SCREENING OF α-GLUCOSIDASE INHIBITORS FROM BLACK SOYBEAN EXTRACT AND STUDY ON THEIR INHIBITORY MECHANISMS

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by

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LIST OF SYMBOLS

K_i	The inhibition constant
K_a	The binding constant
K_{sv}	The fluorescence quenching constant
K_q	The rate constant for biomolecule quenching
n	The number of binding sites
$\Delta H°$	Changes in enthalpy
$\Delta G°$	Changes in free energy
$\Delta S°$	Changes in entropy
R_g	The radius of gyration

LIST OF ABBREVIATIONS

BSSPE	Black soybean seed power extract
MAE	microwave-assisted extraction
UAE	Ultrasonic-assisted extraction
SFE	Supercritical fluid extraction
UV	Ultraviolet Radiation
TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography
LDL	Low-Density Lipoprotein
DPPH	1,1-Diphenyl-2-picrylhydrazyl
MD	Molecular dynamic
pNPG	4-nitrophenyl-alpha-D-glucopyranoside
RMSD	The root means square deviation
RMSF	Root mean square fluctuation

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PENYARINGAN PERENCAT α-GLUKOSIDASE DARIPADA EKSTRAK KACANG SOYA HITAM DAN KAJIAN TERHADAP MEKANISMA PERENCATANNYA

ABSTRAK

Pelbagai kajian telah mendokumentasikan kesan baik ekstrak kacang soya hitam terhadap diabetes. Walau bagaimanapun, sebatian aktif yang terlibat dalam kesan ini masih belum diketahui. Kajian ini bertujuan untuk mengenal pasti perencat α-glukosidase yang terdapat dalam kacang soya hitam dengan menggunakan penurasan ultra-afiniti. Tujuh isoflavon dengan aktiviti perencatan α-glukosidase telah dikenalpasti, antaranya daidzein dan genistein menunjukkan aktiviti perencatan yang jauh lebih unggul berbanding dengan acarbose (IC_{50} = 632.5 ± 70.0 μmol/mL), dengan nilai IC_{50} masing-masing adalah 15.7 ± 0.3 dan 3.2 ± 1.2 μmol/mL. Analisis hubungan struktur-aktiviti menunjukkan korelasi negatif antara darjah glikosilasi isoflavon dan aktiviti perencatan α-glukosidase. Kajian kinetik enzim menunjukkan bahawa kedua-dua daidzein dan genistein bertindak sebagai perencat tak bersaing terhadap α-glukosidase. Tambahan pula, sebatian ini secara berkesan menindas pendafluor intrinsik α-glukosidase melalui mekanisme pemadam statik. Interaksi antara daidzein atau genistein dan α-glukosidase melibatkan pembentukan kompleks secara spontan, yang didorong oleh interaksi hidrofobik dan ikatan hidrogen. Penemuan ini memberi pandangan berharga mengenai mekanisme molekul yang melingkupi aktiviti perencatan α-glukosidase isoflavon kacang soya hitam.

SCREENING OF α-GLUCOSIDASE INHIBITORS FROM BLACK SOYBEAN EXTRACT AND STUDY ON THEIR INHIBITORY MECHANISMS

ABSTRACT

Various studies have documented the beneficial effects of black soybean extract against diabetes. However, the specific active compounds responsible for these effects remain elusive. This study aimed at identifying the α-glucosidase inhibitors present in black soybeans using affinity ultrafiltration. Seven isoflavones with α-glucosidase inhibitory activity were identified, among which daidzein and genistein demonstrated remarkably superior inhibitory activity compared to that of acarbose (IC_{50} = 632.5 ± 70.0 μmol/mL), exhibiting IC_{50} values of 15.7 ± 0.3 and 3.2 ± 1.2 μmol/mL, respectively. An analysis of the structure-activity relationship revealed a negative correlation between the degree of glycosylation of isoflavone and α-glucosidase inhibitory activity. Enzyme kinetic studies revealed that both daidzein and genistein acted as uncompetitive inhibitors of α-glucosidase. Additionally, these compounds effectively suppressed the intrinsic fluorescence of α-glucosidase through the static quenching mechanism. The interaction between daidzein or genistein and α-glucosidase involves spontaneous formation of a complex, which is driven by hydrophobic interactions and hydrogen bonds. These findings provide valuable insights into the molecular mechanisms underlying the α-glucosidase inhibitory activity of black soybean isoflavones.

CHAPTER 1

INTRODUCTION

1.1 Background

Soybean (*Glycine max* (L.) Merrill) is a widely recognized and highly nutritious legume that serves as a significant source of food and nutritional supplements worldwide. It possesses a diverse range of valuable components, including abundant proteins, unsaturated fatty acids, soluble sugars, vitamins, minerals, fiber, polyphenols, flavonoids, and other bioactive compounds, that contribute to human health (Azam et al., 2021; Lokuruka, 2010; Malenčić et al., 2012; Yang et al., 2022). Within the soybean family, seeds can be found in various colors such as yellow, brown, green, and black (Song et al., 2016). Among them, black soybeans have higher average contents of total protein and phenols than green, brown and yellow soybeans (Desta et al., 2022; Kumar et al., 2010; Xu & Chang, 2008). Importantly, black soybeans have higher levels of total isoflavones and flavonoids than yellow soybeans, with significant variations observed in the latter (Kumar et al., 2010; Slavin et al., 2009). Consequently, black soybeans display significantly stronger ferric reducing antioxidant power, DPPH radical-scavenging ability, and oxygen radical absorbance capacity than yellow soybeans (Kumar et al., 2010; Xu & Chang, 2008).

