RESEARCH ARTICLE

Synergistic Activity of *Cinnamomum burmannii* (Nees & T. Nees) Blume and *Aquilaria malaccensis* Lamk. Extracts for Antidiabetic Study

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Abstract

BACKGROUND: Type 2 diabetes mellitus affects glucose metabolism resulting in hyperglycemia. The bark of *Cinnamomum burmannii* (CB) and the leaves of *Aquilaria malaccensis* (AM) are believed to be effective for diabetes treatment. This study evaluated the synergistic effect of CB and AM, the extracts' phytochemical profiles, and the interaction between CB and AM metabolites with α-glucosidase through molecular docking.

METHODS: The dried material was macerated with ethanol and then tested for α-amylase and α-glucosidase inhibitory activities and glucose diffusion inhibition in varied combination proportions. The extract fingerprinting was performed using a UV-Vis spectrophotometer followed by thin layer chromatography to determine the class of secondary metabolite in the extracts. Human maltase-glucoamylase (MGAM) receptor, ligands from acarbose and selected metabolites of CB and AM were studied *in silico* using UCSF Chimera, AutoDockTools, Autodock Vina Wizard (PyRx), and Biovia Discovery Studio software.

RESULTS: The best ratio of CB:AM for α-amylase and α-glucosidase inhibition was 0.75:0.25 mg/mL, with inhibitory activities of 86.36 and 96.38 %, respectively. The best glucose diffusion inhibition was achieved at a ratio of 0.5:0.5 mg/mL CB and AM. The β-caryophyllene of CB and palustrol of AM had a significantly higher binding affinity of -10.7 kcal/mol and -10.2 kcal/mol, respectively than acarbose, which had a binding affinity of -8.1 kcal/mol.

CONCLUSION: A ratio of CB to AM suppresses the activity of diabetes-related enzymes more efficiently. The in silico study suggested that the presence of β-caryophyllene in CB and palustrol in AM supported the synergistic activity.

KEYWORDS: *Aquilaria malaccensis*, *Cinnamomum burmannii*, diabetes mellitus, α-amylase inhibition, α-glucose inhibition, glucose inhibition

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Introduction

Diabetes mellitus (DM) is a multifactorial disease affecting how the body regulates metabolism, mainly how glucose, protein, and fat are regulated. Type 2 DM is characterised by high blood glucose levels, or hyperglycemia, and is caused by insensitivity in the body's production and absorption of insulin.(1) The combination of an unhealthy diet, inactivity, a malfunction in the release of insulin in response to meals, and decreased sensitivity of the target tissues to insulin action causes the blood glucose level to rise in type 2 DM patients.(2)

In the meanwhile, the breakdown of carbohydrates involves the two most essential digestive enzymes, α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20). With the assistance of α-amylase, long carbohydrate chains, such as starch, are hydrolysed into oligosaccharides. While in the small intestine, α-glucosidase hydrolyses oligosaccharides back into monosaccharides, which are then

absorbed by intestinal villi with the assistance of glucose transporters (GLUT) and solute carrier family 2 (SLC2) in the blood vessels. As a result, blood glucose levels rise. (3) Therefore, the inhibitor of these hydrolytic enzymes provides a substitute therapy for DM.

Two different approaches can be used to manage DM. Initially, insulin therapy became the most prevalent therapeutic option. Acarbose, miglitol, and voglibose are currently available enzyme inhibitors in the market.(4) However, undesirable effects, such as enzyme insensitivity, are typically linked with these synthetic drugs. As a result, herbal medicine is favoured as an alternate treatment to regulate glucose metabolism. Herbal medicine is gaining popularity due to the trend toward nature and the numerous studies confirming the safety of herbal medicines.

