The Analysis of Artabotrys hexapetalus Stem Bark and Leaf Ethanol Extract as α-Glucosidase Inhibitor in Relation to Antioxidant Activity, Phenolic, and Flavonoid Contents Using In Vitro Analysis, LC-MS, Machine Learning and Molecular Docking

Dela Rosa
Universitas Indonesia
Berna Elya (berna.elya@farmasi.ui.ac.id)
University of Indonesia Faculty of Pharmacy: Universitas Indonesia Fakultas Farmasi
https://orcid.org/0000-0003-2904-6515
Muhammad Hanafi
National Research and Innovation Agency Indonesia (BRIN)
Alfi Khatib
International Islamic University Malaysia Kulliyyah of Pharmacy
Yuniwaty Halim
Pelita Harapan University: Universitas Pelita Harapan
Muhammad Imam Surya
National Reseach and Innovation Agency Indonesia (BRIN)

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Abstract

Artabotrys hexapetalus is one of *Artabotrys* species that can be found in Asia, such as Sri Lanka, India, China, Malaysia, Indonesia, and other Southeast Asian countries. This plant is used traditionally as a herbal medicine. The aims of this research were to explore the potential of *A. hexapetalus* leaf and stem bark ethanol extract to treat type 2 diabetes (T2D) by inhibiting the activity of α-glucosidase, including its relationship with the antioxidant activity, phenolic content, and flavonoid content. The analysis was done using α-glucosidase inhibition assay, DPPH assay, FRAP assay, TPC, TFC, UHPLC-QTOF-MS/MS, and molecular docking analysis. Results showed that stem bark extract had medium strong α-glucosidase inhibitory activity with IC_{50} value of 47.084 ppm, whereas the leaf extract had weak α-glucosidase inhibitory activity with IC_{50} value of 104.755 ppm. Random permutation in random forest simulation was used to predict the factors that contribute to the α-glucosidase inhibition. For stem bark, the α-glucosidase inhibition activity was influenced by antioxidant activity and phenolic compounds. Nevertheless, the main active compounds that play role in α-glucosidase inhibition in stem bark were likely from terpene groups. Meanwhile, the active compounds in the leaf extract were likely not antioxidants and did not belong to the phenolic and flavonoid group. Comparisons between various analysis were shown to corroborate the random permutation results.

Introduction

One of the common chronic metabolic diseases is type 2 diabetes (T2D). Around 90–95% of diabetes patients have this type of diabetes (Patil et al. 2015). It is indicated by hyperglycemia which is caused by insulin resistance (Piero et al. 2014). T2D is a type of diabetes that occurs when insulin production from the β-cells pancreas is decreasing which leads to hyperglycemia (Sekhon-Loodu and Rupasinghe 2019). One way to cure T2D is through inhibition of α-glucosidase, an enzyme that catalyzes carbohydrate hydrolysis in the intestine. Inhibition of this enzyme will reduce the absorption of glucose and thus, the blood glucose can remain non-increasing (Dirir et al. 2021). This therapeutics method to cure diabetes is quite effective, especially for patients with glucose tolerance disturbance, and can delay the T2D occurrence, especially in newly diagnosed T2D with excessive postprandial glucose (Derosa and Maffioli 2012).

Hyperglycemia which occurs in T2D patient can induce oxidative stress which correlates with reactive oxygen species (ROS) production (Alqahtani et al. 2020). This condition also decreases antioxidative enzymes in the pancreatic cells, making those cells more susceptible to oxidative stress. To help the pancreatic cells from deteriorating, an exogenous antioxidant agent is needed (Archana et al. 2021). Therefore, it is very useful if one compound has both antioxidant and antidiabetic to help curing the T2D patient.

Nature has provided a lot of compounds which have antioxidant activity, which commonly come from flavonoid and phenolic groups (Rasheed and Azeez 2019; Tungmunnithum et al 2018) Meanwhile, the α-

glucosidase inhibitors can come from different groups of compounds, such as terpenoids, flavonoids, phenolics, and alkaloids (Dirir et al. 2021).

One of the *Artabotrys* species is *Artabotrys hexapetalus* (*A. hexapetalus*), which is a native plant of Sri Lanka and Southern India but can still be found in China and Southeast Asia countries, such as Malaysia, Indonesia, or Vietnam (Bailly and Hénichart 2022). Traditionally, it is used to treat malaria, scrofula, kidney pain, cholera, bad breath, bladder diseases, headache, leukoderma, sweating, and vomiting (Tan and Wiart, 2014). From previous research, various compounds have been isolated from this plant such as megastigmane glucoside, flavonoid glycoside (Somanawat et al. 2012), benzylisoquinoline alkaloids (Zhou et al. 2015), neo-lignans, hemiterpenoids (Jingguang et al. 2002), and sesquiterpenoids (Xi et al. 2017). Besides that, some research showed that *A. hexapetalus* had some activities such as cytotoxic (Zhou et al. 2015), antiinflammation (Yeo et al. 2020), antiviral (Xi et al. 2017), anti-plasmodial, and antioxidant (Bailly and Hénichart 2022).

This research aimed to explore the potency of *A. hexapetalus* leaf and stem bark extract as antioxidant and antidiabetic agents especially by inhibiting α-glucosidase. To find the compounds that contributed to antidiabetic activity, random permutation in feature importance analysis and molecular docking were also done in this research. Molecular docking is an important computational tool to predict the interaction between certain compounds and their molecular targets (Pinzi and Rastelli 2019), which in this research means the interaction between active compounds identified from stem bark and leaf extract to α-glucosidase inhibition.

Materials and Methods

Materials

The specimens of *A. hexapetalus* leaf and stem bark were harvested from Bogor Botanical Garden (West Java, Indonesia) with collection number XX.D.130. The seed was originally taken from The Yamanisha Botanical Research Institute located at Oyakesaka no tsujicho 39, Yamashina-ku, Kyoto, Japan and and taxonomically identified by Ruspandi. The reagents used were 1,1-diphenyl-2-picrylhydrazyl (Smart Lab), aluminum chloride (Smart Lab), sodium carbonate (Pudak Scientific), quercetin (Sigma), gallic acid (Merck), ascorbic acid (Merck), sodium acetate (Loba Chemie), ferrous sulphate heptahydrate (Merck), Folin-Ciocâlteu (Merck), ferric chloride hexahydrate (Merck), acetic acid (Merck), 2,4,6-Tris(2-pyridyl)-s-triazine or TPTZ (Sigma), p-nitrophenyl α-d-glucopyranoside (Sigma), α-glucosidase from *Saccharomyces cerevisiae* (Sigma, EC 232-604-7), sodium dihydrogen phosphate monohydrate (Merck), disodium hydrogen phosphate (Merck), ethanol (Smart Lab), and distilled water.

Preparation and Extraction of A. hexapetalus

The leaf specimen was air-dried in the room temperature, powdered, and macerated. The maceration process was done using ethanol solvent with ratio of 1: 5 for 48 h in room temperature and then filtered. It was done twice. The result of this process was referred to as ethanol extract. The solvent (ethanol) was

then evaporated at 45^{o}C using a rotary evaporator (Heidolph Hei-Vap Core). The same process was also done for stem bark specimen.