Numerous in vitro experiments have demonstrated the antibacterial, anti-inflammatory, and antioxidant properties of the polyphenols found in black soybeans (Anjum et al., 2022; Dorjsembe et al., 2022; Xu et al., 2017). Human trials and animal studies have also confirmed the effectiveness of black soybean polyphenols in improving blood pressure, reducing blood lipids, preventing non-alcoholic fatty liver disease, and enhancing vascular function (Dobhal & Raghuvanshi, 2020; Yamamoto

et al., 2022; Yamashita et al., 2020). The antidiabetic effects of black soybeans are remarkable. Gina et al. (2014) discovered that black soybean polyphenol extract reduced fasting blood glucose levels in mice induced by streptozotocin. Additionally, Dobhal & Raghuvanshi (2020) demonstrated the hypoglycemic effect of black soybean powder supplementation on fasting blood glucose levels in postmenopausal women.

Plant extracts have been proven to be effective in reducing blood glucose levels through various mechanisms, including activation of insulin receptors, regulation of liver glucose release, stimulation of insulin secretion, and inhibition of carbohydrate digestion and glucose absorption (Hanhineva et al., 2010). One significant class of hypoglycemic drugs that could lower blood sugar levels is α-glucosidase inhibitors, which hinder the breakdown of linear or branched oligosaccharides into glucose (Priscilla et al., 2014). Currently, acarbose and biguanides are the commonly used α-glucosidase inhibitors (Matsui et al., 2001). However, these medications often lead to unwanted side effects such as flatulence and diarrhea. Consequently, numerous studies have been devoted to the development of natural plant products with fewer adverse reactions (He et al., 2019). Among the various plant extracts, black soybean extracts display α-glucosidase inhibitory activity. Chen et al. (2021) investigated the effects of crude phenolic extracts from black soybeans on α-glucosidase inhibition and observed the effective suppression of enzyme activity. Tan et al. (2017) further divided the black soybean extract into five fractions (I–V) via column fractionation. Among these fractions, III–V exhibited superior α-glucosidase inhibitory effects compared to the crude extract. Although it has been established that black soybean polyphenols possess α-glucosidase inhibitory

effects, the specific active compounds responsible for these effects remain unidentified.

Traditional methods in phytochemistry play a critical role in the exploration and structural characterization of α-glucosidase inhibitors derived from plants. Tang et al. (2020) successfully isolated and identified six phenolic compounds from *Moringa oleifera* seeds with inhibitory activity against α-glucosidase. Özgünseven (2021) used column chromatography to isolate three phenolic compounds from *Potentilla speciosa* var. *specificosa*, which showed their inhibitory effects on α-glucosidase activity. In another study, Ma et al. (2015) identified 21 compounds from the flowers of *Edgeworthia gardneri*; 14 of these were found to exhibit α-glucosidase inhibitory properties through in vitro enzyme inhibition assays. However, this strategy of isolating and evaluating individual compounds for α-glucosidase inhibition is time-consuming and may overlook bioactive compounds of traces amount (Wu et al., 2016). As an alternative, affinity ultrafiltration has emerged as a valuable method for screening bioactive molecules from complex matrices and has been applied in the search for enzyme inhibitors (Wei et al., 2016). Affinity ultrafiltration exploits specific binding between a ligand and receptor to efficiently screen active small-molecule compounds using an ultrafiltration device. This approach offers advantages such as simplicity, cost-effectiveness, and rapid identification (Huang et al., 2023). It has been successfully employed to screen inhibitors of enzymes such as lipase and xanthine oxidase (Liu et al., 2016; Tao et al., 2015).

1.2 Problem statements

Based on iterative extraction and isolation, the conventional bioassay-guided fractionated extraction method for screening inhibitors assessed the effectiveness of components at each extraction and isolation stage through in vitro efficacy evaluations. This process tracked active components until isolating the active monomeric compounds. This conventional approach is characterized by prolonged durations, substantial workloads, lack of precision, and repetitiveness.

Past studies have reported that black soybean crude extract has α-glucosidase inhibitory activity, but the specific bioactive components with α-glucosidase inhibitory activity and their mechanism are still unclear

1.3 Objectives

i. To screen α-glucosidase inhibitors in black soybeans by affinity ultrafiltration.

ii. To elucidate the inhibitory mechanism of black soybean derived inhibitors on α-glucosidase by enzyme kinetic studies, fluorescence quenching and molecular simulation.

1.4 Hypothesis

1.4.1 Hypothesis 1

Affinity ultrafiltration is a screening method based on the specific binding between receptors and ligands, which can rapidly screen enzyme inhibitors from complex plant compound matrices, and there have been many reports on the screening of different enzyme inhibitors from various plant materials. This method can help us to rapidly screen black soybean derived α-glucosidase inhibitors.

