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Anti-diabetic effect of rice extract constituents through the molecular inhibition of α-amylase and α-glucosidase activity

Ramakrishnan Subhashini[1], Thomas Jebastin[2], Raj Sumathy[1], Ariyamuthu Ramathilaga[1], Rithik Selvaraj[1], Najat A. Bukhari[3], Ashraf Atef Hatamleh[3], Anis Ahamed[3]*

[1]*Department of Biotechnology, Dr. G.R. Damodaran College of Science, Coimbatore, Tamil Nadu, India*

[2]*Computer Aided Drug Designing Lab, Department of Bioinformatics, Bishop Heber College (Autonomous), Tamil Nadu, India*

[3]*Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia*

**Corresponding author: subhashini.r@grd.edu.in; subhashinidevaraj2005@gmail.com*

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Abstract: Carbohydrate digestive enzymes like α-amylase and α-glucosidase can be used to treat and manage diabetes. By inhibiting these enzymes, carbohydrate digestion slowed down, lowering the level of glucose entry into the bloodstream and preventing postprandial hyperglycemia. However, the effectiveness of current antidiabetic agents is limited due to their adverse effects. Therefore, the current study explored natural inhibitors from the methanol extract of rice to combat this issue. Through an integrated approach, four different rice cultivars were analysed and found that red rice methanol extract compounds stigmasterol and 1,2-benzenedicarboxylic acid interacted with α-amylase and α-glucosidase. Additionally, further research on stigmasterol directs the structure-activity relationship studies that aid in managing diabetic conditions.

Keywords: Type2 Diabetes mellitus; hyperglycemic; carbohydrate hydrolysing enzymes; antioxidant activity; molecular interaction

Diabetes mellitus (DM) is a chronic health condition characterised by a deficiency in insulin production and resistance. There are two types of DM: T1DM (Type 1 Diabetes mellitus) and T2DM (Type 2 Diabetes mellitus). The disease occurs when the islets of Langerhans of the pancreas do not produce enough insulin to regulate the blood glucose or when the body cannot use the produced insulin effectively. Increased blood glucose levels can cause hyperglycemia, and the condition, when left untreated, leads to life-threatening complications. T2DM is commonly associated with postprandial hyperglycemia, a major health issue. The prevalence of diabetes is increasing globally and is expected to reach 300 million by 2025 (Gupta and Phatak 2003) and 700 million by 2045 (Saeedi et al. 2019).

Carbohydrates are the primary source of energy for the body. However, before being absorbed by the body, the energy source needs to be broken down into smaller units called monosaccharides. This

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process is facilitated by two major enzymes, namely α-amylase and α-glucosidase. The salivary glands produce salivary α-amylase, which is effectively active in the breakdown of carbohydrates while chewing food. Pancreatic α-amylase is present in pancreatic juices and is delivered into the intestine. Here, it converts complex dietary carbohydrates into oligosaccharides and disaccharides. In the final stage of glucose metabolism, α-glucosidase is present at the brush border of human intestinal mucosal cells, playing a crucial role by hydrolysing disaccharides into absorbable monosaccharides, such as glucose (Gong et al. 2020).

Postprandial hyperglycemia is when blood glucose levels remain high after consuming the meal. Managing diabetes, especially T2DM, involves reducing postprandial hyperglycemic conditions by inhibiting carbohydrate hydrolysing enzymes. Thereby reducing the intake of glucose into the bloodstream. Synthetic drugs are available for treating T2DM, such as acarbose, miglitol, sulfonylureas, meglitinides, thiazolidinedione, metformin, etc. These drugs can lower blood glucose levels but may have adverse effects, including irregular low blood sugar, congestive heart failure, and cycles of extreme hunger. Additionally, they can cause gastrointestinal problems like bloating, diarrhea, and abdominal pain (Chiasson et al. 2002, Fujisawa et al. 2005). Also, these drugs are expensive for the lower and middle-class people (http://www.idf.org/diabetesatlas).

Due to the limitations of available antidiabetic drugs, researchers across the globe are looking into identifying natural inhibitors from different sources to control diabetes through different approaches (Ward et al. 2017, Wang et al. 2017, Poulose et al. 2021). Rice is one of the foremost staples and is one of the most consumed foods. Coloured rice cultivars have gained recognition for their nutritional benefits, including their antioxidant properties, high protein content, and rich phytoconstituents. This study aimed to evaluate the efficiency of various rice extracts in inhibiting the α-amylase and α-glucosidase activities, which are crucial for managing blood glucose levels in diabetes, using both *in-vitro* and *in-silico* methods.

MATERIAL AND METHODS

Preparation of samples. Four different cultivars of raw rice samples, red, black, brown, and white rice, were procured from an organic farm for this study. We weighed 10 g of each sample separately and soaked it with 10 mL of methanol at room temperature for two weeks with frequent agitation until the soluble matter was dissolved. Then, the solvent was evaporated to obtain a crude extract. The methanol extract from the red (RRME), black (BRME), brown (BrRME), and white (WRME) rice was labelled and stored at 4 °C for further analysis.

α-amylase inhibition assay. The experiment involved taking rice extracts in varying concentrations ranging from 50–250 µL. 500 µL of 0.5 mg/mL α-amylase solution was added to each tube, and the mixture was incubated at 25 °C for 10 min. Next, 500 µL of 1% soluble starch solution (potato starch dissolved in sodium phosphate buffer of pH 6.9) was added to each tube and incubated again at 25 °C for 10 min. Then, 1 mL dinitrosalicylic acid reagent was added, and tubes were placed in a boiling water bath at 100 °C for 5 min. After this, 10 mL of distilled water was added to each tube, and control was maintained. The resulting absorbance was measured using a UV spectrophotometer at 540 nm and compared against the acarbose with its percentage of inhibition (Jaya Prasad et al. 2019).

