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Discovery of vitexin, isovitexin and catechin as hypoglycemic factors in mung bean via metabolomics combined with in vitro experiments

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Abstract

Mung bean (Vigna radiata *L.*), a food with a medium–low glycemic index, contains a variety of functionally active small molecule metabolites that exhibit hypoglycemic effects. Notably, the hypoglycemic properties of different varieties of mung beans are variable. This study employed nontargeted metabolomics to analyze mung bean varieties with distinct phenotypic differences in the estimated glycemic index (eGI), identifying vitexin, isovitexin, and catechins as significantly relevant metabolites. The functions of these metabolites on the eGI were further validated through enzyme inhibition assays in vitro. The results elucidate the digestion characteristics of mung bean starch, revealing that the three metabolites contribute to an increase in the resistant starch (RS) content and a reduction in the hydrolysis rate of starch, ultimately resulting in a decrease in the eGI. Specifically, vitexin inhibited alpha-glucosidase (AG) activity via an uncompetitive inhibition mechanism (IC_{50} = 50.11 μM), whereas isovitexin functioned through a competitive inhibition mechanism (IC_{50} = 116.14 μM), and catechin exhibited noncompetitive inhibition (IC_{50} = 42.06 μM). Furthermore, the study revealed a notable synergistic effect among these three metabolites in the inhibition of AG activity. This research screened and identified the main metabolites exhibiting hypoglycemic potential in mung beans and confirmed their hypoglycemic effects in vitro, thereby providing a theoretical foundation for the development of functional foods aimed at hypoglycemic applications of mung beans.

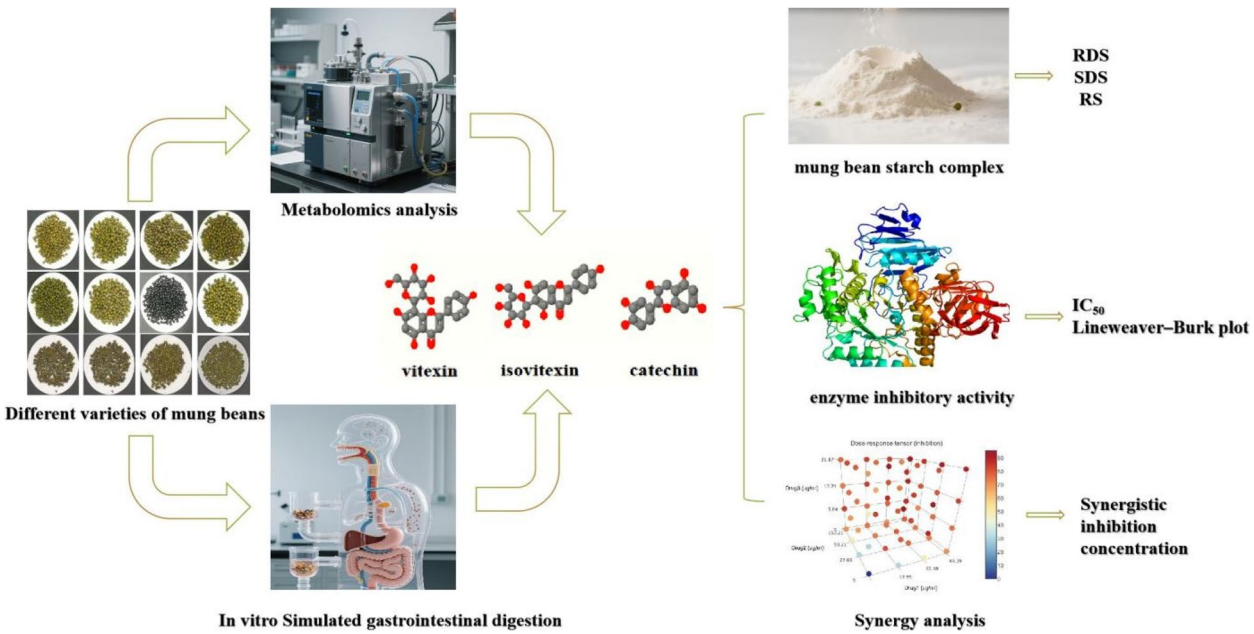
Keywords Digestive properties, Enzyme inhibitory activity, Estimated glycemic index, Mung bean, Natural metabolites

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Graphical Abstract



Introduction

Diabetes is a chronic disease that poses a significant threat to public health. Its incidence has risen markedly in recent years. Among the various types of diabetes, type 2 diabetes mellitus (T2DM) is characterized by long-term abnormalities in insulin secretion and postprandial blood sugar fluctuations, with patients comprising approximately 90% to 95% of the total diabetic population. This condition is regarded as a major public health concern (Zhang et al., 2021). Research indicates that significant fluctuations in blood sugar levels are a contributing risk factor for diabetes. Additionally, a high glycemic index (GI) is associated with an increased risk of chronic diseases, including T2DM (Jayedi et al., 2020; Miller et al., 2014). Consequently, the low-glycemic index (GI) dietary pattern has emerged as an effective intervention for T2DM. Its hypoglycemic mechanism primarily regulates starch digestion, slows the rate of glucose release, effectively reduces insulin demand, and thereby promotes stability in blood sugar levels. Moreover, some studies have indicated that α-glucosidase (AG) is a key target for the treatment of T2DM (Liu et al., 2020). Clinically utilized drugs for lowering blood sugar levels include acarbose, a commonly used α-glucosidase inhibitor. However, these medications may cause gastrointestinal side effects, including severe bloating. The whole-grain diet is recognized as a low-glycemic index dietary pattern. Numerous studies have demonstrated that increasing the intake of whole-grain foods is beneficial for reducing the risk of chronic diseases, including T2DM (Cecilie et al., 2018), which may be due to its high content of polyphenols, alkaloids, alkyl resorcinols and other active compounds (Wu et al., 2019). Among these compounds, polyphenols—including flavonoids, tannins, phenolic acids, and anthocyanins—have been shown to inhibit the activity of α-amylase and α-glucosidase in the digestive system. This inhibition plays a crucial role in regulating blood sugar levels and in the prevention and treatment of T2DM. Other studies have demonstrated notable interactions between starch and polyphenols. This interaction significantly reduces the digestibility of the complex formed by these two components while markedly increasing the quantity of starch residues (Cai et al., 2014), which effectively contributes to a reduction in the glycemic index. Consequently, the selection of natural hypoglycemic active substances from cereal plants that are nontoxic and devoid of side effects represents a crucial strategy for effectively controlling blood sugar and preventing diabetes.

Mung bean is an important edible legume crop known for its balanced nutrient profile and excellent source of numerous bioactive compounds. In addition

to fulfilling basic nutritional requirements, mung beans present properties that may lower blood sugar levels and blood lipids, among other potential health benefits. Flavonoids represent the most abundant class of secondary metabolites in mung beans, with vitexin and isovitexin identified as key functional components contributing to the health-promoting effects of this legume (Luo et al., 2016). These flavonoids are predominantly localized in the seed coat of mung beans. Recent phytochemical analyses have quantified these compounds at 1.49–1.78 mg catechin equivalents/g DW (Hou et al., 2019). Mung bean, classified as a medium–low glycemic index food, represents an effective dietary option for individuals with diabetes. However, the various active compounds present in mung beans exhibit distinct pathways and targets for blood sugar regulation. Consequently, it is essential to screen and identify the particular small molecule metabolites associated with the hypoglycemic effect, as well as explore their mechanisms and impacts.