1.4.2 Hypothesis 2

Enzyme kinetic studies and fluorescence quenching have been widely used to explore the interaction modes between different molecules, which can help us explore the inhibition mode of black soybean α-glucosidase inhibitors.

1.4.3 Hypothesis 3

Molecular simulation is a computer simulation technology that has been widely used in drug screening, verification of intermolecular interaction force types, etc. This technology can help to explain the mode of action between black soybean derived inhibitors and α-glucosidase.

1.5 Research Protocol

This study focuses on elucidating phenolic compounds in black soybean, screening for compounds with α-glucosidase inhibitory effects and delving into their inhibitory mechanisms.

The key steps of this study include two parts, screening of inhibitors and exploration of their inhibitory mechanism.

The first part focused on extraction of polyphenols from black soybean, wherein ethanol solution was employed as the extraction reagent, with ultrasound used as an auxiliary method for extracting polyphenols from black soybean.

Next, screening was done to identify compounds with α-glucosidase inhibitory properties by affinity ultrafiltration, which involves protein and ligand interaction to form a complex that is bigger in size, and thus cannot pass through a membrane of a specific small pore size. And High-performance liquid chromatography technique is combined to detect target inhibitors.

The project proceeded with LC-MS identification of those screened inhibitors, followed by in vitro activity verification. This analysis was conducted by comparing the mass spectrometry data of the screened compounds with reference standards and information available in the literature.

The second part was continued with commercial pure standard of the identified compounds with α-glucosidase inhibitory properties. In this part of the study, the known compounds were subjected to enzyme kinetics study, fluorescence quenching analysis, molecular docking and molecular dynamics simulation, to verify their inhibitory mechanisms.

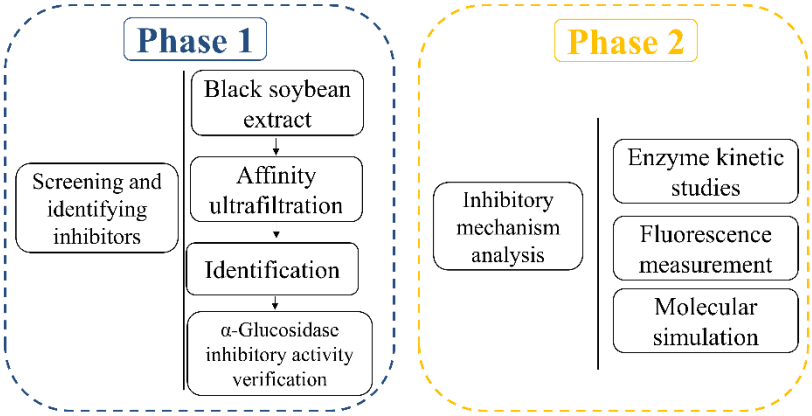

Figure 1.1 Overall method of present study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of black soybean

2.1.1 Introduction of black soybean

Black soybeans, a leguminous plant, are characterized by their black color, oval shape, and rich nutritional content. They are abundant in protein, fat, carbohydrates, dietary fiber, vitamins, and minerals, featuring a high-quality, diverse protein profile inclusive of eight essential amino acids crucial for maintaining normal physiological functions. Furthermore, black soybeans contain phenolic acids, flavanols, isoflavones, anthocyanins, saponins, and other bioactive compounds. Recent medical research has established the diverse bioactive function of black soybean's active components, including antioxidant, anti-inflammatory, anti-tumor, anti-diabetic, and lipid-lowering properties. Black soybeans can be consumed in various forms, such as stew, porridge, and soybean milk, offering not only nutritional benefits but also potential health-promoting and disease-preventive effects.

2.1.2 Development and application of black soybean

Due to its high nutritional value and bioactive function, black soybeans have been widely used in food products.

Slavin et al. (2013) used black soybean in crackers making and found that the contents of total isoflavones and phenols and antioxidant capacity of black soybean crackers were significantly higher than those of yellow soybean crackers. Different from Slavin et al. (2013), Ma et al. (2021) applied black soybeans to the production of noodles, and found that the addition of black soybeans can improve the texture of noodles, change their elasticity, hardness, and chewiness. In addition, black soybeans

are often used in the processing of soybean milk, tofu and other soybean products because of their high protein content and amino acid composition. Guo et al. (2020) compared soy milk made from yellow soybeans and black soybeans in different regions. The results showed that black soy milk had significantly higher phenol content and FRAP, DPPH radical scavenging activity than soybean milk made from yellow soybeans. Besides, black soybeans are also used in the production of soy sauce or soy yogurt, soybean natto (Hsieh et al., 2021; Hu et al., 2010; Lai et al., 2012). There are some anti-nutritional components found in black soybean, and isoflavones in black soybean are mostly in the form of inactive glycosides. Germination or fermentation can improve the accessibility of black soybean, thus improving the nutritional value of black soybean products.

2.2 Main components of black soybean

Due to its rich nutritional value, black soybean has widely been consumed in the Eastern Asian countries for medicinal and functional purposes for millennia. Recent research has demonstrated that black soybeans contain protein, lipids, polysaccharides, trace elements, polyphenols, and other biologically active substances that contribute to human health.

