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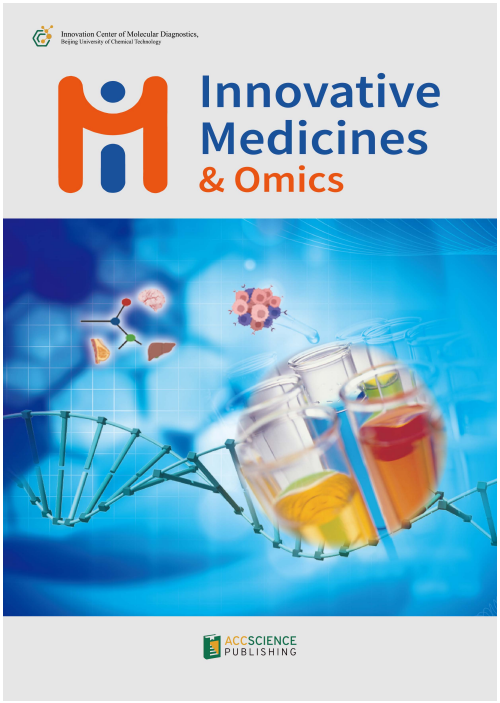

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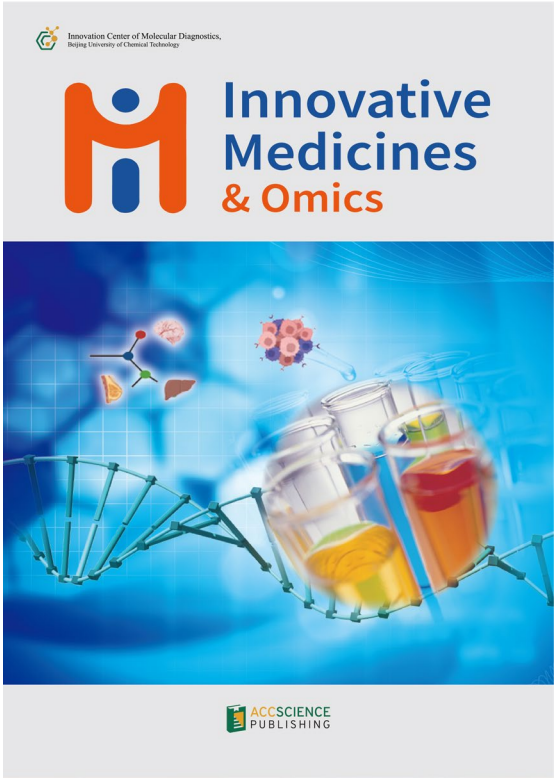

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REVIEW ARTICLE

Incretin mimetics for the management of diabetes and associated comorbidities: An overview

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Abstract

Type 2 diabetes mellitus is commonly associated with various comorbidities that aggravate the disease's overall impact on health. The most prevalent comorbidities of diabetes include obesity, dyslipidemia, hypertension, cardiovascular conditions, and kidney diseases. Incretin mimetics, also known as glucagon-like peptide-1 receptor agonists, mimic incretin hormones to stimulate insulin release in response to food intake. These medications help lower blood glucose by increasing insulin production, reducing glucagon secretion, slowing stomach emptying, and promoting satiety. A key advantage of incretin mimetics is their ability to reduce blood glucose levels without causing hypoglycemia, making them a safer option for many patients. They also promote weight loss, which is particularly beneficial for patients with both obesity and diabetes. Incretin mimetics are typically administered once or twice daily and are often used in combination with other treatments such as metformin or insulin. Evidence suggests that these drugs may reduce the risk of heart attack and stroke, an important consideration given the heightened cardiovascular risk in patients with diabetes. Additionally, incretin mimetics may help preserve pancreatic beta-cell function, potentially slowing the progression of diabetes. However, these drugs are costly and may be unaffordable for low-income individuals. Commonly reported side effects include nausea, vomiting, and diarrhea, which tend to decrease over time. While there have been reports of pancreatitis, current research indicates that incretin mimetics do not increase the risk of pancreatic cancer. Educating patients on proper use and potential side effects is crucial to ensure safe and effective treatment with incretin mimetics.

Keywords: Diabetes; Obesity; Cardiovascular and kidney diseases; Incretin hormones; Glucagon-like peptide-1 receptor agonist; Gastric inhibitory peptide; Incretin mimetics

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by the progressive failure of pancreatic beta-cells (β-cells) to meet the increased the body's increased insulin demand, resulting in relative insulin deficiency and resistance to insulin action.[1] The primary aim of T2DM management is to alleviate symptoms of hyperglycemia and reduce the risk of microvascular and macrovascular complications, such as nephropathy, neuropathy, retinopathy, and cardiovascular disease. Compared to the general population, individuals with T2DM face a significantly higher risk of both fatal and non-fatal cardiovascular events, which contribute to increased mortality rates.[2] The treatment approach for T2DM typically involves lifestyle modifications – such as diet and exercise – along with oral antidiabetic medications and injectable therapies. A strong correlation exists between the severity of hyperglycemia, the metabolic dysfunctions characteristic of T2DM, and vascular damage, all of which heighten the risk of macrovascular complications. Consequently, the development of medications that not only control blood glucose but also address other metabolic risk factors and improve cardiovascular outcomes is of high clinical importance. Key factors contributing to the rising prevalence of T2DM include obesity, aging, and genetic susceptibility.[3]

Insulin resistance, pancreatic β-cell dysfunction, and abnormal glucagon secretion are the key contributors to T2DM progression. At present, T2DM is recognized as a systemic disease in which chronic hyperglycemia causes long-term damage, dysfunction, and failure of multiple organs.[4] Effective management requires continuous patient education on adopting and maintaining lifestyle changes, with treatment plans tailored to each patient's comorbidities and cultural beliefs.[5] T2DM is primarily characterized by insulin resistance in peripheral tissues – such as skeletal muscle and adipose tissue – compounded by pancreatic β-cell failure. The resulting hyperglycemia increases the risk of microvascular complications and cardiovascular diseases, which not only lower patients' quality of life but also contribute to increased economic burden and reduced life expectancy.

For obese patients, achieving stricter glycemic control is necessary, as obesity is strongly associated with T2DM pathophysiology and increased macrovascular risk.[6] Medication, coupled with lifestyle changes, is essential in managing the condition effectively. Obesity is a significant risk factor in both the development and progression of T2DM, as it exacerbates insulin resistance and impairs the body's ability to produce and effectively utilize insulin.[7] Excess adipose tissue in obese individuals contributes to chronic low-grade inflammation, which disrupts insulin signaling pathways. This disruption in insulin sensitivity leads to elevated blood glucose levels, increasing the demand on pancreatic β-cells to produce insulin. Over time, this strain can lead to β-cell dysfunction, further complicating glucose regulation and worsening T2DM progression. Consequently, obesity not only increases the risk of developing T2DM but also accelerates its progression and the onset of related complications, such as cardiovascular disease, kidney failure, and neuropathy. Obesity also complicates T2DM management, making it challenging to control blood glucose through lifestyle changes alone. Obese patients may require higher doses of insulin or other medications to achieve optimal glycemic control. In addition, obesity increases the likelihood of comorbidities such as hypertension and dyslipidemia, further heightening cardiovascular risk in patients with diabetes. Therefore, implementing effective strategies to manage obesity in diabetic patients is critical for preventing and mitigating these complications.[7,8]

Most T2DM treatments target various pathways but are often associated with adverse effects, such as edema, weight gain, hypoglycemia, and reduced insulin sensitivity. However, emerging therapies targeting incretin hormones have emerged as safer alternatives for managing T2DM. The incretin pathway plays a crucial role in regulating blood glucose levels, as it accounts for the majority of insulin secretion by β-cells in response to oral glucose intake.[9] Metformin remains the first-line treatment for managing T2DM, particularly in overweight patients, and is used in conjunction with lifestyle modifications and measures to reduce cardiovascular risk. The American Diabetes Association (ADA) recommends[5] a patient-centered approach to guiding additional T2DM treatments, considering factors such as cost, side effects, weight changes, hypoglycemia risk, and drug efficacy. Add-on therapies to metformin include thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) enzyme inhibitors, insulin, sodium-glucose co-transporter 2 inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists (RAs), luminal glucosidase inhibitors, dietary interventions, and sulfonylureas.[10,11]

In this overview, we summarize the T2DM disease process, its deleterious effects along with comorbidities, the role of incretin hormones, and the development of incretin mimetics as emerging treatment options. Various classes of common incretin mimetic drugs—GLP-1 single, dual, and triple RAs—are discussed, along with their indications and reported side effects.

2. Incretin hormones

Incretin hormones, such as GLP-1 and gastric inhibitory peptide (GIP), also known as glucose-dependent

insulinotropic polypeptide, play a critical role in glucose homeostasis by stimulating insulin release from pancreatic β-cells.[12] These hormones are rapidly degraded by the DPP-4. The "incretin effect" refers to the heightened insulin secretion in response to oral glucose compared to intravenous glucose – a phenomenon notably reduced in patients with diabetes.[13] This reduction is due to a diminished capacity of GIP to induce insulin release, possibly stemming from general β-cell dysfunction or specific abnormalities in the GIP signaling pathway. This decrease in incretin action contributes to impaired glycemic control, particularly in regulating postprandial glucose levels. In contrast, the insulinotropic effects of GLP-1 remain relatively preserved, allowing exogenous GLP-1 to stimulate insulin release, inhibit glucagon secretion, and lower plasma glucose during both fasting and postprandial states.[14] This understanding has propelled the development of incretin-based glucose-lowering medications.

Incretin hormones secreted by the gastrointestinal tract epithelium play a critical role in maintaining normal glucose tolerance by preventing excessive postprandial glucose spikes through glucose-dependent insulin release. The incretin effect is dose-dependent, ensuring stable postprandial glucose levels even as carbohydrate intake varies.[15] To assess the incretin effect, researchers compare insulin responses to oral and intravenous glucose administrations that produce similar glucose excursions. Findings suggest that the incretin effect may account for up to a fivefold increase in postprandial glucose clearance, as evidenced by the differential glucose infusion requirements needed to replicate the glucose levels induced by oral intake.[13]

Oral glucose intake triggers the release of GIP and GLP-1 from the gut, enhancing insulin secretion and facilitating glucose disposal. This role of GIP and GLP-1 in enhancing insulin secretion explains why the incretin effect is closely linked to carbohydrate intake.[15] The amount of carbohydrates affects both incretin secretion and the subsequent insulin response (Figure 1). In healthy individuals, the incretin effect accounts for up to 70% of insulin secretion in response to glucose intake. GLP-1, a 30-amino acid polypeptide, is produced by endocrine L-cells in the mucosa of the distal small intestine and colon, whereas GIP, a 42-amino acid polypeptide, is secreted by endocrine K-cells located in the duodenum and upper jejunum.[16]

Glucagon-like peptide-1 is rapidly degraded by the enzyme DPP-4, with a short half-life of approximately 1.5 minutes, while GIP has a slightly longer half-life of around 7 min. Despite their ability to stimulate insulin secretion, these hormones do not pose a risk of hypoglycemia as their insulinotropic effects are glucose-dependent, activating only at higher glucose concentrations.[16] GLP-1 improves insulin gene expression and synthesis and has trophic and protective effects on β-cells, while also suppressing pancreatic glucagon release in a glucose-dependent manner. Conversely, GIP has been shown to increase glucagon secretion. Both hormones exert insulinotropic effects through G-protein-coupled receptors on pancreatic β-cells.[17]

Beyond their influence on the endocrine pancreas, these incretin hormones play various roles in other physiological systems. GLP-1 receptors are present in several brain regions, where they are believed to stimulate satiety, particularly when combined with GLP-1-induced slowing of gastrointestinal motility through vagal signaling, ultimately reducing food intake and aiding in body weight control. GLP-1 also delays gastric emptying, helping to attenuate postprandial glucose spikes. On the other hand, GIP does not affect gastric emptying, and this effect is specific to GLP-1 RAs, though repeated doses of GLP-1 mimetics may reduce the gastric emptying delay.[18]

The enzyme DPP-4 plays a key role in glucose metabolism by inactivating GLP-1 and GIP, thereby diminishing their effects on insulin secretion and glucose regulation, contributing to higher postprandial glucose levels. DPP-4 inhibitors, such as sitagliptin, saxagliptin, and linagliptin, are used in treating T2DM to inhibit DPP-4 activity, prolonging the action of GLP-1 and GIP. This action enhances insulin secretion and reduces glucagon levels in a glucose-dependent manner, thus improving glycemic control without significantly increasing hypoglycemia risk. DPP-4 inhibitors are often used in combination with other antidiabetic agents, such as metformin, and are generally well-tolerated with minimal side effects.[19]

The GLP-1 receptor, a 463-amino acid protein with eight hydrophobic domains, is expressed in tissues such as the heart, kidneys, gut, pancreatic islets, stomach, and lungs, with a highly conserved N-terminal extracellular domain. On GLP-1 receptor activation, intracellular calcium and cyclic adenosine monophosphate levels rise rapidly, resulting in glucose-dependent insulin secretion. However, DPP-4 quickly inactivates GLP-1, which has a half-life of only 1 – 2 min. Specific amino acid modifications at the C- and N-termini of GLP-1 also enhance receptor engagement and resistance to DPP-4 degradation, thereby prolonging GLP-1's half-life and action.[20]

Only a small fraction of circulating GLP-1 is physiologically active. GLP-1 amide, a key secretory product, represents the active form of GLP-1. Once in the bloodstream, GLP-1 amide has a half-life of <2 min, as it is rapidly cleaved by DPP-4 at positions 8 and 9. This

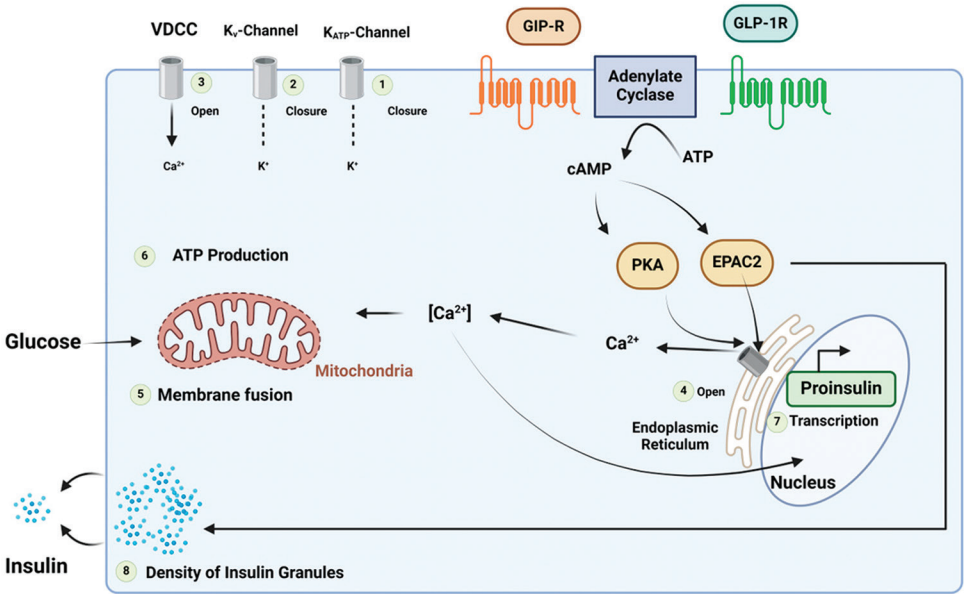


Figure 1. Mechanism of the insulinotropic effects of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1. Reproduced from Al Musaimi[12]

cleavage produces a physiologically inactive N-terminally truncated metabolite that does not bind to GLP-1 receptors. Therapeutically administered GLP-1 and its metabolites confer beneficial glucoregulatory and cardioprotective effects, such as reducing oxidative stress in vascular tissues, protecting β-cells, inhibiting gluconeogenesis, and mitigating oxidative stress in hepatocytes. Both GLP-1 and its metabolites influence vasodilation, enhance cardiomyocyte viability, and support cardiac function. For example, GLP-1 acts through a GLP-1 receptor-dependent mechanism, while certain metabolites may act through GLP-1 receptor-independent pathways in cardiovascular tissues. Therefore, targeting GLP-1 receptor activation or mitigating GLP-1 degradation offers various therapeutic potential, particularly for cardiovascular health.[21]

Research shows that GLP-1 receptors in the heart contribute to myocardial protection. In addition, GLP-1 lowers free fatty acid concentrations in humans and limits postprandial triglyceride increases. It also exhibits diuretic and natriuretic effects by modulating renal Na^+/H^+ exchange, contributing to blood pressure reduction.[22] Other roles of GLP-1 include promoting glucose uptake in muscle tissues, reducing hepatic glucose production, and offering neuroprotection effects. In patients with T2DM, the incretin effect is significantly diminished, which likely contributes to insufficient insulin release to prevent hyperglycemia following oral glucose intake. The attenuated incretin effect in T2DM may be attributed to decreased postprandial secretion, reduced insulinotropic potency, and a diminished insulinotropic action of GIP.[23]

3. Glucagon-like peptide-1 RAs

Incretin mimetics are hormone-like agents used in conjunction with metformin and/or sulfonylurea drugs for managing T2DM. In some cases, GLP-1 RAs are also prescribed for obesity management. These agents mimic incretin hormones, such as GLP-1, stimulating the pancreas to produce more insulin by binding to GLP-1 receptors. Examples include liraglutide, albiglutide, lixisenatide, exenatide, semaglutide, and dulaglutide. While incretin mimetics can be used alongside or as an alternative to insulin, they are not intended to replace antidiabetic medications. According to current consensus protocols from the ADA and the European Association for the Study of Diabetes (EASD), GLP-1 RAs should be used selectively after metformin failure, especially in patients with atherosclerotic cardiovascular disease or those at high cardiovascular risk, even if cardiovascular disease has not been confirmed.[24]

The effects of GLP-1 RAs on fasting and postprandial plasma glucose levels vary by formulation. Long-acting GLP-1 agonists, such as dulaglutide, liraglutide, albiglutide, and extended-release exenatide, primarily lower blood glucose levels by enhancing insulin secretion and reducing glucagon release. Short-acting GLP-1 RAs, like short-acting exenatide and lixisenatide, primarily target postprandial plasma glucose by delaying gastric emptying.[25] GLP-1 RAs are noted for promoting weight loss and present a lower risk of hypoglycemia compared to other anti-hyperglycemic drugs. These agents are resistant to degradation by DPP-4 but require subcutaneous administration due to low oral bioavailability.[26]

Incretin-based mimetics are also considered for patients who have contraindications or intolerance to metformin, or who have not achieved target glycated hemoglobin (HbA1c) levels after 3 months of therapy. This consideration is especially relevant for patients with atherosclerosis, chronic kidney disease, or heart failure. In addition, GLP-1 RAs such as semaglutide and liraglutide are approved for managing obesity in overweight patients with comorbidities. However, high costs and tolerability issues remain significant barriers to their widespread use.[27] These drugs have been shown to reduce total cholesterol, lower systolic and diastolic blood pressure, and promote weight loss by increasing satiety, thereby reducing caloric intake through hypothalamic mechanisms.[28] Cardiovascular disease, particularly atherosclerotic cardiovascular disease, is a leading cause of death among individuals with T2DM, and GLP-1 RAs provide significant cardiovascular benefits, including improved cardiac output, reduced infarction size, and decreased risk for cardiovascular events. These drugs also enhance left ventricular ejection fraction, myocardial contractility, coronary blood flow, and endothelial function.[29]

The most common adverse effect of GLP-1 RAs is mild-to-moderate nausea, typically resolving within a few weeks. Hypoglycemia, a common adverse effect of some antidiabetic therapies, is rare with incretin mimetics, generally occurring only when used in conjunction with sulfonylureas. Notably, concurrent use of insulin and incretin mimetics is not recommended.[20] Other potential adverse effects include vomiting, diarrhea, and, in some cases, acute kidney injury due to volume depletion. Additional side effects may include headaches, infections, moderate tachycardia, dyspepsia, and dizziness.[30] Injection-site reactions, such as erythema and pruritus, are common, particularly with longer-acting formulations. Some patients may develop antibodies against specific GLP-1 RAs, reducing their effectiveness; for instance, exenatide has been associated with a higher likelihood of antibody formation when administered weekly rather than twice daily. As a result, combination therapy with GLP-1 agonists and DPP-4 inhibitors is generally not recommended due to negligible glycemic improvement and an increased risk of hypoglycemia.[31]

Glucagon-like peptide-1 RAs also reduce the formation of oxidized low-density lipoproteins (LDL) and reactive oxygen species (ROS), both of which contribute to atherosclerosis. In addition, GLP-1 RAs inhibit the activation of adhesion molecules and monocytes/macrophages in response to oxidized LDL, further decreasing monocyte accumulation in the vascular wall. GLP-1 agonists such as exenatide and liraglutide stimulate endothelial nitric oxide production, promoting vascular smooth muscle relaxation and endothelium-derived vasodilation.[32] Further, lixisenatide and dulaglutide support the differentiation of monocytes into less proinflammatory M2 macrophages.[33] This activity reduces foam cell formation, limits necrosis within atherosclerotic plaques, and prevents plaque rupture. Semaglutide, in particular, has been found to reduce intraplaque hemorrhage, thereby stabilizing plagues and slowing their progression.[34] Renal benefits of GLP-1 RAs include decreased urinary albumin excretion, prevention of new-onset macroalbuminuria, and a slower decline in estimated glomerular filtration rate (eGFR), with these effects believed to contribute to favorable renal outcomes by delaying the onset of macroalbuminuria.[35]