Plant metabolomics enables comprehensive qualitative and quantitative analyses of metabolite distributions in plants and plays a crucial role in the examination of secondary metabolism. The use of metabolomics technology to investigate phenotypic differences among various plant varieties is essential for evaluating and discriminating plant quality. Given the connection between chemical components and biological activities, metabolomics and bioassays can be utilized to identify key bioactive substances (Guo et al., 2018). For example, He et al. (2023) employed an integrated metabolomics approach to elucidate the nonvolatile properties of beans during soaking, highlighting the dynamic changes in both sexual and volatile metabolites. Importantly, different varieties of mung beans present varying in vitro estimated glycemic indices and distinct hypoglycemic effects. Consequently, a combination of metabolomics and bioassays can be employed to comprehensively and effectively screen for key metabolites that contribute to the reduction in the estimated glycemic index of mung bean.

This study aims to determine the estimated glycemic indices of 20 different varieties of mung bean and employs a metabolomics approach utilizing liquid chromatography and mass spectrometry (LC–MS) to comprehensively analyze the different active ingredients contained in mung bean. Multivariate statistical analysis and difference analysis were employed to identify natural metabolites in mung bean that reduce the starch digestion rate in an in vitro simulated digestion system and test their inhibitory effects on starch digestive enzyme activity. Enzyme kinetics experiments were conducted to investigate the inhibition mechanisms. The interaction between mung bean starch and active ingredients was investigated to determine the effects on the digestive characteristics, followed by an analysis of the drug combination effects between metabolites. To the best of our knowledge, this study represents the first instance of utilizing multiple mung bean cultivars to screen for bioactive components and to investigate the synergistic effects among them.

Materials and methods

Materials

The 414 mung bean accessions representing diverse varieties were provided by the Jiangsu Academy of Agricultural Sciences, Institute of Economic Crops. A starch reagent kit, α-amylase, pepsin, pancreatic enzymes, vitexin (purity >98%), isovitexin (purity >98%), and catechin (purity >98%) were purchased from Solarbio (Beijing, China). p-nitrophenyl α-D-glucopyranoside (pNPG), α-glucosidase, PBS, and DMSO were purchased from Yuan Ye Technology (Shanghai, China), and simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and simulated oral fluid (SOF) were purchased from Coolabor (Beijing, China). Glu reagent kits were purchased from Jiancheng (Nanjing, China).

Static in vitro-simulated gastrointestinal digestion

Following the standardized method for in vitro simulated food digestion (Brodkorb et al., 2019), the total starch content of each mung bean variety was first determined via a total starch reagent kit in 2023. A sample containing 50 mg of available carbohydrates was taken, mixed with an equal volume of oral digestion fluid, and vortexed for 5 min to ensure wetting. Then, 5 mL of gastric fluid was added, and the mixture was transferred to a dissolution cup and incubated at 37 °C for 1 h. After gastric digestion, NaOH was added, and the mixture was mixed well, followed by the addition of 5 mL of intestinal fluid to adjust the digestion system to pH 7. The mixture was incubated at 37 °C, and samples (100 μL) were taken at 0 min, 20 min, 40 min, 60 min, 90 min, and 120 min during intestinal digestion. Each sample was immediately mixed with 900 μL of anhydrous ethanol to terminate the reaction and then centrifuged (5000 r/min for 5 min). A volume of 10 μL of the supernatant was taken and mixed with the glucose reagent kit for detection, followed by incubation at 37 °C for 10 min to measure the glucose concentration at each time point and plot the hydrolysis rate curve. By plotting the hydrolysis rate-time, a simulation equation was generated, with the area under the curve representing the impact of food digestion on blood glucose levels (Ahmed et al., 2000). Using white bread as a reference standard, the relationship between the eGI and hydrolysis index (HI) was defined, with the HI for

white bread set at 100 to calculate the eGI of the samples (Chinedum et al., 2018):

$$eGI = 0.862HI + 8.1981 \quad (1)$$

where eGI is the in vitro simulated glycemic index and HI (%) refers to the ratio of the area under the hydrolysis curve of the test food to that of an equal amount of the standard food (white bread).

Screening and identification of bioactive components

The LC–MS system was selected as the analytical platform for the nontargeted metabolomic analysis of mung bean extract. Chromatographic separation was achieved via an SB-C18 column (2.1 ×100.0 mm id, 1.8 μm, Agilent) maintained at 40 °C. In positive ion mode detection, the mobile phase consisted of pure water containing 0.1% formic acid (phase A) and acetonitrile (phase B). Meanwhile, negative ion mode detection utilized an aqueous solution of 5 mmol/L ammonium acetate (phase A) and acetonitrile (phase B). The elution program was structured as follows: during the initial stage (0—1 min), the proportion of phase B was set at 2%. This was followed by a linear increase in the proportion of phase B to 98% from 1—10 min, which is maintained until 12 min. Finally, from 12–15 min, the proportion of phase B is reverted to 2%. The flow rate was set at 0.35 mL/min, with an injection volume of 2 μL. Metabolomic analysis employs a cloud platform and uses principal component analysis (PCA) to determine the overall relationships among the metabolites. Additionally, orthogonal partial least squares discriminant analysis (OPLS-DA) was conducted to investigate the effects of the natural metabolite distribution across different varieties of mung beans on their estimated glycemic index (eGI) values. A t test and differential analysis were performed, resulting in the creation of a volcano plot to illustrate the upregulation and downregulation of differentially abundant metabolites. By integrating correlation analysis, significance analysis, and specific screening criteria, natural metabolites that were negatively correlated with mung bean eGI values were identified and characterized.

Determination of starch-digesting enzyme inhibitory activity

The in vitro α-glucosidase activity assay was conducted via a spectrophotometric method, with minor modifications to previously reported methods (Xu et al., 2023). All test chemicals were dissolved in DMSO to create a 10 mM stock solution. Gradient dilutions were made via phosphate buffer to generate samples of varying concentrations. In phosphate buffer at pH 6.8, α-glucosidase was prepared at 0.5 U/mL, along with 0.6 mM p-nitrophenyl-α-D-glucopyranoside (pNPG). Different concentrations of the inhibitor (10 μL), enzyme mixture (40 μL), and potassium phosphate buffer (100 μL) were preincubated in a 96-well plate at 37 °C for 10 min. Then, 50 μL of substrate (0.6 mM pNPG) was added to each well, and the mixture was incubated at 37 °C for 20 min. Changes in enzyme activity were detected by measuring the absorbance at 405 nm.

$$\text{AG Inhibition\%} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (2)$$

where Asample is the absorbance value of the sample and Acontrol is the absorbance value of the blank.

Inhibition kinetic analysis

Using a half-dilution method, the substrate concentration of pNPG was set at 0.50, 1.00, 2.00, 4.00, and 8.00 mM. The enzyme concentration was plotted on the x-axis, and the reaction rate was plotted on the y-axis to determine if the inhibition was reversible. The reciprocal of the substrate concentration (1/[S]) was plotted on the x-axis, whereas the initial reaction rate (1/V) was plotted on the y-axis to obtain the Lineweaver–Burk double reciprocal plot. This allowed for the determination of the type of inhibition and the calculation of the Michaelis constant (km) and maximum reaction rate (Vmax).

$$\frac{1}{V} = \frac{Km}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

where V is the initial reaction rate and [S] represents the concentrations of vitexin, isovitexin and catechin.