α-glucosidase inhibition assay. The α-glucosidase inhibitory activity was determined using the method described in the literature (McCue et al. 2005). A mixture containing 50 µL of sample solution, 125 µL of α-glucosidase and 700 µL of phosphate buffer was pre-incubated at 37 °C for 15 min. Next, 125 µL of 5 mmol *p*-nitrophenyl glucopyranoside was added, and the mixture was held at 37 °C for 30 min. The reaction was stopped by adding 1 000 µL of 0.2 mol/L Na_2CO_3. Absorbance was read at 405 nm for a sample, and the acarbose was expressed as a percentage of inhibition (Jaya Prasad et al. 2019).

$$\% \text{ of enzyme inhibition} = [(Ac - As) \div Ac] \times 100$$

where: Ac – absorbance of control; As – absorbance of sample.

Qualitative and quantitative analysis. Preliminary phytochemical analysis was carried out for the RRME using the methods described in Harborne (2001).

Total phenolic content. Generally the molecules in rice with antioxidant activity include phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tococtirenols, γ-oryzanol, and phytic acid. Total phenolic content was measured using the Folin-Ciocalteu method according to Singleton et al. (1999), with some modifications. Here, different concentrations of RRME were taken in aliquots (250, 500, 750, 1 000 µL) and made up to 1 mL using distilled water. 500 µL of Folin-Ciocalteu reagent (1:1)

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and 2.5 mL of 20% sodium carbonate solution were added. The aliquots were incubated in the dark for 40 min at room temperature, and the blank (water) was maintained. The total phenolic content absorbance was read at 765 nm using a UV spectrophotometer. Different concentrations of gallic acid standards (0.1 mg/mL) were used, and the results were expressed as milligram gallic acid equivalents (GAE/0.1 mg/mL).

Total flavonoid determination. Different concentrations of RRME were taken as aliquots (250, 500, 750, and 1 000 µL) and made up to 1 mL with ethanol. Then, 10% of aluminium chloride was prepared, and 100 µL was added to each test tube. After that, 100 µL of 1 mol/L sodium acetate was added, and the tubes were incubated in the dark for 40 min at room temperature. Total flavonoid content was estimated using a UV spectrophotometer at 510 nm. Ascorbic acid (10 mg/mL) was used as standard, and the results were expressed as milligram ascorbic acid equivalents (AAE, 0.1 mg/mL) (Jia et al. 1999).

Gas chromatography-mass spectrometry analysis. Coloured rice cultivars are recognised for their reduced sugar content, high protein levels, and dietary fibre. The red rice methanol extract demonstrated the highest antioxidant properties and enzyme inhibition. Chemical compound analysis was performed using gas chromatography-mass spectrometry (GC-MS, analysis, with a 2 µL sample injected into a Fisons GC8000 gas chromatography coupled to an MD800 mass spectrometer equipped with a quadrupole mass analyser. The injection temperature was 230 °C, and helium flow was 1 mL/min. After a 5 min solvent delay time, the oven temperature was increased at 70 °C/min to 260 °C and cooled to 70 °C. The ion trace integration was done using the mass lab find target method for the characteristic fragment of assigned peaks. Mass spectrum interpretation was conducted using the National Institute Standard and Technology (NIST) database. The spectrum of the unknown component was compared with the spectrum of known components stored in the NIST library. The molecular weight, molecular formula and the compound's name from NIST were recorded (Hemamalini et al. 2013).

Radical scavenging activity. In hyperglycemic conditions, elevated glucose levels lead to glycation, and the resulting glycation-end products generate free radicals. The antioxidant capacity of RRME was assessed for their ability to neutralise these radicals and mitigate oxidative stress. Various concentrations of 1 mg/mL ascorbic acid and RRME extract ranging from 20–100 µL were prepared in different aliquots. These tubes were then made up to 1 mL using ethanol. Then, 1 mL of 0.1 mg/mL 1,1-diphenyl-2-picrylhydrazyl (DPPH) was added to the tubes and incubated for 30 min at 27 °C. The antioxidant assay was determined using a UV spectrophotometer at 517 nm, and the control was maintained (Nickavar et al. 2006).

$$\% \text{ of antioxidant activity} = [(Ac - As) \div Ac] \times 100$$

Ferric reducing antioxidant power assay. Additionally, different concentrations of RRME extract of various fractions ranging from 20–100 µg/mL were added to 2.5 mL of 0.2 mol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution. The reaction mixture was vortexed well and then incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3 000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride. The reducing power of the sample from ferric to ferrous ion was analysed against the standard using a UV-spectrophotometer at 700 nm (Wu et al. 2019).

Pharmacokinetic properties prediction. When selecting a potential drug, the molecular binding of the ligand to protein at its active centre alone is not enough. Other criteria, such as absorption, digestion, metabolism, and excretion (ADME), must be assessed to determine the drug's likeness for reducing the pharmacokinetics-related failure in the drug development process. These properties were calculated by SwissADME. Low molecular weight (MW) signifies that molecules can be easily absorbed orally. Compounds with MW > 500 Da are absorbed *via* an alternate route, generally through the membrane. The logarithm of the octanol/water partition coefficient (Log $P_{o/w}$) should be lower than 5, and the number of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs) should be less than 5 and 10, respectively (Ibrahim et al. 2021, Aneeqa et al. 2024).

