



Combination of citrus peel-derived essential oils with acarbose to inhibit amylolytic enzymes – A potential type II diabetes treatment approach

Kadima Samuel Tshiyoyo^a, Ali Rabbad^a, Abdullahi Ahmed Yusuf^b, Samkelo Malgas^{a,*}

^a Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa

^b Department of Zoology and Entomology, University of Pretoria, Pretoria, South Africa

ARTICLE INFO

Keywords:

α -Glucosidase inhibitor
Type 2 diabetes
Molecular dynamic simulations
In silico analysis
Amylolytic enzymes
Starch digestion
Citrus peel essential oil

ABSTRACT

Type 2 diabetes (T2D) can be managed by inhibiting amylolytic enzymes, α -amylase and α -glucosidase, reducing the impact of dietary carbohydrates on blood glucose elevation. Acarbose, a current α -glucose inhibitor (AGI), has excessive α -amylase inhibition, resulting in side effects associated with large amounts of undigested starch being fermented in the colon. This study evaluated the AGI efficacy of citrus peel-derived essential oils, where they were first tested in silico against the target amylolytic enzymes, and then their AGI activity was tested in vitro. The synergistic effects of the essential oils with acarbose against amylolytic enzymes were also determined. In silico and in vitro data of the efficacy of the essential oils as AGIs correlated positively; lower Ki values correlated with more negative binding affinity. Furthermore, molecular dynamic simulations of the most potent compounds were evaluated and indicated relative flexibility and stability induced upon ligand interactions with the protein. The standard AGI drug, acarbose, had the lowest Ki (0.10 ± 0.01 mg/mL) and more negative binding affinity (-7.5 kcal/mol) than the essential oils for α -glucosidase; however, the essential oils only showed potent inhibition against α -glucosidase, with the most potent essential oils being valencene (Ki = 0.33 ± 0.04 mg/mL), carveol (Ki = 0.53 ± 0.02 mg/mL) and geraniol (Ki = 0.56 ± 0.02 mg/mL). The essential oils and acarbose displayed competitive inhibition of α -glucosidase. Furthermore, a combination of acarbose with carveol or geraniol at a ratio of 12.5 μ g/mL: 2 mg/mL exhibited antagonistic (CI > 10) and synergistic (CI < 0.7) effects on α -amylase and α -glucosidase inhibition, respectively. Carveol or geraniol can be considered as potentially therapeutic in managing T2D, as it may display lowered AGI-associated side effects.

1. Introduction

Diabetes encompasses a series of autoimmune, metabolic, and genetic disorders leading to elevated concentrations of glucose in the blood, a condition known as hyperglycaemia [1]. Hyperglycaemia can lead to significant complications, including damage to the kidneys, retina, nerves, heart, and skin, resulting in serious health problems [1,2]. Diabetes mellitus and its associated disorders are posing a significant burden to the health system. The prevalence of diabetes mellitus has increased significantly and is today one of the leading causes of morbidity and mortality worldwide [3]. A report from the International Diabetes Federation estimated that 537 million adults aged between 20 and 79 were living with diabetes in 2021, with another 240 million living with it undiagnosed [4,5]. These numbers are projected to increase to 643 and 783 million by 2030 and 2045, respectively, due to a reduction in physical activity, an increase in obesity numbers and the

ageing population [4]. Type 1 and type 2 diabetes (T2D) make up the most cases globally, with T2D accounting for up to 90 % of all diabetes cases [6,7].

Carbohydrates are essential to all living organisms and a significant component of our diet [8]. They are obtained from starchy vegetables, beans, rice, milk, and grains. Carbohydrate digestion is connected to T2D as it produces monosaccharides like glucose and increases blood glucose levels. Amylolytic enzymes such as α -amylase (EC 3.2.1.1) and α -glucosidase (EC 3.2.1.3) are responsible for the digestion of complex carbohydrates in our food into simple monosaccharides, which cells required for the energy [9]. The salivary gland and pancreas secrete amylase, which is responsible for the hydrolysis of internal α -1,4 glycosidic bonds in dietary starch into oligosaccharides such as malto-dextrins and maltose [10,11]. Meanwhile, α -glucosidase, found on the brush border of the small intestine, hydrolyses oligosaccharides into absorbable glucose, which enters the bloodstream [12,13]. These

* Corresponding author.

E-mail address: samkelo.malgas@up.ac.za (S. Malgas).

<https://doi.org/10.1016/j.ijbiomac.2025.141504>

Received 15 December 2024; Received in revised form 5 February 2025; Accepted 24 February 2025

Available online 25 February 2025

0141-8130/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

enzymes are essential targets in managing T2D because they affect blood glucose levels.

The current standard α -glucosidase inhibitor (AGI) drug for treating T2D is acarbose, but it can cause gastrointestinal side effects such as abdominal pain, flatulence, and diarrhoea [14]. Strong α -amylase inhibition results in undigested starch fermented by colon bacteria, possibly leading to the reported side effects [15]. Several plants and their phytochemicals have been investigated for their potential AGI activity. Researchers seek natural treatments with improved efficacy and safety profiles to reduce T2D treatment costs [16].

Citrus, belonging to the *Rutaceae* family, is a major fruit crop globally and an essential part of the human diet. Popular for their flavours, aroma, and colours, citrus fruits are crucial in tropical and subtropical regions [17]. The annual production of citrus fruits is 120 million tons, with most consumed while fresh and about one-third processed into juice [18,19]. The increasing demand for processed food comes with the burden of managing generated waste, which is costly and has environmental effects such as greenhouse gas emissions and threatening human health [20,21]. *Citrus* waste contains essential micronutrients and bioactive compounds, including pectin, polyphenols, and essential oils, which offer health-promoting effects [22].

Essential oils (EOs) derived from citrus fruits have various biological activities, such as antimicrobial, antioxidant, and anticancer properties. Due to their aroma and biological activities, they are used in multiple industries, including cosmetics, food, and pharmaceuticals [23–25]. Studies have shown that EOs from medicinal plants have potential antidiabetic activity, but their mode of action is still unknown [26,27]. *Citrus* EOs are found in the peels of *Citrus* fruits, mainly in the exocarp flavedo containing oil sacs [28]. They include a variety of compounds, such as monoterpenes, sesquiterpenes, and other derivatives containing alcohols and aldehydes as functional groups [22,29].

The current study aimed to extract EOs from citrus peels, evaluate their phytochemical composition and analyse their potential as lead antidiabetic agents through the inhibition of amylolytic enzymes. The identified EOs were first computationally tested against these enzymes, and *in vitro* inhibitory activity was evaluated alone and in combination with acarbose against both α -amylase and α -glucosidase; individually and as a cocktail. We also analysed the remaining starch residues after inhibiting the amylolytic enzyme cocktail with the AGIs. This involved measuring the quantity and size distribution of the residues to determine which inhibitor or combination led to more residual starch that could be transported to the colon *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Hog pancreatic α -amylase (10080), potato starch, maltose, acarbose, essential oils (carveol, geraniol, limonene, linalool, nerol and valencene), and dinitrosalicylic acid (DNS) reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). α -Glucosidase from *Saccharomyces cerevisiae* (E-MALTS) and a D-Glucose assay kit containing glucose, glucose oxidase/peroxidase (GOPOD) were purchased from Megazyme (Bray, LEN, Ireland). All other reagents were obtained from Sigma Aldrich and are of purity ranging between 70 and 99 %.

2.2. Citrus peel collection and essential oils extraction

Fresh orange and tangerine *Citrus* fruits were purchased from the local market in Pretoria, South Africa. The fruits were peeled and washed with tap water before being cut to an approximate size of 1.0 × 0.5 cm using a kitchen knife or scissors. The pieces were placed on a blotting paper and dried before extraction. Any unused peels were stored at –20 °C to prevent microbial growth.

Citrus EOs were extracted by hydrodistillation, whereby the distillation setup consisted of a round bottom flask, a heating mantle, a spiral

condenser, a conical flask, and a thermometer. About 60 g of cut peels were transferred into the round bottom flask, mixed with 150 mL of distilled water to cover the peels, and extracted at 95–99 °C for three (3) hours. The distillate was collected in a conical flask and then separated according to polarity using a separating funnel for liquid-liquid extraction. Ten millilitres of hexane and ethyl acetate were added to the mixture to extract hydrophobic and hydrophilic/oxygenated components, respectively. After separation, the fractions (hexane or ethyl acetate) were evaporated overnight. The yellowish EOs were collected and stored in sealed glass vials at 4 °C until further use.

The percentage yield of EOs from the peels was calculated using the following formula (Eq. 1):

$$\text{Yield} = \frac{\text{amount of citrus oil extracted}}{\text{Total amount of citrus peels}} \times 100\% \quad (1)$$

2.3. Chemical profiling of citrus peel essential oils (EOs)

Citrus essential oils were profiled using a Shimadzu QP 2100 SE Gas Chromatograph- Mass spectrometer (GC–MS) (Shimadzu Corporation, Tokyo, Japan) equipped with a Restek Rtx 5MS GC-capillary column (30 m × 0.25 mm × 0.25 μ m) (Restek, Inc., Bellefonte, USA). One milligram of the extract was dissolved in 1 mL of hexane; One microliter of each dissolved extract was injected at 250 °C into the GC–MS in split mode with a split ratio of (1:10) with helium used as a carrier gas at a constant flow rate of 1.0 mL/min. The oven was programmed as follows: 50 °C for 4 min, increased at the rate of 10 °C/min to 150 °C, held for 2 min, then increased at 15 °C to 280 °C and held for 13 min. The ion source was set at 200 °C with the interface at 250 °C, electrical ionisation energy of 70 eV in electro-impact mode and a scan speed of 2000 at 0.30 s. The compounds were tentatively identified by comparing their retention time with those of published mass spectra libraries in the National Institute Standard and Technology 11th edition (NIST11) [30], and Willey 10th edition and retention indices were calculated using a mixture of C13–C44 alkanes.