In several regions of Indonesia, the bark of *Cinnamomum burmannii* (CB) has been used to treat DM. Several bioactivities, including antioxidant, antibacterial, antiviral, anticancer, antitumor, anti-inflammatory, and antidiabetic, have been attributed to the CB bark. Moreover, the bark also contains potential secondary metabolites, especially polyphenolic (phenolic) compounds consisting of catechins, epicatechins, quercetin, and protocatechuic acid. These compounds reduce oxidative stress.(5) Meanwhile, the agarwood plant *Aquilaria malaccensis* (AM) leaves contain potential secondary metabolites, such as phenolic compounds, phenolic acids, steroids, fatty acids, flavonoids, terpenoids, xanthonoids, 2(2-phenylethyl) chromone benzophenone, and nucleosides.(6) These secondary metabolites work as antioxidants, antimicrobials, anti-inflammatory, antipyretic and analgesic properties, and antidiabetic.(7)

The use of polyherbal is one of the local wisdom heritages in Indonesia. Because part of the metabolite content in polyherbal synergises to overcome issues with the absorption of secondary metabolites into cells, polyherbal frequently have a greater therapeutic impact than single herbal.(8) Hence, with different modes of action of the components of polyherbal, they can complement one another in overcoming health problems. In general, several past research evaluated the antidiabetic potential of several types of herbals independently. However, no study has investigated the combination of these two herbals as antidiabetics. Thus, this study researched the potential of combined herbal consisting of CB and AM extracts as an inhibitor of α-amylase and α-glucosidase enzymes, glucose diffusion, and in silico molecular docking to predict the secondary metabolites of both CB and AM which has s good interaction with the enzymes studied.

Methods

Extraction of CB and AM

The bark of CB was obtained from Padang, West Sumatera, Indonesia, while the leaves of AM were collected from Karanganyar plantation, Central Java, Indonesia. Clean bark or leaves with no disease symptoms were selected, cleaned and heated in the oven at 40°C until a constant weight was achieved. Furthermore, samples of CB and AM were pulverised using a blender. About 10 g of the CB powder was then extracted in 100 mL solvents, while 10 g of AM powder was extracted in 130 mL solvents using the maceration method. Solvent for this extraction used ethanol (Merck, Kenilworth, NJ, USA). The crude extract was then evaporated using a rotary evaporator and stored at 4°C for further use. The combination ratio is determined by making a stock concentration of 1 mg/mL for each extract. The combination used for treatment was described in Supplementary 1.

Inhibition of α-amylase Enzyme Activity

A combination ratio of AM and CB extracts was used for the assay, while Acarbose (SigmaAldrich, St. Louis, MO, USA) was a positive control. The treatment was defined as C0, C1, S0 and S1. C treatment is for control treatment in which samples were changed with buffer. C0 was the control without an enzyme, while C1 was the control with an enzyme. S was for sample treatment in which S0 was the sample without enzyme, and S1 was the sample with enzyme. In the treatment without sample, the sample was replaced with distilled water, while for the positive control, acarbose was used, to replace sample. The combination of sample, enzyme and buffer in this analysis was described in Supplementary 2.

The inhibitory activity of α-amylase was evaluated at the absorbance of 540 nm.(9) Data was presented in the form of % inhibition with the following formula:

$$\% \text{ Inhibition} = \frac{((C1\text{-}C0) \text{ absorbance} - (S1\text{-}S0) \text{ absorbance})}{(S1\text{-}S0) \text{ absorbance}}$$

Inhibition of α-glucosidase Enzyme Activity

Set form combination ratio of both AM and CB extracts used for the assay, while acarbose was the positive control. The assay was conducted by combining several components, as described in Supplementary 3. S was for the sample, while C was the control. After following the procedure, the inhibitory of α-glucosidase enzyme activity was determined by measuring absorbance at 405.(10) Data was presented in

the form of % inhibition, as per mentioned in the α−amylase inhibitory assay.

The dialysis bag (MWCO 10 KDa, SigmaAldrich, St. Louis, MO, USA) was filled with 6.25 mL of 0.02 M glucose and mixed with 0.125 mL of sample or positive control (acarbose) with concentration as described in Supplementary 1. The negative control used distilled water to replace sample. The mixture was then put in a 150 rpm shaker for 90 min. Every 30 min, 1 mL of the solution outside the dialysis bag was collected and the glucose concentration was determined using the DNS method.(11)

Secondary Metabolite Profiling

Diluted the extracts of CB and AM in 1 ppm concentration using ethanol. Subsequently, the extract was scanned in a UV-Vis spectrophotometer (ThermoFisher Genesys, Waltham, MA, USA) at a 200-800 nm wavelength with 0.5 nm increment.(12) This procedure was repeated three times. The absorbance results were processed for Principal Component Analysis (PCA) using ChemFlow 20.05 software (INRAE, Paris, France). First, the data were pre-processed using the Savitsky Golay method, followed by PCA analysis which was coloured based on the plant origin, CB and AM.

