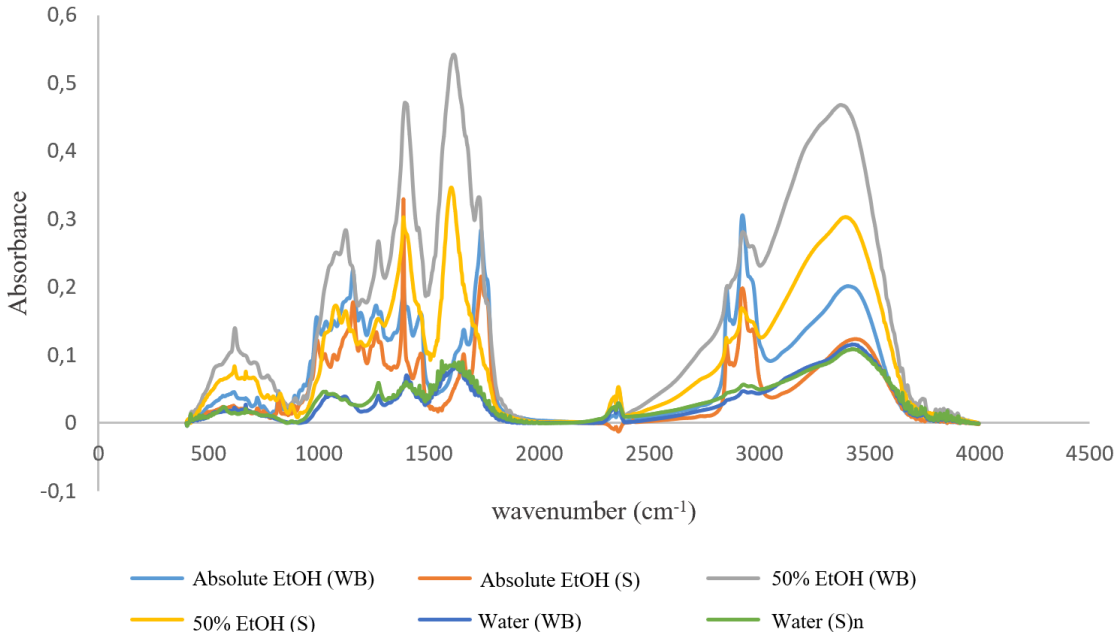

Figure 1. FTIR spectra of *T. diversifolia* leaf extracts from Sleman (S) and West Bandung (WB)

y-variable (IC_{50} value of α-glucosidase inhibitory activity) as the response. A plot of regression coefficients gives information about how significant the x-variable is to the y-variable. When the x-variable has a large regression coefficient, it is essential to the regression model.

Figure 3 shows the PLSR results and provides information regarding the correlation between x- and y-variables, shown by the positive and the negative peaks on the x-axis. The absorption of functional groups that contribute majorly to a particular biological activity, expressed by the IC_{50} values, has a negative regression coefficient (Rafi *et al*., 2021). The results of the PLSR analysis from the absorption bands at the wavenumbers between 4000 and 400 cm^{-1} provided a negative peak at ~3300 cm^{-1}, ~3000 cm^{-1}, ~1650 cm^{-1}, ~1350 cm^{-1}, and ~1100 cm^{-1}. These absorptions corresponded to O-H, CH_3, CH_2, C=C, and C-O and were thought to be responsible for inhibiting the α-glucosidase from the *T. diversifolia* compound.

DISCUSSION

In this study, we used the ultrasonic extraction method because the process is relatively easy, cheap, and fast. Apart from this, the bioactive compounds in the material are not damaged by heating because it is carried out at room temperature. Ethanol was used as the extraction solvent because ethanol, known as a universal extraction solvent, could extract more polar and nonpolar metabolites from the *T. diversifolia* leaves. Mixing ethanol and water can increase the porosity of the cell walls, thereby facilitating the diffusion of the extracted materials out of the cell (Kumar et al., 2021; Plaskova & Mlcek, 2023). Metabolites contained in the *T. diversifolia* leaves from West Bandung tended to be polar because they were extracted more with water solvents. The ANOVA results showed that the percentage of yields for each solvent extraction differed significantly, indicating that the extraction solvent affected the extracted metabolites. Also, the growing location could affect the amount of extracted metabolites in *T. diversifolia,* as we can see that the sample from West Bandung had a higher extraction yield compared to the sample from Sleman.