2.4. Prediction of pharmacokinetic properties

The pharmacokinetic properties of the identified compounds were predicted using the SwissADME online tool [31]. The small molecular-input line-entry systems (SMILES) of the EOs and acarbose were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The SMILES were imported into the SwissADME interface (<http://swissadme.ch/>) before running the program, and the following properties were generated: gastrointestinal (GI) absorption, bioavailability, cytochrome P450 inhibition and blood-brain barrier permeability.

2.5. Molecular docking of EOs against amylolytic enzymes

The compounds were docked in the pocket of two amylolytic enzymes with the AutoDock Vina 1.1.2 scoring engine integrated in PyRx 08 software, an open-source virtual screening tool [32,33]. The preparation of enzymes and their interactions with the compounds were monitored via Discovery Studio Visualizer v21.1.0 (San Diego, CA, USA). The 3D crystal structure of porcine pancreatic α -amylase (PDB ID: 1DHK) was downloaded from the protein data bank PDB (<https://www.rcsb.org/>). However, the 3D crystal structure of *Saccharomyces cerevisiae* α -glucosidase is not yet available; therefore, it was developed through an *in silico*-based homology modelling technique via the SwissMODEL website (<https://swissmodel.expasy.org/>) and following a previously reported method with some modification [34]. The α -glucosidase gene product from *S. cerevisiae* (MAL32_Yeast) was used, and the sequence was obtained from the UniProt website (<https://www.uniprot.org/>) in FASTA format (uniprot ID: P38158). The sequence was submitted to the SwissMODEL interface to generate a model and for genomic analysis. The model template with high sequence similarities, excellent coverage

and good resolution was selected as the defined α -glucosidase 3D structure for molecular docking. Moreover, model-template sequence alignment of the template with the α -glucosidase gene product from *S. cerevisiae* (MAL32_Yeast) was evaluated through SwissMODEL to illustrate the similarity and coverage between the two sequences.

The enzyme 3D structures were refined and prepared using Discovery Studio Visualizer by removing water molecules before loading them into PyRx. On the other hand, the 3D structures of the compounds were downloaded from PubChem and converted to PDB files by Discovery Studio before uploading to PyRx for further energy minimisation and molecular docking.

The PDB files of the compounds and the enzymes were loaded onto PyRx for molecular docking using AutoDock Vina. The following dimensions for the proteins' grid boxes used were (centre: $98 \times 31 \times 18 \text{ \AA}$ and box dimensions: $27 \times 27 \times 27 \text{ \AA}$) for α -amylase and (centre: $1.0 \times 4.6 \times 4.6 \text{ \AA}$ and box dimensions: $27 \times 27 \times 27 \text{ \AA}$) for α -glucosidase. Furthermore, the ligand-receptor complexes were exported into Discovery Studio to generate 2D images showing molecular interactions between the ligand and amino acid residues of the enzymes, clearly illustrating hydrogen and hydrophobic interactions.

2.6. In vitro α -amylase inhibitory determination of EOs

The inhibitory potential of EO extracted from *Citrus* and pure standards against α -amylase were obtained using the following DNS assay method. Briefly, 200 μL of 2 % (w/v) potato starch prepared in 50 mM sodium phosphate buffer (pH 6.9) was mixed with 100 μL of varying concentrations of *Citrus* EOs extract (0–2 mg/mL) or commercial EOs (0–2 mg/mL) or acarbose (0–0.5 mg/mL) in Eppendorf tubes. The inhibitor stocks were prepared in DMSO and diluted in 50 mM sodium phosphate buffer (pH 6.9) during the assay. The mixtures were then incubated for 5 min at 37 °C with agitation at 25 rpm using a Roto-Therm Plus Incubator (Sayreville, New Jersey, USA). The reaction was initiated by adding 100 μL of Hog pancreatic α -amylase solution (12.5 $\mu\text{g/mL}$) prepared using phosphate buffer (pH 6.9); the assay mixtures were incubated at 37 °C for 20 min with constant agitation at 25 rpm. To stop the reaction, 200 μL DNS reagent was added to the mixture, and the resultant solution was incubated at 80 °C for 20 min in an ONiLAB scientific Dry Bath Incubator (Missouri, Texas, USA). Thereafter, 300 μL of the solution was transferred to a 96-well microplate, and the absorbance was measured at 540 nm using a SpectraMax Paradigm microplate reader (San Jose, California, USA). The inhibitor was replaced by the buffer solution for the negative control, while acarbose was used as the positive control. Percentage enzyme inhibition was calculated from the below formula:

$$\%Inhibition = \left[\frac{Absorbance\ of\ negative\ control - Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} \right] \times 100 \quad (2)$$

The IC_{50} values were determined for the EOs fractions, acarbose and pure EOs, and the values were used to select promising compounds for further analysis.

2.7. In vitro α -glucosidase inhibitory determination of EOs

The inhibitory potential of *Citrus* EOs fractions, acarbose and pure was investigated against α -glucosidase using the glucose assay kit (GOPOD format) [35]. Briefly, 100 μL of 2 % (w/v) maltose solution prepared in 50 mM sodium phosphate buffer (pH 6.9) was mixed with 50 μL of varying concentrations of *Citrus* EOs extracts (0–2 mg/mL) or

pure EOs (0–2 mg/mL) and/ acarbose (0–0.125 mg/mL) in a 96-well microplate. The plate was pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of 50 μL of 40 $\mu\text{g/mL}$ α -glucosidase, and the plate was incubated at 37 °C for 20 min in a Roto-Therm Plus Incubator. The plate was incubated at 80 °C for 10 min to stop the reaction in a Labotech EcoTherm Oven (Johannesburg, South Africa). To measure glucose production, 10 μL of the mixtures and glucose (1 mg/mL) were mixed with 300 μL of GOPOD reagent and incubated at 37 °C for 20 min, and the absorbance was measured at 510 nm. The percentage of enzyme inhibition and IC_{50} was calculated and determined as described in Section 2.6.

2.8. Kinetic studies

A 100 μL of varying concentrations of maltose (0–2 %) with 50 μL of selected concentrations of pure EOs (0.5 and 1 mg/mL) or acarbose (0.125 and 0.250 mg/mL) were placed in a 96-well microplate. The mixtures were then preincubated, and the reaction was initiated by the addition of 50 μL of 40 $\mu\text{g/mL}$ α -glucosidase (100 U/mg), and the plate was incubated at 37 °C for 20 min. Glucose production was measured as described in Section 2.7 above.

Kinetic parameters such as the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by generating Lineweaver-Burk plots ($1/V$ versus $1/[Substrate]$) using GraphPad Prism Software (La Jolla, California, United States).

2.9. Correlation between in silico and in vitro studies

Correlation studies between binding affinities and K_i values were conducted using the acarbose, carveol, geraniol, limonene, linalool, nerol and valencene data. Pearson's correlation coefficient was determined by following default settings in GraphPad Prism, and a graph was generated.

2.10. Molecular dynamic simulations

Molecular dynamic (MD) simulations were used further to investigate the interactions between selected compounds and α -glucosidase and to validate the docking study. Prior to MD simulations, UCSF Chimera was used to further prepare the systems by adding hydrogen atoms and AM1-BCC charges [36]. The AMBER 18 Particle Mesh Ewald Molecular Dynamics (PMEMD) single graphic processor unit (GPU) package was then used to carry out the MD simulations [37]. Furthermore, the atomic partial charges of all compounds were generated by using the ANTECHAMBER module, and the 100 ns trajectories at an interval of 1 ps were produced for the free α -glucosidase (apo) system

and all other complex systems [38].

After the MD simulations, the CPTRAJ and PTRAJ modules of AMBER 18 were used to conduct the post-dynamics analysis for each trajectory [39]. The root mean square deviation (RMSD) and the root mean square fluctuation (RMSF) were determined to investigate the system's stability/rigidity and flexibility, respectively. However, to explore the compactness and solvent accessibility of the system, we measured the radius of gyration (ROG) and the solvent-accessible surface area (SASA), respectively. Moreover, the molecular mechanics/generalised-born surface area (MM/GBSA) method was implemented to calculate the binding free energy for each compound [38,40].

2.11. *In vitro* inhibition of amylolytic enzyme cocktail by selected compounds or acarbose alone

In an Eppendorf tube, 200 μL of 2 % (w/v) potato starch was combined with 100 μL of varying concentrations of chosen pure EOs (0–2 mg/mL) or acarbose (0–0.125 mg/mL). The resulting mixture was incubated at 37 °C for 5 min. Next, 50 μL of 50 $\mu\text{g}/\text{mL}$ hog pancreatic α -amylase (50 U/mg) and 50 μL of 80 $\mu\text{g}/\text{mL}$ α -glucosidase (100 U/mg) were added to the tubes to initiate the reaction, and the mixtures were incubated at 37 °C for 20 min with agitation at 25 rpm. The tubes were incubated in a heating block at 100 °C for 5 min to stop the reaction. The IC_{50} value of each compound was estimated.

2.12. *In vitro* inhibition of amylolytic enzyme cocktail by a combination of acarbose and selected compounds

Furthermore, the synergistic effects between acarbose and the selected EOs on the enzyme cocktail were investigated using the same method described in Section 2.10. Briefly, 2 % (w/v) of potato starch was mixed with a combination of acarbose and selected EOs at different ratios (see Table 5). The addition of the enzyme cocktail initiated the reaction, and the inhibitory activities were determined using the DNS (Section 2.6) and GOPOD (Section 2.7) methods.

Compusyn software (Paramus, NJ, USA) was then used to generate the CI-isobologram and to calculate the combination index (CI) based on the method by Chou et al. [41]. The method permits the quantitative determination of combination interaction based on an established algorithm to simulate synergism, additive, and antagonism effects.

2.13. *In vitro* determination of starch residues

The starch-iodine method described by Xiao et al. (2006) was used to quantify starch residues after enzymatic reaction, with some modifications [42]. A spectrum was generated by mixing starch (2 mg/mL) with iodine reagent (0.01 %) in a 96-well plate at a range of 400–650 nm to determine the optimum wavelength and the starch-iodine standard curve was constructed by measuring the absorbance at 610 nm of different starch concentrations (0.03–2 mg/mL) with iodine reagent (0.01 %).