Profile of secondary metabolite was potential from CB and AM extracts were also analysed using TLC Silica Gel GF254 (SigmaAldrich, St. Louis, MO, USA) with a size of 10 × 3 cm, the lower limit was 1.5 cm and the upper limit was 0.5 cm. Variations of the mobile phase, namely ethyl acetate:toluene:acetic acid:water in a ratio of 5:3:2:0.5. TLC plates were visualised under visible light, UV 254 nm, 366 nm, and after spraying with vanillin sulphate for terpenoid detection, $FeCl_3$ for phenolic detection, citroborate for flavonoid detection, and dragendorff for alkaloid detection.(13)

***In silico* Molecular Docking with Secondary Metabolites**

In silico molecular docking was carried out with the target receptor in the form of a protein from the crystal structure from PDB ID: 3TOP, C-terminal subunit of human Maltase-Glucoamylase in complex with acarbose. Acarbose was chosen as the standard ligand (positive control) to represent the commercially available drug, while secondary metabolites tested from AM and CB were based on previous studies.(14,15,16) Ligand was processed in .sdf format from the PubChem source. The target receptor in the form of a protein, namely 3TOP, was obtained from the Protein Data Bank (RSCB PDB) in .pdb format, which was then processed at UCSF Chimera to separate non-standard residues in the form of natural ligands from ligand-free proteins.(17) Process the next protein with AutoDockTools 1.5.7. (Center For Computational Structural Biology, La Jolla, California, USA) in the form of removing water molecules and adding AD4 Type atoms, which are saved in .pdbqt format.(18) The molecular docking procedure was performed using Autodock Vina Wizard in PyRx 0.8 (Sourceforge, Bonita, California, USA), and the binding and interaction between the protein from the receptor and the ligand was visualised in 3D and 2D using Biovia Discovery Studio 20.1.0 (San Diego, California, USA). The parameters used for evaluation in molecular docking were the statistical values of RMSD (Å) and binding affinity (kcal/mol). Both are used as primary validation for suitable and receptor-compatible ligands. The validation used for receptors was the validation of the Ramachandran Plot, which functioned to determine the position of the receptor structures (proteins) such as psi (ψ), Omega (ω), and phi (φ) structures and their stability.

Results

Extraction of CB and AM

Extraction was carried out previously by maceration using ethanol. The result showed that the yield rendement of CB was 10.95%, while AM had 14.13% rendement. Characters of CB and AM extracts have glossy visualisation and slickness that may be due to the content of essential oils (Table 1).

Inhibition of α-amylase Enzyme Activity

Inhibition of α-amylase enzyme activity using a combination ratio of CB and AM extracts as well as the positive control acarbose revealed that sample B with a combination ratio of CB AM 0.75:0.25 mg/mL had the highest inhibition percentage of 86.366±0.451% compared to the single extracts, CB with an inhibition value of 79.397±0.099%

Table 1. The yield of extraction of CB and AM.

Extracts	Simplisia (g)	Extract weights (g)	Rendements (%)	Physical Appearance
CB	10	1.095	10.95	glossy dark brown, paste, wet
AM	10	1.413	14.13	glossy brownish green, paste, wet

and AM with an inhibition value of 77.098±0.521%, while acarbose was 78.143±0.099% (Figure 1).

Inhibition of α-glucosidase Enzyme Activity

Inhibition of α-glucosidase activity using sample B, a combination of CB and AM at a ratio of 0.75:0.25 mg/mL, resulted in the highest inhibition performance of 96.368±0.00870% compared to the single extracts, CB with an inhibition value of 86.747±0.00825% and AM with an inhibition value of 83.133±0.00870%. However, acarbose exhibited the greatest suppression of α-glucosidase activity with an inhibition value of 94.940±0.00636% (Figure 2).