Preparation of complexes and analysis of digestion characteristics

Two grams of mung bean starch was taken from each group, and 0.5% standard product was added. Then, distilled water was added at a solid–liquid ratio of 1:15, and the mixture was stirred until homogeneous (Chou et al., 2020). The mixed solution was placed in a water bath at 25 °C and 80 °C for 30 min and stirred continuously throughout this period. Afterward, the complex was freeze-dried and passed through a 100-mesh sieve for later use. Next, 1 g of the complex was mixed with 1 ml of deionized water, 2 ml of oral digestive juice was added, and the mixture was digested at 37 °C for 1 min. Following digestion, 5 ml of simulated gastric juice was introduced, and the mixture was allowed to react for 30 min. Once the simulated gastric digestion was complete, NaOH was added, and the mixture was stirred evenly. Then, 5 ml of simulated intestinal fluid was added to adjust the pH of the digestive system to 7. The mixture was incubated with shaking at 37 °C at 0, 20, 40, 60, 90, and 120 min, after which 100 μL of the solution

was quickly transferred to a centrifuge tube containing 900 μL of absolute ethanol. For each sample, three parallel measurements were taken, glucose was used as the standard, and a glucose determination kit (GOPOD) was used to measure the glucose concentration in the digestive juice. The digestibility is expressed as the percentage of digested starch content relative to the total starch content at each sampling time point.

$$\mathrm{RDS}(t\%) = 0.9 \times G_{20} \times 100 \quad (4)$$

$$\mathrm{SDS}(\%) = 0.9 \times (G_{120} - G_{20}) \times 100 \quad (5)$$

$$\mathrm{RS}(\%) = (1 - \mathrm{RDS} - \mathrm{SDS}) \times 100 \quad (6)$$

Here, G20 and G120 denote the quantity of glucose generated following 20 and 120 min of starch digestion, respectively. A correction factor of 0.9 is applied when mung bean starch is fully hydrolyzed into glucose.

Synergy analysis

To evaluate the joint effects of vitexin, catechin, and isovitexin, a multidimensional synergy analysis was conducted via SynergyFinder software (https://synergyfinder.fimm.fi/) and the ZIP independence model. This model considers a stochastic process in which two functional factors exert independent effects (Ianevski, 2022). The expected combined effect can be calculated by assessing the probabilities of independent events. This approach facilitates the evaluation of the combined effects of vitexin and catechins, as well as isovitexin, and provides valuable insights into their potential synergistic interactions.

Statistical analysis

The experimental results are expressed as the means and standard deviations of three repeated experiments. Excel and SPSS27 were used for data collection and significance analysis ($P < 0.05$). Origin2019 (OriginLab) and GraphPad Prism 8 (GraphPad Software) were used to process and analyze the original experimental data. The significance level was set at $p < 0.05$.

Results and discussion

Metabolic phenotype analyses

According to the in vitro estimated glycemic index measurement results, 20 groups of mung beans from different varieties were categorized into the HeGI group and the LeGI group for analysis. The in vitro glycemic index was found to vary among the different mung bean varieties, reflecting distinct distributions of active substances. To identify small-molecule metabolites associated with mung beans' eGI values, approximately 558 nonvolatile bioactive compounds were preliminarily identified through metabolomic analysis, including 20 phenolic acids, 45 flavonoids, 38 lipid compounds and other metabolites. It was indicated that the primary nonvolatile bioactive components in mung bean extract are plant secondary metabolites, which have been associated with specific biological activities, such as anti-inflammatory, antioxidant, and antidiabetic effects.

On the basis of the results of the metabolome analysis, principal component analysis (PCA) was initially conducted to gain insights into the overall differences in metabolites between samples across each group, as well as the variability among samples within each group. As shown in Fig. 1A, the first principal component (PC1) was responsible for 25.2% of the total variation, whereas the combined contribution of the first two principal components (PC1 + PC2) accounted for 35.11% of the total variation. Given that all raw mung bean materials were selected, extracted, and tested using identical protocols, observed differences are attributed to varietal variations and should not result in excessive discrepancies. PCA results revealed that although minor inter-group differences were observed in some individual samples, significant overall separation was maintained. Orthogonal partial least squares discriminant analysis (OPLS-DA) was subsequently applied to enhance inter-group separation, minimize intra-group variability, and maximize inter-group differences, thereby facilitating the identification of differential bioactive compounds. Significant differences in active ingredients between groups were demonstrated in Fig. 1B. According to Fig. 1C, R2Y(cum) = 0.994 and Q2(cum) = 0.639, both exceeding 0.5, indicating strong model fitness. Q2 and R2Y P-values were both found to be less than 0.01, confirming that no permutation test-derived random group model outperformed the OPLS-DA model. Figure 1D presents an S-plot showing the covariance (p) and correlation coefficient (p(corr)) between principal component variables and predicted scores, with metabolites closer to the upper right and lower left corners indicating more pronounced differences.

Overall profiles of differentially abundant metabolites in HeGI and LeGI

Variable importance in projection (VIP) scores were utilized as a preliminary screening tool to assess the influence intensity and explanatory capacity of each metabolite component on sample classification and discrimination. This approach was employed to identify differentially abundant metabolites present in various mung bean samples. Generally, a VIP value exceeding 1 is generally considered indicative of substantial differences between groups. The top twenty metabolites

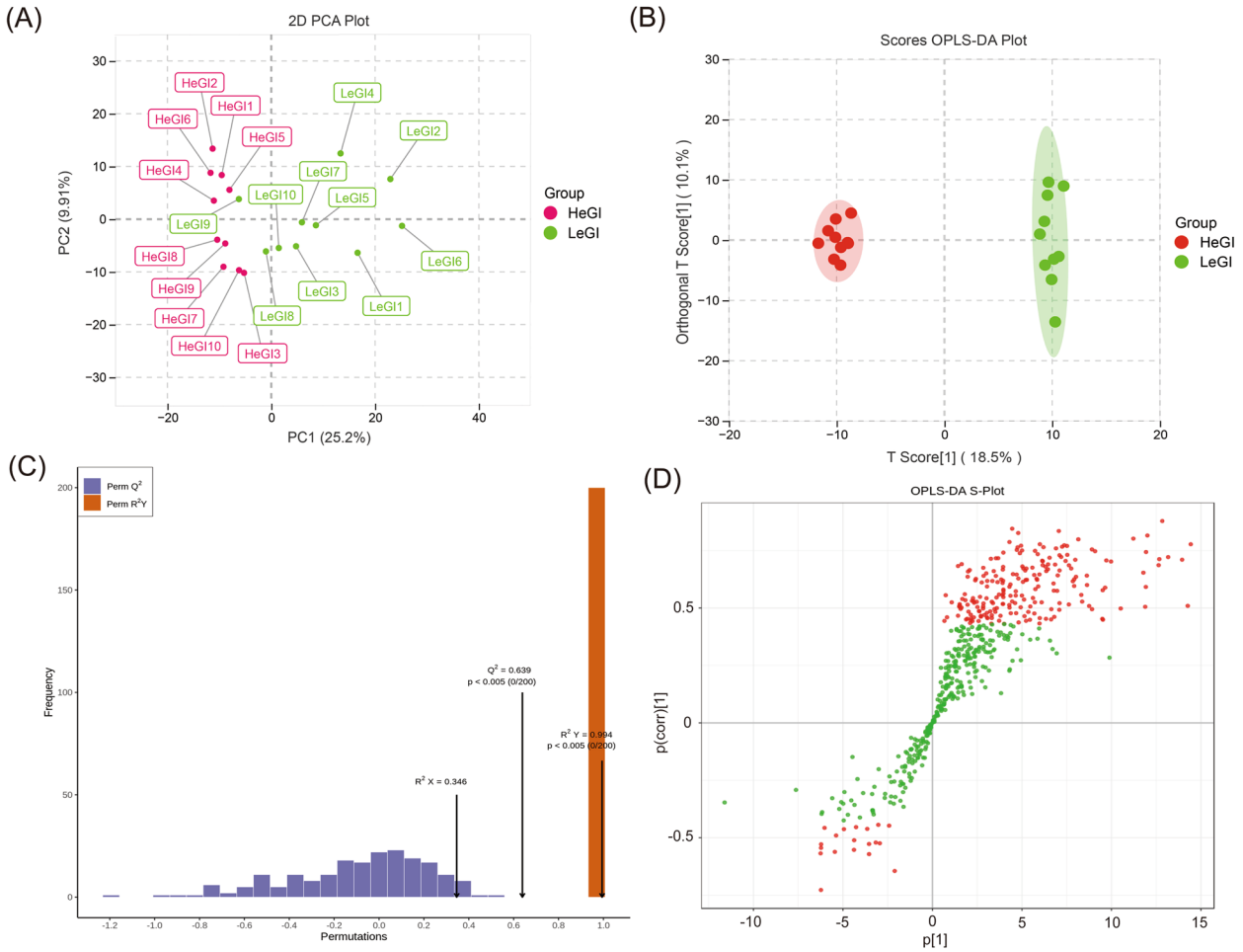


Fig. 1 Multivariate statistics of mung bean metabolites. **A** PCA score plots showing principal component differences; **B** OPLS-DA score plots showing the differentiation of metabolite groups between the high-activity (LeGI) and low-activity (HeGI) groups; **C** Representation of the differentiation effect of 200 randomized permutation combinations; **D** S-plot plots of OPLS-DA showing the differences in metabolites between the HeGI and LeGI groups. HeGI: high-estimated glycemic index; LeGI, low-estimated glycemic index