For patients with high cardiovascular risk or established atherosclerotic cardiovascular disease, the European Society of Cardiology (ESC) recommends GLP-1 RAs or sodium-glucose cotransporter-2 inhibitors as first-line therapy. Risk factors such as albuminuria, eGFR < 60 mL/min, and left ventricular hypertrophy are indicators of likely benefit from GLP-1 RAs. However, despite ESC and ADA guidelines, the actual adoption of GLP-1 RAs remains limited, largely due to their high costs, injection requirements, and contraindications in conditions such as pancreatitis, retinopathy, or medullary thyroid cancer.[36] Increased glucagon secretion plays a central role in the hyperglycemia observed in T2DM, and GLP-1 RAs have proven effective in increasing insulin secretion while inhibiting glucagon production, making them a key therapeutic approach for T2DM, especially for patients unresponsive to traditional therapies.[37]

4. Incretin mimetic classes

Incretin mimetics are broadly categorized into two classes: Exendin-4 analogs and human GLP-1 molecules. Exendin-4, a 39-amino acid peptide, exhibits 53% sequence homology with human GLP-1.[21] The exendin-4-based formulations include exenatide and lixisenatide, while the human GLP-1-based molecules comprise dulaglutide, semaglutide, albiglutide, and liraglutide. These medications for T2DM offer significant therapeutic benefits, such as delaying gastric emptying and inhibiting glucagon production from pancreatic alpha cells during episodes of elevated blood glucose. In addition, GLP-1 RAs promote pancreatic β-cell proliferation and reduce b-cell apoptosis, thereby enhancing insulin secretion and preserving β-cell function over time.[12,38,39] Furthermore, beyond single RAs (such as exenatide, liraglutide, dulaglutide, semaglutide, lixisenatide, and albiglutide), dual RAs (such as tirzepatide) and triple RAs (retatrutide) have been developed to target multiple receptors, such as GLP-1, GIP, and, in some cases, glucagon receptors. This multi-receptor targeting aims to

improve blood glucose regulation and promote weight loss.[21]

4.1. Exenatide

Exenatide (Figure 2) is a GLP-1 RA used as a monotherapy for T2DM in adults. This synthetic form of exendin-4, originally isolated from the saliva of a Gila monster (a venomous lizard species), has an amino acid substitution at position 2, resulting in a structure with approximately 53% homology to native GLP-1. Exenatide mimics GLP-1's effects by binding to and activating GLP-1 receptors on pancreatic β-cells, thereby stimulating insulin synthesis and secretion. Administered through subcutaneous injection, exenatide reaches peak plasma concentrations within about 2 h due to its rapid absorption rate. With a half-life of approximately 2 h, exenatide (marketed as Byetta) requires twice-daily dosing, typically administered 1 h before the morning and evening meals for optimal glycemic control.[40]

An extended-release formulation of exenatide, marketed as Bydureon, is available for once-weekly dosing. Unlike the native GLP-1 hormone, exenatide is resistant to degradation by the ubiquitous enzyme DPP4 and is primarily cleared by the kidneys through glomerular filtration. When used as an add-on therapy to metformin, exenatide has demonstrated significant improvements in glycemic control and reductions in fasting plasma glucose concentrations. Although extended-release exenatide is effective in T2DM management, it lacks cardioprotective properties and has relatively minimal effects on weight loss and HbA1c compared to other GLP-1 RAs.[41] Exenatide is generally well-tolerated, with mild side effects, including nausea, vomiting, diarrhea, and headache. However, it should not be administered to patients with end-stage renal failure due to its renal clearance.[42]

4.2. Liraglutide

Liraglutide (Figure 3) is an acylated analog of the human GLP-1 hormone, designed to mimic the effects of GLP-1 in regulating appetite and glucose metabolism. Marketed as Victoza for T2DM and Saxenda for weight management, liraglutide has about 97% structural homology with native GLP-1 and is produced using recombinant DNA technology. With a half-life of 13 h, it is administered once daily. It has been shown to improve insulin sensitivity, especially in patients with uncontrolled T2DM. The GLP-1 peptide backbone in liraglutide includes a fatty-acyl moiety, which confers resistance to DPP-4 degradation and allows non-covalent binding to serum albumin, thereby extending its half-life. The recommended starting dose is 0.6 mg/day for 1 week, with weekly titration up to a maximum of 1.8 mg/day. Liraglutide has been shown to significantly decrease HbA1c levels, postprandial glycemia, and fasting glycemia. In addition, it is linked to a decreased risk of weight gain and hypoglycemia.[43]

Liraglutide is indicated as an adjunct to diet and lifestyle modifications to improve glycemic control in patients with T2DM. When used concurrently with an insulin secretagogue, consider reducing the dose of the latter to mitigate hypoglycemia risk. Liraglutide has been approved to reduce the risk of major cardiovascular events and improve insulin sensitivity by upregulating glucose transporter-4. In the muscle and liver, it activates AMP-activated protein kinase, which enhances insulin sensitivity and promotes glucose metabolism.[44]

Liraglutide also exerts beneficial effects on oxidative stress and inflammation, contributing to improved insulin sensitivity. It has positive effects on endothelial and cardiac function, which are particularly relevant in T2DN, where low-grade inflammation plays a role in the disease's pathogenesis and related complications,

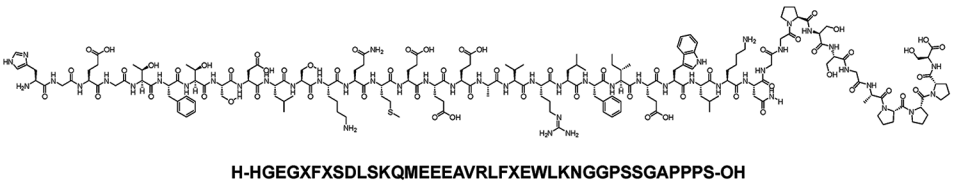


Figure 2. Chemical structure of exenatide. Reproduced from Al Musaimi[12]

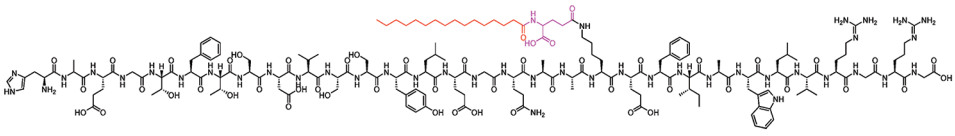


Figure 3. Chemical structure of liraglutide
Notes: Black: peptide backbone; Red: hexadecanoyl; Pink: Glu. Reproduced from Al Musaimi[12]

such as cardiovascular disease. In T2DM patients with cardiovascular disease or at high risk, liraglutide treatment lowers cardiovascular event risk.[45] It is also approved for secondary prevention of cardiovascular disease. Beyond its antidiabetic properties, liraglutide exhibits anti-apoptotic, anti-inflammatory, antioxidant, and neuroprotective effects, potentially making it useful in treating neurological diseases. Notably, liraglutide can traverse the blood-brain barrier. Its anti-inflammatory effects after intracerebral hemorrhage are mediated by GLP-1 amide, a metabolite generated through DPP-4 cleavage of liraglutide.[46] Liraglutide further aids insulin sensitivity by suppressing endogenous glucose production and glucagon release,[47] while its effects on gastric emptying and appetite reduction support weight management.

Victoza, a formulation of liraglutide for T2DM management, is prescribed with an initial dose of 0.6 mg once daily, titrating to 1.2 mg and up to 1.8 mg as needed. It enhances blood glucose control through multiple mechanisms, including increasing insulin secretion, reducing glucagon, slowing gastric emptying, and promoting weight loss through appetite suppression. Common side effects include nausea, vomiting, diarrhea, and headaches, while more serious risks involve pancreatitis and kidney injury. Victoza is contraindicated in patients with a history of medullary thyroid carcinoma or multiple endocrine neoplasia syndrome Type 2 due to an increased risk of thyroid tumors.[48]

In addition to its use in diabetes management, liraglutide is also available as Saxenda for long-term weight management in patients with weight-related conditions. Approved as an adjunct to a reduced-calorie diet and increased physical activity, Saxenda is indicated for individuals with conditions such as hypertension, dyslipidemia, or obesity. Common side effects include headache, constipation, heartburn, malaise, and localized reactions at the injection site. Proper storage is essential: unused pens should be refrigerated at 4–8 °C and protected from light and heat, and they should not be frozen.[49]

4.3. Dulaglutide

Dulaglutide (trade name Trulicity) is a long-acting GLP-1 RA with two modified GLP-1 analogs connected to an Fc fragment of human IgG4 (Figure 4). It is administered subcutaneously once weekly, at any time of the day, with or without food. The initial dose is 1.5 mg, with an option to gradually increase to 3 mg as needed. Dulaglutide has a half-life of 5 days, allowing for its weekly dosing. Patients using dulaglutide alongside insulin should avoid injecting it into the same area. It has demonstrated efficacy comparable to other GLP-1 RAs and is associated with improved glycemic control, as it lowers HbA1c, fasting glucose, and postprandial glucose levels.[50] In addition, dulaglutide supports weight loss and improves pancreatic β-cell function by promoting cellular regeneration, which increases β-cell mass and prevents apoptosis in various cell types, including muscle, lung, and pancreatic β-cells. These regenerative effects help slow the progression of T2DM and delay the need for insulin therapy.[50]

Dulaglutide also provides cardioprotective benefits, including reductions in blood pressure and improvements in lipid profiles, such as lowering LDL cholesterol. It has been shown to increase the number of endothelial cells and endothelial progenitor cells in peripheral blood, reduce persistent inflammation, enhance endothelial function, and ultimately boost arterial elasticity in patients with T2DM. As a second-line therapy, dulaglutide is recommended in addition to diet and exercise for patients who struggle to achieve glycemic control with metformin monotherapy. Dulaglutide has shown particular effectiveness in reducing fasting plasma glucose compared to postprandial blood glucose and acts through a glucose-dependent mechanism, resulting in a minimal risk of severe hypoglycemia. Mild gastrointestinal adverse effects, such as nausea, vomiting, and diarrhea, may occur, typically worsening in the first 2 weeks but diminishing over time. No dose adjustments are required in patients with renal or hepatic impairment when administering dulaglutide.[51,52]

Beyond its role as an antidiabetic agent, a 2023 study highlighted dulaglutide's potential to protect against sepsis-induced lung injury in mice.[53] Sepsis can trigger the excessive release of inflammatory mediators, including cytokines, ROS, and reactive nitrogen species. Through the activation of cyclic adenosine monophosphate/protein kinase A and cyclic guanosine monophosphate/protein kinase G signaling pathways, these reactive nitrogen species and ROS can damage mitochondria, leading to mitochondrial apoptosis.[54] Sepsis-related cardiac damage is also linked to oxidative stress, primarily induced by elevated ROS levels. Activation by lipopolysaccharides (LPS) markedly increases oxidative stress in cardiac cells, as evidenced by heightened nitric oxide generation and reduced glutathione levels. Following dulaglutide treatment, markers of oxidative stress are reduced, implying that dulaglutide may protect cardiac cells from LPS-induced oxidative stress injury. Two key markers of myocardial cell dysfunction, creatine kinase-myoglobin, and troponins, are frequently employed to evaluate acute myocardial infarction. LPS stimulation markedly increases creatine-kinase myoglobin expression in myocardial cells, confirming that LPS is a primary factor in myocardial dysfunction. Dulaglutide significantly decreased this dysfunction, suggesting a protective effect

against sepsis-induced myocardial injury.[55] In sepsis, nitric oxide synthase overactivity leads to excessive nitric oxide production, which, along with superoxide radicals, forms peroxynitrite. Peroxynitrite interferes with cardiac function by modifying cardiac load, downregulating β-adrenergic receptors, impairing Type I calcium channel function, and reducing the efficiency of the mitochondrial electron transport chain complex in cardiac cells.[55] Dulaglutide therapy drastically reduces nitric oxide production and nitric oxide synthase expression induced by LPS, indicating that dulaglutide may inhibit nitric oxide synthase activation driven by inflammatory factors.[56]

4.4. Semaglutide

Semaglutide (Figure 5) is an acylated GLP-1 analog with a modified amino acid structure. It is commonly marketed under various names and formulations: Ozempic and Wegovy as subcutaneous injections (at different doses and strengths), and Rybelsus as an oral formulation.[57] Semaglutide is prescribed as an adjunct to diet and exercise for glycemic control in individuals with T2DM. In addition, it is indicated to reduce the risk of major cardiovascular events, such as non-fatal myocardial infarction and stroke, in patients with a history of cardiovascular disease and T2DM. No dose adjustment is necessary for patients with renal failure or hepatic impairment when administering semaglutide.[58]

Wegovy is approved as a weight loss agent to be used in conjunction with a reduced-calorie diet and increased physical activity in individuals with obesity (body mass index [BMI] ≥30 kg/m^2) or overweight (BMI ≥27 kg/m^2) with at least one weight-related comorbidities. It is also indicated for overweight or obese individuals with existing cardiovascular disease at an increased risk of major adverse cardiovascular events. The growing incidence of obesity has made heart failure with preserved ejection fraction the most common form of heart failure, with its onset and progression significantly influenced by obesity and T2DM. Patients with heart failure and preserved ejection fraction (HFpEF)) are more likely to have T2DM individuals without heart failure, and this association is linked to worse hemodynamic and clinical outcomes, such as higher symptom burden and reduced functional capacity. Few effective treatment options exist for this population.[59]

Ozempic, a semaglutide-based GLP-1 RA, is indicated for T2DM management, with dosing that begins at 0.25 mg weekly for 4 weeks, then increases to 0.5 mg, and may reach 1 mg if needed for glycemic control. Administered subcutaneously, Ozempic enhances insulin secretion, suppresses glucagon release, and slows gastric emptying, helping manage post-meal blood glucose spikes. Its appetite-suppressing effects also contribute to weight loss, making it particularly beneficial for overweight or obese individuals with diabetes. Studies indicate Ozempic effectively lowers HbA1c, improves blood glucose control, and supports weight loss.[60]

Semaglutide, a GLP-1 RA, promotes glucose-dependent insulin release from pancreatic β-cells, inhibits excessive glucagon secretion, and delays gastric emptying. As a weight loss agent, it acts as a physiological regulator of appetite, reducing caloric intake centrally. While it influences insulin secretion, delayed gastric emptying is thought to play a larger role in regulating postprandial hyperglycemia. Semaglutide has demonstrated excellent efficacy in reducing weight and lowering blood glucose levels. Unlike conventional GLP-1 RAs, semaglutide directly affects the brainstem, hypothalamus, and lateral septal nucleus, impacting central neurons,[61] and thus promoting weight reduction. Semaglutide's distinct anorexigenic mechanism may explain its superior weight reduction effects compared to other GLP-1 RAs. This weight reduction effect appears to be independent of common gastrointestinal adverse events,

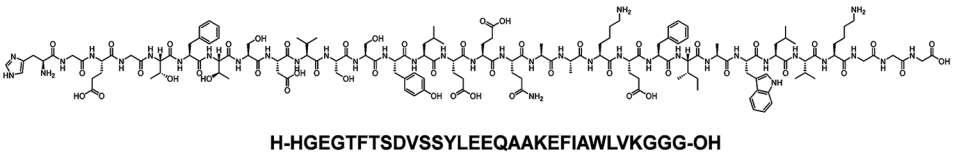


Figure 4. Chemical structure of dulaglutide. Reproduced from Al Musaimi[12]

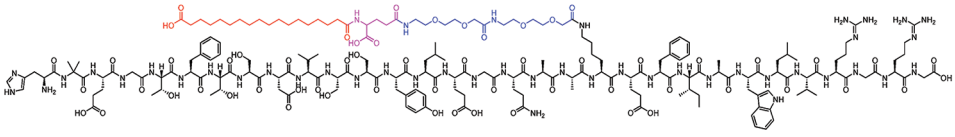


Figure 5. Chemical structure of semaglutide
Notes: Black: peptide backbone; Red: 17-carboxyheptadecanoyl (C18 diacid); Pink: Glu; Blue: 8-amino-3,6-dioxaoctanoic acid (ADO). Reproduced from Al Musaimi[12]

such as nausea and vomiting.[62] In addition, semaglutide lowers LDL cholesterol but, like other GLP-1 Ras, does not increase high-density lipoprotein cholesterol levels. The inhibition of cardiovascular events may be attributed to improved lipid metabolism, increased insulin secretion,[62] and reductions in chronic systemic inflammation.[63] Semaglutide has also shown promise in improving fatty liver by reducing triglycerides and significantly enhancing liver function markers. It is metabolized through the proteolytic breakdown of the peptide framework, followed by β-oxidation of its fatty acid side chain, and is primarily excreted through urine and feces.[64]

Rybelsus, an oral formulation of semaglutide, offers convenience in administration. It similarly delays gastric emptying, aiding in the control of postprandial glucose spikes. The starting dose is 3 mg daily for 30 days, followed by an increase to 7 mg, with the option to go up to 14 mg for additional glycemic control. Rybelsus also promotes weight loss by reducing appetite.[65] Due to nutrient-drug interactions, Rybelsus should be taken on an empty stomach, ideally on waking, with a small amount of water, at least half an hour before any food, liquids, or other oral prescriptions. While gastrointestinal adverse effects are common with GLP-1 RAs, most individuals on oral semaglutide tolerate it well. Counseling on potential sensations of fullness or nausea can improve adherence, as nausea often subsides over time.[66]

Recent publications[67] indicate several new and, in some cases, serious side effects of semaglutide, including hyperemesis gravidarum,[68] psychiatric adverse events,[69] biliary disease,[70] pancreatitis,[71] appendicitis,[72] bowel obstruction,[73] gastroparesis,[74] and elevated liver enzymes.[75]

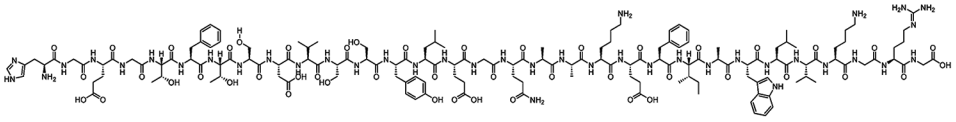
4.5. Albiglutide

Albiglutide (Figure 6), a complex polypeptide, acts as a GLP-1 RA and is commonly marketed under the trade name Tanzeum. Available in 30 mg/pen and 50 mg/pen, it is provided in a lyophilized state and requires reconstitution before use. Albiglutide comprises two GLP-1 molecules fused with human serum albumin, and it is administered as a 30 mg subcutaneous injection once weekly. If the glycemic response is insufficient, the dose can be increased to 50 mg weekly. Albiglutide reaches peak plasma concentrations in approximately 3 – 5 days, with a metabolism similar to human serum albumin in the vascular endothelium and a half-life of up to 5 days. Its mechanism includes slowing gastric emptying and increasing glucose-dependent insulin secretion. Compared to other GLP-1 RAs, albiglutide is associated with fewer episodes of nausea, likely due to its substantial molecular structure that prevents crossing the blood-brain barrier.[76]

Albiglutide is synthesized through recombinant DNA technology, comprising two tandem repeats of a fused 30-amino acid sequence. With only one amino acid alteration, the amino acid sequence of albiglutide closely resembles endogenous human GLP-1, with this alteration conferring resilience against DPP-4 degradation. The fusion of two recombinant molecules with human serum albumin effectively prolongs the plasma half-life of albiglutide.[77]

Albiglutide may have cardioprotective effects by reducing endoplasmic reticulum stress, modulating autophagy, and promoting anti-inflammatory pathways. The indirect beneficial effects of GLP-1 RAs on the ventricles largely stem from their positive impact on inflammation, endothelial function, and glucose uptake, as GLP-1 receptor expression in ventricular cardiomyocytes is relatively low. Furthermore, research suggests that GLP-1 RAs modulate extracellular matrix alterations, potentially attenuating cardiac remodeling post-myocardial infarction. In heart failure, characterized by calcium overload, GLP-1 RA therapy may modulate cytosolic Ca^{2+} levels by inhibiting phosphorylation of the ryanodine receptor 2 and blocking the activation of calmodulin-dependent protein kinase II. Additionally, GLP-1 mimetics demonstrate antioxidative properties, preventing the generation of intracellular and mitochondrial ROS in methylglyoxal-treated cells.[78,79]

Albiglutide is generally well tolerated, though rare adverse events such as pancreatitis and hypersensitivity reactions may occur. To prevent DPP-4-mediated degradation, a Gly8Ala mutation was introduced at the cleavage site of the GLP-1 molecule. Albiglutide employs a unique approach: two DPP-4-resistant GLP-1 RA copies are fused at the N-terminus, with the terminal arginine linked to albumin to enhance stability.[80]



H-HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-OH

Figure 6. Chemical structure of albiglutide. Reproduced from Al Musaimi[12]

4.6. Tirzepatide

Tirzepatide (Figure 7) acts as a dual agonist, targeting both the GIP and GLP-1 receptors. It is marketed as Mounjaro for adults with T2DM to improve glycemic control and as Zepbound for overweight adults with weight-related comorbidities (T2DM, hypertension, and dyslipidemia). By targeting multiple receptors, tirzepatide enhances insulin secretion and reduces glucagon levels, contributing to improved blood glucose management. In addition, it aids in weight loss, which benefits individuals with obesity-related health issues. The development of tirzepatide involved introducing GLP-1 activity into the GIP sequence, resulting in a balanced dual agonist. Tirzepatide exhibits comparable affinity for the GIP receptor to native GIP but binds to the GLP-1 receptor with about 5 times lower affinity than native GLP-1.[81] This higher GIP receptor activity relative to GLP-1 receptor activity contributes to its dose-dependent reduction in HbA1c.