α-Glucosidase Inhibition Assay

The assay followed the method described by Dewi et al. (2014). First, phosphate buffer solution 0.1M (pH 7.0) was prepared by making Na_2HPO_4 solution by diluting 3.59 g Na_2HPO_4 in 100 mL distilled water, and NaH_2PO_4 solution by analogously diluting 1.39 g NaH_2PO_4 in 100 mL distilled water. Then, NaH_2PO_4 solution was added to Na_2HPO_4 solution until pH 7.0 was reached and distilled water was added until total volume was 200 mL. Second, 20 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) was prepared by diluting 150.65 mg PNPG in 25 mL phosphate buffer (pH 7.0). After that, it was diluted to make 5 mM PNPG solution. Third, 0.062 unit of α-glucosidase was prepared by diluting 1.0 mg (62 unit/mg) α-glucosidase in 100 mL phosphate buffer (pH 7.0) which contained 200 mg bovine serum albumin. Then, it was diluted ten times until the concentration of α-glucosidase became 0.0062 unit. Fourth, 0.2 M sodium carbonate (Na_2CO_3) was prepared by diluting 2.12 g Na_2CO_3 in 100 mL of distilled water. After all the solution was prepared, 250 µL of PNPG solution was added to 495 µL phosphate buffer solution. Then, 5 µL sample (with different concentrations) in DMSO was put in the test tube. The mixed solution was added to a reaction tube, homogenized using vortex, and incubated for 15 min at 37^{o}C. Later, 1 mL of Na_2CO_3 was added to the solution to stop the reaction. Finally, the solution was put in the UV spectrophotometer (Thermo Fischer Scientific Varioskan Flash) at wavelength (λ) of 400 nm to measure its absorbance. The solution control was PNPG and phosphate buffer. Positive control solution was using quercetin. DMSO was used as the blank. To calculate the percentage of inhibitory activity, the formula below was used:

$$\%Inhibition = \frac{Abscontrol - Abssample}{Abscontrol} \times 100\%$$

IC_{50} value was obtained by doing a regression of the results of this assay for several sample concentrations, and each concentration was repeated two times.

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Inhibition Assay

The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Inhibition Assay was determined according to González-Palma et al. (2016) with modifications. Initially, the extracts were dissolved in ethanol to make several concentrations. Then, 0.8 mL of sample was reacted with 1 mL of DPPH 0.175 mM. The mixture was then homogenized using vortex and then incubated at room temperature and dark condition for 30 min. Afterwards, UV spectrophotometer (Thermo Scientific Orion Aquamate 8000) was used to measure the absorbance of samples at λ = 517 nm. Control solution consisted of ethanol and DPPH. Positive control used was ascorbic acid and ethanol was used as the blank. The percentage of inhibition towards DPPH was calculated using the following equation:

$$\%DPPHinhibition = \frac{Abscontrol - Abssample}{Abscontrol} \times 100\%$$

IC_{50} value was obtained by doing this assay for several sample concentration, and each concentration was repeated two times.

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power assay was measured according to Tomasina et al. (2012) and Wiliantari et al. (2022). To make $FeSO_4$ calibration curve, 1.68mL FRAP solution (mixture of buffer, TPTZ, and distilled water with a ratio of 10:1:1) was mixed with 0.07 mL $FeSO_4.7H_2O$ with different concentrations in distilled water. The mixture was homogenized using vortex and then incubated at 37°C in the dark condition for 30 min. The absorbance was measured with (Thermo Scientific Orion Aquamate 8000) at λ = 593 nm. To measure the absorbance of the sample, 1.68 mL FRAP solution (mixture of acetate buffer, TPTZ, dan $FeCl_3$ with a ratio of 10:1:1) was mixed with 0.07 mL of sample. The antioxidant activity of this assay is expressed as ferric ion equivalent antioxidant activity (FeEAc), which is the equivalent concentration of Fe^{2+} (usually in mM) required to produce the same absorbance. Ascorbic acid was used as the positive control solution. The analysis results were expressed as Fe^{2+} mol/g extract and can be calculated using the following formula (Rosa et al., 2023):

$$\frac{\mathrm{Fe2+\ mol}}{\mathrm{g}} = \frac{v_s}{1000} \times \frac{y_i - c}{x_i} \times \frac{1}{1000}$$

Here v_s was the volume of the sample in mL, whereas y_i was of Fe^{2+} equivalent's concentration (mM), x_i was extracts' mass (g), and *c* was the intercept value obtained from regression equation from concentration of Fe^{2+} equivalent (mM) and the extracts' mass (ppm).

Total Phenolic Content (TPC)

TPC was conducted based on Chavan et al. (2013). First 0.2 mL of the sample was mixed homogenously with 1 mL of 10% Folin-Ciocalteu solution. Then, the mixture was incubated for 6 min at room temperature and in dark room. Afterwards, 0.8 mL of saturated Na_2CO_3 was added and the mixture was further incubated for 30 min in the room temperature. Finally, the absorbance was measured using UV spectrophotometer (Thermo Scientific Orion Aquamate 8000) at λ = 765 nm. Gallic acid was used to prepare the standard curve and ethanol was used to replace the sample in blank preparation.

The following formula (Rosa et al. 2023) was used to calculated total TPC which was shown as milligram of gallic acid equivalent (GAE) per gram extract.

$$\frac{\mathrm{mg\ GAE}}{\mathrm{g}} = \frac{1}{n} \sum_{i=1}^{n} \frac{y_i - c}{x_i} \times 1000$$

GAE in ppm for sample i was expressed as y_i, and x_i was the extract concentration i in ppm. The number of samples was n, and c represents the intercept value obtained from regression line from GAE (ppm) and extract concentration (ppm).

Total Flavonoid Content (TFC)

TFC was evaluated by colorimetric analysis according to Chang et al. (2002) with modification. The mixture consisted of sample (0.5 mL), ethanol (1.5 mL), 10% aluminum choloride (0.1 mL), 1M sodium acetate (0,1 mL), and distilled water (2.8 mL). The mixture was homogenized using vortex, and incubated in the room temperature for 30 min. UV Spectrophotometer (Thermo Scientific Orion Aquamate 8000) was then used to measure the absorbance at λ = 434 nm. Quercetin was used to make a standard curve and ethanol was used to replace sample in blank preparation. Similar to TPC, TFC was calculated as milligram of quercetin equivalent (QE) per gram extract with the similar formulation was used for calculation.

Liquid Chromatography – Mass Spectrometry (LC-MS-MS) Analysis

Each of the ethanol extracts of *A. hexapetalus* leaf and stem bark was analyzed using LC MS-QTOF instrument (Waters ACQUITY UPLC I-Class and coupled with Vion IMS QTOF MS, USA). 1µl of sample was injected into the column C_{18} (Waters ACQUITY UPLC HSS T3, 2.1 mm x 100 mm with 1.8 µm particle size and 100Å pore size). The column temperature was maintained at 40^{o}C. The mobile phases used were 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The mobile phase composition was changed during the run as follows: 0 min, 1% B; 0.5 min, 1% B; 16.00 min, 35% B; 18.00 min, 100% B; 20.00 min, 1% B. The flow rate was set to 0.6 mL/min. The column system was coupled to QTOF MS equipped with electrospray ionization (ESI) in positive mode. These are the conditios used to operate the column: capillary voltage of 1.5 kV; reference capillary voltage of 3.00 kV; source temperature of 120^{o}C, desolvation gas temperature and flow of 550^{o}C and 800 L/h, respectively, and cone gas flow of 50 L/h. Data were acquired in high-definition MS^{E} ($HDMS^{E}$) mode in the range m/z 50-1500 at 0.1 s/scan. Two independent scans with different collision energies (CE) were alternatively acquired during the run: a low-energy (LE) scan at a fixed CE of 4 eV, and a high- energy (HE) scan where the CE was ramped from 10 to 40 eV. UNIFI software (Waters Corporation, Milford, MA, USA) was used to process the data.