Several factors, such as soil composition, temperature, sunlight, altitude, groundwater, salinity, and soil fertility, could cause differences in the metabolite content in extracts from the two locations. These factors significantly influence the physiological and biochemical responses of medicinal plants, as well as secondary metabolic processes that affect their metabolite content (Qaderi et al., 2023). Several studies have also reported the effect of differences in growing locations on the metabolite content of these plants. Kamilah et al., (2024) reported that differences in altitude affect the metabolite content of *Acalypha indica*. In addition, Li et al. (2024) reported that different growing locations affect the metabolite content of *Lycium barbarum*.

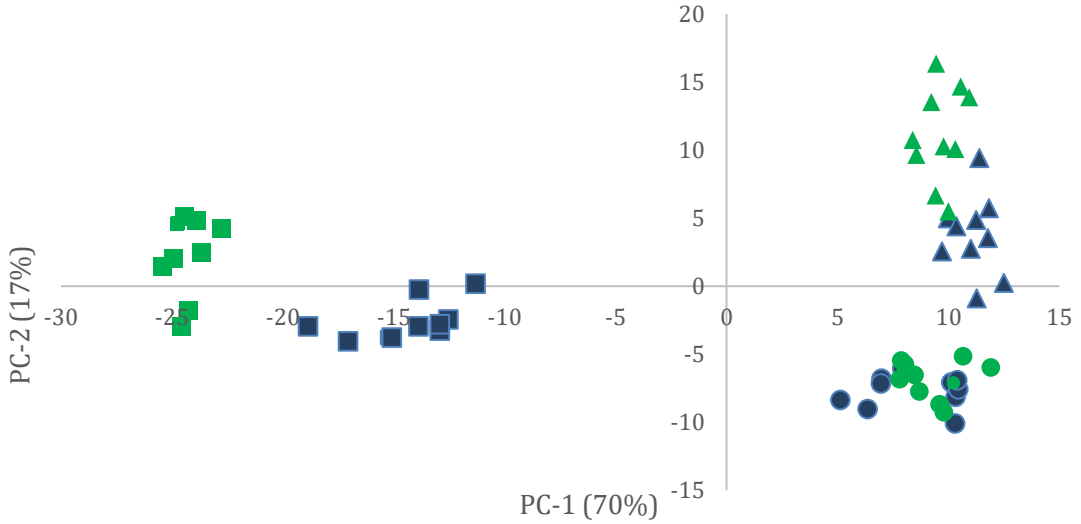

Figure 2. PCA score plot of *T. diversifolia* absolute ethanol (□), 50% ethanol (△), and water (○) extract from West Bandung (blue) and Sleman (green)