Target and ligand preparation. Protein Data Bank (PDB) is a database that mainly contains derived protein structures and is used to retrieve structure data in PDB file format. The three-dimensional crystal structure of α-amylase (PDB ID: 3BAI) and α-glucosidase (PDB ID: 2QMJ) in complex with co-crystallised ligand was retrieved from PDB (http://www.rcsb.org/pdb/home/home.do). The molecular interaction of the selected targets, such as α-amylase, α-glucosidase

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and the standard against GCMS compounds, was performed with AutoDock. Before performing the docking analysis, the bound complex molecule and water molecules were removed while preparing the target (Etsassala et al. 2020). Compounds such as hexadecanoic acid, methyl ester, tetradecanoic acid, 1,2-benzenedicarboxylic acid, oleic anhydride, squalene, stigmasterol, acarbose were downloaded from the PubChem database.

To prepare the receptor for docking algorithms, each atom needs a charge and an atom type to describe its properties for the docking process. The prepared docking-ready format, PDBQT, includes Kollman charges and polar hydrogen atoms only. Next, a grid box is set up in three dimensions (*x*, *y* and *z* coordinates), and AutoGrid is executed. Docking is performed using the Lamarckian genetic algorithm, and the interactions are visualised using Molegro Molecular Viewer (Odder, Denmark).

RESULT AND DISCUSSION

T2DM is a condition where the body becomes insulin resistant, leading to increased blood glucose levels and hyperglycemia. Carbohydrates are broken down into simpler units by digestive enzymes before being absorbed by the body. A strategy to manage T2DM is reducing or inhibiting carbohydrate-hydrolysing enzyme activity to delay digestion, which can decrease postprandial hyperglycemia. By prolonging the overall digestion time, glucose absorption is reduced. Methanol extracts of four different rice cultivars, namely red, brown, black, and white rice, were prepared to investigate their effects on managing this health issue.

Inhibitory effects of the extract on α-amylase and α-glucosidase. α-amylase catalyses the α-(1-4)-D-glycosidic linkages present in the starch, breaking them into polymers of glucose and other small fragments. Extracts were tested for their α-amylase inhibition using the spectrophotometric method. The reducing sugars produced by α-amylase react with dinitrosalicylic acid (DNS) and reduce to a brown-coloured product called nitro-amino-salicylic acid. It was observed that RRME showed a higher inhibition of α-amylase ranging from 47.2% to 93.3% (in 50 to 250 µg/mL) against the standard of 45% to 89.5% (Figure 1). Therefore, RRME was selected for further α-glucosidase inhibitory analysis. The conversion of the substrate 4-nitrophenyl-α-D-glucopyranoside was catalysed, which produces a yellow-coloured product. The RRME demonstrated significant inhibition against α-glucosidase (39.86% to 72.59%) in comparison to acarbose (45% to 89.5%) (Figure 2).

Phytochemical analysis of the RRME. Red rice is a nutritious food that can help reduce the risk of chronic diseases due to the presence of various nutrients such as polyphenols, minerals, fibre, vitamins, and other phytochemicals. These compounds can impact biological functions in different ways. The RRME also contains carbohydrates, proteins, flavonoids, alkaloids, phenols, and glycosides, which indicate its richness in phytochemicals (Table 1). Quantitative analysis of phytochemicals is a useful technique to measure the total content of secondary metabolites such as alkaloids, phenolic compounds, terpenoids, flavonoids, etc. The phytochemical composition of RRME was found to contain 0.45 mg/GAE/mL of phenols and 0.5 mg/AAE/mL of flavonoids. The study conducted in the RRME revealed the presence of

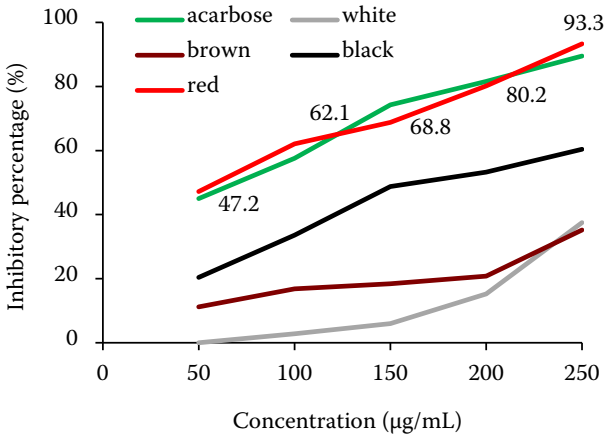

Figure 1. α-amylase inhibition by methanol extract of rice cultivars

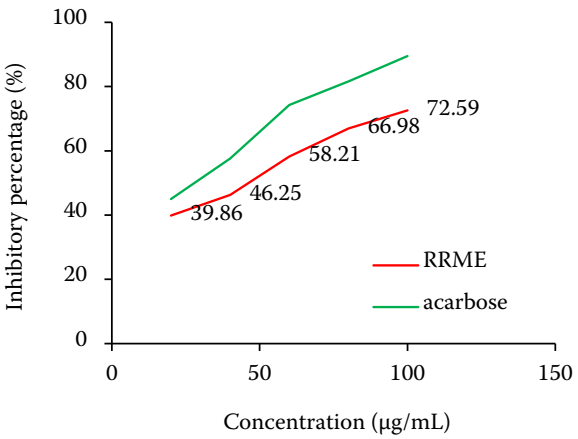

Figure 2. α-glucosidase inhibitory activity of red rice methanol extract (RRME) extract against the standard

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Table 1. Phytochemical analysis of red rice methanol extract (RRME)

Phytochemical	Experiments	Result
Carbohydrates	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
Proteins	Buiret test	+
	Ninhydrin test	+
Alkaloids	Mayer's test	+
	Wagner's test	+
Flavanoids	H_2SO_4 test	+
Phenols	Ferric chloride test	+
	Lead acetate test	+
Steroids	Liebermann Burchard test	–
Saponins	Froth test	–
Tannins	Ferric test	–
Glycosides	Keller-Kiliani test	+

+ – positive; – – negative

47.0 ± 0.7 (GAE/g) phenols and 40.15 mg QE/100 g of flavonoids (Ashokkumar et al. 2020).