Inhibition of Glucose Diffusion

Ability of the extracts to limit glucose diffusion revealed that sample C, combination ratio CB:AM of 0.5:0.5 mg/mL, had the best inhibition of glucose diffusion from the dialysis bag in the first 30 min of 90 min with external glucose concentration of 0.256±0.001 mg/mL. This glucose concentration was lower than that of the single extract, CB, which had a glucose concentration value of 0.263±0.002 mg/mL and AM, which was 0.267±0.001 mg/mL. Positive control acarbose exhibited the least diffusion inhibition compared to combination and single extract treatments, as indicated by the glucose concentration of the outside dialysis bag, which was 0.286±0.001 mg/mL. This experiment's negative control had a dialysate glucose concentration of 0.357±0.001 mg/mL (Figure 3).

Secondary Metabolite Profiling by Spectrophotometer

The absorbance values of the extracts of CB and AM processed using Chemflow software resulted in the spectra, score, and loadings plots. Spectra plots represent the differences in extracts from CB and AM in detail from the wavelength of 200-800 nm, where there was a very significant difference in the spectral results of the two extracts. Then it was seen that there was a clear separation by PC1 with 99% of CB and AM in the score plot, thus representing the possibility of differences in secondary metabolites from their uptake. The loading plot consisted of PC1 99% and PC2 1% (Figure 4).

Secondary Metabolite Profiling by TLC

The metabolites profiling was done by observing the elution results at UV 254 nm, and 366 nm and after spraying with detector reagents in the visible light. The Rf value was determined by the polarity of the eluent and the secondary metabolites to be identified. The more spots, the more secondary metabolite observed in both extracts. CB and AM extracts showed positive reactions for terpenoids, phenolics, and flavonoids but negative for alkaloids. The Rf values for each detected class of secondary metabolites were generally distinct across the two extracts, indicating that they included different secondary metabolites (Table 2, Figure 5).

***In silico* Molecular Docking with Secondary Metabolite**

The studies of molecular docking revealed that secondary metabolites in the form of β-caryophyllene of CB and palustrol of AM have stronger negative binding affinities with values of -10.7 and -10.2 kcal/mol, respectively than the control ligand acarbose (-8.1 kcal/mol) (Table 3). The interaction between the receptor and acarbose yields 9 bonds, whereas -caryophyllene and palustrol have five and four bonds, respectively (Figure 6).

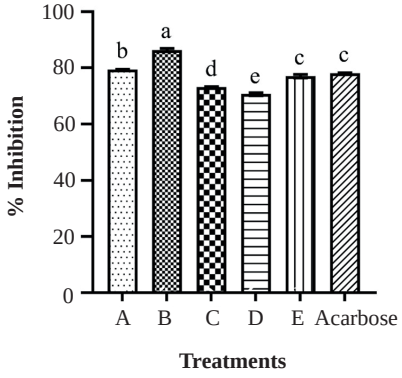

Figure 1. The α-amylase inhibition activity of the combination of CB and AM extracts. Treatments were designed based on the combination of CB:AM at mg/mL (A: 100:0; B: 75:25; C: 50:50; D: 25:75; E: 0:100). The difference in letters shows a significant difference based on Tukey HSD (Honestly Significant Difference) at $p< 0.05$.

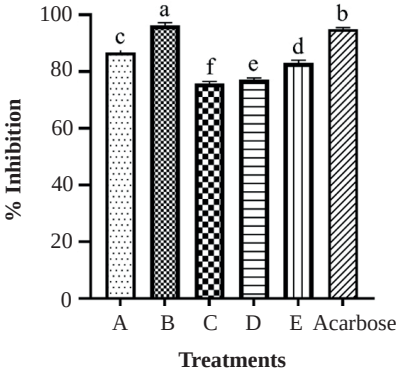

Figure 2. The α-glucosidase inhibition activity of extract combination CB and AM. Treatments were designed based on the combination of CB:AM at mg/mL (A: 100:0; B: 75:25; C: 50:50; D: 25:75; E: 0:100). The difference in letters showed a significant difference based on Tukey HSD (Honestly Significant Difference) at $p<0.05$.