Feature Importance Analysis

In this research, there were several factors (or features in machine learning terminology) which could influence the inhibition of α-glucosidase, such as the TFC, TPC, DPPH inhibition, or the FeEAC (the result of FRAP assay). Unfortunately, the values of these features varied quite large in this research, and consequently, the extract concentration ranges for each of these features were not the same. This made it difficult to do the usual correlation analysis.

To solve this problem, an approach using machine learning method was used, which was using a machine learning method to make a prediction, in this case random forest, and random permutation to find out the most important or dominant feature or variable (Breiman 2001). Random forest (Biau and Scornet 2016) is a method that combines several other machine learning method, namely decision tree. Decision tree is a method that make the prediction by splitting the data according to its features (Myles et al. 2004). In random forest, multiple trees are created by resampling the data with replacement (bagging), creating multiple versions of input data. These multiple input data are then used to create multiple decision trees. It is hoped then by combining several decision trees, the random forest will have better performance than a single decision tree (Biau and Scornet 2016; Breiman, 2001).

Machine learning algorithms need data. For the data, several extract concentrations were chosen: 9 ppm – 330 ppm with increment of 3 ppm for stem bark extract, and 11 ppm – 1745 ppm with increment 2 ppm for leaf extract. These extract concentrations were chosen to make sure that both DPPH inhibition and α-glucosidase inhibition reached 100%, and the concentrations were not too small since extrapolation to small concentrations where changes (for example UV radiation absorption) could not be detected tended to be erroneous. Since not all the above-mentioned features (TPC, TFC, DPPH inhibiton, and FeEAC), including the α-glucosidase inhibition, were measured in these chosen extract concentrations, linear regression was used to estimate the values of the features for those concentrations. The linear regression used the measured values of the features for the extract concentrations of the experiments as the basis for the estimation. Negative values, which were not realistic, were changed to zero. Percentage value more than 100 was analogously changed to 100. To make the values of different features in same range of values, all the feature values were first divided by the mean of the corresponding feature. In this way, all the features had (estimated) values for the same set of extract concentrations. Unfortunately, since these feature values were obtained from linear regression estimation, the correlation analysis on these features values and the α-glucosidase inhibition would always result in perfect correlation, and therefore could not be used. The machine learning methods were then trained on this data (built from estimated feature values by using linear regression for the given extract concentrations) and used to predict the α-glucosidase inhibition. The training and test data were the same in this case since the goal was just to see the dominant features, and not making predictions for unknown data.

The machine learning method used in this research to predict the inhibition percentage of α-glucosidase is random forest, and it was implemented using Orange Data Mining software version 3.34.0 (https://orangedatamining.com/). The parameters used for this random forest implementation in Orange Data Mining were: using 100 decision trees, minimum number of instances that can be split in the decision tree is set to be 5 but not limiting the growth of the decision tree (controlling the complexity of decision trees), using replicable training sets for decision tree, not limiting the number of attributes considered to be split, and not balancing the class distribution. Using these parameters, the random forest had better prediction error, measured in root mean square error (RMSE) than linear regression, which was used as control. The RMSE for random forest was 0.012 when it was used to predict the

training set of stem bark extract data (linear regression RMSE = 0.086), while it was 0.001 for the training set of leaf extract data (linear regression RMSE = 0.089).

The importance of the feature for the prediction was estimated by doing random permutations on the values of the feature (while keeping the others constant) and measuring the change in the prediction error (Breiman 2001). Random permutation would destruct the connection between the feature to the output, resulting in an increase of RMSE. The more important or dominant the feature, the greater the increase of RMSE. The number of random permutations used in this research was 100. The analysis described above was implemented using the Orange Data Mining software version 3.34.0 (https://orangedatamining.com/).

Molecular Docking Study

The molecular docking study was used to predict the binding affinity of the ligand (each of identified secondary metabolites compound from LC-MS/MS analysis) and binding site of the receptor (α-glucosidase from *Saccharomyces cerevisiae*). The 3D ligand structure was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) or ZINC (https://zinc.docking.org/) databases as 3D sdf format. All 3D ligand structures were converted from sdf format to pdb format using Open Babel (version 3.1.1). Then all the ligands were prepared using AutodockFR or ADFR version 1.0 and save as PDBQT.

The *Saccharomyces cerevisiae* α-glucosidase structure was taken from Protein Data Bank (www.rscb.org) using 3A4A model. To repair the missing residue in the 3A4A model, FASTA file of 3A4A model was re-modelled using Swiss Model (https://swissmodel.expasy.org/), resulting in the corrected pdb file of 3A4A. The receptor was prepared with AutoDockTools version 1.5.7 (https://autodocksuite.scripps.edu/adt/) where hydrogen atoms was added to the receptor. The preparations of the receptor (3A4A) and the ligands were continued using the AutodockFR or ADFR version 1.0 (Ravindranath et al. 2015) using the prepare receptor and prepare ligand modules of the tools, where among other charges are added, resulting in pdbqt files. The docking was done using Autodock Vina version 1.2.3 (Eberhardt et al. 2021; Trott and Olson 2009) with several settings: the size of grid box was 185 x 185 x 185 so the box could cover the whole receptor, with the center of the grid box was at the coordinate X = 21.306; Y = -3.222; Z = 18.662 and grid spacing 0.375 Å. For the Autodock Vina docking simulation, exhaustiveness was set to 32, energy range to 4, and number of modes to 50. The docking setting parameters was validated using native ligand redocking. In the native ligand redocking simulation, all the docking parameters are the same as above, but the grid box only covered the active site of the native ligand which is 16 Å x 16 Å x 16 Å with center at coordinate X = 21.1; Y = -7.4; Z = 24.2. This changes in the grid box were to make it easier for the docking simulation to get to the same place as the crystallography result, which was necessary since the native ligand (in this case glucose) seemed to be able to be docked in several places at the receptor (see for example (Dej-adisai et al. 2022) for similar validation design). The resulting RMSD value between the re-docking simulation result and the crystallography position was 0.764 Å, which fulfilled the usual standard (< 2 Å).

Results

α-Glucosidase Inhibition, Antioxidant Activity, TPC, TFC

Table 1 shows the functional properties of *A. hexapetalus* leaf, stem bark ethanol extract, and their positive controls. Related to α-glucosidase inhibition, it could be seen that the IC_{50} value of leaf extract (104.755 ppm) was larger than stem bark extract (47.084 ppm). This means that α-glucosidase inhibition activity of the stem bark extract was about two times stronger than the leaf extract. Nevertheless, both extracts were weaker than the positive control, *i.e.*, quercetin (IC_{50} = 3.082 ppm).