Identification of phytoconstituents in the extract. The GC-MS analysis led to the identification of 19 phytoconstituents in the RRME extract (Figure 3). Compounds that present major and nominal levels are n-hexadecanoic acid and 1-tridecanol (Table 2). All identified compounds, when reviewed, resulted in anti-hyperglycemic (Ibrahim et al. 2019, Liyanaarachchie et al. 2021, Mirmiranpour et al. 2022, Widyawati et al. 2021, 2023), anti-hypoglycemic (Abirami and Gomathi 2022) and diabetic retinopathy (Zhu et al. 2019) activity. The ethanol extract from the red rice contains compounds such as palmitic acid, oleic acid, stearic acid, stigmast-5-en-3-ol, heptadecane, octadecane, hexadecane, linoleic acid, retinoic acid, and others (Ashokumar et al. 2020). All compounds identified in RRME were selected for further study.

Antioxidant assay. The radical scavenging activity assay is a method that measures the ability of antioxidants to neutralise free radicals and reactive oxygen species. These harmful substances can cause metabolic effects, damaging cells. The assay is based on the reduction of DPPH, which has an odd electron and gives a maximum absorbance. When the test sample is mixed with DPPH, the antioxidants present in the test sample donate a hydrogen atom, causing DPPH to be reduced to diphenyl picryl hydrazine, which is in a non-radical form. As a result, the violet colour of the picryl group present in DPPH is lost, and it turns pale yellow (Dontha 2016). The more decolorisation, the better the reducing ability due to the antioxidants in the sample.

The quenching activity of RRME is comparable to the selected standard (Figure 4). The extract is effective in reducing the stable radical DPPH, indicated by the yellow-coloured compound diphenylpicryl hydrazine, which has a strong antioxidant effect. Previous studies reported that the DPPH radical-scavenging activity of the red rice bran extract increases with concentration, which correlates to the present study (Ghasemzadeh et al. 2018).

Ferric reducing antioxidant power (FRAP) assay is a quick and easy method for determin-

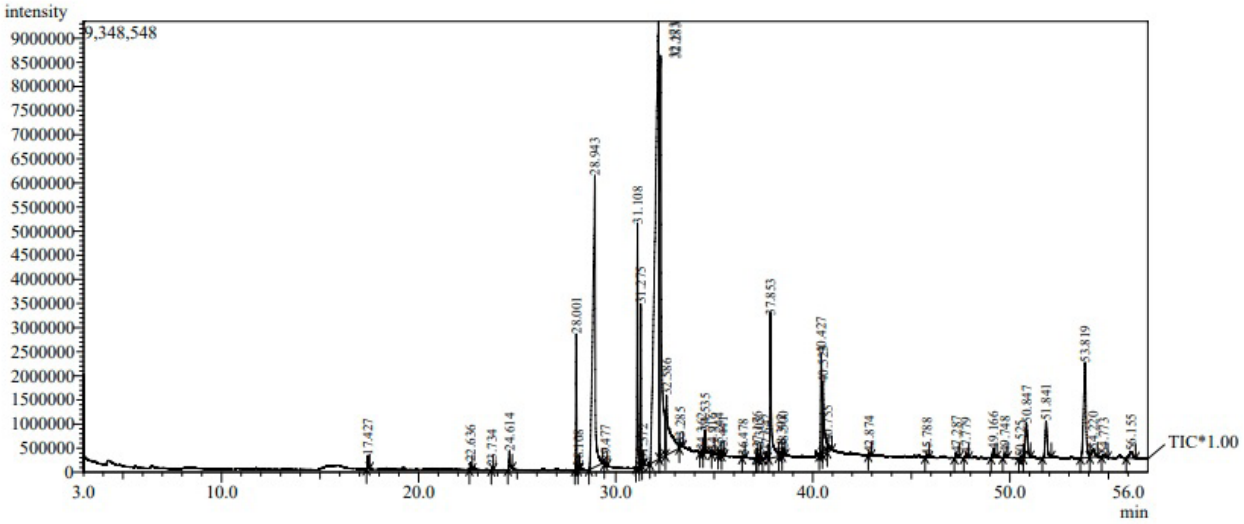

Figure 3. Gas chromatography-mass spectrometry (GCMS) analysis of methanol extract from the red (RRME)

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Table 2. The phytoconstituents in red rice methanol extract (RRME)