Following the enzymatic reaction in Section 2.12, the amount of starch residues was determined using the starch-iodine method and the generated standard curve in Fig. S5A. In a 96-well plate, 30 μL of the reaction mixture was diluted with 120 μL phosphate buffer (pH 6.9) and 150 μL of 0.01 % iodine reagent. Thereafter, the mixture was left for 3 min at room temperature before measuring the absorbance at 610 nm. The reaction blank consisted of the starch residues present in the absence of the enzyme cocktails and was used to calculate % undigested starch following Eq. (3) below.

$$\% \text{Undigested starch} = \frac{\text{starch residues in the assay}}{\text{starch in the blank}} \times 100 \quad (3)$$

2.14. Particle size analysis of starch hydrolysates by the amylolytic enzyme cocktail

The average size of starch particles in the solution after inhibition of enzyme cocktail by acarbose, carveol or a combination of acarbose: carveol (12.5 $\mu\text{g}/\text{mL}$: 2 mg/mL) were estimated by dynamic light scattering (DLS) using a Dual-Light nanoparticle sizer from Genizer nano solution with a standard green laser (30 mW and 570 nm) (Irvine, CA, USA). The intensity of the scattered light was detected at 90° to the incident beam. The measurements were performed in samples after enzymatic reactions and analysed at 25 °C using a 4 mL quartz cuvette. For all the samples, the mean value of three measurements was taken at a photon counting rate of around 40 for a green laser with a delay time of 5 μs . The LPSA software was used to obtain the hydrodynamic diameter

using the cumulant analysis with a repeatability of 5 % and size distribution (polydispersity index, PDI) of the starch.

2.15. Statistical analyses

All experiments were conducted in triplicate with three independent repeats, and the results are presented as the mean \pm standard error of the mean (SEM). The data were first analysed in Excel on Windows 10 before further investigation using GraphPad Prism software version 8 (San Diego, CA, USA). One-way ANOVA was used to determine differences between treatments with Essential oils as grouping variables and concentration as independent variables. All tests were conducted with alpha levels set at $p < 0.05$.

3. Results and discussion

3.1. Chemical constituent of essential oils

The EOs examined in this study were extracted from tangerine (*Citrus reticulata*) and orange (*Citrus sinensis*) by hydrodistillation. The EOs yields were 1.41 % and 0.95 % (w/w) for *C. reticulata* and *C. sinensis* peels, respectively. Previous studies have reported that citrus EOs comprise between 0.5 and 5 % (w/w) of the fresh weight of the citrus peels regardless of the citrus species they are extracted from [43–45]. The current study reported a higher yield per species than other studies that utilised hydrodistillation as an extraction method where the yield obtained was lower than 0.95 % (w/w) [46,47]. We postulate that the differences in extraction yields are due to differences in extraction procedure parameters, such as temperature and time.

Upon EOs extraction, GC–MS analysis was employed to ascertain the chemical composition of extracted citrus EOs (Fig. S1 and Table 1). The retention indices (RI) were used for more reliable comparison across studies; the RI values of some compounds tentatively identified in the current study, such as limonene, linalool, terpineol, carveol, and geraniol, to name a few, were closely similar to RI values from previous studies on essential oils [48–50].

Approximately 38 phytochemicals were identified in *C. reticulata* peels, while 28 were identified in *C. sinensis* EOs extracts from hexane or ethyl acetate extracts. Most essential oils were present in the hexane extract due to their nature as non-polar compounds, with hexane being a non-polar solvent. Ethyl acetate extracts mainly comprise oxygenated terpenes due to the polar nature of ethyl acetate. The major compounds presented in Table 1 include terpinene, limonene oxide and copaene, to name a few, were only identified in *C. reticulata*, while terpin-4-ol, valencene, D-nerolidol and trans-farnesal, to name a few, were only identified in *C. sinensis*.

Most studies investigating the chemical composition of citrus peel-derived EOs have reported hydrocarbon terpenes to be the most abundant, while oxygenated terpenes are less abundant depending on the *Citrus* species and extraction method used [51–53]. Some terpenes found here are similar to those reported in the literature [51,54], with limonene, pinene, carveol, α -terpineol, and linalool as main components of citrus peel EOs extracts.

3.2. Prediction of pharmacokinetic properties of pure essential oils

The pharmacokinetic properties of the identified EOs are the primary consideration in drug development and compound selection. These features, including ADMET properties (Absorption, Distribution, Metabolism, Excretion, and Toxicity), predict the profile of identified or novel compounds with effective medicines for drug-like qualities and to minimise waste of time and resources.

The pharmacokinetic properties of the identified compounds and acarbose were predicted using the SwissADME web server (Table 2). The identified compounds exhibited higher bioavailability scores (>0.5) and intestinal absorption than acarbose. In spite of this, some studies

Table 1
Chemical profiles of essential oils from the two citrus peel cultivars.

	Retention time (min)	Compound	Retention index	Extracts			
				<i>C. reticulata</i>		<i>C. sinensis</i>	
				Hexane	EA	Hexane	EA
1	7.696	α -Pinene	1003	+	-	+	-
2	8.586	β -Pinene	1039	+	-	+	-
3	8.875	β -myrcene	1050	+	-	+	-
4	9.412	α -Terpinene	1072	+	-	+	-
5	9.773	Limonene	1087	+	-	+	-
6	10.209	γ -Terpinene	1105	+	-	-	-
7	10.714	α -Terpinolene	1126	+	+	-	-
8	10.889	Linalool	1133	+	+	+	+
9	11.261	Trans-p-Mentha-2,8-dienol	1148	+	+	-	+
10	11.501	Cis-p-Mentha-2,8-dienol	1158	+	+	-	+
11	11.554	Limonene oxide	1161	+	-	-	-
12	12.218	Terpin-4-ol	1188	-	-	+	+
13	12.425	α -Terpineol	1197	+	+	+	+
14	12.844	Trans-carveol	1223	+	+	-	+
15	12.946	Nerol	1229	+	+	-	+
16	13.020	Cis-carveol	1234	+	+	-	+
17	13.147	Z-Citral	1243	+	-	+	+
18	13.236	Carvone	1249	+	-	-	+
19	13.317	Geraniol	1254	+	+	-	+
20	13.564	E-Citral	1270	+	-	+	+
21	13.572	Linalool oxide	1271	-	+	-	-
22	13.653	Isopiperitenone	1276	-	+	-	+
23	13.698	Perillaldehyde	1279	+	-	-	+
24	13.906	p-Mentha-1(7),8(10)-dien-9-ol	1293	+	+	-	+
25	14.649	Limonene-1,2-diol	1339	-	+	-	+
26	14.941	Neryl acetate	1357	+	-	-	-
27	15.349	α -Copaene	1382	+	-	-	-
28	15.589	Germacrene B or β -copaene	1397	+	+	-	-
29	15.855	Limonen-10-yl acetate	1412	+	+	-	+
30	16.223	Trans-caryophyllene	1434	+	-	-	-
31	16.372	Germacrene D	1442	+	-	-	-
32	17.437	Valencene	1506	-	-	+	+
33	17.48	Farnesene	1509	+	-	-	-
34	17.824	Δ -Cadinene	1534	+	+	-	+
35	18.171	Elemol	1559	+	+	-	-
36	18.268	Nerolidol	1566	+	+	-	-
37	19.151	γ -Gurjunene	1640	-	-	-	+
38	19.831	β -Sinensal	1705	+	+	-	+
39	20.041	Farnesol	1728	+	-	-	-
40	20.115	Dihydrocarvyl acetate	1736	+	+	-	-
41	20.257	Trans-farnesal	1751	-	-	-	+
42	20.365	α -Sinensal	1763	+	+	-	+
43	21.561	D-nerolidol	1902	-	-	-	+

Ethyl acetate (EA); Present (+); Absent (-).

Table 2
Pharmacokinetic properties of acarbose and essential oils identified in the citrus peels.

Compound	GI absorption	Bioavailability	CYP inhibitor	Pgp substrate	BBB permeant
Acarbose	Low	0.17	No (0/5)	Yes	No
Carveol	High	0.55	No (0/5)	No	Yes
Carvone	High	0.55	No (0/5)	No	Yes
Germacrene D	Low	0.55	No (0/5)	No	No
Geraniol	High	0.55	No (0/5)	No	Yes
Limonene	Low	0.55	Yes (1/5)	No	Yes
Linalool	High	0.55	No (0/5)	No	Yes
Nerol	High	0.55	No (0/5)	No	Yes
Terpineol	High	0.55	No (0/5)	No	Yes
Sinensal	High	0.55	Yes (1/5)	No	Yes
Valencene	Low	0.55	Yes (2/5)	No	No

consider a bioavailability score of 0.55 to be good [55]. Most of the identified compounds are not P-gp (permeability glycoprotein) substrates, nor are they inhibitors of cytochrome P450 (CYP), which indicates a low possibility of these EOs interfering with metabolism and excretion/elimination processes in the body. However, germacrene D and valencene were predicted to be able to permeate the blood-brain barrier (BBB).

3.3. Molecular docking of identified EOs against amylolytic enzymes

The modelled structure of α -glucosidase was validated using the SwissMODEL web server to investigate the quality of the modelled protein structure. The oligo-1,6-glucosidase (P53051.1.A) was selected as the template because of its 72.51 % sequence similarities to the *S. cerevisiae* sequence. The template was also chosen because of its high

coverage over the query sequence (1.00) and excellent X-ray crystallographic resolution (1.3 Å), which was better than other templates. Fig. S2 illustrates the comparison protein sequence similarity and identity between the model template and *Saccharomyces cerevisiae* α -glucosidase. Furthermore, the 3D structure of the homology modelling built MALX3 and its corresponding Ramachandran plot showing most of its amino acid residues in the favoured region are presented (Fig. S3). The Ramachandran plot analysis also exhibited 96.38 % of the residues in the favoured region with a molprobtity score of 0.76. It is suggested that a model with a high percentage of Ramachandran-favoured regions and a molprobtity score closer to or <1 is considered to have a high-quality structure [56].

From the pharmacokinetic and ADMET properties in Section 3.2, most compounds were within acceptable range for bioavailability, CYP450 inhibition and Pgp substrate. Therefore, they were subjected to more screening, such as molecular docking. AutoDock Vina analysed the binding affinities (BA) of the identified EOs in kcal/mol and their interactions (HB and VdW) with the target enzymes, as illustrated in Table S1 in the supplementary material.