Table 1
Result of antioxidant activities, TPC, and TFC from *A. hexapetalus* leaf and stem bark

Sample	α-Glucosidase Inhibition (IC_{50}, ppm)	Antioxidant Activity (IC_{50})		TPC (mg GAE/g extract)	TFC (mg QE/g extract)
		DPPH assay (ppm)	FRAP assay (Fe^{2+} mol/g)		
A. hexapetalus leaf extract	104.755	832.110	$6.891 \times 10^{-4} \pm 2.944 \times 10^{-5}$	66.493 ± 3.320	14.346 ± 0.280
A. hexapetalus stem bark extract	**47.084**	**147.339**	**$1.619 \times 10^{-3} \pm 4.781 \times 10^{-4}$**	**81.297 ± 1.782**	**29.681 ± 1.341**
Positive control*	3.082	10.605	$3.131 \times 10^{-1} \pm 6.515 \times 10^{-2}$	-	-

*positive control for α-glucosidase inhibition was quercetin, for DPPH and FRAP assay was ascorbic acid.

One of the assays to measure the antioxidant activity in *A. hexapetalus* leaf and stem bark was using DPPH assay. The results were presented as IC_{50} of the DPPH inhibition, *i.e.*, 832.110 ppm for leaf extract and 147.339 ppm for stem bark extract (Table 1). However, ascorbic acid, as the positive control, had IC_{50} 10.605 ppm. Although there was no universal threshold of IC_{50} for classification of the strength of antioxidant activity, the antioxidant activation strength of stem bark and leaf extracts could be categorized as medium and non-existent respectively, while ascorbic acid could be called a very active antioxidant (Boulmokh et al. 2021).

Another assay to measure the antioxidant activity of *A. hexapetalus* leaf and stem bark was FRAP analysis. FRAP analysis result on Table 1 showed that the leaf and the stem bark ethanol extract of *A. hexapetalus* were $6.891 \times 10^{-4} \pm 2.944 \times 10^{-5}$ and $1.619 \times 10^{-3} \pm 4.781 \times 10^{-4}$ Fe^{2+} mol/g extract, respectively. Meanwhile, the FRAP value for ascorbic acid was $3.131 \times 10^{-1} \pm 6.515 \times 10^{-2}$ Fe^{2+} mol/g which was significantly higher compared to leaf and stem bark. This confirmed the conclusion of the DPPH assay that both leaf and stem bark extracts showed very weak antioxidant activities. The calculation of Fe^{2+}

was done by subtracting of regression line intercept to remove bias (Rosa et al. 2023) because of the non-zero intercept of the calibration regression line of $FeSO_4$ (R^2 = 0.994).

To support the antioxidant activity results, total phenolic content (TPC) and total flavonoid content (TFC) were also measured. The TPC result of *A. hexapetalus* leaf ethanol extract was 66.493 ± 3.320 mg GAE/g extract. This value was lower than the TPC result of *A. hexapetalus* stem bark ethanol extract which was 81.297 ± 1.782 mg GAE/g extract (Table 1).

Furthermore, The TFC result of *A. hexapetalus* leaf ethanol extract was 29.681 ± 1.341 mg QE/g extract while the stem bark ethanol extract was 14.346 ± 0.280 mg QE/, respectively. Comparing the TPC results, it can be concluded that most of phenolics compounds in the leaf and stem bark ethanol extract did not belong to flavonoid group, especially in the stem bark.

Liquid Chromatography – Mass Spectrometry/ Mass Spectrometry (LC-MS/MS) analysis

The identified major compounds in LC-MS/MS for leaf and stem bark extracts (see Table 2, Table 3, Fig. 1, and Fig. 2) were mostly from alkaloid groups, phenolic groups, and terpenoid groups. But not all major compounds were identified. One example was the major peaks for the leaf ethanol extract at retention time interval of 11–14 minutes. The major compounds as can be seen in Table 2 and Table 3 were codeine for the stem bark and 7-O-isopentenyl-8-fagarine for the leaf. Codeine is an alkaloid while 7-O-isopentenyl-8-fagarine which has both flavonoid and alkaloid functional group (furuquinoline alkaloid) (Alara et al. 2020; Sichaem et al. 2015).

Table 2
Identified compounds from Liquid Chromatography – Mass Spectrometry/ Mass Spectrometry analysis for *A. hexapetalus* leaf ethanol extract

R/T	Compound	Formula	Observed MW	Adduct	Response
0.77	Fuzinoside	$C_{15}H_{28}O_{13}$	439.1414	+Na, +K	37339
2.05	Shanzhiside methyl ester	$C_{17}H_{26}O_{11}$	429.1357	+Na, +H	5707
5.68	Moupinamide	$C_{18}H_{19}NO_4$	314.1378	+H	6319
6.17	7-O-Isopentenyl-8-fagarine	$C_{18}H_{19}NO_4$	314.1376	+H	70573
6.53	Forsythoside D	$C_{20}H_{30}O_{13}$	501.1578	+Na, +K	7364
7	Tuduranine	$C_{18}H_{19}NO_3$	298.1429	+H	5367
7.28	d-Isoboldine	$C_{19}H_{21}NO_4$	328.1533	+H	7907
7.32	Apocynoside	$C_{19}H_{30}O_8$	409.1826	+Na, +K	54317
7.86	Sinomenine	$C_{19}H_{23}NO_4$	330.169	+H	14647
7.98	Lucidumoside A	$C_{25}H_{34}O_{12}$	527.2099	+H	11465
8.25	Emodin-8-O-sophoroside	$C_{27}H_{30}O_{15}$	595.1661	+H, +K, +Na	50043
8.31	Roseoside	$C_{19}H_{30}O_8$	409.1828	+Na, +K	6224
9.73	Urolignoside	$C_{26}H_{34}O_{11}$	545.1994	+Na	8740
9.75	Tetrahydrocoptisine	$C_{19}H_{17}NO_4$	324.1217	+H	26455
11.64	**Berberrubine**	$C_{19}H_{15}NO_4$	322.1064	+H	15282
15.31	Berbithine	$C_{19}H_{17}NO_5$	362.0987	+Na, +H	9186
Note: R/T = retention time, MW = molecular weight					

Table 3
Identified compounds from Liquid Chromatography – Mass Spectrometry/ Mass Spectrometry analysis for *A.hexapetalus* stem bark ethanol extract

R/T	Compound	Formula	Observed MW	Adduct	Response
0.79	Fuzinoside	$C_{15}H_{28}O_{13}$	439.1411	+Na, +K	6999
3.22	**Loganic acid**	$C_{16}H_{24}O_{10}$	399.1252	+Na, +K	31795
3.49	Loganin	$C_{17}H_{26}O_{10}$	413.141	+Na	12994
4.32	Aucubin	$C_{15}H_{22}O_{9}$	369.1145	+Na, +K	18634
4.34	Sweroside	$C_{16}H_{22}O_{9}$	381.1145	+Na, +K	62612
4.44	Forsythoside D	$C_{20}H_{30}O_{13}$	501.1577	+Na, +K	6502
4.64	Secoxyloganin	$C_{17}H_{24}O_{11}$	427.1205	+Na	5150
5.1	Isofraxinellone	$C_{14}H_{16}O_{3}$	255.1001	+Na	12622
5.1	Glucosyringic acid	$C_{15}H_{20}O_{10}$	383.0938	+Na, +K	14329
5.7	Moupinamide	$C_{18}H_{19}NO_{4}$	314.1378	+H	474485
5.61	7-O-Methylmorroniside	$C_{18}H_{28}O_{11}$	443.152	+Na	5934
5.67	Scoulerine	$C_{19}H_{21}NO_{4}$	328.1534	+H	23345
6.01	d-Isoboldine	$C_{19}H_{21}NO_{4}$	328.1535	+H, +K, +Na	91197
6.18	7-O-Isopentenyl-8-fagarine	$C_{18}H_{19}NO4$	314.1378	+H, +Na	306579
6.18	Stepharine	$C_{18}H_{19}NO_{3}$	298.1426	+H	24054
6.22	Armepavine	$C_{19}H_{23}NO_{3}$	314.174	+H	208427
6.93	Codeine	$C_{18}H_{21}NO_{3}$	300.1587	+H	587970
6.99	Oxymaistemonine	$C_{23}H_{29}NO_{7}$	432.2014	+H	12032
7.02	Apocynoside	$C_{19}H_{30}O_{8}$	409.1829	+Na, +K	49534
7.35	Leonticine	$C_{20}H_{25}NO_{3}$	328.1896	+H	33578
7.52	Lucidumoside A	$C_{25}H_{34}O_{12}$	549.1946	+Na	12810
7.68	Yadanzioside G	$C_{36}H_{48}O_{18}$	769.2912	+H	24850
7.87	Phenethyl ferulate	$C_{18}H_{18}O_{4}$	299.1268	+H	12470