Mounjaro simultaneously targets the GIP and GLP-1 receptors, promoting insulin secretion and inhibiting glucagon release in a glucose-dependent manner. This mechanism improves blood glucose control and reduces post-meal glucose spikes. Treatment typically begins at a dose of 2.5 mg once weekly for the first 4 weeks, after

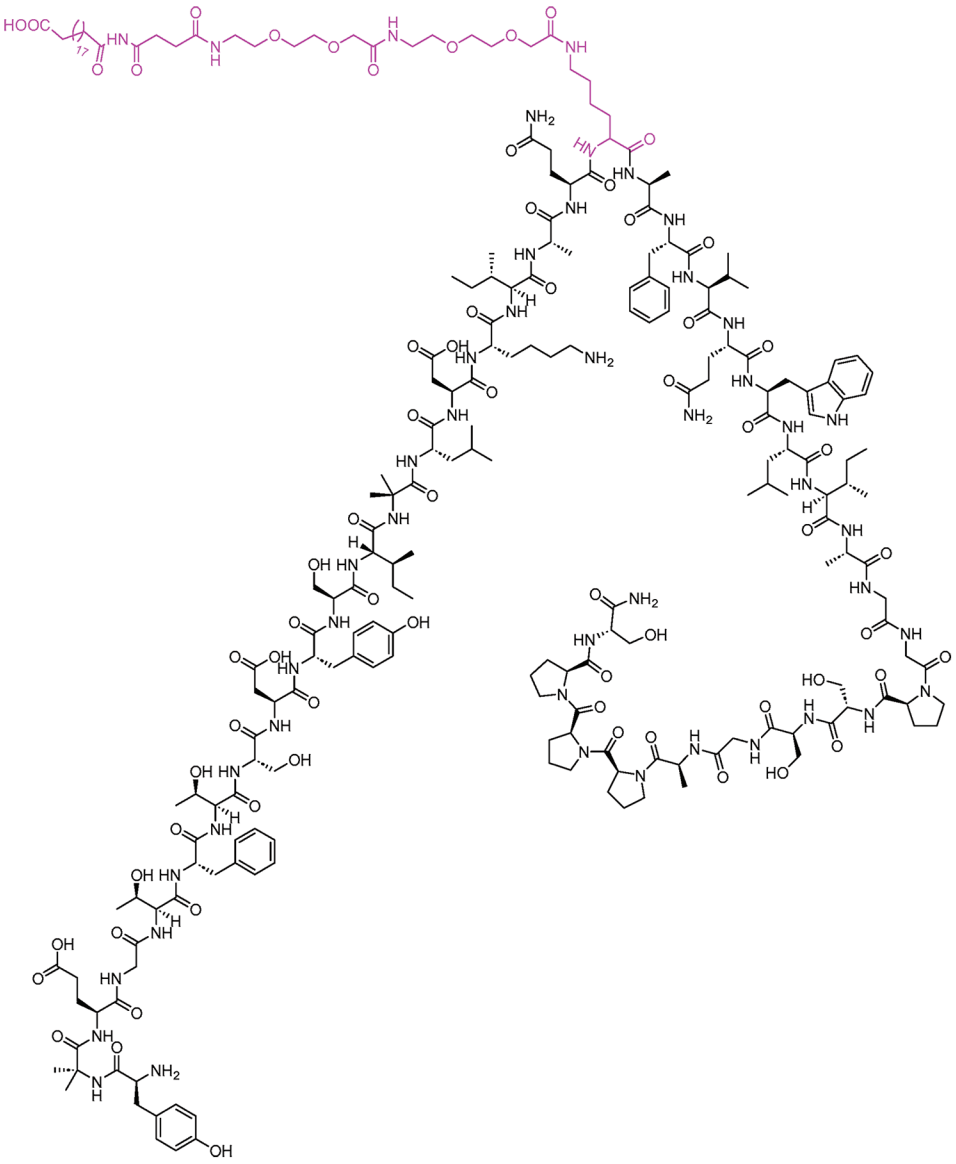


Figure 7. Chemical structure of tirzepatide
Notes: Pink: C20 fatty acid diacidic moiety. Reproduced from Al Musaimi[12]

which the dose can be increased to 5 mg once weekly. Depending on individual patient needs and response, the dosage may be further increased up to a maximum of 15 mg once weekly.[82]

Zepbound is prescribed alongside a calorie-restricted diet and increased physical activity to aid weight management in obese or overweight adults with at least one weight-related comorbidity, such as hypertension, dyslipidemia, T2DM, obstructive sleep apnea, or cardiovascular disease. Zepbound is administered subcutaneously, starting at 2.5 mg once weekly and potentially increasing to a maximum of 15 mg once weekly based on patient response and tolerance. The dual agonist action on pancreatic β-cells promotes insulin production, while GIP activity enhances white adipose tissue function. In addition, signals of both receptor pathways in the brain contribute to a stronger anorexigenic effect, supporting both glycemic control and weight reduction.[81] The most frequently reported side effects include gastrointestinal-related events (nausea, diarrhea, and vomiting) and decreased appetite.[83] Some psychiatric adverse events have also been noted.[84]

4.7. Lixisenatide

Lixisenatide (Figure 8), a GLP-1 RA, is commonly marketed as Adlyxin. It is a peptide composed of 44 amino acids. Similar to exenatide, lixisenatide is derived from exendin-4 but includes six additional lysine residues at its C-terminus, replacing a proline residue. The C-terminal amino acid (position 44) is amidated. Approximately 55% of lixisenatide is bound to plasma proteins, and it binds to GLP-1 receptors with an affinity up to 4 times higher than that of human GLP-1. Following subcutaneous injection, lixisenatide is rapidly absorbed, reaching peak plasma concentration in about 2 h, with a half-life of approximately 3 h.[85]

The primary elimination pathway for lixisenatide is glomerular filtration. Cytochrome P450 isoenzymes remain unaffected by lixisenatide. However, due to its effect on delaying gastric emptying, lixisenatide may slow the absorption of orally administered medications, such as warfarin, ethinyl estradiol, and acetaminophen. Caution is advised when administering lixisenatide with medications that have a narrow therapeutic range, such as warfarin, or with drugs that rely on precise dosing, such as antibiotics. For drugs that require timely absorption, it is recommended to administer them at least an hour before lixisenatide. For oral contraceptives, the administration should occur either 1 h before or 11 h after the lixisenatide injection.[53]

Lixisenatide has a prolonged half-life due to its exendin-4 backbone and is administered once daily at an initial dose of 10 mcg via injection. After 2 weeks, the dose may be increased to 20 mcg. Lixisenatide slows gastric emptying, which significantly impacts postprandial glycemia. As monotherapy, it effectively reduces HbA1c, fasting glucose, and postprandial glycemia. In animal studies, lixisenatide administered peripherally has shown the ability to cross the blood-brain barrier. Its elimination is thought to occur through glomerular filtration, tubular reabsorption, and metabolic catabolism, although the composition of its metabolites and their possible mechanisms of action remain unknown. Lixisenatide is linked to modest decreases in body weight, a considerable reduction in HbA1c (0.5–1%),

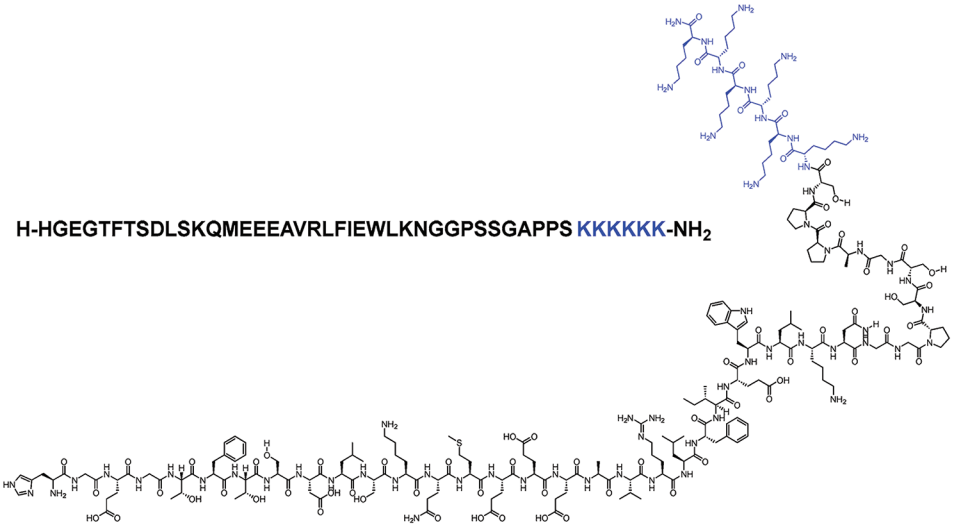


Figure 8. Chemical structure of lixisenatide
Notes: Blue: Differences from exenatide. Reproduced from Al Musaimi[12]

and decreases in postprandial and fasting plasma glucose. Studies have shown that, for HbA1c reduction, lixisenatide is non-inferior to twice-daily exenatide and is generally well tolerated.[86]

The most commonly reported side effects of lixisenatide are gastrointestinal symptoms, such as nausea, vomiting, and diarrhea. These side effects are typically dose-dependent and often diminish in intensity within the first 3 – 6 weeks of treatment. Immunogenic effects have been reported with lixisenatide; patients who develop anti-lixisenatide antibodies may experience a slightly higher frequency of allergic responses and injection site reactions compared to those without antibodies. Routine antibody testing is not required, but patients may consider switching to an alternative antidiabetic therapy if they experience significant glycemic control issues or persistent injection site reactions.[87]

When lixisenatide is introduced to existing regimens, such as basal insulin therapy or sulfonylurea treatment, adjustments to all diabetes medications may be required to mitigate the risk of hypoglycemia. Although mild-to-moderate renal impairment does not necessitate a dosage adjustment, close monitoring is recommended, as these individuals may be more susceptible to adverse effects, such as dehydration, which could deteriorate kidney function. Individuals with severe renal impairment should be constantly monitored for worsening renal function and a higher likelihood of adverse events, especially gastrointestinal issues.[86,88]

4.8. Retatrutide

Retatrutide (Figure 9) is a novel investigational medication designed as a triple-hormone RA, targeting the GLP-1, GIP, and glucagon receptors. This unique mechanism addresses both glycemic control and weight management, making it particularly relevant in the treatment of obesity and T2DM. Recent clinical trials have demonstrated retatrutide's potential to induce significant weight loss and improve metabolic parameters in individuals with obesity and T2DM. By acting on both glucose metabolism and appetite regulation, retatrutide offers a promising avenue for enhanced patient outcomes.[89,90]

In a Phase 2 clinical trial, retatrutide was administered to participants with a BMI of 30 or higher, resulting in substantial weight loss over 24 weeks. Participants receiving retatrutide achieved an average weight reduction of 12 – 15% compared to the placebo group. In addition, improvements in glycemic control were observed, with reductions in HbA1c levels and fasting plasma glucose. These trial results indicate that retatrutide not only promotes weight loss but also has a positive impact on associated metabolic disorders, positioning it as a compelling option for patients with obesity and T2DM.[91,92] The safety profile of retatrutide has been generally favorable, with most adverse events being mild–to-moderate in severity. Common side effects included gastrointestinal symptoms such as nausea, diarrhea, and abdominal pain, which typically resolved with continued treatment. Notably, the incidence of hypoglycemia was low, a significant advantage over some existing therapies for T2DM. Although long-term safety and effectiveness are still under investigation, initial findings suggest that retatrutide may become a valuable addition to the therapeutic option for obesity and T2DM, potentially improving patients' quality of life and metabolic health.[93]

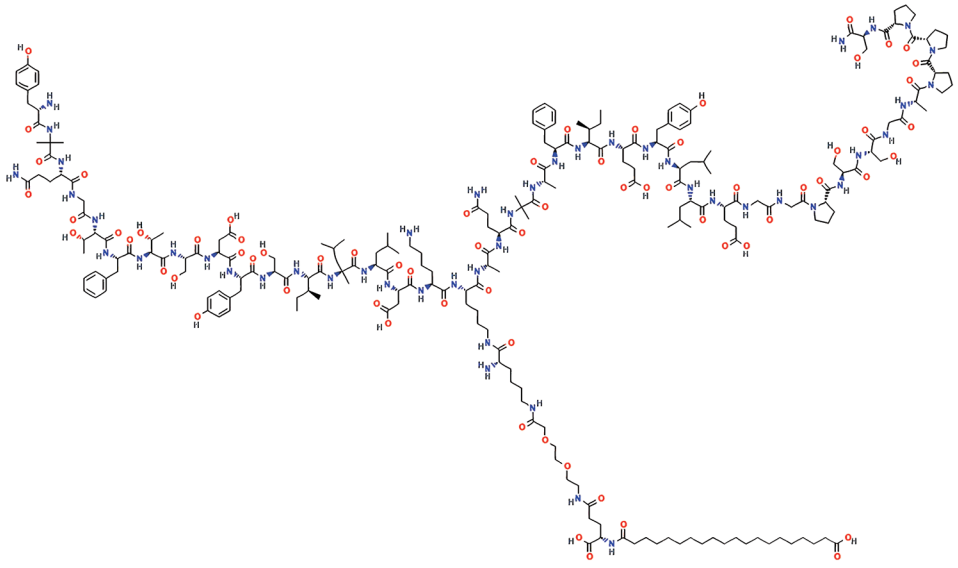


Figure 9. Chemical structure of retatrutide. Reproduced from Caturano *et al.*[94]

5. Conclusion

Diabetes management focuses on alleviating hyperglycemia symptoms and reducing the risk of long-term microvascular and macrovascular complications, such as diabetic nephropathy, neuropathy, retinopathy, and cardiovascular effects. There is a growing demand for medications that not only regulate hyperglycemia but also address additional metabolic risk factors to improve cardiovascular outcomes. The primary causes of T2DM include insulin resistance, pancreatic β-cell dysfunction, and excessive or inappropriate glucagon secretion. The rising prevalence of T2DM can be attributed to obesity, aging, genetic susceptibility, and diverse demographic factors. This chronic condition contributes to both microangiopathy and macroangiopathy, thereby heightening the risk of cardiovascular disease, which leads to increased economic burden and reduced life expectancy.

Obese patients with T2DM require stricter glycemic control and weight management strategies, as obesity is a significant risk factor for both the pathophysiology of T2DM and associated macroangiopathy. Pharmacological interventions, in combination with lifestyle modifications, such as diet and exercise, can effectively manage both obesity and T2DM. GLP-1 RAs have emerged as valuable therapeutic options for T2DM patients, as they target multiple pathophysiological mechanisms of the disease. These agents reduce glucagon secretion, slow gastric emptying, and enhance glucose-dependent insulin secretion, while also suppressing appetite and promoting weight loss. The central and peripheral effects of GLP-1 RAs contribute to reduced food intake, enhanced insulin sensitivity, and improved pancreatic β-cell function.

While GLP-1 RAs offer substantial therapeutic benefits, including improved glycemic control, reduced HbA1c, and decreased fasting and postprandial glucose levels; they are associated with certain side effects. Common adverse reactions include gastrointestinal symptoms such as vomiting, diarrhea, and nausea, which may lead to acute kidney injury due to volume depletion. Other possible side effects include headaches, infections, mild tachycardia, dyspepsia, and dizziness. Erythema and pruritus at the injection site are frequent, particularly with longer-acting agents in this drug class. Immunogenicity, including antibody development against specific GLP-1 RAs like exenatide, may reduce the drug's efficacy and, in rare cases, lead to anaphylaxis or injection site reactions. Contraindications for GLP-1 RAs include pregnancy, hypersensitivity, and certain gastrointestinal conditions such as gastroparesis and inflammatory bowel disease. Recent reports also suggest that GLP-1 monotherapy may increase the risk of new-onset non-proliferative diabetic retinopathy and diabetic macular edema.[95-100]

The use of incretin mimetics in managing T2DM, obesity, and related metabolic disorders offers a promising potential, particularly when used in combination with nutraceuticals, lifestyle modifications, and dietary interventions that enhance physiological responses.[101]

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Writing – original draft: All authors
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Fermentation-derived compounds and their impact on skin health and dermatology: A review

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Abstract

Fermented plant extracts have recently become popular ingredients in various cosmetic and dermal applications, mainly because of their superior biological activities. In fermentation by microorganisms, the concentration of the useful compound is increased or new phytochemicals are introduced, enhancing their biological activity in anti-inflammatory, antibacterial, wound-healing, anti-melanogenic, and antioxidant applications. The fermented state of *Panax ginseng* shows higher anti-wrinkle and whitening effects than its non-fermented counterpart. Black ginseng promotes collagen synthesis and inhibits melanogenesis after fermentation. Fermented extracts of *Magnolia denudata*, organic *Indica rice bran*, and unpolished *Black rice* also show very good antioxidant and skin-whitening activities. Hyaluronic acid, kojic acid, citric acid, glycolic acid, and ascorbic acid are acids produced by the fermentation of natural products, forming smaller molecules size that are more effective than high molecular size and able to penetrate deeply into the skin, maintaining its microbiota balance and providing antioxidant protection. Fermented vitamins include B3, A, and E produced through microbial fermentation with added stability and bioactivity to contribute toward better skin health, promotion of absorption, anti-inflammation, and antioxidant properties. Skin health is modulated by its resident microbiota and their metabolites, among which fermented microorganisms have gained interest as active skincare ingredients. It is known that metabolites from skin-associated bacteria improve skin pigmentation and wrinkle appearance, such as *Nitrosomonas eutropha*, which oxidizes ammonia produced from sweat into nitric oxide derivatives. Similarly, antimicrobial peptide from *Enterococcus faecalis* strain proved to be potent against *Cutibacterium acnes*; hence, anti-acne treatments have been commercialized. In addition, *Vitreoscilla filiformis* found in hot springs significantly ameliorated symptoms of atopic dermatitis and photoaging while *Streptococcus thermophilus* enhanced levels of the natural ceramides, thereby enhancing skin barrier and hydration.

Keywords: Fermented plant extracts; Anti-melanogenic; Antioxidant protection; Skin barrier; Anti-wrinkle effects; Anti-inflammatory; Wound healing; Skin microbiota balance

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1. Introduction

Dermatology and cosmetic science increasingly embrace the use of fermented plant extracts (FPEs) with their profound effects on skin health and beauty. Fermentation is a time-honored process that avails itself of microorganisms in the deconstruction of organic materials into their constituent parts, therefore increasing bioavailability and allowing the creation of new phytochemicals with superior properties. This biotechnological development has had very positive results on the formulation of skincare products that are much more effective than their non-fermented counterparts. Fermentation will enhance the anti-inflammatory, antibacterial, wound-healing, anti-melanogenic, and antioxidant properties of plant materials substantially, which makes them valuable addenda in today's skincare regimens.[1] One impressive example regarding the benefits of fermentation involves the transformation of *Panax ginseng.* This traditional herb shows a more powerful anti-wrinkle and whitening effect when fermented, higher in efficacy to reduce the visible signs of aging and hyperpigmentation than the one that is non-fermented.[2] This improvement is due to an increase in the concentration of bioactive compounds and the generation of new phytochemicals produced through fermentation. Similarly, fermented black ginseng enhanced the synthesis of collagen and reduced melanogenesis, further supporting the maintenance of skin that is young and evenly toned.[3]

Besides plant extracts, other natural materials subjected to a fermentation process also serve beneficial purposes, including extracts from fermented extracts of *Magnolia denudata* (Common name: Yulan Magnolia) Scientific name: *Magnolia denudata*, unpolished black rice (Common name: forbidden rice or purple rice) Scientific name: *Oryza sativa japonica*, which have major antioxidant and skin-whitening properties.[4,5] These fermented materials also have strong protective activity against oxidative stress, one of the major causative agents in skin aging and damage. The increased efficiency of the extracts in question would be in turn a direct result of the fermentation process as a method enabling an increase in active ingredient content and introducing metabolic products not present within raw materials. Another group of valuable metabolites produced by fermentations are fermented acids, such as hyaluronic, kojic, citric, glycolic, and ascorbic acids. With a smaller molecule size than their non-fermented cousins, these acids are well recognized for superior penetration through the skin. This superior level of skin penetration enables them to exhibit deeper therapeutic effects, maintain microbiota balance, and provide antioxidant protection.[6] Moreover, the fermentation process generally enhances the stability of these acids, which may be one more reason for their effectiveness in cosmetic formulations. Besides acids, microbial fermentation of vitamins, such as B3, A, and E has also been shown to improve stability and bioactivity. Fermented vitamins help improve skin health by enhancing better skin absorption, reducing inflammation, and providing defense against oxidation. Fermentation enhances the bioactivity of such vitamin manifold, which thereby allows them to be much more effective in mitigating various skin concerns related to inflammation, dryness, and oxidative damage.[7] However, the role of fermented microorganisms in skin care does not stop at the mere fermentation of plant extracts and acids. Skin metabolites generated by skin-associated bacteria, such as *Nitrosomonas eutropha* and *Enterococcus faecalis*, have provided immense benefits to skin health. *N. eutropha* is known to convert ammonia that is formed from sweat to nitric oxide compounds that are known to be beneficial for toning down pigmentations and wrinkling, while *E. faecalis* excretes out antimicrobial peptides which are active against *Cutibacterium acnes* and hence result in new anti-acne treatments.[3,8] In addition, probiotics, such as *Lactobacillus paracasei* and *Lactobacillus rhamnosus* have other advantages, such as enhancing immune responses, inhibiting inflammation, and improving the skin barrier.[5] This review elucidates the heightened awareness in regard to fermentation playing a better role in skin health and further developments that are likely to emerge on the care of the skin. The aim of this review is to find different functional fermentation products from deferent source as plant, vitamins, amino acids, and microbiome metabolites as shown in (Figure 1).