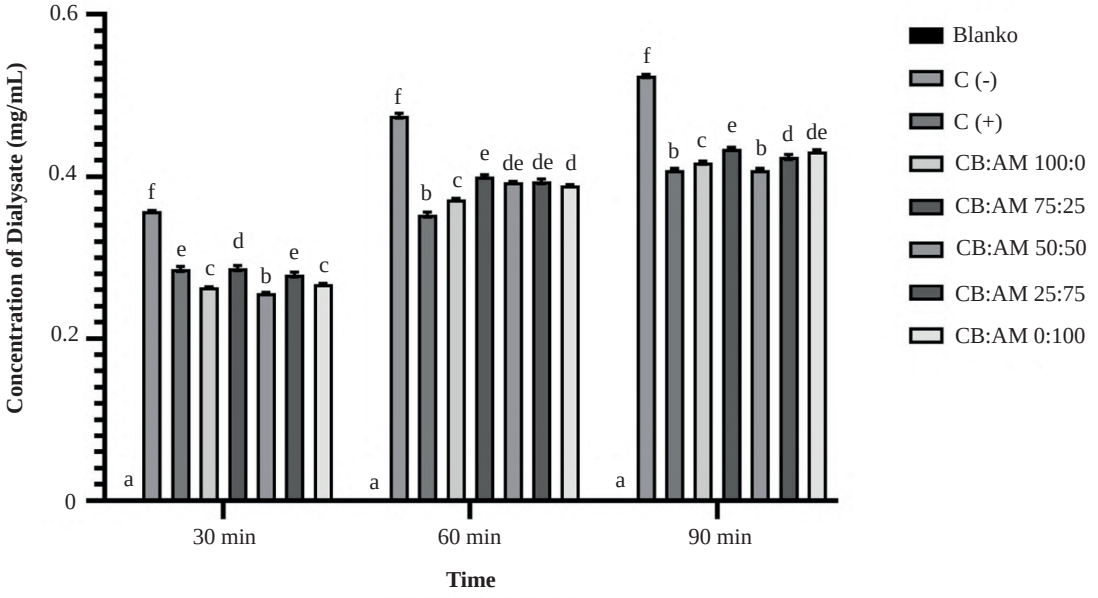

Figure 3. Inhibition of glucose diffusion from the dialysate bag per 30 minutes. Treatments were designed based on the combination of CB:AM at mg/mL. The difference in letters showed a significant difference based on Tukey HSD (Honestly Significant Difference) at $p<0.05$.

Discussion

In this study, the combination of CB and AM ethanol extracts showed better activity of α-amylase and α-glucosidase inhibitors than the single extract and acarbose, the positive control. The best performance to inhibit both enzymes was observed at a ratio of CB:AM 0.75:0.25 mg/mL with an inhibition percentage of 86.366±0.451%. The better performance on the value of inhibition percentage in the combination ratio may be due to the synergism effect between the secondary metabolites of the two extracts. The compounds may reduce the activity of the hydrolytic enzymes; thus, the consumption of the herbals may enable to maintain the blood sugar levels.(19) Secondary metabolites may function as an α-amylase inhibitor by interfering with the enzyme's catalytic action on the α-1,6 glucan linkages in starch.(20) Previous study utilising *Juglans regia* and *Urtica dioica* leaf extracts showed that the highest concentration of 0.8 mg/mL inhibited α-amylase activity by 73% and 76%, respectively.(21) The ethanol extract of *Adenanthera pavoniner* inhibited α-amylase activity by 85% at a concentration of 0.58 mg/mL.(22) A lower activity from this current study has also been reported on the ethanol extract of *Morinda lucida* with inhibit α-amylase by 35% at 5.5 mg/mL.(23) *Graptophylum pictum* extract containing tannin polyphenols showed a 30.68% inhibition of α-glucosidase activity at a concentration of 650 mg/mL.(24) Further research using the ethanol extract of *Strychnos nitida* stem, which is rich in triterpenoids and polyphenols, showed