R/T	Compound	Formula	Observed MW	Adduct	Response
7.88	Sinomenine	$C_{19}H_{23}NO_4$	330.1693	+H, +Na	395882
7.88	Homoarecoline	$C_9H_{15}NO_2$	192.1004	+Na	38823
8.03	Cheilanthifoline	$C_{19}H_{19}NO_4$	326.1378	+H	7685
8.4	Neohesperidin	$C_{28}H_{34}O_{15}$	611.1955	+H	14405
8.17	Curcolone	$C_{15}H_{18}O_3$	269.116	+Na	6017
8.24	Flavanthrinin	$C_{15}H_{14}O_3$	265.0844	+Na	26443
8.24	Danshenxinkun A	$C_{18}H_{16}O_4$	297.1109	+H	43192
8.24	Magnoflorine	$C_{20}H_{23}NO_4$	342.169	+H	195991
8.34	Muramine	$C_{22}H_{27}NO^5$	386.1954	+H	5683
8.55	Norisocorydine	$C_{19}H_{21}NO_4$	328.1532	+H, +K, +Na	52596
8.67	Viscumneoside	$C_{29}H_{32}O_{16}$	637.1746	+H	5851
8.78	Oliveridine	$C_{19}H_{19}NO_4$	326.138	+H, +Na	18257
8.88	**Hydroxytanshi-none ⅡA**	$C_{19}H_{18}O_4$	311.1269	+H	10391
8.98	Coclaurine	$C_{17}H_{19}NO_3$	286.1424	+H	85217
9.08	Menisperine	$C_{21}H_{25}NO_4$	356.1844	+H	7211
9.12	Suffruticoside E	$C_{26}H_{38}O_{17}$	623.2193	+H	8099
10.43	Danshenxinkun B	$C_{18}H_{16}O3$	281.1158	+H	187217
10.43	Tuduranine	$C_{18}H_{19}NO_3$	298.1424	+H	25225
10.78	Toosendanin	$C_{30}H_{38}O_{11}$	597.2303	+Na	7166
10.85	Eucommin A	$C_{27}H_{34}O_{12}$	573.1951	+Na	5087
10.94	Urolignoside	$C_{26}H_{34}O_{11}$	545.1993	+Na, +K	42014
11.4	Sibirioside A	$C_{21}H_{28}O_{12}$	495.1473	+Na, +K	9634
11.08	Mudanpioside D	$C_{24}H^{30}O_{12}$	511.1786	+H	8308

R/T	Compound	Formula	Observed MW	Adduct	Response
11.8	Anonaine	$C_{17}H_{15}NO_2$	266.1184	+H	11551
11.11	Picrasin F	$C_{22}H_{30}O_8$	445.1829	+Na, +K	31491
11.35	Ligustroside	$C_{25}H_{32}O_{12}$	525.194	+H	12469
11.37	Piperyline	$C_{16}H_{17}NO_3$	294.1112	+Na	53767
11.44	Interiotherin C	$C_{30}H_{36}O_{10}$	579.2206	+Na	5960
12.39	N-trans-Coumaroyltyra-mine	$C_{17}H_{17}NO_3$	306.1094	+Na, +H	16415
12.62	Liriodenine	$C_{17}H_9NO_3$	276.0645	+H, +Na	28420
12.87	Thujaplicatin meth-yl ether	$C_{21}H_{24}O_8$	427.1363	+Na	19419
13.14	Lucidumoside C	$C_{27}H_{36}O_{14}$	585.2163	+H, +Na	160187
13.21	15,16-Dihydrotanshi-none	$C_{18}H_{14}O_3$	279.1005	+H	320898
14.81	**Mudanpioside J**	$C_{31}H_{34}O_{14}$	631.2013	+H	7484
14.94	Lucidumoside D	$C_{27}H_{36}O_{13}$	568.21559	+H	7203

Comparing the responses of the compounds for leaf (Table 2) and for stem bark (Table 3), it could be seen that the amount of compounds in stem bark (total response = 3949765) were much larger than in leaf (total response = 321653). Accordingly, even the compound with the largest amount in leaf was a minor compound (response less than 100000).

Molecular Docking

Molecular docking using Autodock Vina were done to see whether the identified compounds could be a potential inhibitor to α-glucosidase based on the free energy binding. The result can be seen in Table 4.

Table 4
Free Binding energy for ligand binding to α-glucosidase from *A.hexapetalus* leaf and stem bark ethanol extract

No.	Compound	Source	Group of secondary metabolite	Response	Free Binding Energy (kcal/mol)
1	**Hydroxytanshinone ⅡA**	Stem bark	Terpene	102384	**-9.817**
2	**Loganic Acid**	Stem bark	Iridoid glycoside	31795	**-9.547**
3	**Mudanpioside J**	Stem bark	Terpene	7484	**-9.544**
4	Danshenxinkun B	Stem bark	Terpene	187217	-9.167
5	Berberrubine	Leaf	Alkaloid	15282	-9.076
6	Ligustroside	Stem bark	Secoiridoid	12469	-9.007
7	Suffruticoside E	Stem bark	Phenolic	8099	-8.970
8	Picrasinoside A	Leaf	Quassinoid glycoside	5177	-8.861
9	Scoulerine	Stem bark	Alkaloid	119086	-8.704
10	Curcolone	Stem bark	Terpene	6017	-8.657
11	Sibirioside A	Stem bark	Phenylpropanoid glycoside	9634	-8.551
12	d- Isoboldine	Leaf Stem bark	Alkaloid	7907 143991	-8.496
13	Moupinamide	Leaf Stem bark	Phenolic	6319 474485	-8.494
14	Danshenxinkun A	Stem bark	Terpene	43192	-8.453
15	Yadanzioside G	Stem bark	Terpene	24850	-8.286