2. Natural-derived functional substances using microbial fermentation

2.1. Plant fermentation effect on skin health

FPEs are practical liquids that are made by the fermentation of fresh plants by microorganisms, mostly bacteria and fungi. Fermentation must be carried out to provide a suitable matrix of biologically active molecules with a range of biological characteristics. Presenting verified data on the biological activity of FPEs for cutaneous and cosmetic applications is the aim as well as some potential applications for the skin. Furthermore, fermentation can increase the antioxidant activity of plant extracts associated with increased concentrations of phytochemicals, mainly by causing microbial hydrolysis or biotransformation to produce antioxidant peptides, antioxidant polyphenols, and antioxidant polysaccharides.[9]

Lactobacillus brevis has been used to assess *P. ginseng* (red ginseng [RG]) and fermented RG (FRG) as

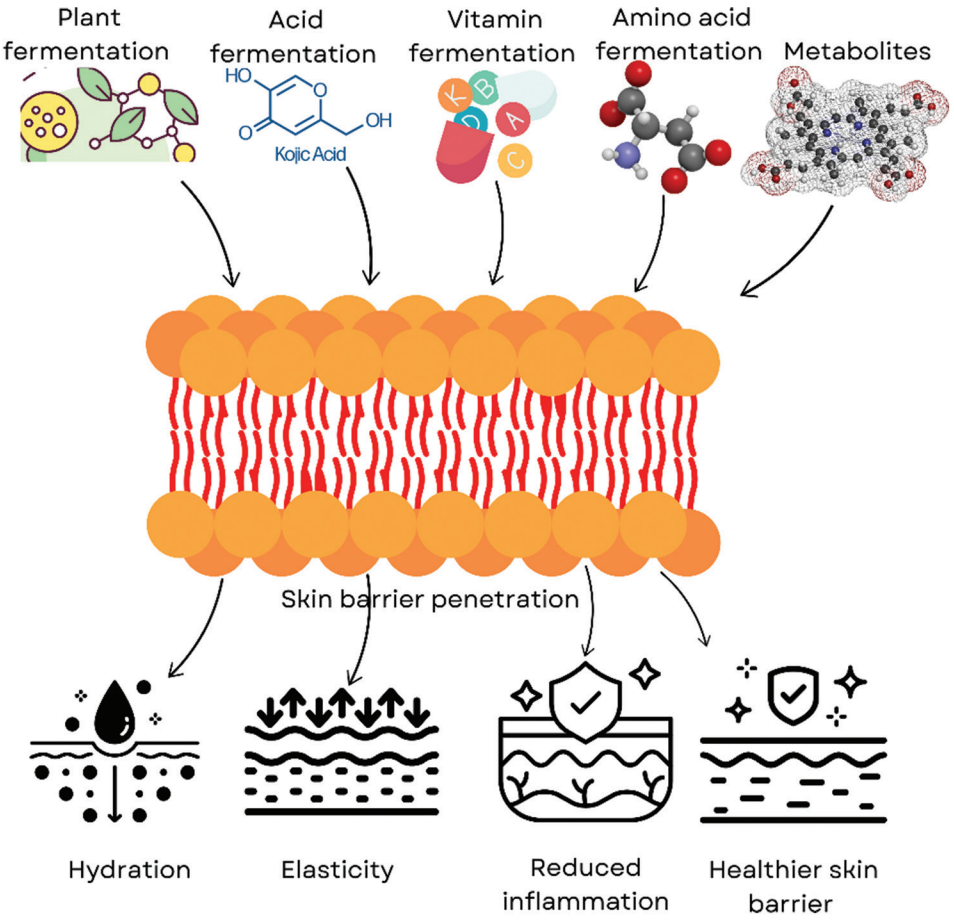


Figure 1. The mechanism of activity of the fermented compounds on skin

cosmetic components, specifically focusing on their anti-aging potential and skin safety. Compared to RG, FRG demonstrates higher concentrations of uronic acid, flavonoids, and polyphenols, as well as elevated antioxidant activity. Notably, no significant changes have been observed in the total ginsenoside content, although FRG exhibits higher levels of ginsenoside metabolites than RG. The tyrosinase inhibitory activity (half-maximal inhibitory concentration [IC^{50}) of FRG (27.63 μg/mL) is more potent than that of RG (34.14 μg/mL). Similarly, the elastase inhibitory activity (IC_{50}) of FRG (117.07 μg/mL) surpasses that of RG (157.90 μg/mL). Both 10% RG and 10% FRG are classified as essentially non-irritating materials in initial skin irritation tests. However, in skin sensitivity assessments, the RG group demonstrated a sensitization rate of 100% and an average irritation score of 1.4, as opposed to the FRG group, which demonstrated a sensitization rate of 20% and an average irritation score of 0.2. After fermentation, FRG possesses elevated levels of ginsenoside metabolites, including Rg3, Rg5, Rk1, compound K, Rh1, F2, Rg2, and flavonoids. Consequently, when compared to RG, FRG has better anti-wrinkle and whitening properties and lower toxicological potency.[10]

Fermented *Panax notoginseng* polysaccharide (FPNP) prevents the decline in antioxidant activity and the expression of the antioxidant enzymes catalase, glutathione peroxidase, and superoxide dismutase, which are typically induced by hydrogen peroxide (H_2O_2) and reduces reactive oxygen species (ROS) and malondialdehyde levels. Moreover, it was reported that FPNP, fermented by *Saccharomyces cerevisiae*, prevented the damage produced by H_2O_2 in collagen and elastin in human fibroblast cells.[11]

Using human fibroblasts (HS68), an *in vitro* experiment assessed FBG as a potential skin-whitening and anti-wrinkle ingredient. At concentrations below 10 μg/mL, FBG did not exhibit cytotoxicity, while at doses up to 100 μg/mL, the bio ferment was harmless to the eyes. In human fibroblasts, it significantly increased the type I procollagen expression level at doses ranging from 0.3 to 10 μg/mL when fermented by *S. cerevisiae*.[12] FBG's anti-wrinkle

action was accompanied by an increase in type I collagen synthesis and a decrease in matrix metalloproteinase-1 activity. Moreover, FBG has skin-whitening qualities as it inhibits tyrosinase, reduces melanogenesis, and decreases dihydroxyphenylalanine oxidation.[13]

The flower petal extract of *Magnolia denudate*, fermented by *Pediococcus acidilactici*, exhibits antioxidant and anti-cancer properties. The concentration of the fermented extract is 3 times higher than its non-fermented counterpart. Moreover, the radical-scavenging activity of fermented *M. denudate* against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) increased from 85.1% to 91.4%, depending on the duration of fermentation.[14]

Fermented organic *Oryza sativa L.* (indica rice bran) has emerged as a key ingredient in skincare products, offering hydration, brightening, and anti-wrinkle benefits. Its effects are comparable to Pitera – a naturally occurring byproduct of sake lees fermentation – but it is free from alcohol-related irritation, making it highly effective for sensitive skin. It is fermented in a clean, safe environment by lactic acid bacteria. In terms of anti-oxidative properties, a 100.0 mg/mL bran fermentation solution demonstrates 71.4% higher DPPH radical-scavenging activity than Vitamin C at the same concentration and chelate Fe^{2+} ions 79.0% more effectively than ethylenediaminetetraacetic acid. Moreover, the superoxide anion-scavenging capacity of 10.0 mg/mL bran fermentation solution was 42.9% higher than butylated hydroxytoluene at the same dosage. The bran fermentation solution also inhibits the formation of dopachrome, a melanin intermediate, in a concentration-dependent manner, with higher concentrations providing greater inhibition. The IC_{50} of the bran fermentation solution and arbutin were 9.23 mg/mL and 0.52 mg/mL, respectively.[15] Fermented unpolished black rice (FUBRS), processed using a mixture of microbes isolated from *loog-pang*, demonstrates superior capacity to reduce melanin concentration in melanoma cells. In addition, it prevents melanogenesis in other melanoma cells by phosphorylating AKT, ERK, and p38. The marked decline in intracellular tyrosinase activity is in line with melanin reduction activity. The human fibroblast and melanoma cell lines are not cytotoxically affected by FUBRS. In addition, it decreases the expression levels of tyrosinase, tyrosinase-related protein 1 (TYRP-1), TYRP-2, and microphthalmia-associated transcription factors in both transcript and protein levels.[16]

A fermented extract of soybean, fermented with *Bacillus bacterium* and processed into a dry powder form, provides the skin with essential nutrients. By incorporating skin-aging inhibitors and whitening agents as active ingredients, the composition moisturizes the skin, improves elasticity, enhances skin tone, and provides antioxidant benefits.[17] Moreover, an extract of *Zanthoxylum schinifolium*, fermented with *L. rhamnosus* A6-5, showed greater antibacterial activity than raw extract when tested against *Propionibacterium acnes* and *Staphylococcus epidermidis*. Similarly, fermented blueberry fruit extract demonstrated antibacterial activity against *Brevibacterium linens*, *P. acnes*, *Bacillus cereus*, and *S. epidermidis* when fermented with particular probiotic bacteria (*Bacillus amyloliquefaciens* and *L. brevis*) and yeast (*Starmerella bombicola*) isolated from fermented starfish.[18]

2.2. Production of acids through fermentation for skin care

In the cosmetics industry, fermented acids are gaining attention by combining innovative and traditional methods to improve skincare regimens. Fermented acids are produced through fermentation, breaking down natural substances into smaller, more potent molecules. This increases the concentration of active chemicals and improves bioavailability, allowing them to penetrate the skin more deeply. Fermented acids have many advantages, including the capability to maintain the skin's microbiota. These compounds assist in minimizing acne, reduce inflammation, and relieve skin problems such as rosacea and eczema by maintaining a healthy balance of bacteria on the skin. Furthermore, the high antioxidant content of fermented acids shields the skin from ultraviolet (UV) rays and free radical damage. Moreover, the fermentation frequently yields novel, health-promoting compounds, including vitamins, peptides, and amino acids that improve the skin's overall appearance and health. These components greatly complement any skincare routine since they increase moisture retention, boost collagen production, and improve skin texture.[19]

Hyaluronic acid (HA) is a glycosaminoglycan widely distributed in humans and other mammalian tissues. It has numerous applications in both the medical and cosmetic industries, however, it excels as a humectant due to its capacity to form viscoelastic hydrogels in aqueous solutions, making it an ideal ingredient as a natural skincare moisturizer. The low yield of this polysaccharide when extracted from animal sources (such as rooster combs) was a key factor that prompted the commercial release of the biotechnologically produced version. Gram-positive endotoxins and proteins present in the membrane of the *Streptococcus* strain used to produce high molecular weight HA are potentially hazardous substances and must be removed through a sequence of filtration processes. These laborious purification steps are avoided by using recombinant *Bacillus subtilis*, which secretes HA into a fermentation medium. The medium is then sprayed and

dried at a high temperature to produce HA powder.[20] HA serves several functions based on its molecular weight.[21] High-molecular HA (about 1 MDa) increases the viscosity and stability of the product film that forms on the skin, hence decreasing transepidermal water loss and improving the hydration of the upper layers of the epidermis. At a lower molecular weight (about 250 kDa), HA interacts with skin cells and extracellular components, providing deeper hydration and wrinkle reduction. This enables HA to function as a signal molecule that triggers the synthesis of natural HA. Reports have suggested that more potent anti-aging effects may arise from molecules with smaller molecular weights.[22]

Another molecule that is becoming increasingly important is kojic acid, which primarily acts as a skin-lightening agent. It is synthesized by strains from the genera *Aspergillus* and *Penicillium*. Kojic acid improves the ability of skin care products to shield users from UV rays and reduces skin hyperpigmentation by inhibiting the production of tyrosinase. According to the Global Industry Analysts, there is a high demand for whitening creams in the Middle East, Asia, and Africa. Moreover, given its economic potential, kojic acid consistently draws attention for its environmentally friendly synthesis, with research continually being conducted to enhance its production.[23]

Citric acid is a member of the class of molecules known as alpha hydroxyl acids (AHAs) and functions as an exfoliating agent in skin care products. AHAs can eliminate dead skin cells from the skin's outermost layer, a process known as exfoliation, leaving the skin radiant and youthful. Over the past few years, there has been a steady increase in demand for cosmetic products containing AHAs, particularly in the Western European and Asian markets. In 1916, research conducted by James Currie enabled the profitable industrial synthesis of citric acid from *Aspergillus niger* using molasses and sucrose as primary carbon sources. He discovered that several strains of *A. niger* can produce significant amounts of citric acid. The two most important findings were: (i) citric acid production could succeed at a pH of approximately 2.5 – 3.5, which inhibited the formation of gluconic and oxalic acid, and (ii) citric acid production increased as sugar content rose.[24] In addition, since the 1960s, scientists have made significant efforts to introduce *Yarrowia lipolytica* yeast as an alternative to conventional fungal technology. *Y. lipolytica* can produce citric acid using a variety of carbon sources. Furthermore, *Y. lipolytica* possesses several desirable properties, such as withstanding lower pH levels, making it more economical and tolerant to high quantities of carbon sources and contaminants found in substrates.[25]

In addition to citric acid, glycolic acid is another AHA known in the skincare industry. Fermentation of glycolic acid breaks it down into smaller molecules, allowing for easier absorption into the skin. This helps with exfoliation, resulting in a smoother and more radiant complexion. Glycolic acid improves skin hydration and barrier function by boosting collagen production, which helps to reduce the appearance of fine lines and wrinkles, giving the skin a more youthful and firmer appearance. It can also introduce beneficial antioxidants that protect the skin from environmental stressors and free radical damage. Currently, glycolic acid is primarily produced using petrochemical resources, which involves the usage of hazardous formaldehyde. Alternatively, a biotechnological production pathway offering a sustainable approach is more desirable. Producing glycolic acid from lignocellulosic biomass feedstocks would be more economically and environmentally viable. Numerous bacteria naturally produce glycolic acid by hydrolyzing glycolonitrile and oxidizing ethylene glycol. However, developing a pathway that allows for the flexible usage of alternative, more abundant carbon sources under standard bioprocessing conditions would be more advantageous, as current production pathways depend on ethylene glycol or glycolonitrile, and specific environmental conditions. One less-explored but promising production pathway is the glyoxylate cycle, where C2 molecules, such as ethanol are naturally converted into glyoxylate, which can be further converted into glycolic acid through metabolic engineering. Other than that, several methods have been proposed for modifying *Escherichia coli*'s glyoxylate cycle to enhance glycolic acid production.[26]

Ascorbic acid, also known as Vitamin C, is a water-soluble essential vitamin and functions as a crucial cofactor for several enzymatic reactions in the body.[27,28] The antioxidative ability of ascorbic acid to scavenge free radicals and minimize oxidative stress has led to its growing use in cosmetic products.[29] Ascorbic acid is produced through a fermentation process that frequently uses several microorganisms. In the first stage of the fermentation, the bacterium *Gluconobacter oxydans* converts D-Sorbitol into L-Sorbose. In the next stage, a mixed culture of *Ketogulonicigenium vulgare* and *Bacillus* species converts L-sorbose into 2-keto-L-gulonic acid, a pre-cursor of ascorbic acid. Together, these microbes perform a two-step fermentation process that effectively produces ascorbic acid.[30]

2.3. Production of vitamins through fermentation for skin care

Fermented vitamins are revolutionizing the cosmetics market with their potent skin benefits and enhanced

bioavailability. The fermentation process, driven by beneficial microbes, transforms the vitamins into more stable and bioactive forms that are particularly effective in skincare products. During fermentation, complex vitamin structures are broken down, enhancing the skin's ability to absorb and utilize the nutrients. The use of fermented vitamins in cosmetics includes two key benefits: (i) improved absorption and (ii) increased effectiveness. Moreover, fermented vitamins exhibit stronger antibacterial and anti-inflammatory properties compared to their non-fermented counterparts, making them valuable in treating various skin issues, such as aging and acne. Research on fermented vitamins in cosmetics can result in novel formulations with improved advantages for skin health, opening doors to more sustainable and effective beauty products.[19]

Niacinamide, also known as Vitamin PP, Vitamin B3, or nicotinamide, is a component of coenzymes involved in hydrogen transfer and has several positive effects on the skin. The topical application of niacinamide helps to cure atopic dermatitis by stabilizing the skin barrier, reducing transepidermal water loss, and promoting the synthesis of ceramides and proteins such as filaggrin, keratin, and involucrin. It has been observed that topical treatment with niacinamide lowered transepidermal water loss in dry skin and improved the synthesis of cholesterol, free fatty acids, and ceramide – the main pathogenic components responsible for barrier failure in atopic dermatitis. The anti-inflammatory properties of niacinamide and its ability to reduce the production of several inflammatory cytokines (interleukin [IL]-1β, IL-6, IL-8, and tumor necrosis factor), make it a promising treatment for acne, rosacea, and other inflammatory skin diseases.[31] As for the production of niacinamide, reports have demonstrated that Vitamin B3 can be produced using recombinant *E. coli* expressing *Rhodococcus rhodochrous* nitrile hydratase.[32]

Another vitamin of interest in skin care applications is Vitamin A, also known as retinoid or β-carotene. Vitamin A can be derived from several sources, such as animal products (meat, dairy products, and fish), colored fruits and vegetables (β-carotenoid or provitamin A), as well as retinol and retinyl ester. In addition to retinol and its natural counterparts, the retinoid family includes numerous synthetic compounds. Retinoids interact with several cellular and nucleic acid receptors, such as the nuclear retinoic acid receptor family, the cellular retinol-binding protein, and the cellular retinoic acid-binding protein types I and II. The mechanism of action of retinoids involves regulating cellular differentiation, which enhances photoaging by promoting epidermal proliferation, leading to epidermal thickening, and stimulating the deposition of glycosaminoglycans. In addition, Vitamin A, as an antioxidant, helps to reduce DNA damage from free radicals, thereby preventing neoplastic transformation and carcinogenesis.[31] In terms of production, Vitamin A or β-carotene is produced by the yeast, *Y. lipolytica*. The production of β-carotene is increased by overexpressing heterologous carotene synthase in *Y. lipolytica*. After screening for the most effective promoter, the modified strain achieved a fermentation yield of 1.5 g/L. By employing fed-batch fermentation and perfectly adjusting the fermentation conditions, the production titer of β-carotene was further increased to 6.5 g/L and 90 mg/g dry cell weight.[33]

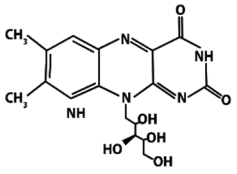
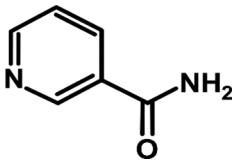
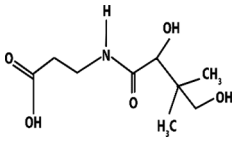
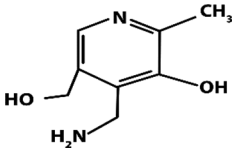
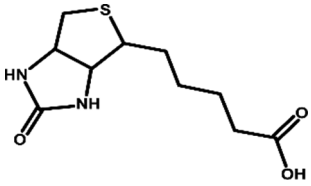
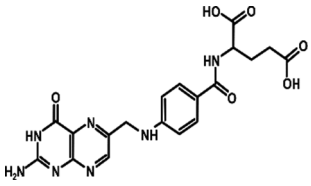
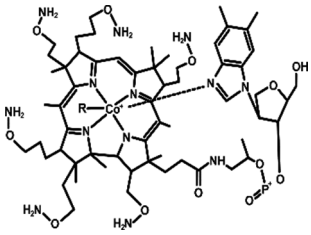
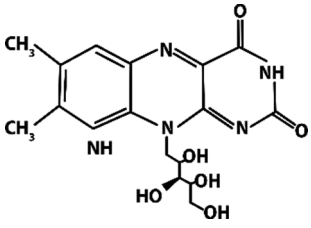
Vitamin E, including tocopherols and tocotrienols, is a group of lipid-soluble antioxidants. Vitamin E serves as the primary defense against lipid peroxidation by inhibiting the synthesis of ROS molecules, thereby preserving the integrity of cell membranes. The antioxidant activity of Vitamin E can be significantly enhanced by ascorbic acid, Vitamin B3, selenium, glutathione, and other biological factors. The potent antioxidant properties of Vitamin E make it an ideal compound for avoiding skin aging, treating several skin disorders such as atopic dermatitis, and melasma, and preventing scar formation. Topical application of Vitamin E is generally safe, with minimal reported side effects and a low frequency of minor irritations. Reports have shown that formulations containing 0.2% α-tocopherol effectively increased Vitamin E levels in the stratum corneum (SC) and decreased lipid peroxidation *in vivo*, thereby protecting the skin from the damaging effects of photoaging and oxidative stress.[34,35] Moreover, it has been recently discovered that the bacterium *Euglena gracilis* is highly effective in creating high-value compounds, such as Vitamin E. It is characterized by rapid growth and a high production of α-tocopherol, which accounts for over 97% of the total tocopherol content in the organism.