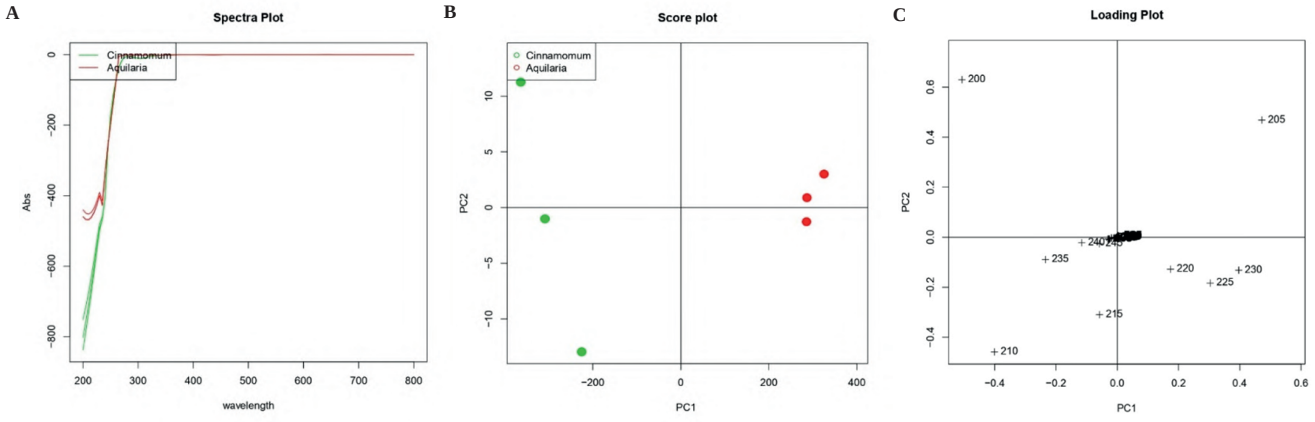

Figure 4. Visualisation of PCA results plot between CB and AM. A: Spectra plot; B: Score Plot; C: Loading Plot.

Table 2. TLC Profile metabolite secondary of CB and AM.

Metabolite Secondary	Extract	Rf	Spots	Spray Reagent	Comparison Compound
Terpenoids	CB	0.167	1	Vanilin sulfate	Thymol blue
	AM	0.250, 0.292	2		
Phenolics	CB	0.158, 0.283, 0.483	3	$FeCl_3$	Folin
	AM	0.429, 0.721, 0.896	3		
Flavonoids	CB	0.417, 0.967	2	Citroborate	Quercetine
	AM	0.450	1		
Alkaloids	CB	0	0	Dragendorff	Caffeine
	AM	0	0		

highest inhibition of the α-glucosidase activity of 11.45% at a concentration of 0.6 mg/mL.(10)

This study also detected the glucose diffusion inhibition action. Secondary metabolites in the colon may bind glucose, slowing the postprandial rise in blood glucose levels.(25) The ratio of CB:AM at 0.5:0.5 mg/mL showed better performance at 30-90 minutes with an external glucose concentration of 0.256±0.001 mg/mL, compared to the single extract and the acarbose with a slightly higher concentration of glucose ranging from 0.263±0.002 to 0.286±0.001 mg/mL. Other study evaluating the glucose diffusion inhibitory properties of aqueous and ethyl acetate extracts of *Caralluma europaea* found that after 30 to 60 minutes of incubation, the external solution of the dialysis bag had a glucose concentration of 1.08 to 1.55 mg/mL.(25) Research using unripe *Musa sapientum* ethanolic extract showed a reduction in glucose concentration in the first 30 min with a concentration of 84.6 mg/mL in the external dialysis solution. The extract contains flavonoids and fibre from ethanol extraction, which is thought to inhibit glucose production.(26)

Due to its polarity, ethanol possesses a broad capacity to extract metabolites; hence, it was anticipated that it would extract numerous CB and AM compounds. Several secondary metabolites in both extracts, such as terpenoids, flavonoids, phenolics, essential oils, and fatty acids, may function in concert to inhibit the enzymes' active sites.(13,15) This study discovered that CB and AM extracts contained phenolics, flavonoids, and terpenoids with different Rf values, as revealed by TLC. Previous findings on the secondary metabolites of CB indicated that terpenoids, particularly ß-caryophyllene, were identified at a greater concentration in CB, whilst palustrol was detected at a higher quantity in AM. Phenolic and flavonoids are