The molecular docking of the compounds and acarbose in the active site of α -amylase (PDB: 1DHK) demonstrated a better binding affinity of acarbose (-8.3 kcal/mol) compared to the selected EO constituents (Table S1). Among the EO compounds, germacrene D (-6.7 kcal/mol) and valencene (-6.7 kcal/mol) exhibited the strongest binding affinity.

Docking the EO constituents in the active site of the modelled 3D structure of yeast α -glucosidase showed that acarbose (-7.5 kcal/mol), valencene (-7.4 kcal/mol), and carveol (-7.2 kcal/mol) had the most negative docking scores, indicating these compounds as the stronger binders to this enzyme (Table 3). In general, the scoring functions used in molecular docking attempt to predict how favourably a ligand binds to a receptor protein, with lower docking scores suggesting greater stability and stronger binding interactions [57]. In addition to the binding affinities exhibited by the compounds against the enzymes' active sites, interactions involved in the binding of the compounds, such as hydrophilic (hydrogen bonds) and hydrophobic interactions (Van der Waals interactions), were also presented (Table s1). Fig. S4 shows the interactions of some selected compounds with α -glucosidase, including the protein residues involved.

3.4. Inhibitory activity and mode of inhibition of acarbose and the selected compounds

Commercially available EOs constituents: valencene, carveol, limonene, geraniol, linalool, and nerol, were selected for further analysis via in vitro inhibition of amylolytic enzymes. These commercial EOs and the citrus-derived EOs extracts were assessed for IC_{50} against α -amylase and α -glucosidase. Citrus EOs and their compounds did not exhibit strong α -amylase inhibitory activity ($IC_{50} > 2$ mg/mL), whereas acarbose had an IC_{50} value of 0.008 ± 0.001 mg/mL. On the other hand, the EOs and some selected compounds exhibited potent α -glucosidase inhibitory activity with IC_{50} values within the concentration range (0.02 to 2 mg/mL). Acarbose had an IC_{50} of 0.11 mg/mL against α -glucosidase,

Table 3

Binding affinity, IC_{50} values, K_i , and mode of inhibition (MOI) of acarbose and commercially available citrus essential oils against α -glucosidase. Values are represented as means \pm SEM (n = 3).

Compound	Binding affinity (kcal/mol)	IC_{50} (mg/mL)	K_i (mg/mL)	Mode of inhibition
Acarbose	-7.5	0.11 ± 0.01	0.10 ± 0.01	Competitive
Valencene	-7.4	0.65 ± 0.01	0.33 ± 0.04	Competitive
Carveol	-7.2	0.54 ± 0.02	0.53 ± 0.02	Competitive
Limonene	-6.5	1.51 ± 0.02	1.10 ± 0.07	Competitive
Geraniol	-5.9	0.71 ± 0.05	0.56 ± 0.02	Competitive
Linalool	-5.8	1.88 ± 0.18	0.90 ± 0.03	Competitive
Nerol	-5.5	1.08 ± 0.04	1.06 ± 0.08	Competitive

whereas the IC_{50} values of the EOs extract were above 1 mg/mL. The IC_{50} values of the selected compounds are shown in Table 3.

The selected compounds have shown inhibition only for α -glucosidase within the concentration range used; therefore, they were analysed further for additional kinetic inhibition experiments against α -glucosidase. Table 3 shows the inhibitor constant (K_i) and the mode of inhibition of these compounds. These data suggested that the selected compounds exhibited competitive inhibition of α -glucosidase. For acarbose, carveol, geraniol, and valencene, as presented in Table S2 (supplementary document), we observed an increase in the K_m value while the V_{max} remained unchanged, which indicates competitive inhibition [8]. The K_i is a better indication of how potent an inhibitor is; the lower the K_i value, the more powerful the inhibitor is [8,58]. Therefore, the order of the potency of the inhibitory compounds is as follows: acarbose > valencene > carveol > geraniol > limonene > linalool > nerol, with acarbose being the most potent and nerol the least potent inhibitor. Valencene, carveol, and geraniol were selected as promising compounds for further studies. The kinetic parameters used to determine the mode of inhibition of acarbose, carveol, geraniol, and valencene are shown in Table S2 in the supplementary data.

The compounds' in vitro inhibitory constants (K_i) correlated positively with their in silico-determined binding affinities; Pearson's correlation coefficient was 0.7708, indicating a highly positive correlation between the molecular docking and in vitro inhibition assay results, as shown in Fig. 1.

A review by Kury et al. (2021) has elaborated on the antidiabetic effects of some essential oil compounds; fewer studies have investigated these compounds' inhibitory action against amylolytic enzymes [59]. The K_i values and the mode of inhibition of these compounds against α -glucosidase have not yet been explored in the literature. However, acarbose has been investigated, and in correlation with the current study, it has been reported to be a competitive inhibitor of α -glucosidase [60,61].

3.5. Molecular dynamic investigation of acarbose and the selected compounds with α -glucosidase

Molecular dynamic simulations were investigated to further examine the binding interactions of the selected compounds and α -glucosidase

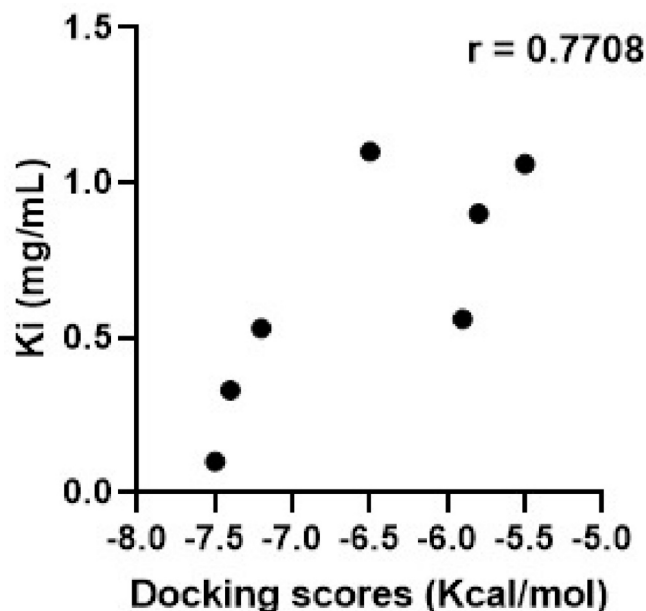


Fig. 1. Graph of AutoDock generated binding affinities vs the inhibition constant (K_i) to show the relationship between molecular docking and in vitro enzyme inhibition results.

Table 4

Total binding energy and conformational dynamics results between selected compounds and α -glucosidase throughout 100 ns.

Compound	ΔG_{bind} (kcal/mol)	Mean of RMSD (\AA)	Mean of RMSF (\AA)	Mean of RoG (\AA)	Mean of SASA (\AA^2)
Apo	–	2.24661	1.15194	24.39143	21,691.71852
Acarbose	–32.8157	2.01614	1.19694	24.46236	21,140.78108
Valencene	–18.4480	1.82515	1.09036	24.54001	21,180.15473
Carveol	–17.6714	2.19487	1.09283	24.5626	21,678.30757
Geraniol	–20.7200	2.27427	1.29004	24.6047	21,708.57684

throughout 100 ns. Among the selected essential oils, valencene, carveol, and geraniol exhibited more potent in vitro inhibitory activity against α -glucosidase, with K_i values of 0.33, 0.53 and 0.56 mg/mL, respectively. The abovementioned compounds were chosen for molecular dynamic investigation, while acarbose (K_i of 0.10 mg/mL) was used for comparison as the standard inhibitor of α -glucosidase. MD was used to determine and generate the total binding energy ΔG_{bind} , the average of RMSD, RMSF, RoG, and SASA. MD parameters are shown in Table 4, and Fig. 2 illustrates the changes over the simulation time.

The ΔG_{bind} was generated and presented in Table 4 for the interaction between α -glucosidase and the selected compounds. Acarbose had a

more negative value, followed by geraniol, valencene and carveol. The order correlated with the docking scores in Table 3, where the more negative value indicates stronger binding affinity and stable complex between ligand and α -glucosidase [62]. In addition, the 2D Ligand-Protein interactions were plotted using LigPlot to determine the type of interactions formed after 100 ns for each compound. As shown in Fig. 3, hydrophobic interactions were predominantly observed in all compounds, with some shifts in the binding key residues for each compound. It was also observed that hydrogen bonds were formed between acarbose with Asp408, carveol with Glu276, and geraniol with Arg356. Despite the lack of hydrogen bond interactions in valencene, it presented stronger binding energy than carveol, which correlates with its smaller K_i value.

RMSD values were measured to analyse the conformational stability and rigidity of the ligand-protein complexes, and the values are presented in Table 4. The mean RMSD value of the apo α -glucosidase was higher than the complexes with acarbose, valencene and carveol. Fig. 2A illustrates the RMSD changes over time throughout the 100 ns. The RMSD initially increased dramatically before maintaining a stable state with slight deviations at different RMSD values and time. The RMSD profile in Fig. 2A shows that the complexes reached equilibrium at values below 2.5 \AA , which indicates the stable state of the complexes; the lower the RMSD, the greater the stability and rigidity [63–65].