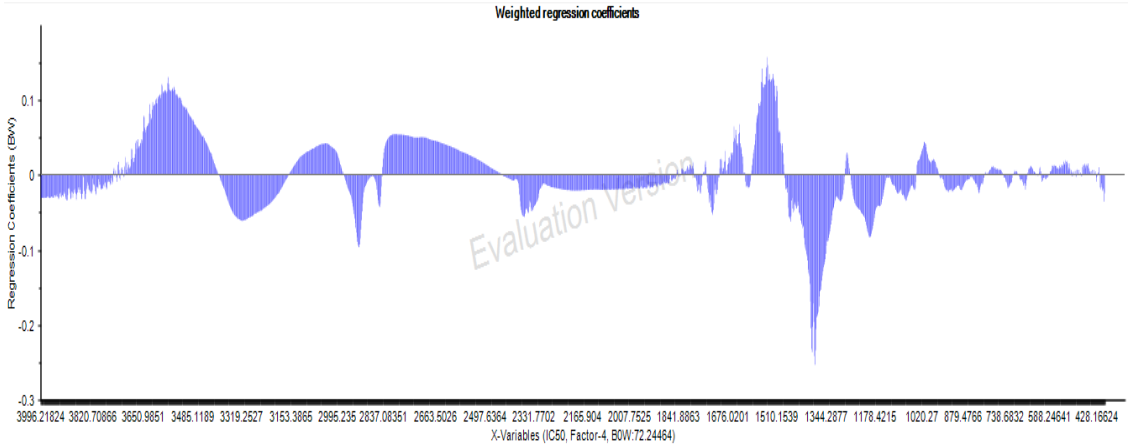

Figure 3. Regression of coefficient plot from PLSR of *T. diversifolia* extract

The absolute ethanol extract of the *T. diversifolia* leaves had more excellent α-glucosidase inhibitory activity than other extracts. As we know, absolute ethanol extracts may contain flavonoid compounds that could inhibit α-glucosidase. Unfortunately, the IC_{50} of all extracts is much lower than the IC_{50} of acarbose. We used acarbose as the positive control because acarbose is an antidiabetic drug that can slow down the absorption of glucose after eating, namely by delaying the hydrolysis of carbohydrates, disaccharides, and glucose absorption, as well as inhibiting the metabolism of sucrose into glucose and fructose (You et al., 2012). There is no significant difference between *T. diversifolia* leaves extract from West Bandung and Sleman for inhibiting α-glucosidase activity, meaning that the composition and concentration of metabolites in the same extract are not significantly different.

FTIR spectra profile for each *T. diversifolia* leaves extract is different, implying that the compounds' composition and concentration differ in each extract. Meanwhile, when considering the geographical origin (West Bandung and Sleman), we found that each extract presented a similar pattern in its respective FTIR spectra, only differing in the absorbance and intensity values. This indicated that the composition of the compounds was not significantly different, and differences occurred in their concentration. From a visual inspection of the FTIR spectra of the sample extracts, we could not distinguish the sample extracts because no peak could be used as the

marker peak for each extract. Therefore, we needed help from a chemometrics analysis, such as PCA, to group the sample extracts.

The grouping between the absolute ethanol, 50% ethanol, and water extract obtained from the PCA could be explained through PC-1, while PC-2 showed the grouping between the 50% ethanol and water extract. Therefore, the initial two PCs could explain the grouping between the three solvents. Plots for the two initial PC values are usually the most useful in the analysis because these two PCs contain the most variation in the data. Based on the geographical origin, each extract could not be grouped because of the similar composition and concentration contained in each extract. The closer the plot values were, the more similar were the characteristics of the chemical compounds contained in the extract, and vice versa.

A predicted functional group from the compound contained in *T. diversifolia* leaves, exhibiting a major contribution to inhibiting α-glucosidase, is O-H, CH_3, CH_2, C=C, and C-O. These functional groups could be attributed to the presence of flavonoid compounds in the extracts, which had the potential to inhibit the activity of the α-glucosidase enzyme (Krysa et al., 2022). Flavonoids have been reported to inhibit the work of the α-glucosidase enzyme. Flavonoids can inhibit the activity of the α-glucosidase enzyme through several mechanisms, such as improving glucose homeostasis and reducing insulin resistance, regulating carbohydrate metabolism, improving cell function and insulin action, and increasing insulin sensitivity in glucose utilization. In addition, the 3' and 4' dihydroxy groups on ring B interacting with the enzyme's active site can inhibit the enzyme's performance (Abou Baker, 2022).

CONCLUSIONS

The FTIR fingerprint profiles of the absolute ethanol, 50% ethanol, and water extracts from *T. diversifolia* leaves had their characteristic profile. All *T. diversifolia* extracts from West Bandung and Sleman could be grouped using PCA based on solvent extraction. However, we did not observe a clear grouping based on the two growth locations. The correlation between the FTIR spectra and the IC_{50} values of the inhibitory activity of α-glucosidase showed that the active compound had O-H, CH_3, CH_2, C=C, and C-O functional groups that were predicted to be the most strongly responsible for the inhibition of α-glucosidase.

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