2.2.1 Proteins

Black soybean has the highest protein content (46.94%) compared with yellow soybean (39.07%), Adzuki bean (26.78%) and Mung bean (27.97%) (Chen et al., 2017). Black soybean protein is a kind of high-quality plant protein, which is mainly composed of four proteins, 2S, 7S, 11S and 15S, of which 7S and 11S are the most abundant, accounting for more than 70% of the total storage protein (Barać et al., 2011). Besides, black soybean is an excellent source of amino acids, which contain

not only nine non-essential amino acids such as asparagine, serine, glutamate, proline, glycine, alanine, tyrosine, histidine and arginine, but also seven essential amino acids such as threonine, valine, methionine, isoleucine, leucine, phenylalanine and lysine (Zhong et al., 2015).

2.2.2 Peptides

Previous studies have shown that dietary protein to be utilized by the body after ingestion, it must undergo hydrolysis to form amino acids or small peptides (Joye, 2019). Enzymatic hydrolysis, acid hydrolysis and fermentation are often used to prepare biological peptides. Generally, different proteases are selected according to the different target products. Commonly used animal proteases include pepsin and trypsin, and plant proteases include papain and fig protease (Tavano, 2013).

Various studies have shown that black soybean peptides have a variety of biological activities. Haiwei (2010) proved that black soybean peptide has the ability of antioxidation and scavenging free radicals. The studies of Chen et al. (2019) have not only revealed that black soybean peptide has antioxidant activity, but also proved that it has anticancer ability. Other scholars have found that black soybean peptide has the ability to protect alcoholic liver injury and improve endoplasmic reticulum stress and insulin resistance (Jang et al., 2010; Ren et al., 2021).

2.2.3 Fats

Fat is one of the major nutrients of black soybean, accounting for about 16% (Choi et al., 2021). Among them, 8 kinds of saturated fatty acid; dominated by palmitic acid C16:0 (10.42%); and 5 kinds of unsaturated fatty acid mainly composed of oleic acid C18:1N9C(22.87%) and linoleic acid C18:2N6C(53.62%), which are the main components of black soybean fat (Zhong et al., 2015). Among them, linoleic acid is an essential fatty acid, which can prevent high cholesterol, improve

hypertension and prevent myocardial infarction, while another essential fatty acid α-linolenic acid can reduce fasting and postprandial glycerol esters, which has the offensive effect of lowering blood pressure.

2.2.4 Polysaccharides

Black soybean is an important source of soybean polysaccharides. However, due to the complex composition of polysaccharides, most studies focus on characterizing the structure and activity of black soybean polysaccharides (BSPS). Liao et al. (2005) have found black soybean polysaccharide can promote the hematopoietic activity of bone marrow, stimulate spleen cells to produce a variety of hematopoietic growth factors, and reconstruct bone marrow inhibited by irradiation and 5-FU. The study of Liao et al. (2001) showed that a high-molecular weight polysaccharide with an a-linked glucan structure is the primary component of black soybean polysaccharides, which have the anti-tumor and immune regulation effects. Liu et al. (2015) further obtained three purified fractions (BSPS-1, BSPS-2 and BSPS3) from black soybean, which were mainly composed of carbohydrate and uronic acid, including arabinose, rhamnose, galactose, glucose and mannose. And the antioxidant assays showed that crude BSPS and its purified fractions had potential scavenging capacity against O^{2-} (superoxide anion) and DPPH radical. A recent study reported that the black soybean polysaccharide (mainly contained of arabinose, galactose, glucose, and galacturonic acid) could increase its antioxidant capacity by covalently binding with proteins (Lee et al., 2018). The study of Sun et al. (2018) suggested that a novel arabinogalactan from black soybean exhibiting potent protective effect on acute liver injury in vivo, which was comparable to that of silymarin (a positive standard).

2.2.5 Polyphenols

Phenolic compounds are the main source of active components in black soybean. Black soybeans are rich in phenolic components, mainly including anthocyanins from seed coats and isoflavones from cotyledons. There are many kinds of anthocyanins in black soybeans, including more than 9 anthocyanins based on delphinidin-3-glucoside and cyanidin-3-glucoside. Many scholars have shown that anthocyanins have antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory and other effects (Lee et al., 2009). In addition, black soybeans also contain two biologically active phenolic substances, phenolic acids and flavonoids. The phenolic acids in black soybeans are mainly gallic acid protocatechuic acid, protocatechualdehyde, 2,3,4-trihydroxybenzoic acid, vanillic acid, chlorogenic acid, sinapic acid and so, and the flavonoids are mainly epicatechin, quercetin, kaempferol and catechins. Studies have shown that the above compounds can play antioxidant, antibacterial, hypolipidemic, hypoglycemic and other effects (Correa et al., 2010; Xu & Chang, 2008b). Isoflavones are the characteristic flavonoids of soybean plants. Isoflavone in soybean exhibits multiple health effects such as anticancer, anti-tumor, antidiabetic, antimicrobial, anti-obesity activities, preventative effect against Cardiovascular disease Senile dementia and inhibition of inflammatory.