In addition to the vitamins mentioned, many other vitamins are produced through microbial fermentation and exert beneficial effects on the skin. A summary of these vitamins and their respective functions on skin health is tabulated in Table 1.[36]

2.4. Production of amino acids through fermentation for skin care

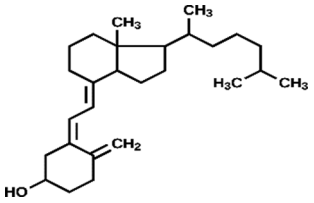
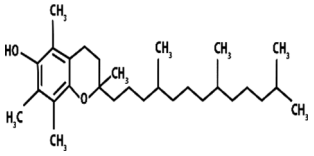
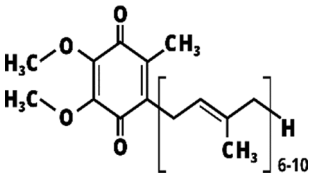
Fermented amino acids are revolutionizing the fields of nutrition and skincare. Fermentation increases the bioavailability and efficacy of amino acids, allowing better absorption by the body than their synthetic counterparts, thereby improving overall skin health and general wellness. Fermented amino acids help strengthen the skin's protective barrier, enhance moisture retention, and

Table 1. Examples of other vitamins produced by microorganisms and their respective functions on skin

Vitamin	Chemical structure	Microbes involved	Function on skin	Description of benefits
Vitamin B2 (Riboflavin)		*Staphylococcus epidermidis, Corynebacterium* spp.	Acts as an antioxidant, supports cellular energy production, enhances skin integrity, and repair	Riboflavin protects skin cells from oxidative damage and helps in repairing skin exposed to environmental stressors, such as UV rays.[37]
Vitamin B3 (Niacin/ Niacinamide)		*Staphylococcus epidermidis, Cutibacterium acnes*	Functions as an anti-inflammatory agent, improves skin hydration, helps reduce redness	Niacinamide helps in building keratin, keeps skin firm and healthy, improves skin barrier function, and reduces signs of aging.[38]
Vitamin B5 (Pantothenic acid)		*Propionibacterium* spp., *Staphylococcus* spp.	Functions as an anti-inflammatory agent, enhances wound healing, maintains skin hydration	Pantothenic acid supports the synthesis of coenzyme A which is important for cell regeneration and soothing irritated or damaged skin.[39]
Vitamin B6 (Pyridoxine)		*Cutibacterium acnes, Streptococcus* spp.	Reduces skin irritation, enhances immune defense	Pyridoxine supports skin health by helping enzymes that regulate metabolism and protein production, providing protection against pathogens.[40]
Vitamin B7 (Biotin)		*Propionibacterium* acnes, *Corynebacterium* spp.	Improves skin barrier function, supports anti-inflammatory processes	Biotin maintains skin hydration, elasticity, and smoothness, aiding in the formation of fatty acids essential for skin health.[41]
Vitamin B9 (Folate)		*Lactobacillus* spp., *Bifidobacterium* spp.	Promotes cell renewal, supports DNA synthesis, helps maintain youthful skin	Folate is essential for cell division, and in the skin, it can promote faster regeneration, beneficial for healing wounds and blemishes.[42]
Vitamin B12 (Cobalamin)		*Propionibacterium* acnes, *Staphylococcus* aureus	Regulates microbiota, essential for DNA synthesis and cell metabolism	Cobalamin supports cellular energy production and may help control inflammation and acne by regulating skin microbial balance.[43]
Vitamin K2 (Menaquinone)		*Staphylococcus epidermidis, Bacillus* spp.	Supports blood clotting, provides antioxidant benefits	Menaquinone helps with wound healing due to its role in blood clotting and provides antioxidant protection, which may reduce wrinkles and sunspots.[44]

(Cont'd...)

Table 1. ***(Continued)***

Vitamin	Chemical structure	Microbes involved	Function on skin	Description of benefits
Vitamin D3 (Cholecalciferol)		UV-activated synthesis on skin with *Staphylococcus* spp.	Enhances skin immunity, reduces inflammation, strengthens skin barrier	Cholecalciferol, produced in response to sunlight, is vital for skin immunity, protecting against pathogens and inflammation.[45]
Vitamin E		Skin microbiota contribution not fully established	Acts as an antioxidant, protects skin from UV damage, promotes healing	Though primarily acquired through diet, Vitamin E on the skin provides photoprotection and repairs cell membranes damaged by free radicals.[46]
Coenzyme Q10		*Cutibacterium acnes* and other anaerobes	Acts as an antioxidant, helps in collagen production, protects skin cells from oxidative stress	Although not a vitamin, coenzyme Q10 functions similarly by protecting the skin from oxidative stress and maintaining cellular energy levels. [47]

Abbreviation: UV: Ultraviolet.

promote a youthful appearance, making them valuable in skincare applications. They are renowned for their ability to boost collagen synthesis, a crucial process in maintaining skin elasticity and reducing the appearance of wrinkles and fine lines. Furthermore, fermentation is both sustainable and environmentally friendly, aligning with the growing demand for eco-friendly and clean beauty products. Harnessing the power of fermentation enables the production of potent and natural ingredients that benefit the skin and contribute to a more sustainable planet. The production of fermented amino acids frequently uses two bacteria: *Corynebacterium glutamicum* and *E. coli*.[48] These bacteria can create a wide range of amino acids, and their productivity as amino acid producers has increased by applying different metabolic engineering modifications. For instance, genetically engineered *C. glutamicum* is utilized to manufacture high yields of glutamic acid or lysine (up to 50% w/w), whereas *E. coli* has been modified to produce aromatic amino acids such as L-tryptophan, L-phenylalanine (L-Phe), and L-tyrosine (L-Tyr).[49]

C. glutamicum is an aerobic, non-pathogenic Gram-positive soil bacterium primarily used in amino acid production. Amino acids produced by *C. glutamicum*, such as L-glutamate, L-lysine, L-serine, L-proline, L-arginine, and L-isoleucine, have a favorable effect on the skin. The bacterium primarily prefers glucose as a carbon source in amino acid production but can also utilize other sugars such as sucrose, fructose, ribose, mannose, and maltose.[50] Glutamic acid is an amino acid involved in the central nervous system, known for its ability to enhance the antioxidant activity of the skin, regulate redox balance, prevent apoptosis, and play a vital role in the development of fibroblasts and keratinocytes. It is also recognized for its beneficial effects in treating various conditions such as large wounds, surgical wounds, and wounds from radiation and chemotherapy. Despite these benefits, glutamic acid's low water solubility (7.5 g/L at 20°C) limits its applications in cosmetics and ointments at high doses.[51]

Arginine, a semi-essential amino acid known for its role in skin immunity, wound healing, collagen synthesis, and cell division, is metabolized by the enzymes nitric oxide synthase II or arginase I. It has been reported to play a role in the management of serious wounds, including burns, diabetic wounds, bedsores, and UV-induced erythema.[52]

L-lysine has been noted for its beneficial effects in managing pityriasis rosea (PR), a skin condition primarily affecting young individuals aged 10 – 35. PR typically presents as an erythemato-papulosquamous eruption on the trunk and extremities in otherwise healthy individuals. The condition generally resolves on its own within six to eight weeks but may persist for three to six months, sometimes causing reversible skin discoloration. L-lysine can reduce the frequency of yearly symptoms and accelerate lesion healing, offering potential benefits in managing PR.[53]

Serine, an amino acid containing a hydroxyl group,

is found in keratin and naturally occurring moisturizing agents. Its ability to interact actively with water molecules suggests that it could improve the skin's ability to retain moisture when effectively delivered into the skin's SC.[54]

L-proline is a crucial amino acid for collagen synthesis, providing stability to skin, bones, tendons, and connective tissues. As humans age, collagen levels decline, leading to changes in skin texture. L-proline supports collagen synthesis, aiding skin repair and maintaining its structural integrity. It plays a key role in the four stages of wound healing – hemostasis, inflammation, proliferation, and maturation – facilitating the growth of new, healthy tissue in damaged areas. I addition, L-proline contributes to anti-aging by promoting collagen production to help delay or repair age-related skin changes. It also supports the healing of burns, cuts, and wounds by boosting collagen synthesis.[55-57]

L-isoleucine is an amino acid used in the treatment of atopic eczema. It stimulates epithelial cells to produce antimicrobial peptides, strengthening the skin's innate immune response. L-isoleucine, used topically, has been demonstrated to enhance skin barrier function, lower inflammation, and reduce eczema symptoms, including itching and redness. Research has shown that L-isoleucine-containing creams, used in combination with ceramides and rhamnosoft, significantly improved clinical outcomes for individuals suffering from mild-to-moderate eczema.[58,59]

E. coli, is a naturally occurring Gram-negative aerobic bacteria mostly found in plants and the gut flora of mammals. It produces numerous amino acids, including the aromatics, L-Phe, L-Tyr, L-methionine (Met), L-cysteine (Cys), and L-threonine (Thr). Although there is no direct evidence linking the availability of these amino acids and the production of skin proteins, these volatile amino acids play a crucial role as melanin pre-cursors. Melanin is the primary cutaneous photoprotective pigment preventing DNA damage and certain forms of skin cancer by absorbing harmful UV rays from the sun.[60,61] Melanocytes, the specialized cells responsible for the creation of melanin, contain the enzymes tyrosinase (a copper enzyme) and Phe (a tetrahydrobiopterin-dependent enzyme).[62] After activation, Phe hydroxylase in human melanocytes converts Phe into Tyr. Phe hydroxylase can be activated by ROS and the oxidant condition produced following UV exposure.[63] Tyrosinase then further oxidizes Tyr to produce melanin.[64] Melanosomes, the subcellular organelle of melanocytes, transport the melanin to keratinocytes.[65] Freckles and moles are areas of the skin with a higher concentration of these melanocytes and melanin. The amount of Phe and Tyr required for melanin production depends on sun exposure and skin type. The type and amount of melanin determine an individual's tanning response and overall skin health.[66]

Met is a sulfur-containing amino acid required to synthesize glutathione and Cys. Sulfur is needed to sustain the skin's elasticity and strength. Met serves as a pre-cursor to glutathione, an effective antioxidant that shields the skin from UV ray damage and oxidative stress, which can cause pre-mature aging. Moreover, it serves as an essential amino acid in collagen and elastin synthesis, preserving the firmness and elasticity of skin, while promoting skin restoration and wound healing. In addition, Met can improve the skin's natural barrier against environmental stressors while helping to keep the skin clear through detoxification. Furthermore, in the context of Cys in keratins, it can be obtained endogenously from oral consumption of Met. Cys is crucial in producing the disulfide bridges found in integumentary skin structures. These sulfur-containing amino acids and other sulfated molecules provide sulfur, which is needed for several purposes.[67] The body requires an adequate intake of Met and Cys to produce glycosaminoglycans and skin structural polysaccharides. Besides that, Met can serve as an antioxidant complex to treat inflammatory cutaneous lesions and acne by acting as a zinc carrier rather than a sulfur provider.[68]

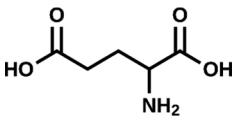
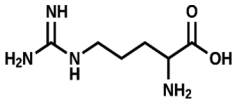
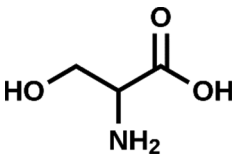
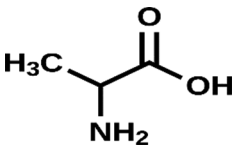
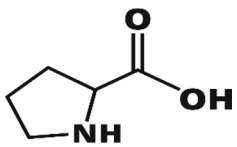
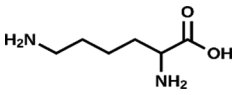
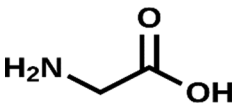
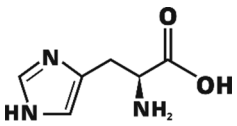
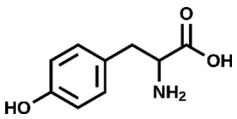
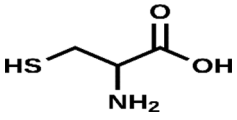
Thr, produced from the formation of collagen and elastin, aids in preserving the moisture balance of the skin, giving it a smoother, more youthful appearance. However, there is limited information regarding the dietary requirement for maintaining skin or any other tissues. Thr and serine are amino acids essential in maintaining the moisture of the SC. Both amino acids can be phosphorylated by many kinases, and this phosphorylation is crucial for proper structural function and regulation of skin metabolism.[69]

Other than the mentioned amino acids, various types of amino acids possess functional effects on skin health, as shown in Table 2.[70]

3. Benefits of microorganism-derived metabolites on the skin

The skin is home to 1,000 species of commensal bacteria, viruses, fungi, and other microorganisms. It has been shown that metabolites, such as glycerol and fatty acids produced by naturally occurring skin microorganisms, benefit skin health. As a result, probiotic cosmetics and skin-domesticating microorganisms and their metabolites are common constituents in skincare products. Currently, traditional Japanese fermented ingredients are employed as fermented cosmetics

Table 2. Amino acids and their respective beneficial compounds for skin health

Amino acid	Chemical structure	Microbes involved	Function on skin	Description of benefits
L-glutamic		*Staphylococcus epidermidis, Corynebacterium* spp.	Functions as a moisturizer promotes hydration	L-glutamic acid helps retain moisture and maintain the skin's natural hydration barrier.[38]
L-arginine		*Cutibacterium* acnes, *Staphylococcus* spp.	Acts as an antioxidant, promotes wound healing	L-arginine plays a role in collagen synthesis, supporting wound repair and skin elasticity.[39]
L-serine		*Staphylococcus* spp., Micrococcus luteus	Hydrates skin, maintains elasticity	L-serine is essential for skin hydration and softness, contributing to the skin's natural moisturizing factor.[71]
L-alanine		*Staphylococcus epidermidis, Corynebacterium* spp.	Strengthens skin barrier, enhances moisture retention	L-alanine provides hydration and supports the skin barrier, helping protect against environmental stress.[44]
L-proline		*Cutibacterium* acnes, *Corynebacterium* spp.	Synthesizes collagen, improves skin texture	L-proline aids in collagen formation, enhancing skin firmness and reducing the appearance of fine lines.[72]
L-lysine		*Cutibacterium* acnes, *Staphylococcus* aureus	Acts as an anti-inflammatory agent, supports skin repair	L-lysine contributes to collagen formation, aids in wound healing and inflammation reduction.[73]
L-glycine		*Corynebacterium* spp., Micrococcus luteus	Provides hydration, enhances skin texture	L-glycine supports collagen production, maintains hydration, and improves overall skin texture.[74]
L-histidine		*Staphylococcus epidermidis, Corynebacterium* spp.	Acts as an antioxidant, enhances moisture retention	L-histidine is an antioxidant that hydrates the skin, promoting softness and resilience.[75]
L-tyrosine		*Propionibacterium* acnes, *Staphylococcus* spp.	Regulates skin pigmentation	L-tyrosine is a pre-cursor for melanin synthesis, regulates pigmentation, and protects against UV radiation.[76]
L-cysteine		*Cutibacterium* acnes, *Staphylococcus* aureus	Acts as an antioxidant, supports skin healing	L-cysteine helps with collagen production and provides antioxidant protection, aiding skin regeneration.[77]

Abbreviation: UV: Ultraviolet.

after using these ingredients as food for more than 1,300 years. It is anticipated that fermented cosmetics will continue to expand as a category of products in the natural skincare product market and offer many health benefits.[78]

N. eutropha is an ammonia-oxidizing bacterium that oxidizes sweat-derived ammonia to create nitric oxide and nitrogen on the skin.[79] Clinical trials on topical treatment of *N. eutropha* showed improvement in pigmentation and wrinkles between the eyebrows and on the forehead.[80] This

discovery prompted the creation of a mist lotion with *N. eutropha*, currently available for purchase and has been demonstrated to improve the appearance and texture of skin. *E. faecalis* is a type of lactic acid bacterium found in the intestines and is known to benefit human health. Studies have demonstrated that the antibacterial peptides produced by *E. faecalis* possess antibacterial activity against *C. acnes*, confirming its potential as an effective new anti-inflammatory drug for acne. This has led to the commercialization of *E. faecalis* in skincare cosmetics and other therapies, intended to reduce inflammation associated with acne.[81]

Vitreoscilla filiformis is a fungus present in hot spring water and hydrothermal vents. The fungal extract was observed to balance the skin's immunological homeostasis. According to a previous study, patients with mild to moderate atopic dermatitis showed a reduction in the level of pruritus upon application of ointments containing *V. filiformis* twice daily to the site of inflammation.[82] Moreover, an extract of *V. filiformis* cultured in Vasi Volcanic Mineral Water is known to fortify the skin against exposome attack, mend damaged skin, regenerate the skin barrier, and avoid photoaging through increased immunological response. The results showed that *V. filiformis* is highly effective in repairing damaged skin, reducing photoaging, and enhancing immunological function by restoring the skin barrier.[83] Moreover, clinical trials have proven the benefits of fungus in balancing the skin flora and relieving itching in skin prone to allergies and the condition. Consequently, body washes and lotions containing *V. filiformis* cultures have been commercialized in the market.[84]

Another lactic acid bacterium, *Streptococcus thermophilus*, sometimes known as the cheese and yogurt seed, was observed to elucidate positive effects on skin health. An increase in ceramide levels was observed in healthy individuals who were given creams containing *S. thermophilus*. Ceramides are essential in maintaining the barrier and moisture of a healthy SC.[85] In addition, atopic dermatitis patients who received topical treatment of *S. thermophilus* also demonstrated elevated ceramide levels in their skin, followed by improvements in erythema, scaling, and pruritus.[86]

L. paracasei, a lactic acid bacterium inhabiting the digestive tract and oral cavity, was reported to improve atopic dermatitis and benefit the skin.[87] Furthermore, an investigation on the impact of *Pueraria lobata* extract fermented with *L. paracasei* on the skin's immune response revealed differential expression of seven skin-related proteins and a moisturizing effect on the skin upon treatment. Hence, *L. paracasei* can be used as a nutricosmetic resource for its hydrating and anti-inflammatory properties. In addition, it has been discovered that *L. paracasei* produces the same antioxidant and bioactive peptides, which reduce wrinkles and may be utilized as a more advantageous probiotic for

Table 3. Effects of microbiome metabolites on skin health

Microorganism	Metabolite	Effect on skin
Nitrosomonas eutropha	Nitric oxide compounds	Improves skin pigmentation and reduces wrinkle appearance by oxidizing sweat-derived ammonia.[92]
Enterococcus faecalis	Antimicrobial peptides	Effective against *Cutibacterium* acnes, leading to reduced acne and commercialization of anti-acne treatments.[93]
Vitreoscilla filiformis	Unknown metabolites	Reduces symptoms of atopic dermatitis and photoaging due to its anti-inflammatory properties.[84]
Streptococcus thermophilus	Ceramide synthesis enhancement	Increases ceramide levels, supporting skin barrier function and hydration.[94]
Lactobacillus paracasei	Unknown metabolites	Improves immune response, reduces inflammation, and enhances skin barrier function.[95]
Lactobacillus rhamnosus	Unknown metabolites	Reduces inflammation and promotes skin barrier repair.[96]
Bifidobacterium breve	Polysaccharide A	Modulates immune responses and enhances skin hydration by stimulating T-regulatory cells.[97]
Lactobacillus plantarum	Lipoteichoic acid	Reduces skin sensitivity, provides anti-inflammatory effects, and improves skin barrier function.[98]
Cutibacterium acnes	Short-chain fatty acids	Modulates skin pH and inhibits pathogenic bacteria, promoting a healthy skin microbiome.[99]
Staphylococcus epidermidis	Phenol-soluble modulins	Enhances skin defense mechanisms by promoting antimicrobial peptide production.[100]
Lactobacillus reuteri	Reuterin	Displays antimicrobial activity against skin pathogens and promotes skin barrier function.[101]
Bacillus subtilis	Surfactin	Provides anti-inflammatory and wound-healing properties.[102]

the skin.[88] *L. rhamnosus* is a probiotic known to enhance the intestinal barrier's performance and is anticipated to be used in cosmetics.[89] Research has indicated that topical application of *L. rhamnosus* lysate to a human epidermal model for 16 days increased the expression of skin barrier proteins and promoted SC formation, which may have beneficial effects in preserving skin barrier function.[90] Although more investigation is necessary, these results imply that topical probiotics could have potential applications in skincare products. In addition, *L. rhamnosus* showed a high inhibition of tyrosinase and melanin synthesis and increased collagen production when co-cultured with *L. paracasei*.[91] Besides the mentioned microorganisms and metabolites, there are many other examples of microorganism-derived metabolites affecting the skin, as shown in Table 3.