S. No	Constituent	Molecular formula	Area (%)
1	1-Dodecanol	$C_{12}H_{26}O$	0.25
2	1-Tridecanol	$C_{13}H_{28}O$	0.13
3	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	2.07
4	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.42
5	1,2-benzenedicarboxylic acid	$C_8H_6O_4$	0.15
6	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	14.69
7	Oleic anhydride	$C_{36}H_{66}O_3$	2.57
8	Squalene	$C_{30}H_{50}$	0.05
9	Stigmasterol	$C_{29}H_{48}O$	1.52
10	9,12-Octadecadienoic acid methyl ester (Z, Z)-, 2,3-dihydroxypropylester	$C_{19}H_{34}O_2$	4.01
11	Octadecanoic acid	$C_{18}H_{36}O_2$	4.47
12	4-Octadecenal	$C_{18}H_{34}O$	0.11
13	Fumaric acid	$C_4H_4O_4$	0.37
14	3-Cyclopentylpropioinc acid, 2-dimethylaminoethyl ester	$C_8H_{14}O_2$	0.16
15	Heptanoic acid	$C_7H_{14}O_2$	0.13
16	Tetradecanal	$C_{14}H_{28}O$	0.14
17	4,5-Diamino-2-hydroxypyrimidine	$C_4H_6N_4O$	0.29
18	1-Heptatriacotanol	$C_{37}H_{76}O$	0.04
19	Stigmast-5-en-3-ol, (3 beta)	$C_{29}H_{50}O$	4.70

ing total antioxidant activity *in vitro* (Figure 5). his method relies on the reduction of TPTZ (2,4,6-tripyridyl-s-triazine) with ferric chloride hexahydrate ($FeCl_3 6H_2O$), which causes the solution to turn a slight brown colour by reducing blue ferrous complexes. The FRAP assay treats antioxidants in the samples as reductants that decrease the activity of the extract as the concentration increases, similar to the findings reported using red rice ethanol extract (Krishnanunni et al. 2015).

Drug likeness properties of compounds. All compounds in this study have an MW less than 500 g/mol except for oleic anhydride and the standard acarbose. The HBD and HBA of compounds exhibit favourable pharmacokinetic parameters for oral bioavailability. LogS indicated only acarbose and 1,2-benzenedicarboxylic acid are soluble in water. The other moderately and poorly soluble compounds are predicted based on their lower negative values. The partition coefficient between n-octanol and water can be achieved experimentally but is challenging for many compounds. Using computation tools, ILogP criteria facilitate the drug interactions with their biological targets. The estimated values

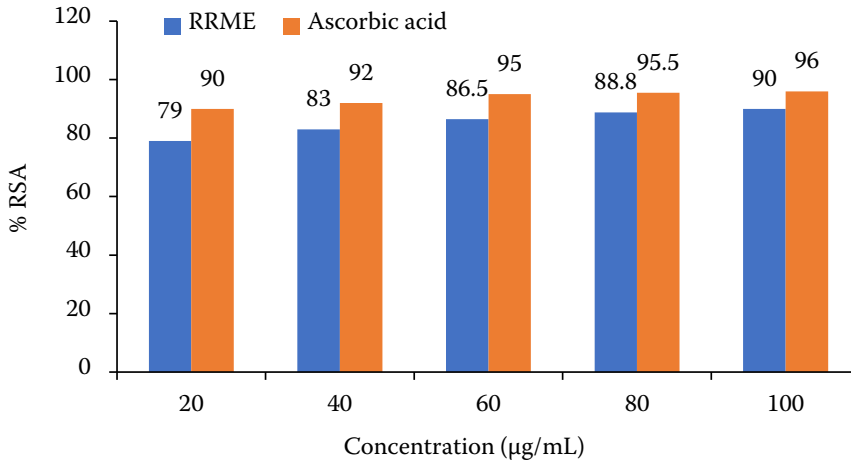

Figure 4. Radical scavenging activity of red rice methanol extract (RRME)

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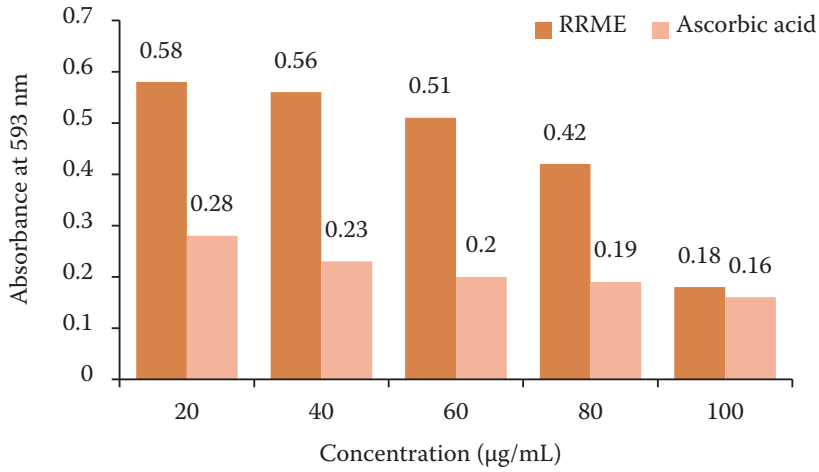
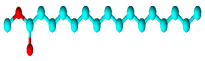
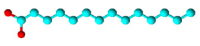
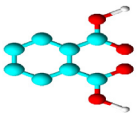
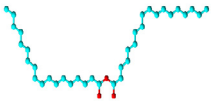
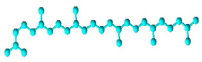
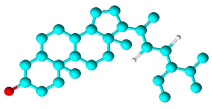
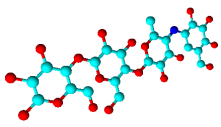


Figure 5. Ferric reducing antioxidant power (FRAP) activity of red rice methanol extract (RRME)

Table 3. Drug likeness properties of the compounds

Constituent	Structure	Molecular weight (g/mol)	HBD	HBA	Log $P_{o/w}$	LogS	GI absorption	Rule of five violations
Hexadecanoic acid, methyl ester		270.45	0	2	4.41	–5.18	high	0
Tetradecanoic acid		228.37	1	2	3.32	–4.31	high	0
1,2-benzene-dicarboxylic acid		166.13	2	4	0.60	–1.57	high	0
Oleic anhydride		546.91	0	3	8.71	–10.47	low	1
Squalene		410.70	0	0	6.37	–8.69	low	0
Stigmasterol		412.69	1	1	5.01	–7.46	low	0
Acarbose		645.60	14	19	0.63	2.13	low	3