2.2.6 Trace nutrients

Besides the above components, black soybeans also contain saponins, vitamins (thiamine, riboflavin, niacin, pantothenic acid, folic acid, vitamin C, E, etc.), minerals (phosphorous, potassium, iron, sodium, etc.) and so on (Chung et al., 2017; Kim et al., 2014; Kumar et al., 2010; Lokuruka, 2010; Saha et al., 2008; Y. Zhong et al., 2015). Both vitamins and mineral elements are essential components for the human body. For instance, potassium contributes to maintaining normal heart function, while iron aids

in oxygen transportation and helps prevent anemia (Abbaspour et al., 2014; Weaver, 2013). Vitamin C and E are potent antioxidants, protecting against damage from free radicals, with the latter benefiting the cardiovascular system (Clarke et al., 2008; Pullar et al., 2017). Besides, the vitamins B complex primarily play a crucial role in the nervous system, maintaining the normal function of neurons (Agnew-Blais et al., 2015; McGarel et al., 2015). Although they are present in relatively small amounts, the important physiological activities of these trace components should not be overlooked and are equally deserving of in-depth research.

2.3 Research progress of soybean isoflavone

The term isoflavones first appeared in the 1940s in connection with fertility problems in sheep. It wasn't until 1995 when the discovery of the cholesterol-lowering ability of soy protein drew worldwide attention that isoflavones began to be widely discussed (Messina, 2010). Isoflavone is one of the flavonoids, mainly found in legumes, belonging to plant secondary metabolites.

2.3.1 Extraction and identification

2.3.1(a) Extraction

Isoflavones contain multiple hydroxyl groups, giving them a certain polarity between water and ethanol. They can be dissolved in some weakly polar solvents, such as acetone, methanol, and ethanol. There are four main types of soy isoflavones: isoflavone aglycone, glucoside, acetyl-glucoside, and malonyl-glucoside. With increasing glycosylation of the isoflavone hydroxyl group, the polarity gradually increases. Isoflavone aglycones are the least water-soluble, being basically insoluble in water, while isoflavone glucosides, acetyl-, and malonyl-glucosides are soluble in water (Křížová et al., 2019; Rostagno et al., 2003; Wang et al., 2013).

The table 2.1 delineates four distinct prevalent techniques for isoflavone extraction, expounding upon the underlying principles, advantages, and limitations associated with each method.

Table 2.1 An overview of diverse extraction techniques

Method	Principle	Advantages	Limitations	Ref.
Solvent extraction	Isoflavones feature several hydroxyl groups, which render them moderately polar, with properties intermediate between water and ethanol. They are soluble in mildly polar solvents like acetone, methanol, and ethanol.	Versatility, simple operation	Excessive solvent usage, long extraction times	Kaufmann et al. (2002)
Ultrasonic-assisted extraction	Utilizes ultrasound to disrupt plant cell walls, thereby accelerating the dissolution of compounds in organic solvents.	Efficient	Potential for sample damage	Shen et al. (2023)
Microwave-assisted extraction	Efficient and homogeneous extraction of target compounds through microwave irradiation.	Highly efficient and fast, solvent saving	High equipment costs, potential for sample instability	Mandal et al. (2007); Zhang et al. (2011)
Supercritical fluid extraction	Employing a supercritical fluid as the extracting agent for targeted extraction.	Mild conditions to protect the target	High equipment costs, complex operation	Herrero et al. (2010)

2.3.1(a)(i) Solvent extraction

Solvent extraction is the most used extraction method for soy isoflavones extraction from soybeans or soy products, but other auxiliary extraction methods such as ultrasound-assisted extraction and carbon dioxide supercritical extraction can also be used. Most studies use different proportions of organic reagents, such as methanol, ethanol, and acetonitrile, as extraction solvents （Lee et al., 2004; Rostagno et al., 2003; Rostagno et al., 2007）

2.3.1(a)(ii) Ultrasonic-assisted extraction

Ultrasonic-assisted extraction (UAE) is a widely used method that employs ultrasound to disrupt plant cell walls and expedite the dissolution of compounds in

organic solvents. Ultrasound's high-speed cavitation and stirring action can significantly enhance isoflavone extraction efficiency. UAE is frequently combined with other extraction methods to further optimize yield (Rostagno et al., 2003).

2.3.1(a)(iii) Microwave-assisted extraction

Microwave-assisted extraction employs microwave heating to rapidly and uniformly extract target compounds in the solvent. This technique is gaining popularity due to its rapid extraction and high efficiency, and is widely employed in natural products, pharmaceuticals, and food extraction.

Numerous studies have focused on optimizing microwave-assisted extraction (MAE) parameters for isoflavone extraction. Careri et al. (2007) identified microwave power, extraction time, and acid concentration as key factors influencing isoflavone recovery. Rostagno et al. (2007) investigated the impact of solvents, temperature, extractant dosage, and extraction time, finding that optimal conditions yielded isoflavones with no degradation and good reproducibility. Additionally, Terigar et al. (2010) utilized continuous MAE to extract genistein, daidzein, and glycitein from soybean powder, demonstrating that the optimized MAE method doubled the extraction yield of isoflavones compared to traditional solvent extraction methods.

2.3.1(a)(iv) Supercritical fluid extraction

Supercritical fluid extraction (SFE) utilizes supercritical fluids as extractants to extract active ingredients, preserving the active components and nutrients of raw materials. SFE has been applied to extract isoflavones from diverse soybean products, including soybean flour, soybean cake, soybean meal, tofu, and miso (a fermented product) (Kang et al., 2018; Kao et al., 2008; Lummaetee et al., 2017; Rostagno et al., 2002) while having a low destructive effect on microorganisms and biologically active components (Herrero et al., 2010).