with VIP values ≥ 1 were identified as follows: 5a,11a-Dehydrooxytetracycline, Peonidin-3-glucoside, Azelaic acid, Amino-7-oxononanoate, Diosmin, Vitexin, Catechin, Apiole, (+)−6a-Hydroxymaackiain, Epicatechin, Isovitexin, Rutin, Naringenin-7-O-beta-D-glucoside, Perakine, Delphinidin 3-rutinoside, Hesperetin, Baicalein, and Xanthurenic acid. To further identify bioactive components in mung beans, components were screened using a variable importance in projection (VIP) threshold of ≥ 1 and a fold change (FC) of ≥ 2 or ≤ 0.5. As demonstrated in Fig. 2, 94 upregulated metabolites and 5 downregulated metabolites were observed in the low-eGI group compared to the high-eGI group. A unit variance scaling procedure was applied to quantify the original relative contents of the screened bioactive components.

Identification and analysis of candidate bioactive components

The estimated glycemic index (eGI) is primarily influenced by two factors: alterations in starch digestion characteristics and the inhibition of starch digestive enzyme activity. Consequently, relevant active ingredients associated with these mechanisms need to be systematically identified and screened. To isolate small molecule metabolites that can significantly reduce the eGI of mung bean effectively, bioactive compounds exhibiting negative correlations with eGI were prioritized for investigation. However, upregulated metabolites across different varieties of mung bean cannot fully represent their biological activities. Thus, integration of multi-study evidence is required. Therefore, integration of multi-study evidence is required for accurate screening of bioactive substances, along with supplementary technologies to evaluate their blood glucose-lowering effects. As illustrated in Table 1,

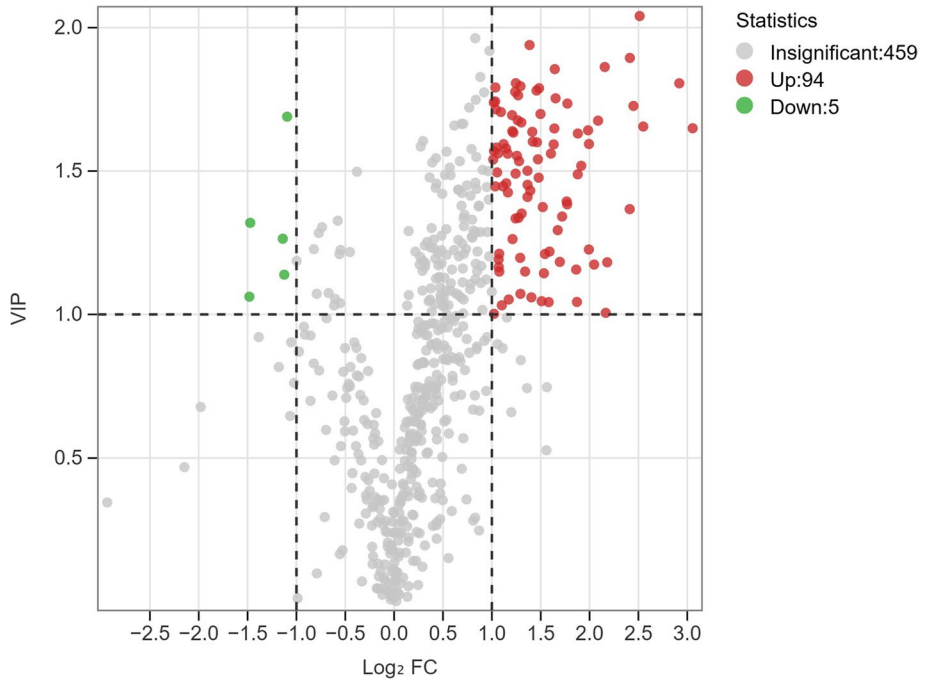


Fig. 2 Volcano plot of mung bean differentially abundant metabolites (based on Variable Importance Projection with fold change).FC:Flod Change VIP:Variable Importance in Projection

Table 1 Top 20 metabolites found in mung bean varieties as evaluated by VIP

NO	Formula	Compounds	VIP	FC	PCCs	*P*value
1	$C_{22}H_{22}N_2O_9$	5a,11a-Dehydrooxytetracycline	2.041	5.707	−0.350**	0.000606
2	$C_{22}H_{23}O_{11}$	Peonidin-3-glucoside	1.939	2.613	−0.369**	0.000172
3	$C_9H_{16}O_4$	Azelaic acid	1.894	5.325	−0.097	0.003670
4	$C_9H_{17}NO_3$	8-Amino-7-oxononanoate	1.863	4.457	−0.081	0.002142
5	$C_{28}H_{32}O_{15}$	Diosmin	1.855	3.125	−0.385**	0.003359
6	$C_{21}H_{20}O_{10}$	Vitexin	1.806	2.370	−0.466**	0.001847
7	$C_{15}H_{14}O_6$	Catechin	1.806	7.559	−0.392**	0.005075
8	$C_{12}H_{14}O_4$	Apiole	1.791	2.050	−0.288**	0.001665
9	$C_{16}H_{12}O_6$	(+)−6a-Hydroxymaackiain	1.789	2.795	−0.375**	0.001010
10	$C_{15}H_{14}O_6$	Epicatechin	1.781	2.746	−0.392**	0.001274
11	$C_{21}H_{20}O_{10}$	Isovitexin	1.776	2.360	−0.408**	0.000353
12	$C_{27}H_{30}O_{16}$	Rutin	1.764	2.409	−0.234*	0.000894
13	$C_{21}H_{22}O_{10}$	Naringenin 7-O-beta-D-glucoside	1.754	3.144	−0.282**	0.006331
14	$C_{21}H_{22}N_2O_3$	Perakine	1.743	2.046	−0.245*	0.002550
15	$C_{27}H_{31}O_{16}$	Delphinidin 3-rutinoside	1.735	3.419	−0.332**	0.003317
16	$C_{16}H_{14}O_6$	Hesperetin	1.727	5.463	−0.342**	0.012960
17	$C_{15}H_{10}O_5$	Baicalein	1.716	2.054	−0.342**	0.010597
18	$C_{10}H_7NO_4$	Xanthurenic acid	1.706	2.132	−0.267**	0.000588
19	$C_{15}H_{10}O_4$	7,4′-Dihydroxyflavone	1.699	2.824	−0.378**	0.003634
20	$C_8H_{14}N_2O_5$	gamma-Glutamylalanine	1.695	2.309	−0.204*	0.000394

FC Flod Change, *VIP* Variable Importance in Projection, *PCCs* Pearson correlation coefficient, *P*value Probability value

* representsa statistically significant difference at the $p < 0.05$ level

** represents a statistically significant difference at the $p < 0.01$ level

the top 20 substances with fold change (FC) > 1 and significant variable importance in projection (VIP) values were identified. Among these, 18 substances showed significant negative correlations with eGI, with 12 belonging to polyphenols and flavonoids. Based on upregulation ratios, flavonoids including vitexin, catechin, epicatechin, isovitexin, rutin, naringenin-7-O-beta-D-glucoside, delphinidin-3-rutinoside, hesperetin, and baicalein-7, 4'-dihydroxyflavone were significantly upregulated ($p < 0.05$), among which vitexin, catechin, and isovitexin exhibited the strongest correlations. Vitexin and isovitexin have been recognized as the primary active compounds in mung bean that inhibit starch-digesting enzymes (Hou et al., 2019). It has been demonstrated that vitexin extracted from Moso bamboo leaves can effectively delay starch digestion (Yang et al., 2014). Both vitexin and isovitexin have been shown to significantly inhibit α-glucosidase activity at physiologically relevant doses without observable toxicity. Additionally, potent antioxidant properties have been reported in cellular experiments, suggesting their potential as ideal antiglycation agents. Isovitexin has also been found to stimulate insulin secretion, contributing to its hypoglycemic effects (Folador et al., 2010). Catechin, which is known to regulate glucose metabolism and reduce postprandial blood glucose levels (Hana et al., 2018), was therefore selected for further validation based on experimental data and literature evidence.