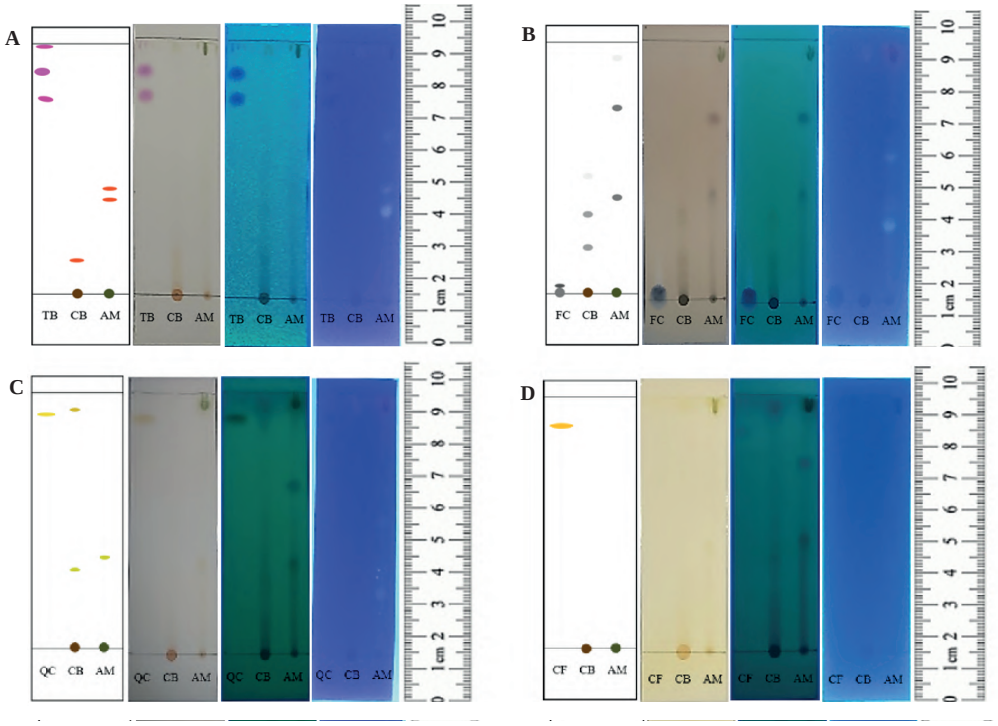

Figure 5. Detection of secondary metabolite content in the CB and AM extract. A: Terpenoids; B: Phenolic; C: Flavonoids; D: Alkaloids in visible light, UV 254 nm, and 366 nm. Note: TB: Thymol blue; FC: Folin; QC: Quercetine, CF: Caffeine.

Table 3. Binding affinity of receptor-ligand (kcal/mol).

Treatments	Binding Affinity (kcal/mol)	Molecule Weight (g/mol)	RMSD (Å)
Control			
Acarbose	-8.1	645.60	0
CB			
β-caryophyllene	-10.7	204.35	0.495
Catechin	-7.7	290.27	1.875
Cinnamaldehyde	-6.6	132.16	0
Mellilotine	-6	148.16	1.518
AM			
Palustrol	-10.2	222.37	0.728
Squalene	-6.8	410.70	0
Calarene	-6.3	204.35	0
9-decanoic	-5	172.26	0

secondary metabolites with strong antioxidant qualities and antidiabetic characteristics that can lower blood sugar levels and block the synthesis of glucose.(27) Alkaloids were not detected in this study. This finding is consistent another study on metabolite fingerprinting of AM extract using TLC.(13) This may be attributable to low environmental stress, growing location, growth, and species variety.(28) Similarly, for CB extract, a previous study that detected the secondary metabolites of CB using TLC reported the presence of phenolic, flavonoids and steroid-triterpenoids but no alkaloids.(29) Further metabolite fingerprinting was performed using UV/Vis spectrophotometer. The spectra of both extracts displayed a considerable difference, highlighted by the score plots of PCA, which clearly distinguished between the two extracts. The loading plots revealed that CB's significant wavelengths were 500-600 nm (flavonoids and flavins group) (30), whereas AM's were 200-400 nm (triterpenoid and phenolic groups) (31).