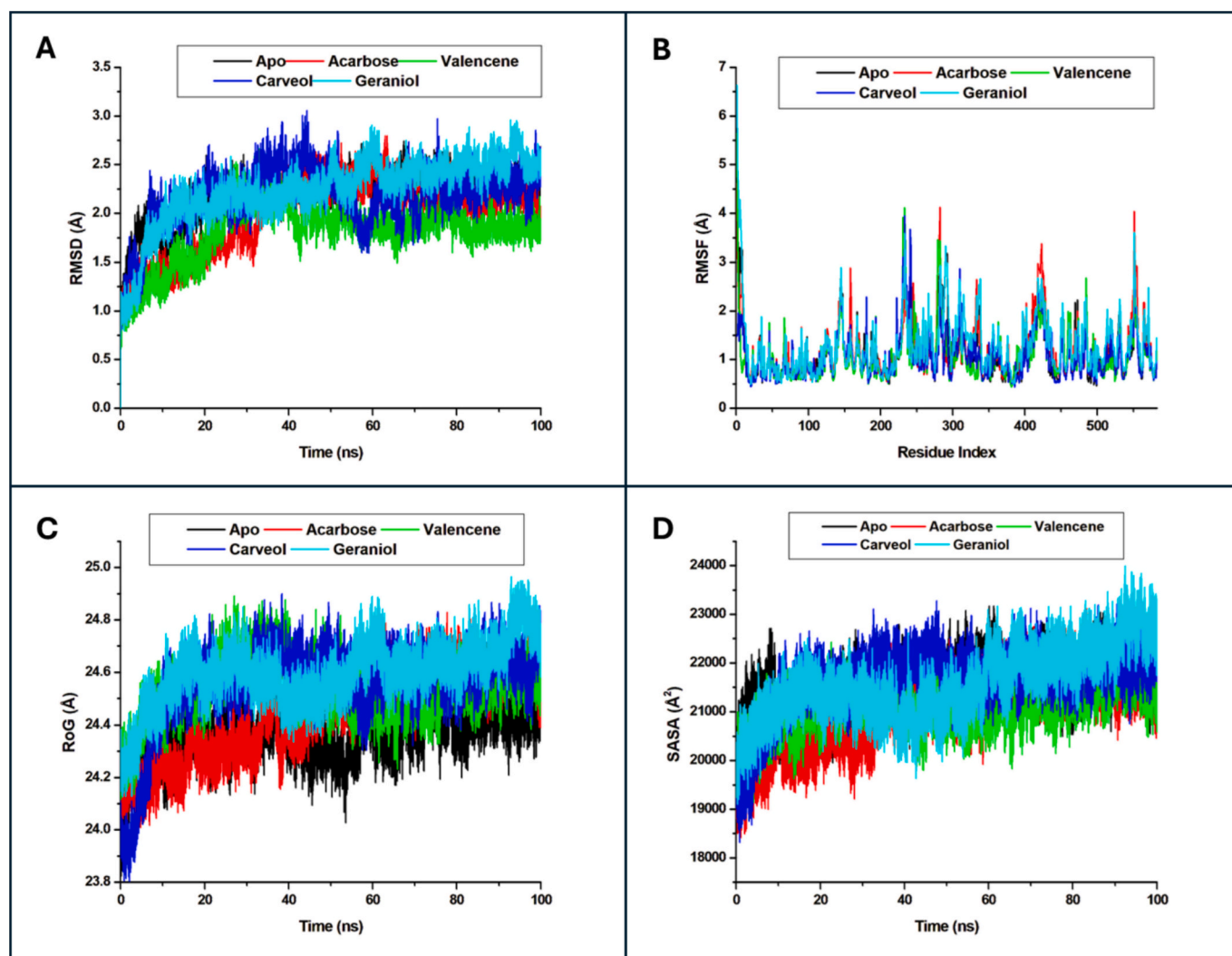


Fig. 2. Molecular dynamic simulation for α -glucosidase and complexes of α -glucosidase with selected compounds throughout the 100 ns of the simulation time. Analysis of (A) root mean square deviation (RMSD); (B) root mean square fluctuations (RMSF); (C) radius of gyration (RoG) and (D) solvent accessible surface area (SASA).

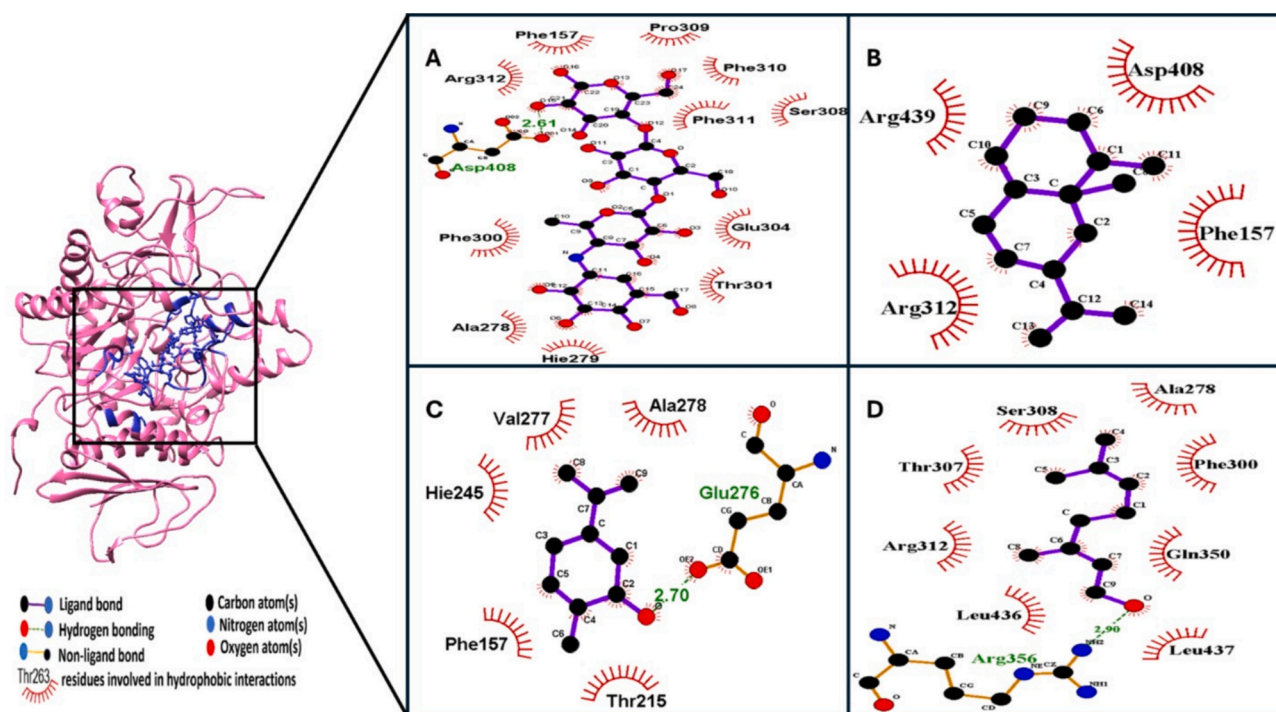


Fig. 3. Ligand-protein (α -glucosidase) interactions after 100 ns derived from MD simulations. The figure illustrates the spatial arrangement and interaction types between the surrounding protein residues and ligands; (A) acarbose, (B) valencene, (C) carveol, and (D) geraniol.

Therefore, the order of complex stability and rigidity in this study is as follows: valencene > acarbose > carveol > geraniol.

RMSF values were measured to analyse the flexibility of the protein backbone throughout 100 ns. A high RMSF value indicates more flexibility and mobility and less stability between the ligand and protein, while a low RMSF value is associated with the residue maintaining a stable position, being more rigid and having less fluctuation [66,67]. Fig. 2B illustrates the RMSF values to indicate the flexibility of individual residues of α -glucosidase over time. The active site of α -glucosidase is found around residues shown in Figs. 2 and 3 [67]. Complex with acarbose illustrated the lowest RMSF in the active site region (Fig. 2B), which indicates more stability/rigidity and less fluctuation and correlates with acarbose as a potent competitive inhibitor of α -glucosidase (Table 3).

RoG was measured to analyse the structural compactness of α -glucosidase and the liability of the complexes. A low RoG value indicates tight packing associated with a more stable and compact structure, while a high RoG value indicates less tight packing, greater flexibility and less stable configuration [68]. Fig. 2C illustrates that the RoG of the complexes initially increased rapidly before reaching a stable state. The mean RoG values of the complexes (Table 4) show that apo α -glucosidase has the lowest value than ligand-protein complexes, showing conformational changes upon ligand binding to α -glucosidase. The order of complex stability and compactness in this study is as follows: acarbose > valencene > carveol > geraniol.

SASA is measured to quantify the surface area of α -glucosidase accessible to solvent molecules [69]. SASA helps to understand the stability, molecular interactions and function of proteins relevant to protein folding, ligand binding, and conformational changes [70]. The apo α -glucosidase has a higher mean SASA value than the complex with acarbose, valencene and carveol, indicating that α -glucosidase has a rigid binding affinity with these ligands, corresponding to folded and stable structure compared to apo α -glucosidase. In contrast, the complex with geraniol has a higher SASA value than the apo α -glucosidase, which indicates less stable/rigid binding affinity, and unfolded structure compared to the apo α -glucosidase.

The MD parameters in Table 4 correlated positively throughout the simulation study. Complex with geraniol has the highest mean values for the measured parameters compared to the apo α -glucosidase and other complexes. Complexes with acarbose, valencene, and carveol exhibited a more stable structure than geraniol in terms of rigidity, compactness, and fluctuation.

3.6. Inhibition of the amylolytic enzyme cocktail by acarbose or potent EOs

The inhibition of an amylolytic enzyme cocktail by acarbose, carveol, geraniol, and valencene was assessed by determining the IC_{50} value that inhibited the production of the reducing sugar through the DNS method and the IC_{50} that inhibited the amount of glucose released through the GOPOD method, shown in Fig. 4. Acarbose inhibited the activity of the enzyme cocktail at lower concentrations with IC_{50} values of 2.50 and 2.10 μ g/mL for the inhibition of α -amylase and α -glucosidase activities, respectively. The result shows that acarbose strongly inhibits the activities of both enzymes, and its action may lead to the accumulation of undigested starch in the colon associated with abdominal discomfort [71]. Therefore, a slight inhibition of α -amylase and potent inhibition of α -glucosidase is preferable because it leads to high production of total reducing sugars and a low amount of glucose released; furthermore, it reduces the accumulation of undigested starch and its associated side effects [15,72].

Carveol, geraniol, and valencene did not inhibit α -amylase activity at the highest inhibitor concentration, with some EOs showing slight activation of the enzyme instead of inhibition. On the other hand, the promising compounds show a dose-dependent inhibition of α -glucosidase activity, with IC_{50} values of 1.32, 1.63, and 1.70 mg/mL for carveol, geraniol, and valencene, respectively. The above result is ideal because it minimises adverse effects associated with undigested starch and reduces the amount of glucose released. Therefore, these compounds could be potential antidiabetic agents for managing type 2 diabetes.