HBD – hydrogen bond donors; HBA – hydrogen bond acceptors; GI – gastrointestinal

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Table 4. Molecular docking analysis of the compounds against α-amylase

S. No.	Compound name	Binding energy (kcal/mol)	Interactions	Bond length (Å)
1	Acarbose	–4.6	Hie305, Asp300, Asp 197, Lys200 (2)	1.87, 1.82, 2.03, 2.18, 2.36
2	Stigmasterol	–5.9	Asp300	2.01
3	Stigmast-5-en-3-ol, (3 beta)	–4.5	Asp197	1.91
4	Squalene	–3.9	–	–
5	1-Heptatriacotanol	–3.7	Leu237	1.78
6	Oleic anhydride	–3.2	–	–
7	4,5-Diamino-2-hydroxypyrimidine	–3.0	Glu233 (2), Asp300 (2)	2.17, 2.09, 1.79, 2.14
8	Heptanoic acid	–1.6	Arg195, Hie299,	2.18, 2.02
9	Hexadecanoic acid methyl ester	–1.0	Lys200	1.91
10	n-Hexadecanoic acid	–0.9	Lys200 (2)	1.62, 2.09
11	Tetradecanal	–0.8	Arg195, Hie299	2.35, 2.15
12	Fumaric acid	–0.8	–	–
13	4-Octadecenal	–0.6	–	–
14	Tetradecanoic acid	–0.4	Lys200 (2), Ile235	2.46, 2.05, 1.96
15	1-Tridecanol	–0.2	Asp300	1.92
16	1-Dodecanol	–0.1	Hie305	1.84

of ILogP were less than 5 and adhered to the rule of thumb by three compounds in Table 3. High lipophilicity contributes to immobilisation within a given layer and reduced penetration of these compounds through the blood-brain barrier. Also, these three compounds are predicted to be absorbed more in the gastrointestinal tract than other compounds. In addition, oleic anhydride (MW) and acarbose (MW; HBD and HBA) violated the Rule of Five (Table 3).

Molecular docking analysis. As clinical and laboratory trials for designing new drugs are expensive, time-consuming, and prone to errors, bioinformatics techniques are increasingly utilised. *In-silico* screening is one such innovative approach that can help identify drug-like compounds. Molecular docking is a vital technique that allows the examination of the binding affinity of the small molecules with the receptor in the three-dimensional space.

The structural arrangement of the selected compounds was verified through PubChem, and all structures were prepared using the ChemSketch. Both standard and test compounds are prepared in .mol format, which is then converted to .pdb format and retrieved the three-dimensional crystal structure of α-amylase (PDB ID: 3BAI) and α-glucosidase (PDB ID: 2QMJ) from PDB database (Maurus et al. 2008, Sim et al. 2008). The predicted active site positions of α-amylase and α-glucosidase are used in interaction analysis. The docking parameter file is then prepared with Lamarckian Genetic Algorithm to perform the docking process. This resulted in interactions of the compounds with the key residues, along with the binding free energy value. The < –7.0 kcal/mol binding affinity indicates the best docking score, and interacting residues remain closer (Kashyap et al. 2023).

Docking experiments have uncovered several crucial insights into the interactions between red rice metabolites and alpha-amylase. Inhibiting this enzyme, a target for therapies for type 2 diabetes mellitus, may help modulate postprandial blood glucose levels. Among the red rice metabolites found, stigmasterol had the strongest docking score of –5.9 kcal/mol for α-amylase. This docking score assesses the binding energy; a lower score indicates higher binding affinity. Docking pose analysis revealed that stigmasterol establishes important hydrogen bonds with α-amylase amino acid residues in the active site. These hydrogen bonds are crucial for stabilising the ligand-protein complex, effectively blocking the enzyme's activity. In contrast, the well-known α-amylase inhibitor acarbose had a docking score of –4.6 kcal/mol. This value is less favourable than stigmasterol, though they still exhibit a high binding affinity. This suggests that stigmasterol may inhibit α-amylase more effectively than these current inhibitors. In the current

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study, the binding energy between each compound and the target is < –7 Kcal/mol. Stigmasterol and acarbose interacted with active sites, revealing that they commonly had the Asp residue but at different positions (Table 4, Figure 6).

The significant binding affinity and stabilising interactions of stigmasterol imply that it might be viable for controlling type 2 diabetes mellitus. When consumed after a meal, alpha-amylase inhibitors help control blood sugar levels by delaying the decomposition of carbohydrates into glucose. Stigmasterol's increased binding affinity compared to acarbose shows its prospective utility in this capacity. The most prevalent phytosterols in the human diet are sitosterol, stigmasterol, and campesterol, which may be found in free form, as fatty acid/cinnamic acid esters, or as glycosides metabolised by pancreatic enzymes. Accumulating research suggests that phytosterols and diets enriched with them may manage glucose and lipid metabolism, as well as insulin resistance (Prasad et al. 2022). In a preceding *in silico* investigation, it was observed that stigmasterol, present in twig extracts of *Paederia foetida* Linn, exhibited a low binding energy with α-amylase, suggesting its potential as an antidiabetic compound (Tan et al. 2019). In addition oleic acid and hexadecanoic acid present in *Leucaena leucocephala* (Lam) has anti α-amylase activity (Renganathan et al. 2021). Oleic acid and hexadecanoic acid also present in RRME.