Chandra and Nair (1996) demonstrated the potential of supercritical fluid extraction (SFE) for extracting daidzein and genistein from black soybean products. Subsequent studies have optimized SFE conditions and compared them to traditional solvent extraction methods to identify the most effective extraction parameters. Kao et al. (2008) noted that while the total isoflavone yield from the modified SFE method was lower than that obtained by solvent extraction, it was more suited for extracting acetyl glucosides and aglycones. Additionally, Zuo et al. (2008) observed that increasing particle size and extraction pressure led to improved isoflavone recovery.

2.3.1(b) Identification

2.3.1(b)(i) Chromogenic methods

Chromogenic methods rely on the chemical reaction between the analyte and a reagent to produce one or more colored products. This approach offers advantages such as simplicity and high sensitivity. The chromogenic reagent can interact with specific functional groups on the compound (e.g., double bonds, hydroxyl groups, aldehyde groups) to produce distinct colors. By observing the color change of the chromogenic reagent, researchers can make preliminary inferences about the presence or absence of the target product.

Chromogenic methods were initially used for the determination of soybean isoflavones, but their application is limited due to their narrow range and inability to quantify multiple isoflavones simultaneously. This approach provides an incomplete representation of the isoflavone content in the analyzed samples and is not suitable for comprehensive flavonoid profiling.

2.3.1(b)(ii) Ultraviolet spectrophotometric method

Ultraviolet spectrophotometry is an analytical technique used to measure the absorbance of a substance within the 190-800 nm range, enabling both qualitative and quantitative analysis.

Ultraviolet spectrophotometry can determine the total isoflavone content in soybean samples by exploiting the strong UV light absorption of hydroxyl groups and aromatic rings of isoflavones. Typically, this method employs a specific isoflavone standard concentration to quantify the total isoflavone content. However, it should be noted that ultraviolet spectrophotometry cannot distinguish between individual isoflavone monomers such as genistein, daidzein, and glycitein due to their similar UV absorption wavelengths and additive absorbance. Consequently, this method offers an approximation of the total isoflavone content rather than a comprehensive breakdown of individual isoflavone components.

2.3.1(b)(iii) Thin layer chromatography

Thin-layer chromatography (TLC) is an adsorption chromatography technique that separates components based on their differing affinities for a stationary phase (adsorbent) and a mobile phase (solvent). The interaction between the components of the mixture and both phases, through processes such as adsorption, desorption, re-adsorption, and re-desorption, leads to their separation according to their relative affinities for the two phases. TLC can be conducted on any surface that can form an interface, and the selection of the adsorbent and solvent system is depending on the specific mixture being separated (Bele & Khale, 2011).

2.3.1(b)(iv) High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is an analytical technique widely utilized for separating and identifying compounds in complex mixtures, with

applications in diverse fields such as drug analysis, pesticide residue analysis, and food quality control (Reuhs, 2017).

HPLC separates components based on their distribution coefficients between a mobile phase and a stationary phase. As the sample passes through the column, components interact with both phases through adsorption and desorption, resulting in different retention times for each component, enabling their separation. The separated components are then detected by a detector, converting their concentration into an electrical signal, recorded and displayed as a chromatogram, providing a visual representation of the separation results (Kupiec, 2004).

2.3.2 Structure

Soy isoflavones are a class of isoflavonoid compounds that share a core structure of 3-benzopyrone, which consists of two benzene rings connected by a three-carbon side chain. Soy isoflavones have various substituents, including hydroxyl, methoxy, and sugar groups, which are attached to different rings and carbon positions. These different substituents contribute to the diverse biological activities and medicinal properties of soy isoflavones. There are four main types of soy isoflavones: aglycones, glucosides, acetyl-glucosides, and malonyl-glucosides (Křížová et al., 2019).

2.3.3 Bioactivities

Isoflavones exhibit antioxidant, antitumor, and anti-inflammatory properties, and show promise in preventing metabolic syndrome, a cluster of conditions that heighten the risk of cardiovascular disease and type 2 diabetes in individuals with obesity and hypertension. Isoflavones may aid in the prevention of metabolic syndrome by enhancing blood pressure, glucose control, lipid profiles, and arterial health.

2.3.3(a) Anti-oxidation

Reactive oxygen species (ROS) and free radicals are major contributors to aging, cancer, and cellular damage in the human body. Isoflavones have been shown to significantly reduce lipid peroxidation and enhance the activity of antioxidant enzymes (Dixit et al., 2012). For example, Takahashi et al. (2005) found that black soybean extract exhibited antioxidant activity against LDL oxidation and DPPH radicals. Additionally, Xu and Chang (2008) demonstrated that black soybean isoflavone extract possesses both ORAC and FRAP antioxidant activities.

2.3.3(b) Anti-tumor

Isoflavones have been shown to be effective in preventing the growth and proliferation of various types of cancer cells through multiple mechanisms, including:

Inhibiting tumor cell metastasis, delaying cancer cell pathological changes, inducing tumor cell apoptosis, inhibiting gene expression, providing antioxidant protection and so on.