4. Conclusion

FPEs are superior among the reviewed skincare ingredients, due to their increased biological activity and varied benefits. In these extracts, fermentation enhances anti-inflammatory, antibacterial, and antioxidant properties, and accelerates wound-healing processes, making these fermented extracts more potent than their non-fermented counterparts. For example, fermented *P. ginseng* is remarkably effective in anti-aging and skin whitening, while fermented *M. denudata* and rice bran have significant antioxidant and skin-brightening properties. Fermentation enhances the bioavailability of bioactive phytochemicals toward better skin hydration, collagen synthesis, and barrier function. The integration of microorganisms and their metabolites in skincare products highlights the tendency of industry productions to shift toward more advanced natural solutions. Probiotic strains, including *N. eutropha, E. faecalis*, and *V. filiformis*, impart beneficial properties such as skin texture improvement, anti-inflammation, and barrier support. Fermented acids, vitamins, and amino acids enhance the functionality of ingredients, thereby increasing hydration, skin lightening, and exfoliation. These advancements within fermentation technology would enable the development of innovative skincare products, offering better and more sustainable solutions for achieving healthier, brighter skin.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

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Ethics approval and consent to participate

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ACCSCIENCE PUBLISHING

Innovative Medicines & Omics

REVIEW ARTICLE

Innovative approaches in kidney disease management: Advances in therapeutics and treatment strategies

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Abstract

The health system is burdened by kidney disease (KD), which has considerable economic consequences. The aging population and the rise in Type 2 diabetes and hypertension are the main contributing causes. KD is also associated with an increased risk of cardiovascular diseases (CVDs) morbidity, early mortality, and reduced quality of life. Recent studies estimate that more than 850 million people worldwide are affected by kidney-related illnesses each year. Of these, about 3.9 million individuals are going through dialysis or kidney transplantations, neither of which provides an ultimate solution. Alternative therapeutic approaches through medications include the use of angiotensin-converting enzyme inhibitors and Angiotensin II receptor blockers, renin inhibitors, anti-inflammatory medicines, and bioactive phytocompounds isolated from several plants. Plants contain numerous bioactive compounds that are thought to provide a variety of health benefits, including potential nephroprotective properties. In this review, recent advancements in kidney disease (KD) research will be highlighted, including newly identified causes, renal pathophysiological alterations, and current therapeutic approaches.

Keywords: Kidney disease; Phytocompounds; Nephroprotective; Anti-inflammatory

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1. Introduction

Acute kidney injury (AKI) is a syndrome characterized by reduced urine production and the accumulation of nitrogen metabolism end products, such as urea and creatinine in renal tubules.[1] At the initial phase of injury and inflammation, circulating immune cells (T- and B-cells) infiltrate the kidney, drawn in by cytokines, chemokines, and damage-associated molecular patterns (DAMPs) generated by wounded cells. DAMPs contribute to a pro-fibrotic environment by interacting with activated monocytes/macrophages, damaged tubular epithelial cells (TECs), and endothelial cells. This environment stimulates pericytes to proliferate and differentiate into myofibroblasts, which causes extracellular matrix (ECM) protein deposition, renal fibrosis, and progression to chronic kidney disease (CKD). TECs may have a pro-fibrotic phenotype and become

atrophic as a result of aberrant repair pathways. Pro-fibrotic cytokines, including connective tissue growth factor and transforming growth factor-β (TGF-β), may be produced by G2/M-arrested TECs through activation of JNK signaling. Cell cycle arrest may dictate the course of damage, whereas favorable cell cycle events may determine the healing. Dysregulated and inefficient tubular repair has been associated with the converging of tubular cells toward a pro-fibrotic and senescent phenotype, sustained inflammation, and ECM deposition.[2,3]

It has been noted that regardless of the cause, patients with AKI are more likely to develop CKD, end-stage renal disease (ESKD), and premature mortality.[4] At present, 850 million individuals are suffering from KD and its related illnesses, including the 3.9 million receiving regular dialysis or kidney transplantation.[5] Globally, the anticipated number of individuals with diabetes in 2015 was 415 million, or 8.8% of the total population. This is more than double the 4.6% (151 million) estimated in 2000, and by 2040, the figure is predicted to rise to 10.4% (642 million). One well-known example of a chronic multisystemic illness linked to an increased risk factor of CVD is CKD. According to clinical and experimental evidence, CKD increases oxidative stress and promotes an inflammatory state, both of which are critical factors in the development of CVD in uremia.[6,7]

CKD is marked by vasculopathy, renal interstitial fibrosis, tubular atrophy, and glomerulosclerosis, leading to impaired kidney regeneration. Renal fibrosis is histologically indicative of the onset of KD, albeit the underlying mechanisms are yet unknown.[8] Over the last few decades, research on animals and molecules has expanded our knowledge of the pathophysiology of AKI, identifying oxidative stress, endothelial damage, mitochondrial injury, and innate immunity as primary causes.[9] Oxidative stress is thought to be a major factor in the development of endothelial impairment, as excessive reactive oxygen species (ROS) activate intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK). Furthermore, the uremic endothelium exhibits a proinflammatory phenotype, characterized by increased synthesis and expression of adhesion molecules, which have been found to be important factors in endothelial activation and damage.[7] Many signaling pathways that maintain homeostasis are routinely activated by the creation of reactive species. However, the excessive generation of reactive species can be highly detrimental. As mitochondrial damage increases, the electron transport chain becomes less effective, which also results in a drop in ATP production and an increased ROS creation. Impaired mitochondrial respiration is an indication of an imbalanced aerobic metabolism and increased oxidative stress in patients receiving hemodialysis and those with CKD.[10] The etiology of CKD is influenced by hampered cellular antioxidant mechanisms, which also affect signaling processes that lead to senescence and death of renal cells, renal fibrosis, and reduced renal cell regeneration.

This review gives an update on the discovery of new antioxidant drugs for CKD and discusses the sources of ROS, transcription factors, and signaling mechanisms impacted by the oxidative stress-related pathway during the development of renal fibrosis. Ongoing research worldwide is exploring various causes of KD and contemporary prevention measures (Figure 1), which are outlined in this review article.

2. Newly identified causes of CKD

2.1. Mitochondrial dysfunction

Despite the fact that mitochondria have long been associated with the pathobiology of AKI, interest in how this cellular organelle contributes to the development of AKI and CKD is expanding. Mitochondrial fragmentation has been related to cell loss in the kidney and other organs. The mitochondrial fission protein dynamin-related protein 1 (DRP1), which constricts and cleaves mitochondria and induces fragmentation, was specifically deleted in the proximal tubules, preventing renal ischemia-reperfusion damage and promoting epithelial recovery. Furthermore, DRP1 deletion in the proximal tubules after ischemia-reperfusion slowed the development of kidney damage and fibrosis, suggesting that DRP1 in the proximal tubules increases the kidneys' vulnerability to AKI and that activation of the protein contributes to maladaptive repair over time.[11,12]

2.2. Cell death pathway

The control of cell death is another crucial function of mitochondria, in addition to their well-known involvement in cellular metabolism. Recent research suggests that mitochondrial permeability transition is also an important mediator of AKI and the subsequent progression to CKD. These pathways include necroptosis, ferroptosis, and apoptosis. Studies have shown that the absence of caspase-3, a key pro-apoptotic enzyme, leads to significant kidney abnormalities in mice, highlighting the critical role of tubular cell death in AKI. Recent research suggests that ischemic conditions lead to reduced microvascular loss in mice but exacerbate tubular damage, accompanied by elevated levels of the necroptosis marker, receptor-interacting protein kinase 3 (RIPK3).[13] Ischemia induces apoptosis by causing oxidative stress, mitochondrial dysfunction, and the production of

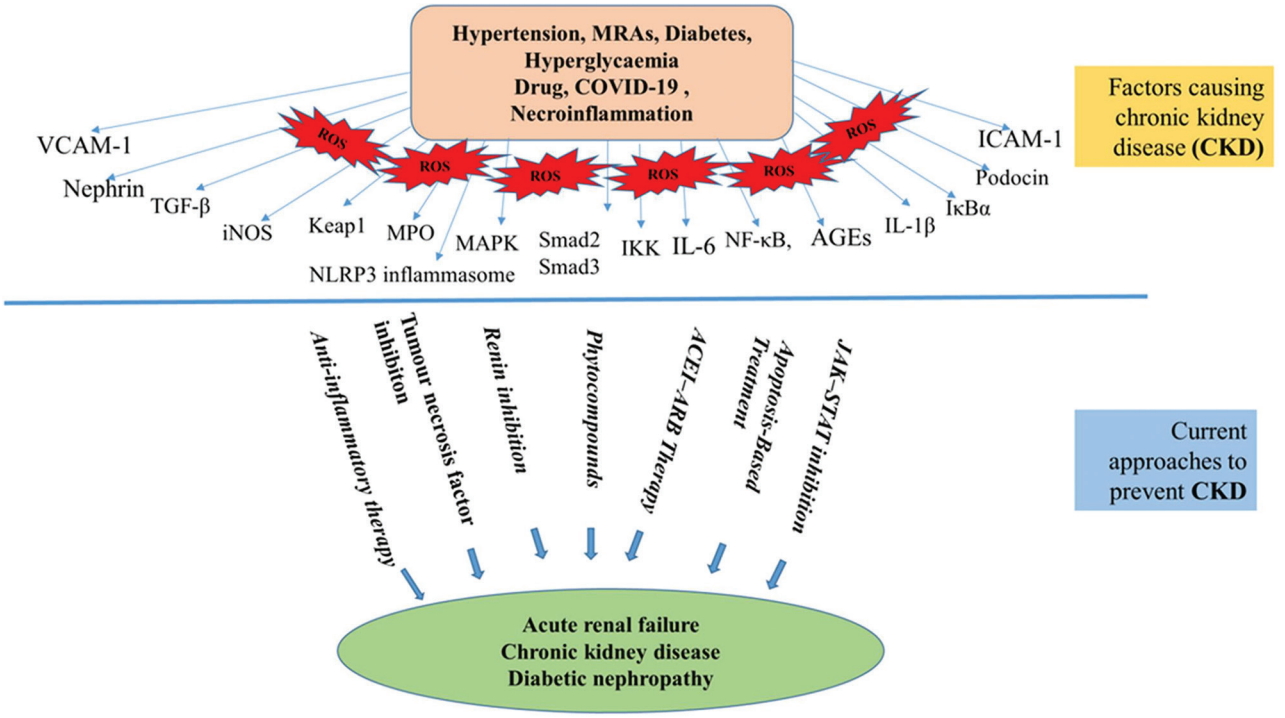

Figure 1. Recent advancement in the causative factors and preventive strategies for kidney disease
Abbreviations: MRAs: Mineralocorticoid receptor antagonists; VCAM-1: Vascular cell adhesion protein 1; ROS: Reactive oxygen species; TGF-β: Transforming growth factor-β; KEAP1: Kelch-like ECH associated protein 1; MPO: Myeloperoxidase; SMAD2: Suppressor of mothers against decapentaplegic 2; SMAD3: Suppressor of mothers against decapentaplegic 3; IKK: Ikappa B kinase; IL-6: Interleukin-6; NF-κB: Nuclear factor-kappa B; AGEs: Advanced glycation end-products; IL-1β: Interleukin-1β; IκBα: Ikappa B-alpha; ICAM-1: Intercellular adhesion molecule 1; NLRP3: NLR family pyrin domain containing 3; ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin II receptor blocker; JAK: Janus kinase; STAT: Signal transducer and activator of transcription.

pro-apoptotic proteins. Reperfusion triggers a series of reactions, including immune cell activation, inflammation, and the production of ROS. Apoptosis is particularly likely to occur in proximal tubular cells during renal ischemia-reperfusion injury (IRI). These cells, which participate in solute reabsorption, exhibit significant metabolic activity. Apoptosis is the final outcome of oxidative stress, ATP depletion, and mitochondrial dysfunction experienced by proximal tubular cells during IRI. As apoptotic cells emit DAMPs, immune cells get activated, and proinflammatory cytokines are produced, which ultimately cause damage to renal tissue.[14]

2.3. Inflammation

Necroinflammation, in which inflammation and kidney damage are both amplified in an auto-amplification cycle, is a defining feature of controlled necrosis. Necroinflammation can be initiated by a few necrotic cells that trigger the innate immune system. This can lead to further cell necrosis and inflammation, which can ultimately lead to organ failure. Damaged cells that survive renal cell injury also release different kinds of proinflammatory cytokines and chemokines, which, in conjunction with resident macrophages, dendritic cells, and the innate immunity response from infiltrating neutrophils, lymphocytes, and monocytes, intensify the inflammatory milieu. As a result, inflammation plays a crucial role in the pathophysiologic component of AKI.[15,16]

2.4. Acute respiratory distress syndrome (ARDS) related AKI

AKI is observed in 35% – 50% of patients who develop ARDS, and it dramatically increases intensive care unit mortality by about twofold. Renal injury can be caused or worsened by several factors, such as ARDS and its associated mechanical breathing procedures. These factors can be broadly categorized into five groups which include hyperinflammation, acid-base dysregulation, poor gas exchange (hypoxia/hypercapnia), hemodynamic consequences, and neurohormonal impacts. Immunosuppressed patients, especially those with T cell-mediated immunity dysfunction, are more susceptible to severe viral infections due to a reduced immune system. Depending on the specific situation and severity of the

sickness, immunosuppression in transplant recipients with suspected or confirmed COVID-19 should be altered promptly. After the onset of ARDS, significant AKI frequently manifests in COVID-19 patients, indicating that lung-kidney crosstalk is the primary mechanism causing kidney injury.[17,18]

2.5. Role of SARSCoV2 in KD

Angiotensin-converting enzyme 2 (ACE2), a homolog of ACE, reduces vasoconstriction induced by the renin-angiotensin system by converting Angiotensin II to angiotensin 1 – 7. There are two types of ACE2: membrane-bound ACE2 and soluble ACE2. SARS-CoV-2 attaches to ACE2 on the host cell membranes. The ability of coronaviruses to enter cells depends on their ability to attach to cellular receptors and prime their S proteins for entry by host cell proteases. As a result, the activity of the protease transmembrane protease, serine 2 (TMPRSS2) to cleave the viral spike protein and the expression of ACE2, are essential for cell invasion. Both podocytes and the apical brush borders of the proximal tubules in the kidneys express ACE2. ACE is expressed in renal endothelial cells, whereas ACE2 is not. Recent human tissue RNA-sequencing data show that the expression of ACE2 in kidney tissue is about 100 times higher than in pulmonary tissue. The proximal tubules of the kidney have been shown to express TMPRSS2.[19] Antibodies against ACE2 are produced when ACE2 binds to the SARS-CoV-2 spike protein, causing a conformational shift in proteins that serve as a target for autoantibody development. After antigen-presenting cells process complexes of SARS-CoV-2 and soluble ACE2, antibodies may cause type 2/3 hypersensitivity reactions, in addition to Type 4 hypersensitivity reactions. Type 2 hypersensitivity responses during SARS-CoV-2 infection trigger the production of immunoglobulin M against ACE2, which targets ACE2 in kidney cells and causes renal impairment.[20] Recent research revealed that SARS-CoV-2 entered host cells through the novel CD147-spike protein pathway. The transmembrane glycoprotein CD147, which is widely expressed, has been linked to numerous kidney illnesses, including CKD. It is significantly expressed on inflammatory cells and proximal TECs.[21,22] According to Legrand *et al.*, the enhanced production of inflammatory cytokines by resident and immune kidney cells is likely a factor contributing to tissue damage in COVID-19 patients. In COVID-19, nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream signaling components are likewise suppressed in the lungs. Inflammatory mediators such as tumor necrosis factor (TNF) and FAS can directly harm renal endothelial and epithelial cells by binding to specific receptors they express. These associations, observed in laboratory models of sepsis, are supported by plasma cytokine levels in patients with sepsis-associated AKI.[23] Human COVID-19 infection is caused by the interaction of the viral spike protein's receptor-binding domain with the cell surface ACE2. The spike protein is then cleaved by proteases, such as TMPRSS2, in a proteolytic manner. When the virus interacts with CD147, which is expressed on the proximal convoluted tubules of the nephron and inflammatory cells; it can cause acute tubular necrosis, collapsing glomerulopathy, protein leakage from Bowman's capsule, and mitochondrial dysfunction.[24]

2.6. Impaired renal reflex in AKI

The pathophysiology of renal disorders is thought to be influenced by renal sympathetic nerve activity. The intrarenal release of adenosine, triggered by tissue ischemia, increases the activity of both afferent renal sensory neurons and efferent renal sympathetic nerve activity. Equally significant in the etiology of AKI is the effects of efferent RSNA, which include decreased renal blood flow and oxygen delivery, as well as increased renal workload. In hypotensive and hypovolemic conditions, an elevation in RSNA causes acute renal vasoconstriction. This results in glomerulotubular dysfunction, hormonal changes, and the development of renal ischemia.[25] In contrast to angiotensin-converting enzyme inhibitor (ACEI)/angiotensin II receptor blockers (ARB) monotherapy, short-term use of mineralocorticoid receptor antagonists (MRAs) in combination with ACEIs/ARBs was not associated with a lower risk of cardiovascular or renal outcomes in patients with diabetic KD and hypertension. A real-world clinical problem for MRA-ACEI/ARB combination therapy is indicated by the risk of hyperkalemia and the brief duration of the combination medication. Numerous pathophysiological conditions, including diabetes, hypoxia, ureteral blockage, cirrhosis, and renal IRI, have been linked to this defective inhibitory renorenal reflex.[26] Nitric oxide (NO), which functions as both a neurotransmitter and neuromodulator, is one of the several neurotransmitters in the brain that alter sympathetic nerve activity. Inducible nitric oxide synthase (iNOS) and neuronal NO synthase (nNOS)-induced endogenous NO synthesis seem to affect blood pressure and sympathetic nervous system activity differently. This is thought to be caused, at least in part, by the differential release of neurotransmitters in the rostral ventrolateral medulla, including inhibitory gamma-aminobutyric acid and sympatho-excitatory glutamate. Cyclic 3′-5′ guanosine monophosphate-dependent processes are suggested in the control of neuronal activity by microinjection of exogenous NO. The inhibition of Angiotensin II release also mediates

the effects of NO system activation within the central sympathetic nervous system.[27]

3. Current therapeutic approach of CKD

3.1. Combining ACEI and ARB therapy

According to recent studies, since the RAS is clearly involved in the development of renal disease, more complete RAS blockade may be able to halt its progression. In contrast to ACEI/ARB monotherapy, short-term use of MRAs, such as spironolactone or eplerenone, in combination with ACEIs/ARBs was not associated with a lower risk of cardiovascular and renal outcomes in patients with diabetic KD and hypertension. Given the short duration of combination therapy and the risk factor of hyperkalemia, MRA-integrated ACEI/ARB combination therapy may face practical therapeutic challenges.[26,28] To investigate this, several trials investigating the combination of an ACEIs and ARBs have been performed. Although they have distinct mechanisms of action, ACEIs and ARBs both disrupt the RAS. ACEIs inhibit Angiotensin-I from converting to Angiotensin II, whereas ARBs prevent Angiotensin II from binding to Angiotensin II Type 1 receptors. Analyses of ACEIs and other ARBs have revealed that they are equally effective in reducing blood pressure.[29] By maintaining peritubular capillary perfusion through efferent arteriolar vasodilation and boosting the renal medullary plasma flow by decreasing the filtration fraction, ACEIs/ARBs could mitigate tubular damage following AKI insults. Angiotensin II blockade has been demonstrated to lessen the development of acute tubular necrosis or damage as well as tubular ischemia. In addition, ACEIs/ARBs are advised to slow the course of kidney deterioration in diabetic nephropathy. In addition, ACEIs/ARBs lower CVD-related mortality, such as myocardial infarction and congestive heart failure. The use of ACEIs/ARBs is generally supported by evidence, as they protect the kidneys and heart and lower all-cause mortality. Our present meta-analysis findings support their timely use following AKI and consistent with previous reports. Profibrotic pathways may directly damage essential organs if the RAAS is activated, and AKI has a major effect on the functioning of injury/repair pathways in distant organs. Following AKI and CKD, we hypothesize that using ACEIs/ARBs may enhance organ function and avoid maladaptive repair.[30-32] The Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET), which included 25,920 individuals with vascular diseases and a higher risk of diabetes, evaluated the benefits of ACEI ramipril, ARB telmisartan, and their combination. The majority of patients included in ONTARGET did not exhibit microalbuminuria and/or macroalbuminuria at baseline. Therefore, it was not possible to determine the renal benefit of combined ACEI/ARB treatment for patients with proteinuria. Due to hypotensive symptoms, 784 participants (mostly those on combination therapy) permanently stopped receiving randomized therapy throughout the research. Compared to patients receiving monotherapy, the combination treatment group had a considerably higher number of patients reaching the primary renal outcome of dialysis, doubling of serum creatinine, or death. Acute renal failure was the primary cause of many dialysis episodes, and it was more common in individuals with normotension. These unsatisfactory but not totally unexpected findings highlight the safety concerns related to ACEI/ARB treatment.[33] The abrupt transition to sodium-glucose cotransporter-2 inhibitors and MRAs for reducing albuminuria, followed by a return to ACEIs and ARBs, resulted in greatly reduced hyperkalemia and potassium levels, as well as a dramatically lowered the urinary albumin-to-creatinine ratio when dapagliflozin and eplerenone were taken as adjuvants to ACEIs or ARBs. These recent trials imply that dapagliflozin with eplerenone is a desirable combination to help individuals with CKD reduce the course of their illness.[34] RAS blocker medications increased the risk of hyperkalemia, hypotension, and cough, but they also improved the outcomes for patients with non-dialysis CKD. ACEIs were more likely than ARBs and other antihypertensive drugs to be the most effective therapy for renal events, cardiovascular outcomes, and causes of mortality in patients with diabetic KD, and non-dialysis CKD. ARBs outperformed ACEIs in preventing the risk of cardiovascular and renal events, but they were less effective than ACEIs in lowering all-cause mortality.[35-37]

3.2. RAS and renin inhibition

ACEIs and ARBs are RAS inhibitors that slow the progression of mild to severe CKD. According to some research, discontinuing RAS inhibitors in individuals with severe chronic renal disease may result in an increase in estimated glomerular filtration rate or a slowing of its decline.[38] Evidence does not support the combination therapy of aliskiren and losartan among non-diabetic CKD patients generally, and aliskiren does not provide extra renoprotection over a 144-week period in individuals with non-diabetic KD. However, KD responders could potentially benefit from direct renin inhibition, making it a more targeted treatment option for specific subgroups of CKD patients, based on some positive short-term outcomes.[39] In particular, proinflammatory chemicals and stress hormones seem to increase the synthesis of kynurenine and its downstream metabolites, which may affect insulin action and favor the onset of diabetes mellitus and its complications, including nephropathy.