MGAM is used as a receptor because it has a role like the α-glucosidase enzyme, which can hydrolyse the

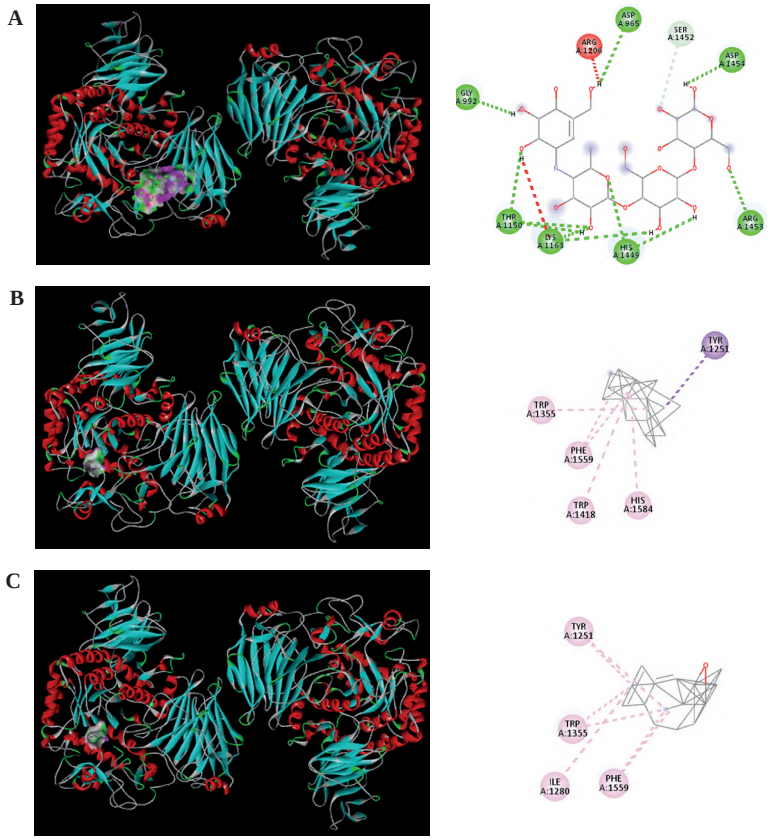

Figure 6. Molecular docking visualisation of 3TOP receptor with ligand. A: Acarbose; B: β-caryophyllene; C: Palustrol.

α-1,4 bond of glucose in the form of oligosaccharides and the presence of catalytic receptor terminals in the form of carboxyl and amino acids.(32) This receptor is used as a target with standard type 2 DM control/therapy, namely acarbose which can inhibit the formation of enzyme products in the brush border intestine.(33) The RMSD value is an essential parameter and validation in measuring and calculating the predictive docking process. The RMSD value for validation is generally from 0 to 3 Å.(29) The RMSD values of β-caryophyllene, palustrol, and acarbose were 0.495; 0.728; and 0 Å, which means the value is accepted and becomes a potential candidate for alternative antidiabetic therapy. Binding affinity in Table 3 supported by the number of bonds, shows the strength and stability of the bond between the receptor and the ligand. The lower or more negative binding affinity, the stronger the ability of the ligand to bind to the receptor is related to the alignment of the binding energy and its stability.

The binding site of acarbose to MGAM is observed at the pocket binding position. While β-caryophyllene and palustrol are shown to occupy the same binding pocket location, the two metabolites occupy distinct positions relative to acarbose, albeit inside the same receptor region. The difference in the position of the pocket binding causes the difference in the binding affinity results and molecular weight of β-caryophyllene and palustrol is 204.35 and 222.37 g/mol, which is smaller than that of acarbose with a molecular weight of 645.6 g/mol. This may make it simpler for the two metabolites to enter the receptor pocket, bind, and fit. Therefore, the secondary metabolites β-caryophyllene and palustrol are possible candidates for diabetic treatment.

Conclusion

A correct ratio of CB to AM suppresses the activity of diabetes-related enzymes more efficiently than the conventional drug acarbose. The profiling of secondary metabolites using TLC and UV-Vis spectrophotometer demonstrated that the CB extract is distinct from that of AM. The *in silico* study suggested that the presence of β-caryophyllene in CB and palustrol in AM supported the synergistic activity.

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Authors Contribution

AAB conducted the investigation and in drafting the manuscript. LH performed conceptualisation *in silico* and provided technical assistance. NW was involved conceptualisation and drafting. TRN involved supervisor, resource, conceptualisation in experimental, and analysis data. All authors discussed the results and commented on the manuscript.

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