Stigmasterol, the phytosterol present in soybean oil, is efficient in the treatment of T2DM by targeting glucose transporter4 (GLUT4) (Wang et al. 2017). Accumulation of cholesterol leads to defects in insulin secretion, causing hyperglycemia and dyslipidemia. This phytosterol with anti-atherosclerotic potential can be used as a strategy to protect β-cell function (Ward et al. 2017). The results imply that stigmasterol and, conceivably, other red rice metabolites might be studied further as novel medicinal agents. These natural substances could provide a more effective or complementary method for managing type 2 diabetes mellitus.

In the docking investigations targeting α-glucosidase, distinct binding patterns were discovered for the red rice metabolites, which give valuable insights into their potential as therapeutic agents. Acarbose had the greatest binding affinity among the investigated pharmaceuticals, with a docking score of –7.0 kcal/mol. This score shows a very high interaction between acarbose and α-glucosidase. The molecular interactions investigation indicated that acarbose forms essential hydrogen bonds with numerous critical residues in the α-glucosidase binding site. Specifically, hydrogen bonds were created with residues Glu114, Arg283,

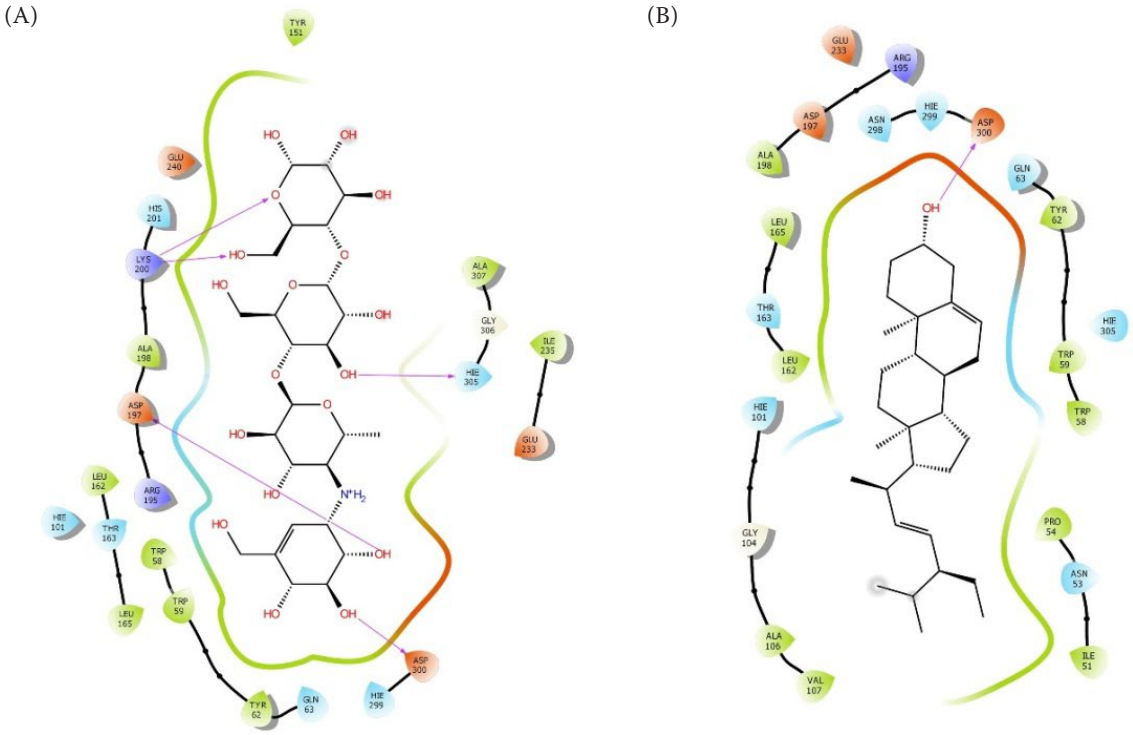

Figure 6. Molecular interaction of α-amylase with (A) acarbose and (B) stigmasterol

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Table 5. Molecular docking analysis of the compounds against α-glucosidase

S. No.	Compound name	Binding energy (kcal/mol)	Interactions	Bond length (Å)
1	Acarbose	–7.0	Glu114 (2) , Arg283 (2), Ala509, Lys776, Asp777, His645	1.83, 1.94, 1.98, 2.30, 2.46, 1.92, 2.02, 1.86
2	1,2-benzenedicarboxylic acid	–6.1	Ser521, Ser288	1.64, 2.07
3	4,5-Diamino-2-hydroxypyrimidine	–5.6	Arg520 (2) , Ile523	2.47, 2.21, 2.05
4	Tetradecanoic acid	–3.0	Asp777, Leu286	2.36, 1.86
5	Heptanoic acid	–2.7	Ile523	1.85
6	1-Dodecanol	–2.5	Lys776, Arg520	2.04, 1.77
7	Stigmast-5-en-3-ol, (3 beta)	–2.5	Arg283	2.28
8	Stigmasterol	–2.4	Asp777	2.06
9	n-Hexadecanoic acid	–2.3	Lys776, Lys513	2.81, 2.28
10	Fumaric acid	–2.1	Met567, Ile523	2.55, 2.13
11	1-Tridecanol	–1.9	Arg520, Lys776	2.80, 2.02
12	Hexadecanoic acid methyl ester	–1.8	Lys513	2.74
13	Squalene	–1.3	–	–
14	4-Octadecenal	–0.9	Met567	2.23

Ala509, Lys776, and His645. These interactions are critical for stabilising the acarbose α-glucosidase complex, enhancing its inhibitory influence on the enzyme's activity.