Studies have shown that isoflavones have various anticancer effects. Among them, isoflavones significantly contribute to the mitigation and prophylaxis of prostate and breast cancer. This potentially stems from their structural resemblance to estrogen, enabling isoflavones to modulate estrogen metabolism through binding to estrogen receptors and impeding estrogen attachment, thereby impeding hormone-associated cancers (Banerjee et al., 2008).

In animal experiments, several experiments have confirmed that isoflavones can reduce the incidence and size of prostate cancer, which may be by preventing the onset of prostatic intra-epithelial neoplasia (PIN) or impeding their advancement to carcinomas. In addition, a number of experiments have also proved that Isoflavones primarily demonstrated a capacity to hinder or postpone chemically-induced

mammary carcinogenesis in prepubertal or premenopausal adult female rats. It is worth noting that there are other situations where isoflavones may also have negative effects. In human experiments, on the whole, it has been proved that the intake of soybeans can reduce the risk of breast cancer, and the risk of premenopausal women is significantly lower (Steiner et al., 2008). Besides, in a 12-month clinical trial, Miyanaga et al. (2012) found that those who ate pure isoflavones showed a lower incidence of prostate cancer.

In addition to that, Zuo et al. (2008) found that soybean isoflavones reduce the nuclear gene expression of tumor cells, inhibit the expression of cyclooxygenase and nitric oxide synthase, and induce apoptosis of HT-29 tumor cells; Moreover, isoflavone genistein have demonstrated the capability to inhibit enzyme activity and protein levels associated with the invasion process of human glioblastoma (U87MG) cells, while significantly suppressing the proliferation and triggering apoptosis in the hepatocellular carcinoma cell line Hepa1-6 (Puli et al., 2006; Sanaei et al., 2018).

2.3.3(c) Lipid metabolism regulation

Obesity is a major risk factor for type 2 diabetes, atherosclerosis, cardiovascular disease, and certain types of cancer. It is closely associated with hyperinsulinemia, insulin resistance, and abnormal lipid metabolism.

Animal studies have demonstrated the beneficial effects of isoflavones on lipid metabolism. For example, Kawakami et al. (2005) showed that isoflavone aglycone reduced liver and serum total cholesterol levels and liver triglyceride levels in rats, while both isoflavone aglycone and glucoside reduced serum triglyceride levels in rats. Similarly, Yousef et al. (2004) found that isoflavones caused a significant decrease in the levels of low-density lipoprotein, total cholesterol, very low-density lipoprotein, plasma total lipids, and triglyceride, while the level of high-density lipoprotein

increased in male rabbits. Additionally, studies have shown that soy isoflavones are beneficial in improving lipid profiles in postmenopausal women (Barańska et al., 2021).

2.3.3(d) Anti-diabetic potential

2.3.3(d)(i) Type 2 diabetes mellitus and α-glucosidase

Diabetes is a chronic condition that affects how human body processes blood sugar (glucose). There are two main types of diabetes: Type 1 and Type 2. In Type 1 diabetes, the body's immune system attacks and destroys the cells in the pancreas that produce insulin, a hormone that helps regulate blood sugar. In Type 2 diabetes, the body becomes resistant to insulin or the pancreas does not produce enough insulin to maintain normal blood sugar levels (Oyetayo et al., 2019). Both types of diabetes can lead to high blood sugar levels, which can damage blood vessels and organs and lead to serious health complications such as heart disease, stroke, kidney disease, and nerve damage. Management of diabetes typically involves a combination of medication, lifestyle changes, and regular monitoring of blood sugar levels.

α-Glucosidase, also known as α-D-glucoside hydrolase, is a class of enzymes that break down complex carbohydrates into simpler sugars, such as glucose. It is found in the small intestine and plays a key role in the digestion and absorption of carbohydrates (Oyetayo et al., 2019).

2.3.3(d)(ii) α-glucosidase Inhibitors

α-Glucosidase inhibitors are medications used to treat Type 2 diabetes by slowing down the digestion of carbohydrates, which helps prevent spikes in blood sugar levels after meals. The most common α-glucosidase inhibitors include acarbose and miglitol, which can cause side effects such as, bloating and diarrhea. As a result,

there is growing interest in developing natural plant-based inhibitors with fewer side effects.

2.3.3(e) Others

In addition to their main physiological effects, soy isoflavones have also been shown to play a role in radiation protection, prevention of dementia, slowing of aging, prevention of osteoporosis, antibacterial activity, reduction of blood pressure, and anti-fatigue effects. (Chen et al., 2022; Dixit et al., 2012; Liu et al., 2012; Wang et al., 2010; Wei et al., 2012; Yang et al., 2010, 2011)

2.4 Research progress of affinity-ultrafiltration

2.4.1 Introduction of affinity-ultrafiltration

Traditional methods in phytochemistry play a critical role in the exploration and structural characterization of α-glucosidase inhibitors derived from plants. However, this strategy of isolating and evaluating individual compounds for α-glucosidase inhibition is time-consuming and may overlook trace active components. As an alternative, affinity ultrafiltration is a method for targeted screening of active ingredients of interest from complex mixtures. This technology exploits the specific binding between ligands and receptor to efficiently screen active small-molecule compounds using an ultrafiltration device, and then combines it with liquid chromatography-mass spectrometry (LC-MS) to identify the structure of the active ingredients. Compared with traditional screening methods, affinity ultrafiltration has the characteristics of rapidity, high sensitivity, and strong selectivity, and is especially suitable for screening potentially active small molecule compounds from complex systems (Oyetayo et al., 2019).