Progressive renal insufficiency has been associated with decreased tryptophan levels and kynurenine accumulation due to inflammation and impaired kidney function in diabetic individuals. Proteinuria and albuminuria are signs of several kidney illnesses, and a few clinical and experimental studies have looked into the potential link between the kynurenine pathway and these conditions. Kynurenine aminotransferases are the enzymes responsible for converting kynurenine into its downstream metabolites, and RAS inhibitors can reduce their activity, hence reducing the synthesis of kynurenic acid in kidney homogenates. These findings could be clinically significant because kynurenic acid has been linked to the extent of renal function loss in patients with kidney illness.[40] However, recent research has shown that RAS inhibitors may cause common adverse effects such as anemia, hyperkalemia, and functional renal insufficiency.[41]

3.3. Anti-inflammatory therapy

Renal failure in individuals with diabetes and inflammation has long been linked. Growing evidence from both animal and clinical trials suggests that endothelin Type A receptor antagonists may have a role in the treatment of diabetic renal diseases (DRD). Vasoconstriction, mesangial proliferation, podocyte damage, inflammation, and fibrosis are all linked to increased renal endothelin expression in DRD. In DRD patients, the expression of endothelial adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), vascular adhesion protein 1 (VAP-1), and vascular cell adhesion protein 1 (VCAM-1) is increased, and this increase is associated with the severity of the illness. These molecules are crucial for leukocyte adhesion to the endothelium; therefore, blocking them may affect leukocyte trafficking and reduce inflammation in DRD.[42]

3.4. TNF inhibition

The results are consistent with previous research suggesting that in diabetes, hyperglycemia-induced formation of advanced glycation end-products (AGEs) triggers macrophage production of TNF. However, it is unknown whether TNF or its receptors play harmful functions in the development of KD and diabetic nephropathy. TNF receptor-deficient animals treated with TNF-neutralizing antibodies have lessened disease severity in experimental rodent models of renal disease.[43] Despite anti-TNF drugs being used clinically for more than 20 years, few studies have looked into their clinical activity in renal illness. Such studies have been limited in size and mostly concentrated on focal segmental glomerulosclerosis and lupus nephritis, leaving their potential involvement in various types of CKD development largely unanswered.

3.5. Janus kinase inhibitors and signal transducer and activator of transcription (JAK/STAT) inhibition

JAK and STAT are important intracellular mediators of growth hormone, erythropoietin, pro-epidermal growth factor, and inflammatory cytokines such as interleukin(IL)-6, IL-23, IL-12, interferon, and its cognate receptor.[44] According to recent clinical trials, autoimmune inflammatory diseases such as rheumatoid arthritis and ulcerative colitis can be effectively treated with JAK inhibitors such as tofacitinib and baricitinib. In diabetic KD, the JAK/STAT signaling pathway and the documented clinical anti-inflammatory activity of JAK inhibitors have prompted a Phase II investigation to assess their clinical effectiveness in renal illness. In this trial, patients with proteinuria in diabetic KD who were already on ACEIs and ARBs were treated with the JAK1 and JAK2 inhibitor baricitinib for 24 weeks. The results showed a 30 – 40% reduction in albuminuria with baricitinib treatment. However, a side effect associated with this class of drugs – decreased hemoglobin level – was observed. The extent to which these effects on albuminuria decreased translate into long-term advantages for renal function and mortality remains to be determined.[45,33]

3.6. Apoptosis-based treatment strategies in AKI

Recent research indicates that a variety of pathways contribute to both apoptosis and programmed necrosis-induced cell death following apoptosis, including in AKI. Suppressing both processes may be necessary to completely avoid AKI. This is noteworthy because caspase inhibitors may affect autophagy and proinflammatory necroptosis, two other processes involved in cell death and survival. Phosphorylation and activation of p53 have a major function in the pathogenesis of vancomycin-induced AKI, as well as nephrotoxicity caused by folic acid, aristolochic acid, and cisplatin. Ferroptosis, cell cycle arrest, autophagy, metabolism, fibrosis, and both necrotic and apoptotic cell death are among the processes in which p53 is implicated in the kidney. Based on experimental studies, the protection against ischemia and cisplatin-induced AKI is due to the pharmacological suppression or proximal tubule-specific p53 deletion.[46,47] The p53 gene is targeted by a small interfering RNA known as teprasiran, and in a randomized Phase 2 clinical trial, teprasiran provided protection against AKI in high-risk, on-pump patients undergoing heart surgery.[48]

3.7. Phytomedicinal therapeutic approach of KD

Phytocompounds are naturally occurring groupings of different substances that are present in plants and fruits that have several health-beneficial effects, including anti-

inflammation, anti-oxidative, anti-diabetic, anticancer, and nephroprotective action. Phytocompounds, including flavonoids, have the ability to both directly and indirectly reduce renal damage. Significant biological benefits of flavonoids in CKD include reducing oxidative stress, immunological modulation, antioxidant actions, anti-inflammation, anti-apoptosis, gut microbiota regulation, anti-diabetic, and antihypertensive; they also help to relieve renal fibrosis.[49] In addition, they serve as intermediaries for the activation of the Nrf2 antioxidant action, which lowers oxidative stress.[50]

3.7.1. Troxerutin

Troxerutin, a derivative of the naturally occurring bioflavonoid and found in tea and coffee. The ability of troxerutin to reduce drug-induced nephrotoxicity has been investigated in earlier research. Troxerutin reduces the oxidative stress induced by cisplatin and methotrexate by inhibiting lipid peroxidase and nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX-1), restoring superoxide dismutase (SOD), GSH, and glutathione peroxidase (GPx) levels, and activating the Nrf2/HO-1 signaling pathway.[51] Long-term administration of 2,2,4,4-tetrabromodiphenyl ether (PBDE-47) increased Kelch-like ECH associated protein 1 (KEAP1) levels, leading to Nrf2 ubiquitination and degradation, which in turn decreased Nrf2 activity and its downstream genes in the kidneys of mice, including catalase, GPx, SOD, and heme oxygenase 1 (HO-1). Nevertheless, troxerutin enhanced Nrf2 activity, prevented the negative effects of PBDE-47 and partially mimicking the action of carbobenzoxy-l-leucyl-l-leucinal (MG132). In the liver of mice, PBDE-47 was found to increase caspase-3 action and the levels of B-cell lymphoma 2 (Bcl-2)-associated X (Bax) and Bcl-2.[52] Activated TGF-β has been linked to the pathogenesis of diabetic KD. TGF-β triggers receptor activation through autocrine and paracrine pathways, initiating a signaling cycle that ultimately regulates the production of ECM, leading to the impaired mesangial cell function. As nephropathy progresses, TGF-β builds up in mesenchymal cells and influences the synthesis of ECM proteins, such as collagen I and collagen II. TGF-β inhibits E-box repressors like δEF1 and SMAD interacting protein 1 (SIP1), which regulate collagen gene expression. The role and target of certain kidney-dwelling microRNAs, such as miR-192, miR-194, miR-204, and miR-215, in the setting of nephropathy have received significant attention. Since miR-192 has been shown to target SIP1, the low levels of SIP1 observed in diabetics may validate the interaction between elevated TGF-β and miR-192, leading to low levels of SIP1 in renal tissue. Troxerutin's effects on the kidney in a diabetic rat model appear to be mediated by decreased levels of miR-192, a crucial miRNA involved in the development and exacerbation of nephropathy, and the increase of SIP1. Further research is required to assess troxerutin's effects on collagen levels and ECM proteins, to evaluate its potential as a natural preventive component that can help avoid renal problems[53] Similarly, research has found that troxerutin may reduce cisplatin-induced kidney cell death in rats by increasing microtubule-associated protein 4 (MAP4) expression and activating the PI3K/AKT signaling pathway, one of the most effective intracellular pathways for enhancing cell survival.[54] In addition, troxerutin has been demonstrated to prevent renal damage caused by drug-induced cytotoxicity in rat models by increasing the antioxidant defense system and reducing lipid peroxidation.

3.7.2. Fisetin

Fisetin, a flavonoid is isolated from a variety of fruits, vegetables, seaweeds, and persimmons, as well as strawberries, apples, and onions. After being given orally to mice, it can penetrate the blood-brain barrier and accumulate in the brain. Fisetin is rapidly bio-transformed through conjugative metabolism, mostly by glucuronidation, sulfation, and methylation in the liver, and is eliminated through urine and feces. Cytochrome P450 enzymes are among the Phase I and II metabolic enzymes involved in the metabolic process. *In vitro* research demonstrated that fisetin, like other flavonoids, inhibits a number of cytochrome P450 enzymes, potentially leading to drug interactions when combined with other medications. Fisetin inhibits myeloperoxidase (MPO) activity, inflammatory cytokines, and renal production of iNOS, thereby protecting the kidney from drug-induced renal impairment.[55] In the context of ureteric obstruction, TGF-β is essential for cell development, proliferation, differentiation, apoptosis, immunological response, and renal fibrosis. TGF-β1 binds to its receptor, TβRII, causing phosphorylation of TβRI and activation of TGF-β1 downstream effectors, including suppressor of mothers against decapentaplegic (SMAD). Canonical pathway involves receptor-regulated SMADs (R-SMADs), such as SMAD2/3, which are both overexpressed in human fibrotic kidneys, and responsible for TGF-β1 signaling transduction. Non-canonical SMAD-independent pathways, including Rho-like GTPase, PI3K/AKT, Jun N-terminal kinases (JNKs), and MAPK, also regulate gene transcription, promoting apoptosis and the epithelial-to-mesenchymal transition (EMT). In experimental models, fisetin injections (25 mg/kg) administered intraperitoneally one hour prior to surgery and every other day for seven days. In addition, fisetin pretreatment (40 μM) dramatically decreased TGF-β1-

induced phosphorylation of SMAD2/3 in human kidney-2 (HK-2) cells. By modifying TGF-β1/SMADd3 and STAT3 signaling, fisetin helps to improve kidney fibrosis.[56,57] Fisetin also reduces the release of inflammatory cytokines, AGEs, ROS, and NLR family pyrin domain containing 3 (NLRP3) inflammasome – factors associated with diabetic nephropathy. When the NLRP3 inflammasome is activated in mice, podocyte proteins such as nephrin and podocin are lost, accompanied by mitochondrial dysfunction. Tubular injury in animals has been linked to increased high glucose-induced EMT and the involvement of SMAD3, p38 MAPK, extracellular signal-regulated kinase 1 (ERK1), and ERK2 signaling pathways. Fisetin treatment reduced the expression of fibronectin, collagen, and vascular endothelial growth factor A (VEGFA) while increasing matrix metalloproteinases 2/9. This was primarily caused by inactivating the TGF-β/SMAD2/3 pathways, which inhibits the production of ECM in the kidney. Both *in vitro* and *in vivo* experiments demonstrated that fisetin effectively protects against kidney fibrosis.[58] Fisetin shows significant potential as a senolytic medication with a variety of therapeutic applications, although human data remain limited currently. Carefully supervised clinical investigations are necessary to demonstrate whether fisetin's beneficial and senolytic properties can be translated into human use. According to a recent cohort study sub-analysis, serum levels of senescence-associated secretory phenotype factors, MMP-3 and MMP-9, platelet-derived growth factor AA, IL-6 and IL-8, monocyte chemoattractant protein-1 (MCP-1), and growth differentiation factor 11 and 15, dropped between baseline and follow-up visit in healthy individuals who self-dosed with 100 mg/day of fisetin.[59] (Figure 2).

3.7.3. Kaempferol

Kaempferol, a flavonoid widely distributed in vegetables and fruits, including broccoli, tea, and grapes, exhibits antioxidant and anti-inflammatory properties. In HK-2 cells, lipopolysaccharide (LPS) upregulated the production of TNF-α and IL-1β, demonstrating its ability to induce inflammation. However, the administration of kaempferol considerably decreased the LPS-induced apoptosis in HK-2 cells.[60] LPS also induced STAT3 and NF-κB, which subsequently increased procalcitonin expression, a validated blood biomarker in septic patients. Kaempferol played a crucial anti-inflammatory role in

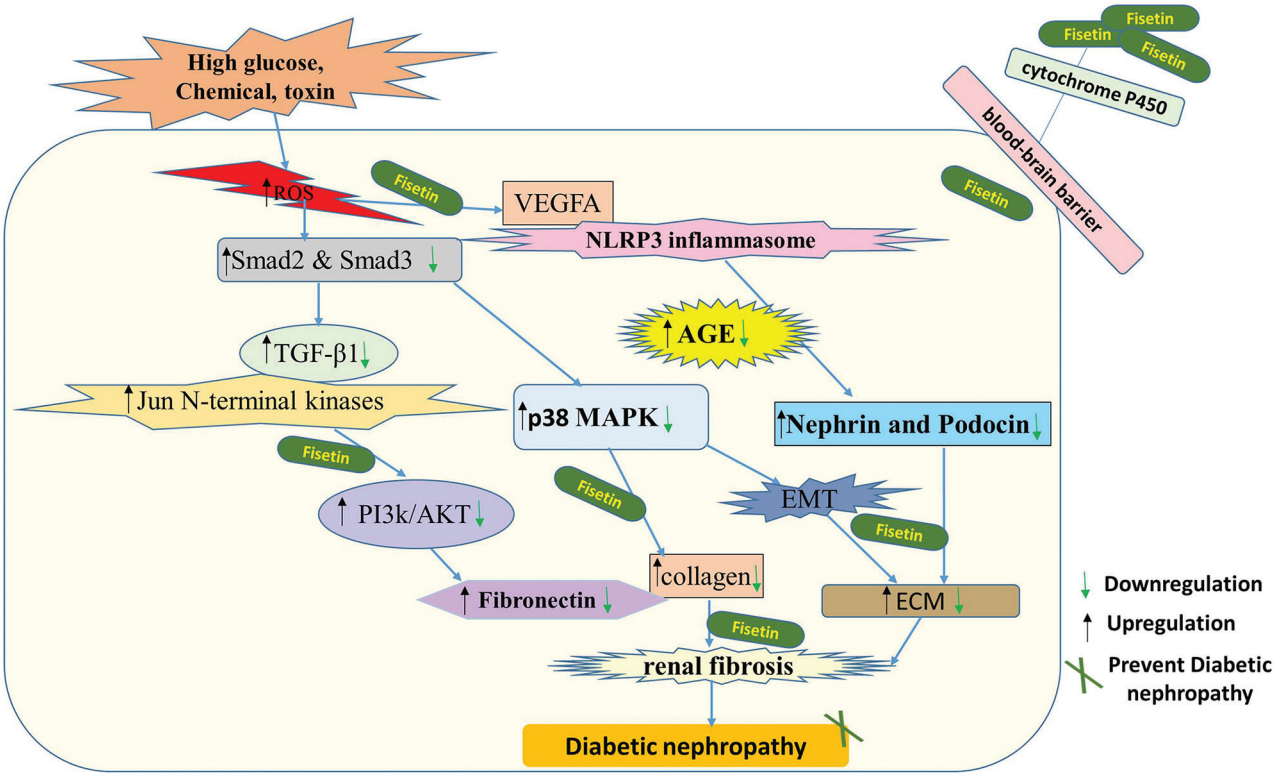

Figure 2. Mechanism of action of fisetin in the protection of diabetic nephropathy
Abbreviations: VEGFA: Vascular endothelial growth factor A; ROS: Reactive oxygen species; NLRP3: NLR family pyrin domain containing 3; AGE: Advanced glycation end-product; MAPK: Mitogen-activated protein kinases; TGF-β1: Transforming growth factor-β1; EMT: Epithelial-to-mesenchymal transition; ECM: Extracellular matrix.

sepsis models by specifically inhibiting cyclooxygenase-2 (COX-2) and ameliorating liver damage in animal studies. Moreover, kaempferol decreased the excessive production of TNF-α, IL-1β, IL-6, ICAM-1, and VCAM-1 in LPS-treated groups. Kaempferol reduces COX-2 expression while simultaneously inhibiting the production of MCP-1, ICAM-1, and VCAM-1.[61] Oxidative stress is triggered by major signaling pathways, such as the MAPK signaling cascades. Activation of these proteins alters stress response pathways unique to particular cell types and conditions, causing apoptosis through phosphorylation of JNK and P38. As a biological mediator between oxidative stress and the pathogenic processes, ASK1 could be a potential therapeutic target to stop oxidative stress-related kidney damage. By blocking the ROS-mediated MAPK signaling pathway, kaempferol lessens drug-induced renal tubular damage.[62] The cytoplasm contains the inhibitory protein IκBα, while cisplatin-mediated ROS trigger signaling cascades involving p53, MAPK, and NF-κB. It has been demonstrated that phosphorylation of IκBα contributes to the activation of NF-κB, which translocates to the nucleus and activates inflammation-related genes, causing damage to renal cells. Kaempferol modulates NF-κB levels by preventing IκB kinase (IKK) phosphorylation and IκBα degradation, thereby reducing the risk of cisplatin-induced kidney damage.[63] According to Yuan *et al*., calcium oxalate (CaOx) crystal deposition and crystal-induced renal TEC injury are the main factors to the development of CaOx nephrolithiasis. Excess ROS generated during oxidative stress is regulated by NOX. Renal oxidative stress and inflammation have been associated with elevated NOX2 expression. The activation of the NOX isoenzyme suppresses the oxidative and inflammatory damage produced by the crystals, as well as the generation of adhesion molecules, by downregulating the NOX2 signaling pathway. Kaempferol may have a significant role in reducing the quantity of CaOx crystals that deposit in the renal cell.[64] Nevertheless, kaempferol treatment decreased production of proinflammatory cytokines such as TNF and MPO, which lessens leukocyte infiltration and kidney damage. In addition, kaempferol regulated NF-kB levels, inhibited the activation of the IKK, and reduced drug-induced renal inflammation.