Inhibition of eGI and starch digestive enzymes by metabolites

Following the screening of the most prominent differentially abundant metabolites,solutions of each metabolite (1 mM concentration) were prepared and introduced into the in vitro digestion system to assess their eGI-modulating effects. As shown in Fig. 3A, vitexin, isovitexin, and catechin were observed to reduce mung bean eGI by 26.23%, 32.59%, and 16.25%, respectively. The direct introduction of metabolites into the system was designed to minimize interactions between metabolites and mung bean starch, thereby isolating their enzyme-inhibitory effects on eGI regulation. As illustrated in Fig. 3B-D, all three metabolites exhibited varying degrees of in vitro inhibitory activity against AG, with a clear dose-dependent effect. The IC_{50} value of vitexin against α-glucosidase was 50.11 μM, while the IC_{50} value for catechin was 42.06 μM, and that for isovitexin is 116.14 μM. Compared with the results of previous studies, the inhibitory activity observed in this study was notably more pronounced. However, the comparative trends among the three studies remain similar, which may be attributed to variations in experimental conditions and differences in the accuracy of the testing instruments (Ni et al., 2020) Structural diversity among polyphenolic flavonoids has been shown to influence starch-digestive enzyme binding specificity and inhibition efficacy (Wang et al., 2024). A comparison of these results indicates that catechin has stronger inhibitory effects on α-glucosidase than both vitexin and isovitexin do. In this study, the three metabolites may exhibit varying enzyme inhibitory activities by binding to key amino acid residues in AG that are capable of catalyzing the substrate (pNPG) (Şöhretoğlu et al., 2017). From the perspective of molecular configuration, catechin was identified as the most potent AG inhibitor in this study, which may be explained by its flavanol structure. The characteristic 2-phenylchroman moiety of catechins has been proposed to interact with AG's active site through hydrogen bonding, van der Waals forces, and π-alkyl interactions, potentiating its inhibitory effects (Tiji et al., 2021).Vitexin and isovitexin are C-glycoside isomers of apigenin and are distinguished by their differing glycosylation positions. Specifically, vitexin is glycosylated at the C8 position, whereas isovitexin is glycosylated at the C6 position. These variations result in distinct steric hindrance effects. For example, the aromatic ring structure of vitexin may facilitate π–π stacking interactions with the enzyme's active site (Wang, et al., 2024), a capability that is lacking inovitexin. Additionally, the position of the glycosylation can influence the intermolecular forces between the metabolite and the enzyme, including hydrogen bonds and van der Waals forces. Consequently, these differences contribute to the divergent biological activities and pharmacological effects of vitexin and isovitexin. Hydroxylation at the C-3' position of the B-ring in apigenin, vitexin, and isovitexin enhances α-glucosidase inhibitory activity (Li et al., 2009a). However, B-ring C-3' hydroxylation, a shared feature among these compounds, has been demonstrated to enhance α-glucosidase inhibition (Li et al., 2009b).

Effects of metabolites on the digestive properties of mung bean starch

The mechanisms underlying natural metabolite-starch interactions and their functional modulations have been extensively investigated in recent years. It has been established that specific plant secondary metabolites can significantly alter starch's physicochemical properties through molecular interactions. For instance, flavonoids such as quercetin and catechin have been shown to form amylose complexes via hydrogen bonding and hydrophobic interactions, resulting in reduced in vitro starch digestibility and crystallinity (Beta & Corke, 2004; L. Zhang et al., 2011). Similarly, starch-thymol encapsulation systems have been demonstrated to prolong active compound release (Pozo-Bayon et al., 2008), while proanthocyanidin-starch complexes enhance molecular

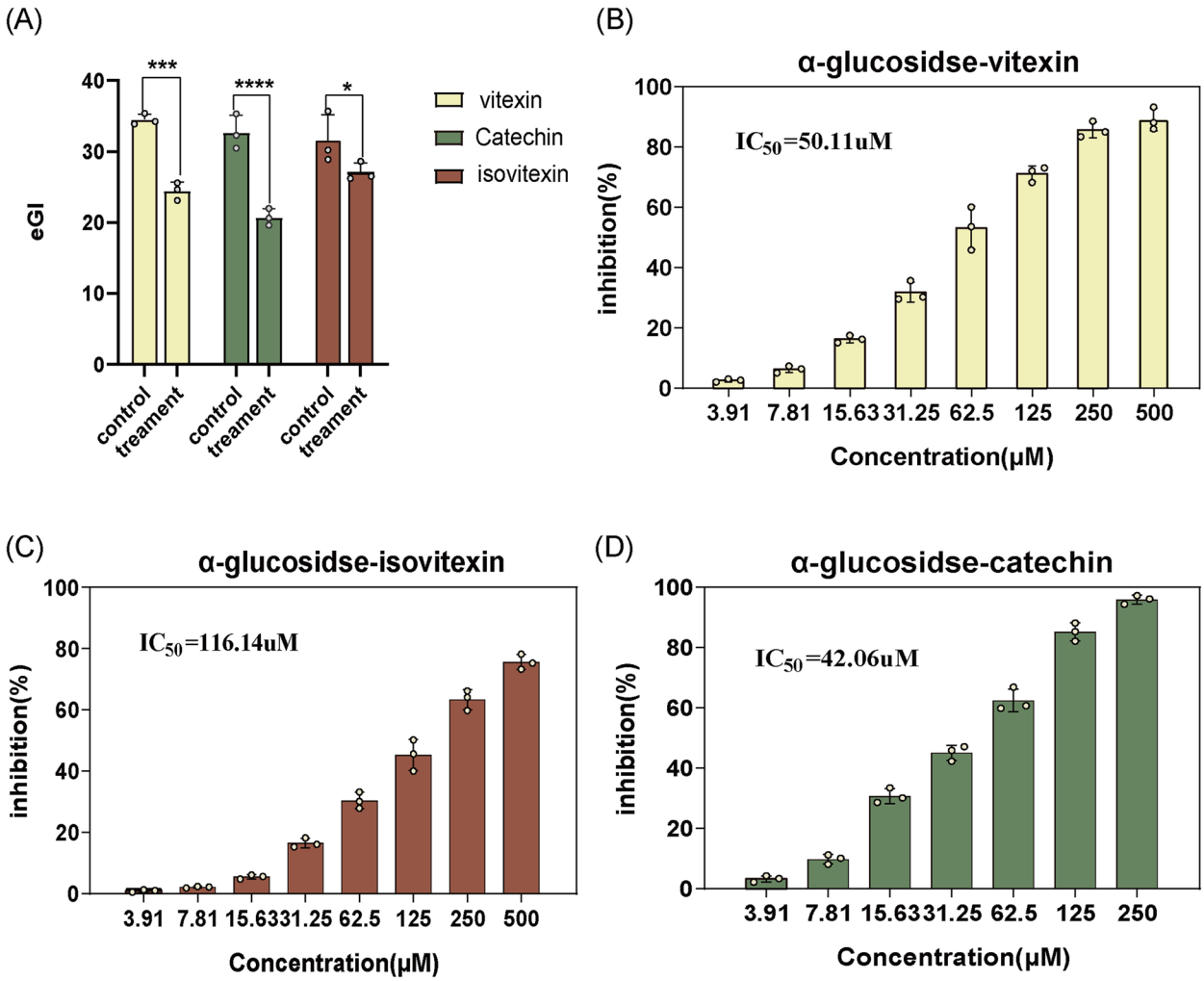


Fig. 3 Effects of different metabolites on eGI and inhibitory effects on a-glucosidase. **A** eGI assay results of metabolites added to digestive solution. **B** Inhibitory effects of vitexin on α-glucosidase. **C** Inhibitory effects of isovitexin on α-glucosidase. **D** Inhibitory effects of catechin on α-glucosidase. Data are presented as the means ± SEMs.*represents a statistically significant difference at the $p < 0.05$ level,*** reprents a statistically significant difference at the $p < 0.001$ level, **** reprents a statistically significant difference at the $p < 0.0001$ level