No.	Compound	Source	Group of secondary metabolite	Response	Free Binding Energy (kcal/mol)
16	Tuduranine	Leaf Stem bark	Alkaloid	5367 25225	-8.242
17	Roseoside	Leaf	Phenolic	6224	-8.219
18	Oliveridine	Stem bark	Alkaloid	18257	-8.174
19	Armeparvine	Stem bark	Alkaloid	309949	-8.012
20	Toosendanin	Stem bark	Terpene	7166	-7.987
21	Aucubin	Stem bark	Iridoid	18634	-7.892
22	Oxymaistemonine	Stem bark	Alkaloid	12032	-7.844
23	7-O- Methylmorroniside	Stem bark	Iridoid glycoside	5934	-7.815
24	Flavarithine	Stem bark	Phenanthrene	26443	-7.762
25	Menisperine	Stem bark	Alkaloid	7211	-7.719
26	Magnoflorine	Stem bark	Alkaloid	195991	-7.700
27	Picrasin F	Stem bark	Quassinoid glycoside	31491	-7.684
28	Neohesperidin	Stem bark	Flavonoid glycoside	14405	-7.666
29	Lucidumoside A	Leaf Stem bark	Iridoid glycoside	11465 12810	-7.632
30	Codeine	Sterm bark	Alkaloid	587970	-7.595
31	Shanzhiside methyl ester	Leaf Stem bark	Phenolic	5707 21613	-7.583

No.	Compound	Source	Group of secondary metabolite	Response	Free Binding Energy (kcal/mol)
32	Forsythoside D	Leaf Stem bark	Phenylethanoid glycosides	7364 6502	-7.544
33	Piperyline	Stem bark	Pyrolidine	53767	-7.532
34	15,16-Dihydrotanshinone	Stem bark	Terpene	320898	-7.509
35	Sinomenine	Leaf Stem bark	Alkaloid	14647 395882	-7.436
36	Sweroside	Stem bark	Iridoid	62612	-7.410
37	Anonaine	Stem bark	Alkaloid	11551	-7.384
38	Eucommin A	Stem bark	Phenolic	5087	-7.361
39	Coclaurine	Stem bark	Alkaloid	85217	-7.353
40	Interiotherin C	Stem bark	Phenolic	5960	-7.314
41	Cheilanthifoline	Stem bark	Alkaloid	7685	-7.191
42	Leonticine	Stem bark	Alkaloid	33578	-7.167
43	7-O-Isopentenyl-8-fagarine	Leaf Stem bark	Flavonoid Alkaloid	70573 306579	-7.165
44	Tetrahydrocoptisine	Leaf	Alkaloid	26455	-7.163
45	Phenethyl Ferulate	Stem bark	Phenolic	12470	-7.715
46	Uroglinoside	Leaf Stem bark	Flavonoid glycoside	8740 42014	-6.943
47	Loganin	Stem bark	Iridoid	12994	-6.880

No.	Compound	Source	Group of secondary metabolite	Response	Free Binding Energy (kcal/mol)
48	Norisocorydine	Stem bark	Alkaloid	52596	-6.844
49	Lucidumoside D	Stem bark	Iridoid glycoside	7203	-6.803
50	Homoarecoline	Stem bark	Iridoid	38823	-6.719
51	Fuzinoside	Leaf Stem bark	Phenolic	37339 6999	-6.676
52	Liriodenine	Stem bark	Alkaloid	28420	-6.653
53	Muramine	Stem bark	Alkaloid	5683	-6.231
54	Lucidumoside C	Stem bark	Iridoid glycoside	160187	-6.471
55	Secoxyloganin	Stem bark	Alkaloid	5150	-6.218
56	Stepharine	Stem bark	Alkaloid	24054	-6.152
57	Apocynoside	Leaf Stem bark	Phenolic	49534 54317	-6.003
58	Thujaplicatin methyl ether	Stem bark	Phenolic	19419	-5.926
59	Isofraxinellone	Stem bark	Terpene	12622	-4.964
60	Glucosyringic Acid	Stem bark	Tanin	14329	-4.426

From the Table 4, it could be seen that hydroxytanshinone A had the highest affinity to α-glucosidase receptor with a binding energy of -9.817 kcal/mol. Following Morris et al. (2008), a ligand with the energy binding ≤ -9 kcal/mol could be classified to have high-affinity binding to the receptor. In stem bark, beside hydroxytanshinone A, there were several other compounds with high affinity, *i.e.*, loganic acid (-9.547 kcal/mol), mudanpioside J (-9.544 kcal/mol), danshenxinkun B (-9.167 kcal/mol), and ligustroside (-9.007 kcal/mol). The structure of hydroxytanshinone IIA, loganic acid, and mudanpioside J can be seen in Fig. 3.

In leaf, there was only one compound found with high affinity (binding energy ≤ -9 kcal/mol). The compound in the leaf with the highest affinity was berberrubine (-9.0758 kcal/mol).

Feature importance analysis results

The results of the feature importance analysis using random permutations of the features used in random forest prediction of the α-glucosidase inhibition could be seen in Fig. 3(a) and 3(b) for leaf and stem bark extracts, respectively. The mean and standard deviations for the increase in RMSE could also be seen in the Fig. 3.

For the leaf extract, the increase in RMSE was the largest for total phenolic content, followed by FeEAC, DPPH inhibition, and lastly by total flavonoid content. The same order was also observed for the stem bark extract. It was interesting to note that the values of the RMSE increase for DPPH inhibition and FeEAC were nearly the same for both leaf and stem bark extracts, highlighting the fact that these two features measured the same phenomenon: antioxidant activity. Total phenolic content was a bit more dominant in influencing the α-glucosidase inhibition activity than the antioxidant activity (both DPPH inhibition and FeEAC), although only by a small margin. Total flavonoid content clearly did not influence the α-glucosidase inhibition activity.

Since the differences among the increase of RMSE between total phenolic content, DPPH inhibition, and FeEAC were small, it could be concluded that these three features had the same influence in predicting α-glucosidase inhibition activity. They were clearly more influential than total flavonoid content, but they could not be said the main factors that influence α-glucosidase inhibition activity, since this feature importance analysis only compared the given or measured features. There could be other factors that were more dominant, but not measured in this research.

Discussion

Feature importance analysis indicates that total flavonoid content was not influential in determining the α-glucosidase inhibitory activity. This means that the active compounds which contributed to inhibition of α-glucosidase activity in the leaf and stem bark of *A. hexapetalus* most probably did not belong to flavonoid group.

Antioxidant activities of both the leaf and stem bark extracts could be classified as non-existent and medium, respectively. On the other hand, the α-glucosidase inhibition activities of leaf and stem bark extracts were weak and medium strong, respectively. Therefore, for the leaf extract, it was highly unlikely that active compound for α-glucosidase inhibition activity is also antioxidant. Since the feature importance analysis also grouped together the influence of total phenolic content in the same category as the antioxidant activity, it could be further concluded that the active compounds which contributed to inhibition of α-glucosidase activity in the leaf extract were not of phenolic group either.

For the stem bark extract, since the antioxidant activity was medium while the α-glucosidase inhibition was also medium, the feature importance analysis result suggested that there was a high probability that the active compounds which contributed to inhibition of α-glucosidase activity in the stem bark extract could have antioxidant activity. Analogous to the leaf extract, the feature importance analysis also predicted that the active compound which contributed to inhibition of α-glucosidase activity in the stem bark extract could belong to phenolic compound with a high probability.

From the analysis LC-MS/MS data of stem bark and leaf extract, only one flavonoid compound (7-O-isopentenyl-8-fagarine) was identified with relatively high amount in stem bark (response = 306579) and in leaf (response = 70573). In this study, compound with response less than 100000 were considered minor. This lack of flavonoid compound result corresponded well to the TFC as well as the features importance analysis. The amount of flavonoid was higher in stem bark than in leaf (Table 1, 2, and 3).