The red rice metabolite 1,2-benzenedicarboxylic acid demonstrated modest binding affinities, with a docking score of –6.4 kcal/mol. Although this score is less favourable than acarbose, it nevertheless suggests a substantial binding interaction (Table 5, Figure 7). Examination of the binding mechanisms of 1,2-benzenedicarboxylic acid showed that this metabolite similarly forms hydrogen bonds with a α-glucosidase residues. Still, the precise residues and the intensity of these interactions may vary from those of acarbose. The intermediate binding affinity means that although it may not be as potent as acarbose, it can competitively inhibit α-glucosidase activity. In addition, oleic acid in other pigmented

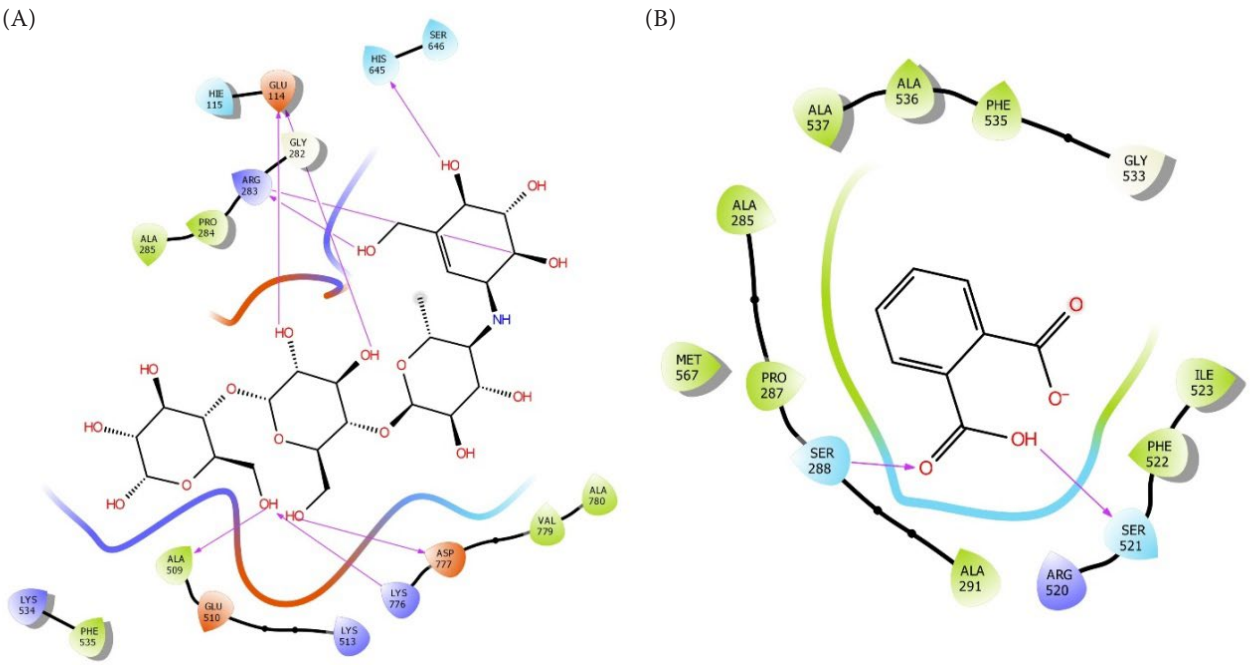

Figure 7. Molecular interaction of α-glucosidase with (A) acarbose and (B) 1,2-benzenedicarboxylic acid

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rice is reported to have anti-glucosidase activity (Su et al. 2013).

The results underscore the medicinal potential of red rice metabolites as efficacious inhibitors of α-glucosidase. Despite acarbose having a greater binding affinity, the intense interaction seen with 1,2-benzenedicarboxylic acid emphasises the potential of these natural compounds. Red rice metabolites might be further researched and perhaps produced as additional or alternative therapies to current α-glucosidase inhibitors. The ability of these metabolites to inhibit α-glucosidase implies they might help control type 2 diabetes mellitus by adjusting postprandial blood glucose levels. This corresponds with the treatment approach of α-glucosidase inhibitors to manage blood sugar levels after meals. Also, a study demonstrated the presence of this compound in the seaweed *Gelidium spinosum* reduced hyperglycemic effects in streptozotocin-induced rats (Poulose et al. 2021). 1,2-benzenedicarboxylic acid is commonly known as phthalic acid or phthalates (Nam et al. 2020).

The current study explored the chemical constituent in the red rice methanol extract for its anti-diabetic activity through *in vitro* and *in silico* approaches by inhibiting carbohydrate hydrolysing enzymes such as α-amylase and α-glucosidase. Among four different rice extracts, the red rice methanol extract showed the highest inhibition of α-amylase and α-glucosidase. Molecular docking studies of identified metabolites, 1,2-benzenedicarboxylic acid exhibited notable binding affinity against α-glucosidase. At the same time, stigmasterol demonstrated promising interaction with α-amylase, surpassing the efficacy of the conventional drug acarbose in binding affinity. However, it is noteworthy that acarbose still exhibited lower binding affinity than rice metabolites against α-glucosidase. Although *in vitro* studies demonstrated that acarbose yielded marginally better results than the active metabolites, the significant binding affinity of specific rice metabolites against key enzymes implicated in T2DM underscores their potential as preventive supplements against the onset of the disease. In conclusion, the findings suggest that incorporating these active metabolites from red rice into daily supplementation could serve as a promising strategy for T2DM prevention. Further research and clinical trials are warranted to validate these findings and explore the full therapeutic potential of these rice metabolites in mitigating T2DM and related metabolic disorders.

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