crosslinking density, concurrently lowering digestion rates and improving thermal stability (Althawab et al., 2023). Polyphenol-starch complexes have been particularly recognized for their efficiency in postprandial glycemic regulation (Chai et al., 2013). As evidenced in Figs. 4A and B, the digestion kinetics of mung bean starch and its complexes were characterized by an initial rapid hydrolysis rate followed by stabilization after 60 min. Notably, the introduction of metabolites led to a decrease in the final hydrolysis rate. Comparative analysis revealed that catechin had a more pronounced effect on reducing the starch hydrolysis rate than did vitexin, followed by isovitexin, which aligns with previous experimental findings regarding α-glucosidase activity. Additionally, under certain high-temperature conditions, metabolites can effectively attenuate the rate of starch hydrolysis. As shown in Table 2, under heat treatment without the addition of metabolites, the rapidly digestible starch content of mung bean starch decreased slightly by 4%, whereas the resistant starch content increased by 3.69%. The incorporation of various metabolites increased the contents of slow digestible starch (SDS) and resistant starch (RS) in mung bean starch at both room and high temperatures, especially after gelatinization, and the effects of these three metabolites on reducing the hydrolysis rate of starch became more obvious. Under pretreatment conditions at room temperature and 80 °C, the content of RS increased by 5.48% and 8.85% with the addition of vitexin, 2.57% and 5.02% with the addition of isovitexin, and 7.4% and 13.21% with the addition of catechin. This

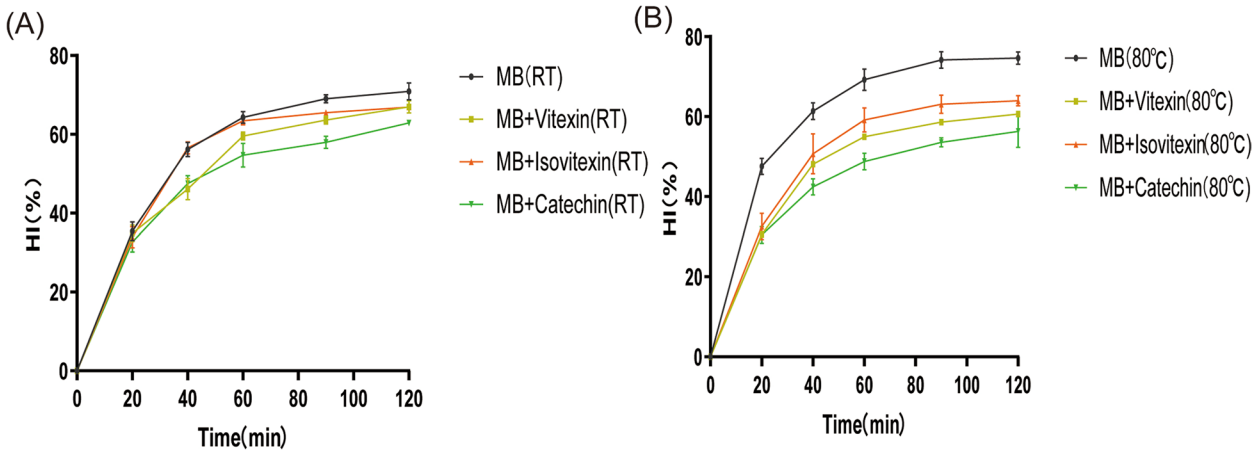


Fig. 4 Hydrolysis profile of mung bean starch. **A** Hydrolysis profiles of the mung bean starch + metabolites at room temperature. **B** Hydrolysis profile of the mung bean starch + metabolites at 80 °C. The data are shown as the means ± SEMs

study demonstrated that under specific conditions, these metabolites can interact with starch molecules to form more stable complexes, which also induce structural changes in starch granules. At lower reaction temperatures, the interaction between starch and metabolites is relatively limited, primarily involving physical adsorption and some weak interactions. However, at elevated temperatures, mung bean starch undergoes a degree of gelatinization, leading to the absorption of water by starch granules, which swell and rupture, thereby releasing starch molecules. This process enhances the contact between starch and metabolites, resulting in stronger physical adsorption. The interaction occurs mainly by forming covalent bonds and hydrophobic interactions that encapsulate the metabolites within the helical lumen of starch and attach it to the surface of starch chains. The starch molecules within this complex become more compact and resistant to hydrolysis by digestive enzymes.

Table 2 Individual digested fragments of mung bean starch and complexes

Sample		RDS (%)	SDS (%)	RS (%)
MBS	25 °C	36.27 ± 1.36	33.972 ± 0.71	30.52 ± 1.66
	80 °C	32.59 ± 1.24	34.72 ± 0.93	33.69 ± 0.93
MBS + Vitexin	25 °C	33.56 ± 1.37	31.44 ± 0.88	36.00 ± 1.08
	80 °C	30.59 ± 1.45	30.04 ± 0.61	39.37 ± 1.45
MBS + Isovitexin	25 °C	33.04 ± 0.97	33.87 ± 0.57	33.09 ± 1.67
	80 °C	31.22 ± 1.42	33.23 ± 0.87	35.54 ± 1.43
MBS + Catethin	25 °C	33.04 ± 1.07	29.84 ± 0.69	37.12 ± 1.22
	80 °C	24.09 ± 1.39	32.18 ± 0.78	43.73 ± 1.12

The experiment was repeated three times, and the results are presented as mean ± standard deviation

RDS rapid digestible starch, *SDS* slow digestible starch, *RS* resistant starch

Concurrently, the formation of enzyme inhibitor-starch complexes enables sustained release within the intestinal tract, enhancing absorption efficiency and bioavailability. This gradual release mechanism prolongs the inhibitory activity against starch-digestive enzymes (α-amylase and α-glucosidase), effectively decelerating starch hydrolysis and subsequently attenuating postprandial hyperglycemia (Takahama & Hirota, 2018).