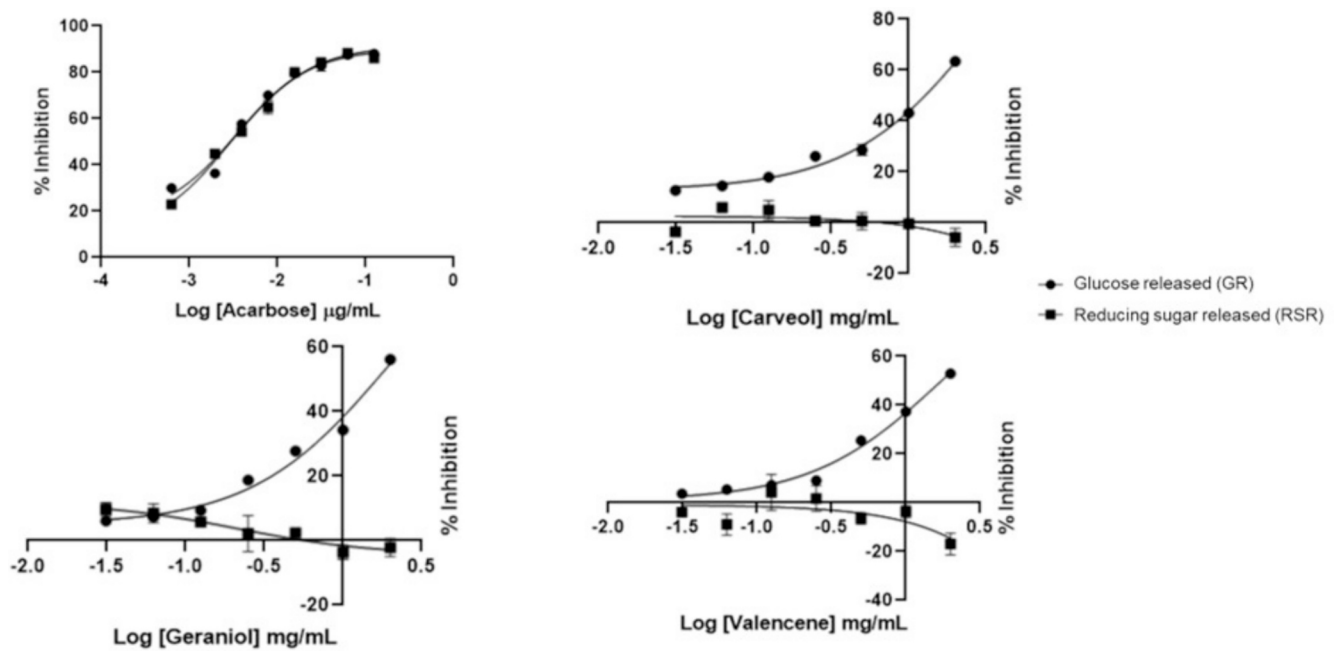


Fig. 4. Inhibition of amylolytic enzyme cocktail (α-amylase and α-glucosidase) by acarbose (top left), carveol (top right), geraniol (bottom left), and valencene (bottom right). Two curves are shown in each plot, representing the inhibition of total reducing sugars (TRS) and glucose released (GR). Values are represented as means ±SEM (n = 3).

Table 5

Combination study of acarbose and essential oils on the inhibition of the amylolytic enzyme cocktail with an emphasis on the inhibition of total reducing sugars and glucose released. Values are represented as means ±SEM (n = 3).

Combination points	Acarbose (μg/mL): EOs (mg/mL)	Acarbose: carveol				Acarbose: geraniol				Acarbose: valencene			
		Reducing sugars released		Glucose released		Reducing sugars released		Glucose released		Reducing sugars released		Glucose released	
		% inhibition	CI	% inhibition	CI	% inhibition	CI	% inhibition	CI	% inhibition	CI	% inhibition	CI
1	12.5: 2	67.51	>10	78.21	0.62	64.98	>10	81.25	0.38	26.67	>10	57.98	3.65
2	12.5: 1	63.82	>10	76.17	0.66	69.70	>10	77.64	0.52	49.68	5.09	66.83	1.55
3	12.5: 0.5	69.82	>10	74.34	0.72	72.45	>10	77.35	0.50	63.93	2.56	67.66	1.35
4	3.125: 2	41.16	>10	68.60	0.77	36.35	>10	66.70	0.79	0.90	>10	56.32	1.67
5	3.125: 1	39.54	>10	59.96	1.09	42.55	>10	62.83	0.80	20.01	6.31	49.72	2.08
6	3.125: 0.5	37.14	>10	58.87	0.93	44.06	>10	57.98	0.97	36.04	2.48	50.54	1.64
7	0.625: 2	14.51	>10	65.17	0.72	11.90	3.03	62.89	0.73	0.26	>10	53.53	1.22
8	0.625: 1	16.71	>10	53.50	0.96	15.32	2.32	52.02	1.03	2.52	>10	44.14	1.41
9	0.625: 0.5	19.81	>10	46.88	0.96	20.49	1.81	47.26	0.94	10.36	3.16	33.34	2.17

CI: combination index.

Table 6

Summary of synergistic effects of combined acarbose and essential oils observed during the inhibition of the amylolytic enzyme cocktail.

Combination points	Acarbose (μg/mL): EOs (mg/mL)	Acarbose: carveol		Acarbose: geraniol		Acarbose: valencene	
		Effect		Effect		Effect	
		Reducing sugars released	Glucose released	Reducing sugars released	Glucose released	Reducing sugars released	Glucose released
1	12.5: 2	Very strong antagonism	Synergism	Very strong antagonism	Synergism	Very strong antagonism	Strong Antagonism
2	12.5: 1					Strong Antagonism	Antagonism
3	12.5: 0.5		Moderate synergism			Antagonism	Moderate Antagonism
4	3.125: 2				Moderate synergism	Very strong antagonism	Antagonism
5	3.125: 1		Nearly additive			Strong Antagonism	
6	3.125: 0.5		Nearly additive		Nearly additive	Antagonism	
7	0.625: 2		Moderate antagonism	Antagonism	Moderate synergism	Very strong antagonism	Moderate antagonism
8	0.625: 1				Nearly additive		
9	0.625: 0.5		Antagonism			Antagonism	Antagonism

3.7. Combined effects of acarbose and potent EOs against the amyolytic enzyme cocktail

The combination effects of the selected compounds against the amyolytic enzyme cocktail were assessed by mixing acarbose with each promising EOs at different inhibitory potency ratios. An approximation of IC_{30} (0.625 $\mu\text{g/mL}$), IC_{50} (3.125 $\mu\text{g/mL}$), and IC_{75} (12.5 $\mu\text{g/mL}$) of acarbose were each mixed with IC_{30} (0.5 mg/mL), IC_{50} (1 mg/mL) and IC_{75} (2 mg/mL) of carveol, geraniol or valencene, yielding nine combinations. A combination index (CI) and a CI-isobologram were then generated from the results (Fig. S5 and Tables 5 & 6). According to drug synergism studies, $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive, and antagonism effects, respectively [41].

Combining acarbose with carveol, geraniol, or valencene exhibited an antagonistic effect on α -amylase inhibition, with the combination index (CI) being higher than 1.45. No CI-isobologram was generated due to all the points being outside the graph. Acarbose combined with carveol had a powerful antagonistic effect with CI higher than 10. The observed result is ideal as these drug combinations lead to the reduction of the amount of undigested starch by reducing the strong α -amylase inhibitory activity of acarbose and producing more reducing sugars.

On the other hand, combining acarbose with carveol or geraniol exhibited a synergistic effect for α -glucosidase inhibition, and some were nearly additive with CI values lower than 1 (Table 5). Fig. S5A and B clearly show the presence of all nine points inside the graph to promote synergistic or nearly additive effects on α -glucosidase inhibition. The outcome correlates with the ideal results expected because it reduces glucose released to the bloodstream, thus possibly minimising post-prandial hyperglycaemia. However, the combination of acarbose with valencene exhibited antagonistic and additive effects on α -glucosidase inhibition, with most CI values higher than 1.20.

Combining acarbose with carveol and geraniol (12.5 $\mu\text{g/mL}$: 2 mg/mL) showed the ideal results because of the desired antagonistic effect on α -amylase inhibition, promoting the production of reducing sugars, and a more substantial synergistic effect on α -glucosidase inhibition, reducing the amount of glucose released.

3.8. Quantification of undigested starch

The starch-iodine method was used to further quantify the amount of starch residues after the enzymatic reactions. This method was used to identify further which of the two ideal combinations is associated with minimal starch residues. The percentage of undigested starch was

calculated using the formula in Eq. (3) (see 2.12). Fig. 4 illustrates the % undigested starch along with % inhibition of reducing sugar released (RSR) and glucose released (GR) for the combination of acarbose with carveol and geraniol at the ratio (12.5 $\mu\text{g/mL}$: 2 mg/mL), which showed stronger antagonistic and synergistic effect for RS and glucose production, respectively. The combinations were compared to the impact of the single compounds (Fig. 5).

Acarbose had the highest amount of undigested starch (92 %), possibly associated with its strong inhibition of RSR, leading to less starch digestion. Carveol and geraniol exhibited no inhibition of RSR, with increased α -amylase activity, leading to less undigested starch (3 and 17 %, respectively). Both combinations of acarbose with carveol or geraniol led to a slight increase in the inhibition of GR from 65 % to 67 % and 69 % for the combination with carveol or geraniol, respectively. Combining acarbose with carveol significantly decreased undigested starch from 92 % to 33 %, whereas combining acarbose with geraniol decreased undigested starch to 79 %. A similar study investigated the effect of flavonoids and acarbose on the digestibility of starch; the authors reported a decrease in starch due to the presence of acarbose because of its strong inhibitory activity on α -amylase [73].

Other studies have speculated that in addition to the inhibition of α -amylase, the hydrophobic interactions between the inhibitor and the helical structure of starch may also affect the digestibility of starch [73–75]. However, the authors reported no binding interaction between acarbose and starch, thus concluding that the effect was solely due to the strong inhibitory activity of acarbose on α -amylase.