In the leaf extract, there was no phenolic compounds whose response more than 100000. The three phenolic compounds with the largest amount were: fuzinoside (response = 37339), apocynoside (response = 54317), and emodin-8-sophoroside (response = 50043). In stem bark, there was only one phenolic compound with response more than 100000, *i.e.*, moupinamide (response = 474485). It is noticeable that even one phenolic compound identified from stem bark extract has higher response than the total of three largest phenolic compounds in leaf. This corresponded to Table 1 as well.

The response of total phenolic compound was larger than the response of total flavonoid compound. In stem bark, the total response of total phenolic compound (phenolic only and flavonoid) and flavonoid groups was 971446 and total flavonoid only was 362998, respectively (see Table 3). Even for minor compounds in the stem bark, the phenolic compounds were more prevalent than the flavonoid. This corresponded with the observation that TFC was lower than total TPC in stem bark (Table 1). Analogously, in the leaf, the total response of total phenolic compound (phenolic only and flavonoid) was 198841 and total flavonoid compounds 93718, respectively. This corresponded analogously to the differences of TPC and TFC in leaf extract as shown in Table 1. Because the differences in the responses of phenolic and flavonoid compounds (Table 3) and the differences of the TPC and TFC (Table 1) were not proportionally similar for stem bark and leaf, it could be concluded that some phenolic compounds were not yet identified in stem bark and leaf extract.

As can be seen from Table 1, the inhibition of α-glucosidase was stronger in stem bark extract (IC_{50} = 47.084 ppm). This inhibition of α-glucosidase of the stem bark extract was also stronger compared to several other plants, *e.g., K. senegalensis* stem bark (82.2 ± 1.0 ppm) and *Z. spina-christi* stem bark (92.3 ± 4.3 ppm) (Andrew et al. 2013). This indicated that stem bark of *A. hexapetalus* had higher antidiabetic activity compared to those plants. Molecular docking simulation as explained before verified this observation. In stem bark, there were several high affinity compounds, but only hydroxytanshinone A and danshenxinkun B were major compounds. These two compounds belonged to terpene group. The other high affinity compounds were minor and belonged to iridoid glycoside (loganic acid) (Dzydzan et al. 2020), terpene glycoside (mudanpioside J) (Ding et al. 2000), and secoiridoid glycoside (ligustroside) (He

et al. 2001). The feature importance analysis predicted that there were some active compounds which contributed to inhibition of α-glucosidase activity from phenolic group in the stem bark extract. Further investigation on the results of molecular docking showed that for stem bark extract there were some phenolic compounds which were quite active (see Table 4): suffruticoside E (-8.9705 kcal/mol), although a minor compound (response = 8099). Best on the prediction from feature importance analysis, it could be concluded that some active α-glucosidase inhibitor from phenolic compounds were not yet identified in stem bark extract.

Whereas in leaf extract, there was only one compound high affinity (energy binding ≤ 9 kcal/mol). The compound with the highest affinity was berberrubine, an alkaloid (Lam et al. 2016) which was also a minor compound. This showed there were no phenolic or flavonoid compounds which were active to α-glucosidase in the leaf extract, corresponding to the result of feature important analysis.

As can be seen in Table 1, the antioxidant activity was higher in the stem bark than in leaf. It could be explained by the LC-MS/MS analysis. In the stem bark, some major compounds were reported to have antioxidant activity, namely moupinamide (Shukla et al. 2021), sinomenin (Yang et al. 2022), 15,16 dihydrotanshinone (Jiang et al. 2019), danshenxinkun B (Kang et al. 1997), and lucidumoside C (He et al. 2001). From the molecular docking analysis, only one of these antioxidant compounds had also high affinity to α-glucosidase: danshenxinkun B. This affirmed the prediction of feature important analysis that some of the active compounds that contributed to α-glucosidase inhibition in stem bark extract had also antioxidant activity.

In the leaf extract, none of the five most affluent compounds was reported to have antioxidant activity. This confirmed the conclusion made by combining feature important and DPPH analysis above.

Conclusions

Stem bark extract of *A. hexapetalus* showed medium strong α-glucosidase inhibition with IC_{50} of 47.084 ppm. Feature importance analysis predicted that some of the active compound in stem bark would also be antioxidant and belong to phenolic groups but not flavonoid. This prediction was corroborated by molecular docking and LC-MS/MS as well as TPC and TFC analysis especially for the antioxidant and flavonoid conclusion. There were some active phenolic compounds identified, but since the identified responses were not high enough, it was suggested that some active phenolic compounds were not yet identified in the stem bark extract. Stem bark extract also showed medium strong antioxidant activity with IC_{50} 147.339 ppm. The main active compounds for α-glucosidase inhibition in stem bark were likely from terpene group, i.e. hydroxytanshinone IIA.

For leaf extract, the α-glucosidase inhibition activity was weak with IC_{50} 104.755 ppm. The antioxidant activity was practically nonexistence (IC_{50} = 832.110 ppm). Like stem bark, feature important analysis predicted that the contribution of phenolic group and antioxidant activity to α-glucosidase inhibition was similar while flavonoid group did not contribute at all. Since the antioxidant activity was very weak, it

could be concluded that there would be no active α-glucosidase inhibitor from phenolic groups in the leaf extract. These predictions and conclusions were confirmed by molecular docking, TPC, TFC, and LC-MS/MS analysis.

The major compounds for both stem bark and leaf extracts were mostly from alkaloid group. This corroborated the TPC and TFC results which were not very high for both extracts.

Declarations

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Authors' contributions

D.R performed the experiment and drafted the manuscript, B.E designed the experiment and re-viewed the article, M.H collected the sample and performed LC-MS/MS analyses, Y.H, M.I.S and A.K reviewed and did critical reading of the manuscript. All authors have read and agreed to the published version of manuscript.

Conflict of interest

All authors have none to declare

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Figures

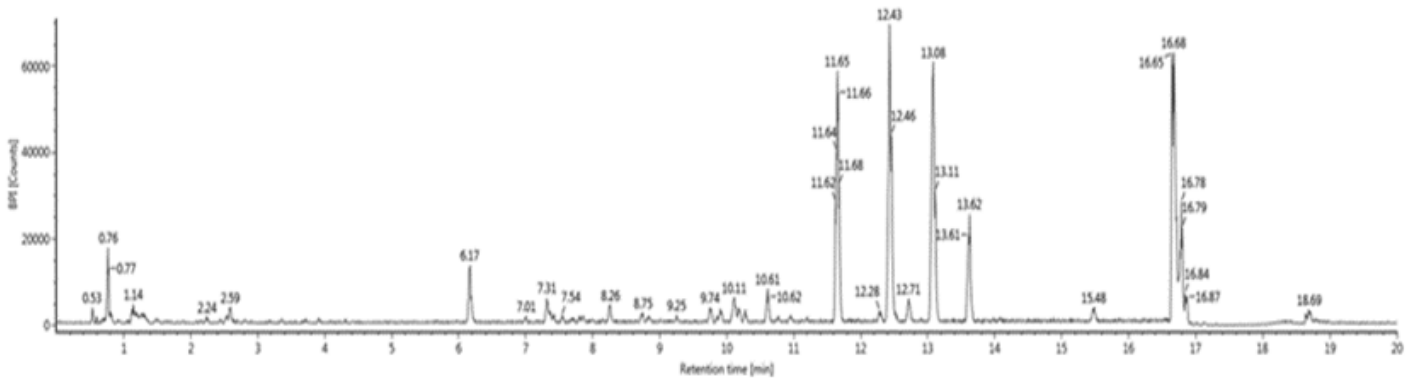

Figure 1

LC-MS/MS result of *A.hexapetalus*leaf ethanol extract (BPI =Base Peak Intensity)