Inhibitory types of metabolites

Naturally derived plant polyphenols have been widely recognized for their potent inhibitory effects on digestive enzymes, coupled with low toxicity profiles (Carlos-Amaya et al., 2011). The binding mechanisms of flavonoids to enzymatic targets have been extensively documented, demonstrating diverse interaction modalities (Zhu et al., 2020). Specifically, α-amylase and α-glucosidase inhibition has been observed in natural flavonoids such as apigenin, myricetin, and quercetin, mediated through distinct competitive mechanisms (Sun & Miao, 2019; Zeng et al., 2016). To elucidate the reasons for the differences in the α-glucosidase inhibitory activities of vitexin, isovitexin, and catechin, the specific values of the kinetic parameters are presented in Table 3. Discrepancies in numerical outcomes compared to prior studies were attributed to methodological variations in error tolerance and parameterization, though consistent trends were maintained (Ni et al., 2020). Lineweaver–Burk plots were generated to clarify the type and mechanism of α-glucosidase inhibition. Figure 5A presents the Lineweaver–Burk plot after the addition of different concentrations of isovitexin. In this case, Vmax remains constant while the km value increases, suggesting that isovitexin inhibits α-glucosidase activity

Table 3 Kinetic parameters of α-glucosidase and α-amylase inhibition

Sample	Concentration(μM)	1/km(μM)	1/Vmax(μM/min)	Inhibition Type
Vitexin	0	7.147 ± 0.0812	2.592	Uncompetitive
	31.35	8.343 ± 0.0698	3.033	
	62.5	9.787 ± 0.031	3.527	
	125	15.03 ± 0.062	5.515	
Isovitexin	0	9.843 ± 0.135	1.377	Competitive
	15.625	10.06 ± 0.095	1.130	
	31.35	9.878 ± 0.133	0.8285	
	15.625	10.77 ± 0.371	0.6820	
Catethin	0	9.631 ± 0.336	0.2586	Noncompetitive
	15.625	12.91 ± 0.338	0.2818	
	31.35	20.25 ± 0.456	0.2749	
	62.5	33.15 ± 0.347	0.2675	

The experiment was repeated three times, and the results are presented as mean ± standard deviation

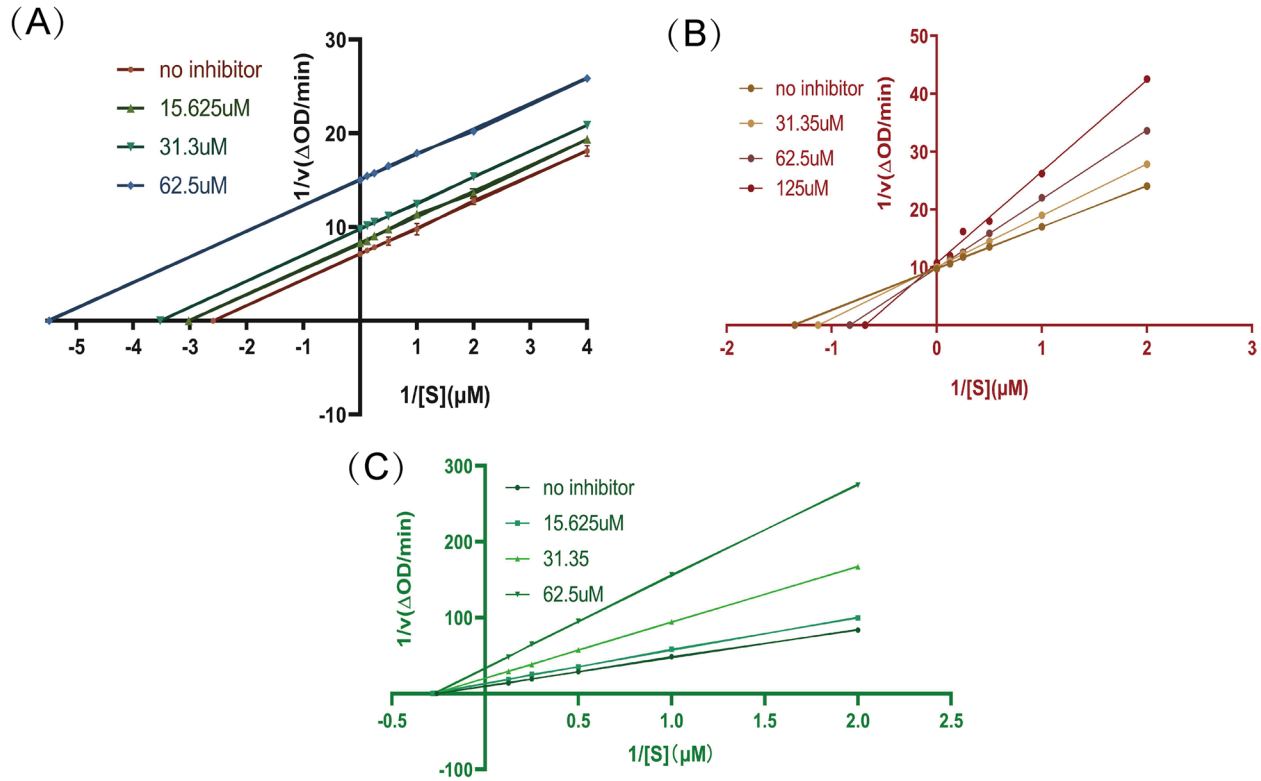


Fig. 5 Kinetics of the inhibitory effects of different metabolites on α-glucosidase: **A** ilhibition kinetics of α-glucosidase and Vitexin; **B** Inhibition kinetics of α-glucosidase and Isovitexin; **C** Inhibition kinetics of α-glucosidase and Catechin

through competitive inhibition, directly competing with the substrate for the enzyme's binding site and preventing the substrate from being catalyzed. Figure 5B shows the Lineweaver–Burk plot following the addition of various concentrations of vitexin, where the four lines are nearly parallel. As the concentration increased, both Vmax and km decreased simultaneously, indicating that vitexin exerts an uncompetitive inhibitory effect on α-glucosidase by binding to the enzyme–substrate complex and thereby inhibiting the enzyme's catalytic activity. Figure 5C shows the Lineweaver–Burk plot following the addition of various concentrations of catechins, where the km value remains unchanged while Vmax decreases. This observation indicates that catechin

inhibits α-glucosidase activity in a noncompetitive manner, binding to both the enzyme and the enzyme–substrate complex, thus inhibiting enzyme activity. These different inhibition mechanisms suggest that the binding sites and inhibition methods of these three compounds exert distinct effects on α-glucosidase activity. A synergistic inhibitory effect may therefore be postulated through combined application of these compounds.

Interaction analysis of vitexin, isovitexin and catechin

The therapeutic synergy achieved by combining pharmacologically distinct agents targeting a unified disease pathway enhances treatment efficacy. This combinatorial approach demonstrates three principal advantages: (1) augmented therapeutic outcomes through complementary mechanisms, (2) reduced dosing requirements, and (3) delayed drug resistance development. Such synergistic strategies have been systematically integrated into contemporary diabetes management protocols (Chou, 2006). Different enzyme inhibitors can produce specific synergistic or antagonistic effects. The combined use of certain inhibitors with α-glucosidase inhibitory activity can regulate blood sugar through multiple mechanisms, demonstrating a notable synergistic effect on hypoglycemic function. The occurrence of a synergistic effect may also be the superposition of the effects of action sites between the combined substances, thus enhancing the composition in a certain way. In terms of biological efficacy, the action sites of these substances can be diverse or the same. Relevant studies indicate that flavonoids alone can reduce blood sugar; however, their efficacy is significantly lower than that of flavonoid crude extract complexes. Clinical trials have demonstrated that flavonoid compounds in grape juice extract, including proanthocyanidins and quercetin derivatives, exhibit dual therapeutic efficacy through α-glucosidase inhibition (resulting in significant anti-diabetic activity) and PPAR-α activation (mediating potent anti-hyperlipidemic effects) (Cao et al., 2017). The inhibitory effect of the extract of colored grains on the activity of α-glucosidase in the intestinal tract of mice was attributed to the synergistic effect between the phenols (Parizad et al., 2019). Additionally, studies have shown that the combination of puerarin and pumpkin polysaccharides can significantly lower blood sugar levels in diabetic mice and effectively regulate their oxygenation balance (Chen et al., 2019). Research has revealed a synergistic effect between vitexin and acarbose on the inhibition of α-glucosidase activity (Ni et al., 2020), while the combination of catechin and acarbose results in the antagonistic inhibition of α-glucosidase (B. W. Zhang et al., 2017).