3.9. Particle size determination of the starch hydrolysate

The average particle size of hydrolysate from the inhibition reaction with acarbose, carveol and a combination of acarbose and carveol was

Table 7

Average particle size and polydispersity index of starch hydrolysates generated by the amyolytic enzyme cocktail in the absence or presence of lone inhibitors or their combination. Values are represented as means \pm SEM (n = 3).

Sample (hydrolysate)	Average particle size (nm)	Polydispersity (PDI)
Starch only	163.62 \pm 15.38	0.47 \pm 0.01
No inhibitor	5.16 \pm 0.34	0.29 \pm 0.03
Acarbose as inhibitor	128.59 \pm 18.16	0.44 \pm 0.02
Carveol as inhibitor	7.30 \pm 1.15	0.21 \pm 0.01
Acarbose + carveol	36.17 \pm 3.88	0.59 \pm 0.01

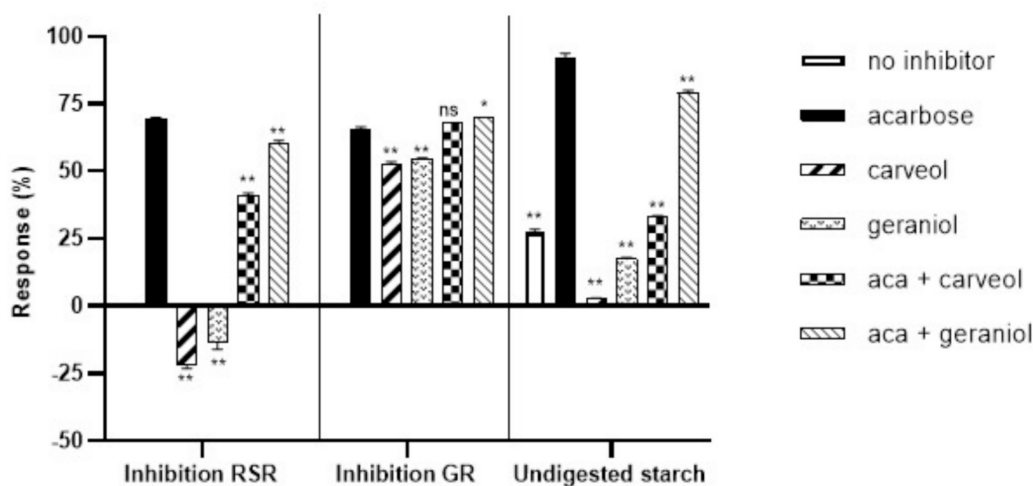


Fig. 5. Amyolytic enzyme cocktail response to inhibitors with respect to % inhibition of reducing sugar released (RSR), % inhibition of glucose released (GR), and % undigested starch by acarbose (12.5 $\mu\text{g/mL}$), carveol (2 mg/mL), geraniol (2 mg/mL), a combination of acarbose: carveol (12.5 $\mu\text{g/mL}$: 2 mg/mL), and a combination of acarbose: geraniol (12.5 $\mu\text{g/mL}$: 2 mg/mL). Values are represented as means \pm SEM (n = 3).

used to validate the amount of undigested starch. As indicated in Table 4, the average particle sizes were 129 and 36 nm for the reaction with acarbose alone and in combination with carveol, respectively. The average particle sizes were compared to the starch solution alone, which had an average size of 164 nm. The average size of a starch solution is reported to range between 40 and 300 nm depending on conditions and aggregations [76].

The data in Table 7 correlated with the % undigested starch in Fig. 5, where acarbose had a high undigested starch compared to the combination with carveol. The large particle size in acarbose is due to the undigested starch associated with strong inhibition of α -amylase. In contrast, a significant decrease in average particle size was observed in the combination of acarbose with carveol, associated with decreased inhibition of α -amylase and hydrolysis of starch into smaller molecules.

4. Conclusion

The current study highlights the AGI activity of EOs derived from citrus peels using in silico and in vitro approaches. The EOs demonstrated a more potent inhibitory effect on α -glucosidase compared to α -amylase, which could help reduce side effects linked to undigested starch while lowering glucose release. MD simulations revealed that carveol, geraniol, and valencene had stable and flexible interactions with α -glucosidase, leading to compact complex formation. The combination of acarbose with either carveol or geraniol produced antagonistic and synergistic effects on α -amylase and α -glucosidase, respectively, potentially alleviating side effects associated with undigested starch and enhancing therapeutic outcomes. Overall, the findings suggest that citrus peel-derived EOs could be promising antidiabetic agents. Furthermore, these compounds can be sourced from citrus peel waste, aligning with circular economy principles and offering a cost-effective solution for increased accessibility. However, additional in vitro and in vivo studies are needed to validate these results.

CRedit authorship contribution statement

Kadima Samuel Tshiyoyo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ali Rabbad:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis. **Abdullahi Ahmed Yusuf:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Samkelo Malgas:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly: Free AI Writing Assistance to improve grammar and the tone of the language in the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Funding

This study was supported by the competitive support for unrated Researchers grant No 138084 awarded by the National Research Foundation (NRF), South Africa.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge the Center of High-Performance Computing (CHPC), Cape Town, South Africa, for the platform to conduct the computational part of the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.141504>.

Data availability

Data will be made available on request.

References

- [1] A.M. Egan, S.F. Dinneen, What is diabetes? *Medicine* 47 (1) (2019) 1–4.
- [2] F. Quondamatteo, Skin and diabetes mellitus: what do we know? *Cell Tissue Res.* 355 (2014) 1–21.
- [3] V. Mohan, et al., Epidemiology of type 2 diabetes: Indian scenario, *Indian J. Med. Res.* 125 (3) (2007) 217–230.
- [4] IDF, International Diabetes Federation Diabetes Atlas 2021, 2021.
- [5] N.G. Forouhi, N.J. Wareham, Epidemiology of diabetes, *Medicine* 50 (10) (2022) 638–643.
- [6] G.R. Kokil, et al., Type 2 diabetes mellitus: limitations of conventional therapies and intervention with nucleic acid-based therapeutics, *Chem. Rev.* 115 (11) (2015) 4719–4743.
- [7] X.-Y. Deng, et al., Synthesis and bioactivities evaluation of oleanolic acid oxime ester derivatives as α -glucosidase and α -amylase inhibitors, *J. Enzyme Inhib. Med. Chem.* 37 (1) (2022) 451–461.
- [8] D. Voet, J.G. Voet, *Biochemistry*, John Wiley & Sons, 2021.
- [9] M. Vihinen, P. Mantsila, Microbial amylolytic enzyme, *Crit. Rev. Biochem. Mol. Biol.* 24 (4) (1989) 329–418.
- [10] S. Tiwari, et al., Amylases: an overview with special reference to alpha amylase, *J. Glob. Biosci.* 4 (1) (2015) 1886–1901.
- [11] M. Jones, et al., *Cambridge International AS and a Level Biology Coursebook With CD-ROM*, Cambridge University Press, 2014.
- [12] A.E. Martin, P.A. Montgomery, Acarbose: an α -glucosidase inhibitor, *Am. J. Health Syst. Pharm.* 53 (19) (1996) 2277–2290.
- [13] I. Sensoy, A review on the food digestion in the digestive tract and the used in vitro models, *Curr. Res. Food Sci.* 4 (2021) 308–319.
- [14] N. Turner, et al., Repurposing drugs to target the diabetes epidemic, *Trends Pharmacol. Sci.* 37 (5) (2016) 379–389.
- [15] M. Tolmie, M.J. Bester, Z. Apostolides, Inhibition of α -glucosidase and α -amylase by herbal compounds for the treatment of type 2 diabetes: a validation of in silico reverse docking with in vitro enzyme assays, *J. Diabetes* 13 (10) (2021) 779–791.
- [16] B.B. Petrovska, Historical review of medicinal plants' usage, *Pharmacogn. Rev.* 6 (11) (2012) 1.
- [17] Z. Zou, et al., Antioxidant activity of Citrus fruits, *Food Chem.* 196 (2016) 885–896.
- [18] J. Cui, et al., The structure–property relationships of acid-and alkali-extracted grapefruit peel pectins, *Carbohydr. Polym.* 229 (2020) 115524.
- [19] N. Liu, et al., A review of chemical constituents and health-promoting effects of citrus peels, *Food Chem.* 365 (2021) 130585.
- [20] P. Chavan, A.K. Singh, G. Kaur, Recent progress in the utilization of industrial waste and by-products of citrus fruits: a review, *J. Food Process Eng.* 41 (8) (2018) e12895.
- [21] N. Mahato, et al., Biotransformation of citrus waste-I: production of biofuel and valuable compounds by fermentation, *Processes* 9 (2) (2021) 220.
- [22] H. Bora, et al., Citrus essential oils (CEOs) and their applications in food: an overview, *Plants* 9 (3) (2020) 357.
- [23] T. Nakatsu, et al., Biological activity of essential oils and their constituents, *Stud. Nat. Prod. Chem.* 21 (2000) 571–631.
- [24] C. Ambrosio, et al., Unraveling the selective antibacterial activity and chemical composition of citrus essential oils, *Sci. Rep.* 9 (1) (2019) 1–13.
- [25] N. Mahato, et al., Citrus essential oils: extraction, authentication and application in food preservation, *Crit. Rev. Food Sci. Nutr.* 59 (4) (2019) 611–625.
- [26] K. Majouli, et al., Antioxidant activity and α -glucosidase inhibition by essential oils from *Hertia cheirifolia* (L.), *Ind. Crop. Prod.* 82 (2016) 23–28.
- [27] M.O. Salazar, et al., New α -glucosidase inhibitors from a chemically engineered essential oil of *Origanum vulgare* L., *Ind. Crop. Prod.* 156 (2020) 112855.
- [28] A. Sadka, et al., Primary metabolism in citrus fruit as affected by its unique structure, *Front. Plant Sci.* 10 (2019) 1167.
- [29] Q. Lu, et al., Peel oils from three Citrus species: volatile constituents, antioxidant activities and related contributions of individual components, *J. Food Sci. Technol.* 56 (10) (2019) 4492–4502.
- [30] S. Stein, et al., The NIST mass spectral search program for the nist/epa/nih mass spectra library, in: *Standard Reference Data Program of the National Institute of Standards and Technology*, Gaithersburg, MD, US, 2011, 2021.