2.4.2 The general process of affinity ultrafiltration

The general process of affinity ultrafiltration involves incubation, ultrafiltration, centrifugation, and analysis of differential components, followed by separation and purification. First, the crude extract solution is incubated with the target enzyme solution. The mixture is then passed through a filtration membrane with a specific pore size. The target substance, due to its interaction with the immobilized affinity ligand, selectively binds to the ligand while other unwanted substances pass through the membrane. This allows for the separation and concentration of the target substance. After the target substance has been captured on the solid support, it can be further analyzed and purified using various techniques, such as HPLC.

2.4.3 Application of Affinity ultrafiltration

Affinity ultrafiltration is widely used in drug discovery and biopharmaceutical production to isolate and purify proteins, enzymes, antibodies, and other biomolecules of interest.

2.4.3(a) Protein Separation and Purification

Affinity ultrafiltration is widely used to isolate and purify specific proteins from complex mixtures. Using an affinity ligand that selectively binds to the target protein allows for the removal of unwanted impurities, resulting in a higher purity of the desired protein.

As early as 2002, it was used to purify immunoglobulins (Castilho et al., 2002). Moreover, it has been used to purify bovine serum albumin, ovalbumin, bovine pancreatic phospholipase A2 (Rao & Zydney, 2006; Teke & Telefoncu, 2008).

In addition to conventional membrane separation, new techniques have also been developed for the efficient separation of proteases, such as Immobilized Metal Affinity Chromatographic Membrane, which has been used to separate trypsin with

recoveries up to 72.3% (Hamzah et al., 2018). Various studies have found that affinity ultrafiltration screening can lead to higher productivity and cost savings in the purification of proteases.

2.4.3(b) Drug Discovery

Affinity ultrafiltration is widely used in drug discovery to screen and identify small molecules or compounds that bind to specific target proteins. By coupling affinity ligands with high-throughput screening methods, potential drug candidates can be rapidly identified and evaluated for their binding affinity. Compared with protein purification, affinity ultrafiltration is more mature in the screening of enzyme inhibitors from natural plants, and has been widely used in the screening of α-glucosidase, xanthine oxidase, COX-2, Topo I, Topo II, tyrosinase, amylase, lipase, neuraminidase, thrombin, and other enzyme inhibitors (Feng et al., 2023; Lan et al., 2021; Li et al., 2022; Rakotondrabe et al., 2022; Tian et al., 2022; Wang et al., 2023; Xu et al., 2024).

Mainly used for extracting active components from terrestrial herbs or plants, there have been reports of screening different enzyme inhibitors from the leaves of *Acanthopanax senticosus* Harms, *Saussurea laniceps* Hand.-Mazz, *Andrographis paniculata*, *Radix Astragali*, *Curcumae Rhizoma*, *Puerariae flos*, and other plants. (Chen et al., 2022; Feng et al., 2023; Jiang et al., 2015; Lan et al., 2021; Liu et al., 2016; Zhou et al., 2012). There are also a few studies focusing on aquatic plants (Wang et al., 2023) or fish (Zhong et al., 2021), Chinese red yeast rice (Jin et al., 2016), and animal-sourced TCM *Poecilobdella manillensis* (Huang et al., 2021).

Affinity ultrafiltration is a versatile technique that facilitates the isolation, purification, and analysis of specific molecules. It has enabled advancements in diverse fields such as medicine, biotechnology, and food research.

2.5 Analysis of the interaction between Inhibitors and enzymes

Enzymes are catalytic protein molecules whose catalysis is closely related to their active sites (Welborn & Head-Gordon, 2019). The active site of an enzyme is composed of a binding site, which specifically binds to the substrate, and a catalytic site, which cleaves and forms bonds (Yabukarski et al., 2020).

Under normal conditions, the substrate is recognized and bound by the binding site of the enzyme and hydrolyzed into a new product under the catalytic action of the enzyme. When substrate analogs are present in the reaction, they compete with the substrate for binding to the enzyme, thus reducing or delaying the production of the product while acting to inhibit the enzyme's catalytic effect (Lopina, 2017). It is because of this inhibitory effect that substrate analogs called enzyme inhibitors. Exploring the binding (interaction) between inhibitors and enzymes helps us to better understand the mechanism of inhibition of enzymes by inhibitors. In the present study, enzyme kinetic studies, fluorescence quenching, and molecular simulation were used to clarify the inhibition mechanism of the inhibitor on the enzyme.

2.5.1 Kinetic analysis of inhibition of α-glucosidase activity

Enzyme inhibition kinetics is the study of the binding of inhibitors to enzymes, which involves determining the parameters that describe the interaction between the enzyme, substrate, and inhibitor, such as the inhibition constant (K_i) and the type of inhibition. This information helps in understanding the mechanism of inhibition, optimizing enzyme activity, and designing inhibitors for therapeutic purposes.

The mechanism of action of enzyme inhibitors involves the step of forming low- or inactive inhibitor-enzyme intermediate complexes. The spatial structure of the enzyme molecules is not destroyed in the whole process of inhibition, but only