The combined use of different types of enzyme inhibitors affects the catalytic efficiency of the enzyme, resulting in mixed competitive inhibition. Given that the binding sites for competitive and non-competitive inhibition differ, the synergistic effect warrants further investigation. To assess the combined effects of vitexin, isovitexin, and catechins, which were screened and identified from mung beans, we evaluated their inhibitory effects on α-glucosidase activity at three different concentrations. The inhibitor concentrations of vitexin (Drug1) required to achieve enzyme inhibitory activities of 30%, 50%, and 70% were 12.59 μg/ml, 22.18 μg/ml, and 49.39 μg/ml, respectively. For isovitexin (Drug 2), the concentrations needed to reach 30%, 50%, and 70% inhibitory activities were 27.82 μg/ml, 50.21 μg/ml, and 153.21 μg/ml, respectively. Similarly, the inhibitor concentrations for catechin (Drug3) corresponding to 30%, 50%, and 70% enzyme inhibitory activities were 5.04 μg/ml, 12.21 μg/ml, and 21.87 μg/ml, respectively. The synergistic inhibition results were analyzed via SynergyFinder, which calculates the Bliss synergistic score of the combination, thereby illustrating the relationship between the inhibition rates of the three metabolites. The dose–response 3D diagram is presented in Fig. 6A, and the scoring results are shown in Fig. 6B. These results indicate that each pair of metabolites has a certain level of synergistic efficiency. However, the scores for the three metabolites that act together are not as high as those observed for the individual pairs. The increase in the inhibition rate was not particularly pronounced. Notably, the highest scores were attributed to the combination of vitexin and catechin. This synergy may arise from their distinct inhibitory mechanisms, which engage different active sites of alpha-glucosidase simultaneously. In summary, combinations of these three metabolites, whether in pairs or collectively, can effectively and synergistically inhibit AG activity, thereby slowing the digestion of starch. The highest inhibitory efficiency was achieved at concentrations of 12.59 μg/ml for vitexin, 27.82 μg/ml for isovitexin, and 5.04 μg/ml for catechin, resulting in a zip score of 14.302. Notably, bioavailability is intrinsically linked to the hypoglycemic potential of metabolites. Accumulating evidence indicates that co-ingestion of different flavonoids compounds may enhance their bioavailability (Kamiloglu et al., 2021). Substantial evidence demonstrates that co-administration of flavones (e.g., apigenin) with flavonols (e.g., quercetin) significantly enhances bioavailability through glycoside-aglycone interactions. Building on this established mechanism, the structural configuration of vitexin (apigenin-8-C-glucosides) and isovitexin (apigenin-6-C-glucosides) in conjunction with catechin (a flavonol derivative) in our study suggests potential bioavailability potentiation via analogous glycoside-flavonol synergy (Ravisankar et al., 2019). Critically, in vivo hypoglycemic evaluation fundamentally differs from in vitro

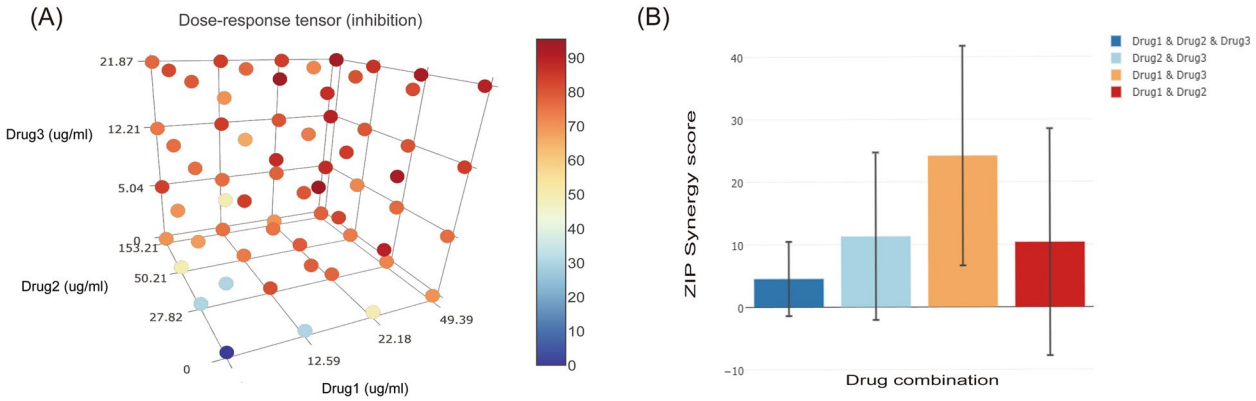


Fig. 6 Drug–drug interaction analysis of data obtained from the α-glucosidase inhibition assay via SynergyFinder software. **A** Inhibitory effects of different concentrations of vitexin, isovitexin, and catechin on α-glucosidase; **B** ZIP score graph. Drug1:vitexin Drug2:isovitexin Drug3:Catechin

digestion studies by requiring comprehensive assessment of both enzymatic inhibition and complementary bioactivities (e.g., antioxidant and anti-inflammatory effects). Building upon formulation strategies developed for classical polyphenols including catechin, epicatechin, and rutin, the three natural metabolites identified in this study exhibit dual synergistic potentiality: enhanced glycemic control through improved enzyme inhibition efficacy and bioavailability, and multi-pathway regulation via potent redox modulation and inflammatory suppression. This dual-action mechanism provides critical insights for developing next-generation antidiabetic formulations (Hamza et al., 2021).

Conclusion

The results of this study indicate significant differences in the in vitro estimated glycemic indices among different varieties of mung beans, as well as variations in the distributions of their active substances. Using a nontargeted metabolomics approach based on LC–MS, we comprehensively detected bioactive compounds in various mung bean varieties and revealed differences between the low-eGI and high-eGI groups through multivariate statistical analysis, identifying a total of 94 upregulated bioactive compounds. Then, by setting the screening conditions of VIP, FC and significant correlation coefficients, we screened out vitexin, isovitexin and catechin, which are significantly related to the eGI.

The experimental results revealed that these three metabolites can inhibit the activity of glucosidase and have a certain synergistic effect. Under certain conditions, they can combine with mung bean starch to form a complex, increase the SDS and RS contents, and alleviate carbohydrate digestion. These findings suggest that vitexin, isovitexin, and catechin could serve as natural additives in low-GI functional foods. Future clinical trials should validate their bioavailability and long-term efficacy in human subjects. Additionally, incorporating these compounds into staple foods (e.g., mung bean noodles or fortified bread) may offer practical dietary solutions for glycemic control. In conclusion, we validated their eGI-lowering effects through in vitro digestion experiments and explored the underlying mechanism, which provided a theoretical basis for developing low-GI dietary products related to glycemic regulation.

Abbreviations

T2DM	Type 2 diabetes mellitus
MB	Mung bean
AG	Alpha-glucosidase
eGI	Estimated glycemic index
HeGI	High-estimated glycemic index
LeGI	Low-estimated glycemic index
RS	Resistant starch
SDS	Slow digestible starch
RDS	Rapid digestible starch
MBS	Mung bean starch
LC–MS	Liquid chromatography and mass spectrometry
pNPG	P-nitrophenyl-α-D-glucopyranoside
PCA	Principal component analysis
OPLS-DA	Orthogonal partial least squares discriminant analysis
MBS	Mungbean stearch
FC	Flod Change
VIP	Variable Importance in Projection
PCCs	Pearson correlation coefficient
*P*value	Probability value
MBS	Mung bean starch

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Authors' contributions

Renzhe LV and Jinyang Liu are co-first authors. R. L., X.C., X.Y., L.W., and Y.L. formulated the research goals and aims; R.L., and J.L., completed the data curation and formal analysis; R.L. performed the research; S.L. and D.G. assisted with the experiments; R.L. wrote the original draft; J.L., and Y.L. revised the original draft.

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Data availability
Data will be available upon request.

Declarations

Ethics approval and consent to participate
There are no human subjects and experimental animal involved in this study.

Consent for publication
All authors agreed to submit this manuscript to this journal for publication.

Competing interests
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.

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