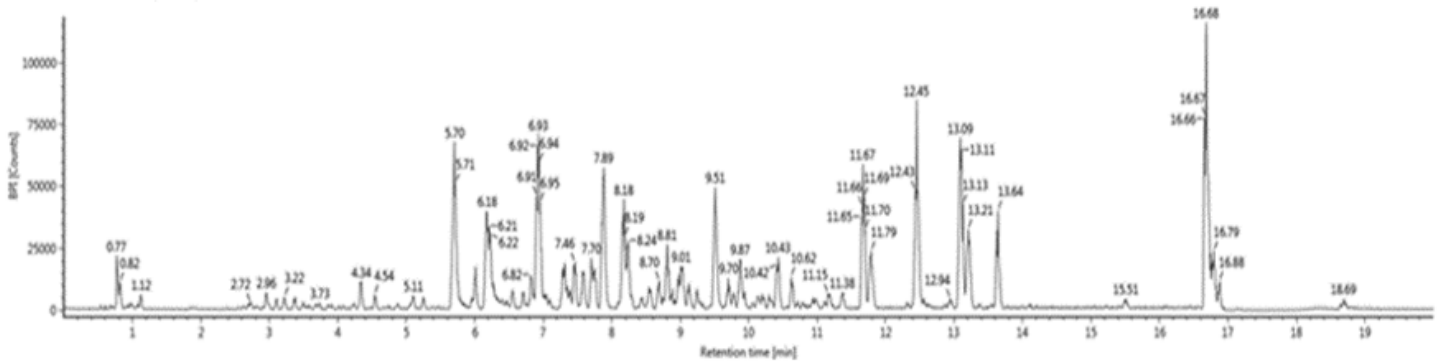

Figure 2

LC-MS/MS result of *Artabotrys hexapetalus* stem bark ethanol extract (BPI =Base Peak Intensity)

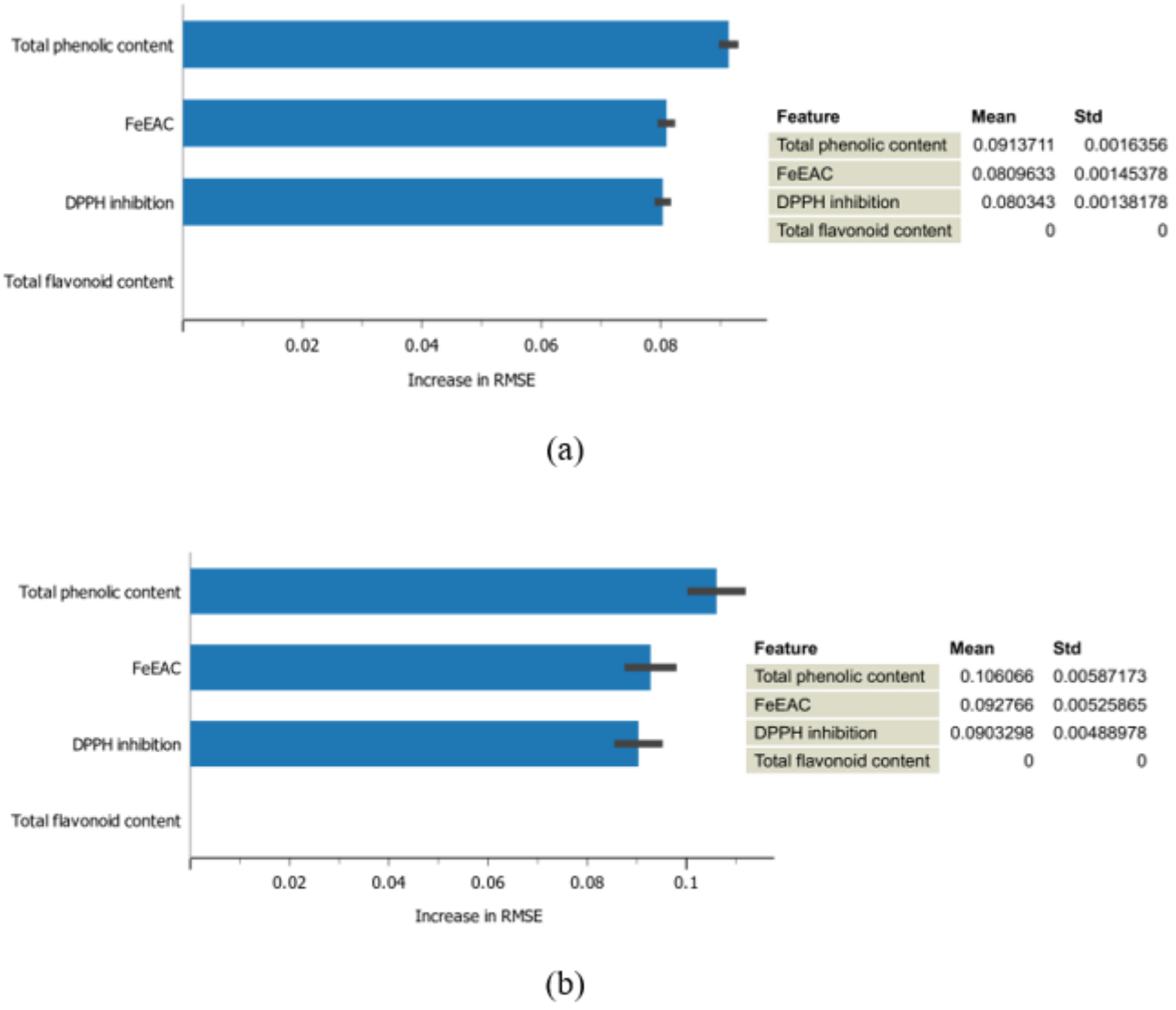

Figure 3

Result of random permutation of features used in random forest prediction of α-glucosidase inhibition for (a) leaf extract and (b) stem bark extract

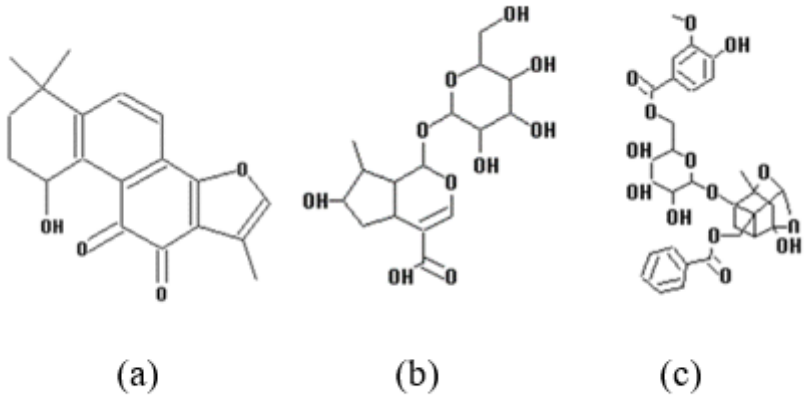

Figure 4

Figure 3. Structure of several active compounds (a) hydroxytanshinone II A (b) loganic acid (c) mudanpioside J

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