- [31] A. Daina, O. Michielin, V. Zoete, SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, *Sci. Rep.* 7 (1) (2017) 42717.
- [32] G.M. Morris, et al., AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, *J. Comput. Chem.* 30 (16) (2009) 2785–2791.
- [33] S. Dallakyan, A.J. Olson, Small-molecule library screening by docking with PyRx, *Chem. Biol. Methods Protoc.* (2015) 243–250.
- [34] M. Yousuf, K. Mammadova, L. Khan, Homology modeling of α -glucosidase enzyme: 3D structure prediction, *Bioinform. Rev.* 4 (11) (2018) 09–14.
- [35] Megazyme, GOPOD REAGENT ENZYMES (for the Preparation of GOPOD Determination Reagents), 2019.
- [36] E.F. Pettersen, et al., UCSF Chimera—a visualization system for exploratory research and analysis, *J. Comput. Chem.* 25 (13) (2004) 1605–1612.
- [37] D.A. Case, et al., The Amber biomolecular simulation programs, *J. Comput. Chem.* 26 (16) (2005) 1668–1688.
- [38] A.H. Rabbad, et al., Microbes, not humans: exploring the molecular basis of Pseudouridimycin selectivity towards bacterial and not human RNA polymerase, *Biotechnol. Lett.* 41 (2019) 115–128.
- [39] E. Odoro-Kwateng, A.H. Rabbad, M.E. Soliman, The juxtaposition of allosteric and catalytic inhibition in PLK1: tradeoff for chemotherapy and thermodynamic profiles of KBJK557 and BI 6727, *J. Comput. Biophys. Chem.* 23 (3) (2024) 379–401.
- [40] A. H. Rabbad, C. Agoni, M.E. Soliman, Structural insights into the role of pseudouridimycin binding in disruption of bacterial RNA polymerase bridge helix conformational arrangement, *Curr. Pharm. Biotechnol.* 24 (4) (2023) 562–569.
- [41] T.-C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, *Pharmacol. Rev.* 58 (3) (2006) 621–681.
- [42] Z. Xiao, R. Storms, A. Tsang, A quantitative starch? Iodine method for measuring alpha-amylase and glucoamylase activities, *Anal. Biochem.* 351 (1) (2006) 146–148.
- [43] B. Singh, et al., Insights into the chemical composition and bioactivities of citrus peel essential oils, *Food Res. Int.* 143 (2021) 110231.
- [44] F. Brahmī, et al., Chemical and biological characterization of essential oils extracted from citrus fruits peels, *Mater. Today Proc.* 45 (2021) 7794–7799.
- [45] B. Satari, et al., Process optimization for citrus waste biorefinery via simultaneous pectin extraction and pretreatment, *BioResources* 12 (1) (2017) 1706–1722.
- [46] D.P. Bhandari, et al., Volatile compounds and antioxidant and antimicrobial activities of selected citrus essential oils originated from Nepal, *Molecules* 26 (21) (2021) 6683.
- [47] K.L. Vasquez-Gomez, et al., Characterization of Essential Oils From the Skin of *Citrus limetta* Risso, *Citrus limetta* Sp., *Citrus reticulata* and *Citrus sinensis* Grown in Northeastern Peru, 2023.
- [48] M.-F. Hérent, V. De Bie, B. Tilquin, Determination of new retention indices for quick identification of essential oils compounds, *J. Pharm. Biomed. Anal.* 43 (3) (2007) 886–892.
- [49] M. Saeidi, et al., Comparative volatile composition, antioxidant and cytotoxic evaluation of the essential oil of *Zhumeria majdae* from south of Iran, Iran. *J. Basic Med. Sci.* 22 (1) (2019) 80.
- [50] Z. Benchama, et al., Chemical composition and antibacterial activity of the essential oils of *Asphodelus microcarpus* Salzm. & Viv. growing wild at different sites in Morocco: ADME-Tox, molecular docking and molecular dynamics simulations, *J. Essent. Oil Res.* (2024) 1–17.
- [51] W. Zhang, et al., Peel essential oil composition and antibacterial activities of *Citrus x sinensis* L. Osbeck 'Tarocco' and *Citrus reticulata* Blanco, *Horticulturae* 8 (9) (2022) 793.
- [52] K. Ghadiri, et al., Response surface methodology for optimization of supercritical fluid extraction of orange peel essential oil, *Pharm. Biomed. Res.* 6 (4) (2020) 303–312.
- [53] C. Li, et al., Variation in compositions and biological activities of essential oils from four citrus species: *Citrus limon*, *Citrus sinensis*, *Citrus paradisi*, and *Citrus reticulata*, *Chem. Biodivers.* 19 (4) (2022) e202100910.
- [54] S. Javed, et al., Phytochemistry, GC-MS analysis, antioxidant and antimicrobial potential of essential oil from five citrus species, *J. Agric. Sci.* 6 (3) (2014) 201.
- [55] H.D. Sympli, Estimation of drug-likeness properties of GC-MS separated bioactive compounds in rare medicinal *Pleione maculata* using molecular docking technique and SwissADME in silico tools, *Netw. Model. Anal. Health Inform. Bioinform.* 10 (1) (2021) 14.
- [56] W. Liu, H. Wang, F. Meng, In silico modeling of aspalathin and nothofagin against SGLT2, *J. Theor. Comput. Chem.* 14 (08) (2015) 1550056.
- [57] M. García-Ortegón, et al., DOCKSTRING: easy molecular docking yields better benchmarks for ligand design, *J. Chem. Inf. Model.* 62 (15) (2022) 3486–3502.
- [58] H.-Y. Kim, et al., Anti-diabetic effect of the lupinalbin a compound isolated from *Apios americana*: in vitro analysis and molecular docking study, *Biomed. Rep.* 14 (4) (2021) 1–5.
- [59] L.T. Al Kury, et al., In vitro and in vivo antidiabetic potential of monoterpenoids: an update, *Molecules* 27 (1) (2021) 182.
- [60] M. Ni, et al., Inhibitory mechanism of vitexin on α -glucosidase and its synergy with acarbose, *Food Hydrocoll.* 105 (2020) 105824.
- [61] K.S. Tshiyoyo, In silico docking and ADMET studies on clinical targets for type 2 diabetes correlated to in vitro inhibition of α glucosidase and pancreatic α -amylase by curcumin, 18 α glycyrrhetic acid, rosmarinic acid, and quercetin, in: *Biochemistry, Genetics and Microbiology*, University of Pretoria, Pretoria, South Africa, 2022.
- [62] M. Uzzaman, et al., Medicinal and toxicological investigation of some common NSAIDs: a computer-aided drug design approach, *J. Mol. Struct.* 1292 (2023) 136124.
- [63] S. Ahmed, et al., Molecular docking and dynamics simulation of natural compounds from betel leaves (*Piper betle* L.) for investigating the potential inhibition of alpha-amylase and alpha-glucosidase of type 2 diabetes, *Molecules* 27 (14) (2022) 4526.
- [64] N. Wu, et al., Elucidation of protein–ligand interactions by multiple trajectory analysis methods, *Phys. Chem. Chem. Phys.* 26 (8) (2024) 6903–6915.
- [65] S. Rampogu, et al., Molecular docking and molecular dynamics simulations discover curcumin analogue as a plausible dual inhibitor for SARS-CoV-2, *Int. J. Mol. Sci.* 23 (3) (2022) 1771.
- [66] S. Ghahremanian, et al., Molecular dynamics simulation approach for discovering potential inhibitors against SARS-CoV-2: a structural review, *J. Mol. Liq.* 354 (2022) 118901.
- [67] M. Asadi, et al., Synthesis, α -glucosidase inhibitory activity, and molecular dynamic simulation of 6-chloro-2-methoxyacridine linked to triazole derivatives, *Sci. Rep.* 14 (1) (2024) 17338.
- [68] M.Y. Lobanov, N. Bogatyreva, O. Galzitskaya, Radius of gyration as an indicator of protein structure compactness, *Mol. Biol.* 42 (2008) 623–628.
- [69] N.F.S.K. Anuar, et al., Molecular docking and molecular dynamics simulations of a mutant *Acinetobacter haemolyticus* alkaline-stable lipase against tributyrin, *J. Biomol. Struct. Dyn.* 39 (6) (2021) 2079–2091.
- [70] E. Durham, et al., Solvent accessible surface area approximations for rapid and accurate protein structure prediction, *J. Mol. Model.* 15 (2009) 1093–1108.
- [71] Y. Dong, et al., Reducing the intestinal side effects of acarbose by baicalein through the regulation of gut microbiota: an in vitro study, *Food Chem.* 394 (2022) 133561.
- [72] B. Mabate, et al., A combination approach in inhibiting type 2 diabetes-related enzymes using *Ecklonia radiata* fucoidan and acarbose, *Pharmaceutics* 13 (11) (2021) 1979.
- [73] J.-P. Yang, H. He, Y.-H. Lu, Four flavonoid compounds from *Phyllostachys edulis* leaf extract retard the digestion of starch and its working mechanisms, *J. Agric. Food Chem.* 62 (31) (2014) 7760–7770.
- [74] W. Shen, Y. Xu, Y.-H. Lu, Inhibitory effects of Citrus flavonoids on starch digestion and antihyperglycemic effects in HepG2 cells, *J. Agric. Food Chem.* 60 (38) (2012) 9609–9619.
- [75] U. Takahama, S. Hirota, Fatty acids, epicatechin-dimethylgallate, and rutin interact with buckwheat starch inhibiting its digestion by amylase: implications for the decrease in glycemic index by buckwheat flour, *J. Agric. Food Chem.* 58 (23) (2010) 12431–12439.
- [76] S. Chakraborty, et al., Solution properties of starch nanoparticles in water and DMSO as studied by dynamic light scattering, *Carbohydr. Polym.* 60 (4) (2005) 475–481.