3.7.4. Other bioactive compounds

Quercetin, a flavonol has been shown to protect DNA by lowering oxidative stress. In kidney damage caused by ionizing radiation, quercetin inhibits neutrophil infiltration and subsequent release of proinflammatory biomarkers, reducing oxidative stress-related DNA damage and apoptosis. Moreover, quercetin reduced oxidative stress, ROS, and thiobarbituric acid induced by lead exposure, preventing nephrotoxicity.[65] Similarly, quercetin's anti-apoptotic and antioxidant properties protect against kidney damage caused by titanium dioxide nanoparticles. In addition, quercetin treatment improved kidney function by increasing serum SOD and lactate dehydrogenase levels, and total antioxidant activity, demonstrating nephroprotective properties. This activity is believed to be caused by quercetin's ability to decrease the production of malondialdehyde and its capacity to remove ROS. Nrf2 and HO-1 are primarily activated by free radicals and ROS. The Nrf2/HO-1 pathway may be crucial for boosting the antioxidant moieties of glutathione (GSH), SOD, and GPx in relation to nephrotoxicity. In animals with copper sulfate-induced[66] and gentamicin-induced kidney damage, quercetin significantly increased the mRNA expression of HO-1 and Nrf2 when administered at a dose of 50 mg/kg.[67-69] The Food and Drug Administration has classified quercetin as "Generally Recognized as Safe" for use as a dietary supplement due to its well-established safety and tolerability profile in humans. Another study reported that myricetin is a bioactive phytocompound that has historically been used to treat a variety of ailments, including malaria, dysentery, diarrhea, and hypertension. Different parts of the plant, such as its fruits, bark, and leaves, have been utilized in these treatments. Other reported uses include antihypertensive and vasodilatory properties, analgesic and anti-inflammatory properties, antimalarial activity, and antidiabetic properties.[51] Flavonoids are potential substances to explore further for the development of innovative CKD therapy agents. However, the dearth of clinical studies implies that further research is required before flavonoids can be applied in medical treatments. Finding the metabolites produced after dosage and increasing bioavailability is also essential, as they could increase the advantages of flavonoids.[70] A flavone called luteolin, which is naturally present in various plants, has several pharmaceutical properties, like anti-inflammatory effects. It also lessens kidney damage caused by mercuric chloride. Luteolin decreases total TNF-α expression and several other indicators of inflammation by blocking NF-kB and activating the Nrf2 pathway.[71] In the Middle East, it has been used as traditional medicine since ancient times. The primary aglycones found are rhamnocitrin, kaempferol, quercetin, and rhamnetin. Renal colic and its associated symptoms were treated by the ancient Egyptians with a fruit decoction that also relieved prostatic pain, urolithiasis, and kidney inflammation. Raising urine pH and citrate concentration, reducing urine oxalates, and protecting renal epithelial cells from calcium oxalate monohydrate crystals have been shown to limit the oxalate formation associated with the formation of kidney stones.[72,73] Among the Lespedeza species, *Lespedeza*

capitata is less studied, although extracts from its leaves and stems, as well as the roots of *Asparagus racemosus* (family Asparagaceae), are used for urinary tract and KD due to the presence of bioactive compounds, including quercetin, apigenin, resveratrol, quercetin-3-D-galactoside, 3,3',4'-trihydroxyflavone (synonym 5,7-dideoxyquercetin), and 6-methyldihydroquercetin.[74-76]

3.8. Glucagon-like peptide 1 receptor agonists (GLP-1RAs)

The human GLP-1RAs are stimulated by the pharmaceutical class of peptides known as GLP-1RAs. There is debate regarding whether GLP-1RAs affect glomerular hemodynamics. GLP-1RAs may reduce glomerular hyperfiltration by reducing vasoconstriction induced by endothelin-1 and Angiotensin II. However, in theory, tubule-glomerular feedback would cause vasoconstriction of the pre-glomerular arteriole in response to reduced proximal salt reabsorption. However, the current study found that exenatide had a net vasodilatory impact on pre-glomerular arterioles, indicating a greater direct vasodilation effect. According to these results, GLP-1RAs are renal vasodilators and proximal diuretics that, in healthy individuals, have a negligible effect on tubule-glomerular feedback. It is probable that GLP-1 protects the renal system from damage caused by oxidation because GLP-1R activation stimulates the cyclic adenosine monophosphate–protein kinase A pathway, which results in antioxidative actions.[40] GLP-1RAs also decreased the expression of a number of inflammatory markers in rats in a diabetic nephropathy model, including collagen I, alpha-smooth muscle actin, tubulointerstitial TNF-alpha, MCP-1, fibronectin, and prevented tubulointerstitial lesions. These biomarkers have all been linked to diabetic nephropathy.[77]

4. Conclusion

Research on the prevention and protection of slow-progressing renal illnesses has been carried out globally. At present, dialysis and kidney transplantation are the primary treatments for KD, but these options are very expensive and have a number of drawbacks. In light of these challenges, further research is required to prevent and treat ESKD and to prolong the lives of KD patients. The current research on KD shows potential for opening new pathway to reduce the global burden of KD.

This review primarily highlights that ACEIs, ARBs, renin inhibitors, apoptosis-based treatment strategies, phytomedicines, JAK/STAT inhibition, and TNF inhibition may offer nephroprotective effects well beyond their main indications for diabetic nephropathy, kidney cancer, AKI, and CKD. Moreover, combining these therapies with a specific administration route could enhance their effectiveness, as they may provide additive nephroprotective effects. Future research focused on molecular pathway will be necessary to determine the effect of these treatments.

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Conflict of interest

The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

Innovative Medicines & Omics

PERSPECTIVE ARTICLE

Medicinal cannabis delivery systems: A perspective

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Abstract

In recent years, cannabis derivatives have been proposed for the treatment of various medical conditions, including pain, inflammation, epilepsy, sleep disorders, multiple sclerosis, anorexia, schizophrenia, neurodegenerative diseases, nausea, and cancer. While the benefits of cannabis derivatives, primarily cannabinoids, have been demonstrated and continue to be studied, their use presents various challenges associated with their low water solubility, rapid metabolism, erratic and poor bioavailability, and erratic pharmacokinetics, which directly affect their efficacy. In this context, a great deal of research is being carried out to overcome these drawbacks by designing delivery systems capable of improving solubility/bioavailability, potency, and efficacy, while addressing the purity and quality issues required by the pharmaceutical industry. This article aims to critically review the major trends and challenges in designing controlled-release cannabinoid delivery systems and their potential application in the pharmaceutical industry.

Keywords: Cannabis; Cannabinoids; Drug delivery systems

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1. Introduction

Cannabis plant (*Cannabis sativa* L.) is one of the oldest plants cultivated for food, fiber, and medicine. Native to western Asia, it can grow in all habitats, from sea level to temperate zones and alpine foothills. Cannabis has been used for thousands of years to treat conditions such as asthma, epilepsy, fatigue, glaucoma, insomnia, nausea, pain, and rheumatism.[1] First botanically classified in 1753 by Carl Linnaeus, *C. sativa* L. is an annual plant belonging to the *Cannabaceae* family. Later, in 1785, Jean Baptiste Lamarck discovered another species which he named *Cannabis indica*. At present, the Missouri Botanical Garden recognizes 13 species, namely *C. sativa*, *C. indica*, *Cannabis americana*, *Cannabis chinensis*, *Cannabis erratica*, *Cannabis foetens*, *Cannabis generalis*, *Cannabis gigantea*, *Cannabis intersita*, *Cannabis kafiristanica*, *Cannabis lupulus*, *Cannabis macrosperma*, and *Cannabis ruderalis*.[2] Chemical composition of these species has been widely studied, and approximately 500 compounds have been identified, including cannabinoids, terpenes, flavonoids, alkaloids, stilbenes, phenolic amides, and lignanamides.[3-5]

Cannabinoids are the most abundant and exclusive metabolites of these species and the most important because they are capable of interacting with an entire system of endogenous receptors (endogenous cannabinoid system). In addition, they are terpenophenolic in nature and are generally concentrated in the resin produced in the plant's trichomes, especially in the female inflorescences. There are around 70 known terpenophenolics, of which tetrahydrocannabinol (THC) is the most studied.[3] Cannabinoids are synthesized and accumulated as cannabinoid acids, and it is not until the drying and storage process that the acids are gradually decarboxylated until they reach their final form, such as THC or cannabidiol (CBD).[6] Around 120 terpenes have also been identified in these plant species.[3,7] These metabolites are responsible for the flavor of the different varieties. Some of these terpenes are pharmacologically active and could produce synergistic effects with cannabinoids.[8] Flavonoids are aromatic compounds and can be found in free form or conjugated with a glucoside. More than 20 of these metabolites are produced,[3,7] mainly found in the plant leaves, and some studies suggest that they modulate the action of cannabinoids.[8] Other chemical components also present in the plant are alkaloids, although they are found in lower proportions. These are nitrogenous compounds that usually present biological activity at low doses and can be derived from amino acids. Due to the low concentration of alkaloids present in these species, their pharmacological evaluation is difficult. Finally, it also contains stilbenoids, lignanamides, and phenolic amides. Stilbenoids are phenolic compounds which are presumed to have certain pharmacological activities, such as antibacterial, antifungal, anti-inflammatory, antineoplastic, neuroprotective, cardio- and vasculo-protective, and antioxidant effects. Phenolic amides have also been reported to have cytotoxic, anti-inflammatory, antineoplastic, and analgesic activities, whereas some lignanamides have shown cytotoxic activity.[3]

2. Medicinal cannabis delivery systems

Given the low bioavailability of cannabinoids, their clinical use is limited. In this sense, the pharmaceutical industry is focused on improving this point to optimize the different formulations. At present, a wide variety of cannabis products are commercially available to be administered in several routes, namely oral, transdermal, pulmonary, and transmucosal administration. Various release systems have been proposed for each administration route to increase their efficacy. Table 1 shows the main advantages and disadvantages of each route of administration.

2.1. Oral administration

Although oral administration of cannabinoids has been shown to have poor bioavailability, mainly due to poor absorption and degradation in the stomach or pre-systemic metabolism in the liver, various formulations have been proposed to counteract this disadvantage. For example, several encapsulation systems have been proposed to protect cannabinoids, such as micelles, soft capsules, and tablets.[9-11] Another alternative to improve oral bioavailability is the combination with lipids, which has been shown to increase the cannabinoid absorption rate. In the case of long-chain triglycerides, it has also been shown that this combination can cross the liver, reducing the possibility of pre-systemic metabolism.[12] Cyclodextrins or their derivatives have also been proposed to form inclusion complexes that significantly increase cannabinoid solubility.[13] Another approach to improve cannabinoid solubility is their derivatization, known as prodrugs. An example is the CBD/naproxen combination developed by Claritas Pharmaceuticals (USA), which is being tested

Table 1. Main advantages and disadvantages of oral, transdermal, pulmonary, and transmucosal administration of cannabis products

Route of administration	Advantages	Disadvantages
Oral	Patients prefer oral administration for its simplicity.	This route causes slow and erratic absorption and degradation by stomach acids. Bioavailability is reduced by the pre-systemic metabolism in the liver.
Transdermal	Transdermal delivery bypasses the first-pass metabolism effect, resulting in increased drug bioavailability. Besides, it can provide continuous drug release. Topical application is suitable for localized pathologies.	Local irritation and limited skin penetration of drugs.
Pulmonary	Highly effective, rapid distribution to the central nervous system, and higher systemic bioavailability.	Inhaling harmful combustion products can cause bronchitis and chronic coughing. Vaporization systems and nebulization formulations lack practicality.
Transmucosal	Transmucosal delivery bypasses the first-pass metabolism effect, resulting in increased drug bioavailability.	The use of transmucosal excipients may cause mouth ulcers. The bioavailability of intranasal delivery systems could be improved.

in various pathologies.[14] Although the aforementioned formulations partially counteract the difficulties associated with oral administration of cannabinoids, there are other routes of administration that could increase efficacy through more direct uptake and transport of the drug into the bloodstream, also avoiding hepatic metabolism. These alternative administration routes are described in sections 2.2, 2.3, and 2.4.

2.2. Transdermal administration

Dermal formulations can be transdermal (for systemic effects) and topical (for localized effects on the skin). Transdermal drug administration has the advantage of avoiding the first-pass metabolism effect. In addition, a sustained release is achieved over time, preventing the adverse effects of high drug concentrations. Topical application suits localized symptoms associated with peripheral neuropathic pain, dermatological disorders, and arthritis. However, this administration route has several disadvantages such as limited skin penetration of drugs and local irritation. Particularly, cannabis is highly lipophilic, a property that hampers its release through the skin. In this sense, numerous researchers have developed synthetic cannabinoid prodrugs to obtain more skin-permeable products and demonstrated the efficiency of this strategy.[15,16]

2.3. Pulmonary administration

Since ancient times, cannabis has been consumed in the form of cigarettes, both for recreational and therapeutic purposes. Its compounds have very high bioavailability, reaching a maximum concentration in plasma after approximately 7.5 min. In addition, this concentration can remain constant for about 4 h with rapid distribution to the central nervous system. Unfortunately, this route of administration has the problem of being able to cause conditions such as bronchitis and chronic cough associated with the harmful products generated during smoking.[15] One method that can mitigate these adverse effects is the use of vaporization, which involves heating cannabis to the temperature at which cannabinoids are vaporized, below the combustion temperature. This avoids the formation of harmful compounds associated with the combustion process.[17]

2.4. Transmucosal administration

Through transmucosal administration, drugs can be rapidly absorbed into the bloodstream due to the vascularity of the oral mucosa. This route of administration is non-invasive and has the advantage of being able to bypass the first pass through the liver. In this way, pre-systemic metabolism is avoided. Although the oral route is the most commonly used intramucosal route of administration, there are other more effective administration modes such as intranasal, oral transmucosal, and rectal routes. In this context, a multitude of cannabis products have been developed to be administered by this route, such as sublingual disintegrating thin films, sprays, orally disintegrating tablets, buccal mucoadhesive tablets, films and patches, chewing gum, and lozenges.[15] These formulations have been shown to increase the bioavailability of cannabinoids, but have not been developed with the ability to release cannabinoids at sites of interest such as tumors or disease sites, and also show various adverse effects. In this context, there is currently a growing interest in the development of nano-formulations of cannabis compounds for cancer therapy, pain management, and treatments for various other pathologies.

3. Trends, perspectives, and conclusion

The usefulness and efficacy of a drug delivery system depend on several factors such as solubility, half-life, metabolism, distribution, and ease of use. In this regard, many novel proposals have been developed in recent years to improve the performance of the above-mentioned systems. Particularly, nanotechnology is an example of a novel proposal for medicinal cannabis delivery systems. Nanoparticles have been extensively studied in controlled release systems. Their main attributes are: (i) improved encapsulation efficiency and increased drug solubility; (ii) presence of surface functional groups that improve stability and internalization; (iii) increased bioavailability, improved pharmacokinetics, and minimal clearance from the body; and (iv) controlled drug release properties. Examples of nanoparticle-based delivery systems for cannabinoids are nano-emulsions, dendrimers, micelles, liposomes, and biodegradable polymers. As mentioned above, the delivery of cannabinoids in lipids has several advantages which, in combination with nanotechnology, make it highly attractive in the design of delivery systems. In this context, systems based on nanomicelles, nanoemulsions, and nanostructured lipid carriers have been proposed. Another alternative is the formation of self-emulsifying delivery systems, which can improve the bioavailability, dissolution, and stability of cannabinoids. These systems consist of mixtures of solvents, surfactants, and oils with the ability to spontaneously disintegrate upon contact with an aqueous phase, such as the fluids present in the gastrointestinal tract. On the other hand, polymer-based drug delivery systems have also been proposed to modulate the release of cannabis compounds. In particular, poly(lactic-co-glycolic acid) has been widely studied for encapsulating cannabis-based compounds due to its biocompatible and biodegradable properties.[15] The use of nanotechnology

to develop cannabis-based formulations has shown to have several advantages over other formulations, such as increased efficacy, tumor targeting, and reduced systemic toxicity. However, each proposed formulation has been studied or has remarkable performance for a specific type of cannabinoid. In this sense, further developments using nanotechnology applicable to a specific cannabinoid or group of cannabinoids are needed, accompanied by appropriate pre-clinical and clinical studies.

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Conflict of interest

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

Author contributions

Conceptualization: All authors
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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

High inhibitory activity of flavonoids from *Sophora japonica* L. flower buds against α-amylase and α-glucosidase: A mechanistic insight

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Abstract

In this work, a method was developed to screen compounds with enzyme activity inhibition *in vitro* using chromatographic analysis combined with activity difference analysis (CAADA). The flower buds of *Sophora japonica* L. (FBSJ) were found to contain abundant flavonoids. These flavonoids were then screened for their high inhibitory activity against α-amylase and α-glucosidase using fingerprint and activity difference analysis. Consistent conclusions were drawn from multiple techniques, including the reported technique, IC_{50} data, and CAADA. The inhibitory mechanism was further analyzed through enzyme inhibition kinetics, circular dichroism, fluorescence spectrometry, molecular docking, and molecular dynamics (MD). Among the six flavonoid components studied, quercetin was found to act as a competitive inhibitor against α-amylase, while kaempferol showed a mixed manner of inhibition against α-glucosidase. Molecular docking and MD simulations demonstrated that quercetin and kaempferol have higher binding energies and bound more tightly to their targets. In general, flavonols exhibited higher inhibitory activity than their corresponding flavonol glycosides against both α-amylase and α-glucosidase. Quercetin and kaempferol in FBSJ showed potential as inhibitors of α-amylase and α-glucosidase. This study not only presented a novel method for screening compounds with high activity but also provided a theoretical basis for studying the application and mechanism of flavonoids against α-amylase and α-glucosidase in FBSJ.

Keywords: *Sophora japonica* L.; Flavonoids; α-amylase; α-glucosidase

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1. Introduction

Flavonoids, polyphenolic compounds typically present in plants, play a crucial role in human nutrition, and numerous studies have thoroughly documented their various biological functions.[1] These compounds demonstrate multifunctional anti-diabetic properties by influencing certain cellular signaling pathways, thereby enhancing multiple facets of carbohydrate metabolism, including α-glycosidase activity, glucose

transport, aldose reductase activity, and cellular glucose uptake.[2-4] A practical strategy for controlling postprandial hyperglycemia involves using α-glucosidase and α-amylase inhibitors.[5] Implementing dietary strategies that incorporate flavonoids can help establish effective dietary recommendations for healthy individuals and aid in relieving symptoms for patients suffering from Type 2 Diabetes Mellitus.[6]

The flower buds of *Sophora japonica* L. (FBSJ), a form of traditional Chinese medicine (TCM), are common known as Flos Sophorae Immaturus, which are utilized for treating various ailments, including hypertension and arteriosclerosis. Flavonoids, such as quercetin, kaempferol, genistein, rutin, and isorhamnetin, have been isolated from *S. japonica* L.[7,8] The components found in TCM are intricate, with numerous chemicals contributing to its therapeutic efficacy. This intricacy poses challenges in identifying the specific active constituents of TCM. Nevertheless, high-performance liquid chromatography (HPLC)-based fingerprint spectra have been employed to thoroughly analyze the diversity and concentrations of compounds in botanical materials, thereby assessing the quality of TCM products.[9,10] Circular dichroism (CD), when paired with fluorescence quenching methods, is frequently used to examine how flavonoids interact with proteins, offering a crucial understanding of protein-ligand interactions, as well as the conformational and structural changes of flavonoids in relation to proteins.[11] Furthermore, molecular simulation methods, including molecular docking and dynamics, have become increasingly significant in studying interactions and have contributed to exploring the mechanisms behind inhibitor-enzyme interactions, enhancing our comprehension from a molecular biology standpoint regarding the efficacy of these interactions.[12]

Various screening methods have been developed and applied in a wide range of fields. However, these methods often require specialized software and complex data processing. Therefore, there is an urgent need to develop a simple and feasible screening and comparison method. Chromatographic analysis combined with activity difference analysis (CAADA) was applied. This article aimed to assess the inhibitory potentials of the extract against α-amylase and α-glucosidase and to develop a new screening method using compounds found in FBSJ. The compounds that exhibited significant inhibitory effects were verified, and the inhibitory mechanism was preliminarily studied.

2. Materials and methods

2.1. Materials

FBSJ was collected from the campus of Henan University. Porcine pancreas α-amylase and yeast α-glucosidase were purchased from Bomei Biochemical Co. (Hefei, Anhui Province, China). 4-Nitrophenyl-α-D-glucopyranoside (pNPG) was purchased from Beijing Kuerhuaxue Co (Beijing, China). Rutin, quercetin, kaempferol, isorhamnetin, kaempferol-3-O-rutinoside, and narcissoside were purchased from Chengdu Alfa Biotechnology Co (Chengdu, China). Acarbose tablets were purchased from Bayer Healthcare Co (Leverkusen, Germany).

2.2. Determination of flavonoids in samples

The active components of *S. japonica* L. were extracted using ethanol (5 g FBSJ: 100 mL solvent) at various concentrations of 30%, 50%, 70%, and 90%, as well as with ethyl acetate. Flavonoid standards were dissolved in methanol, while the flavonoid content in the samples was extracted using ethanol. These flavonoids were subsequently separated with a Waters HPLC system (Milford, Massachusetts, USA) equipped with a C18 Agilent column. The gradient elution was performed under the following conditions: (A) and 0.05% phosphoric acid (B), with the following time intervals: 0 – 10 min at 24 – 40% (A); 10 – 20 min at 40 – 50% (A); 20 – 35 min at 50 – 75% (A); 35 – 40 min at 75 – 80% (A); 40 – 50 min at 80 – 95% (A); 50 – 55 min at 95 – 95% (A); and 55 – 80 min at 95 – 24% (A). Both qualitative and quantitative analyses of the samples were conducted using standard solutions. The data were imported into the "Traditional Chinese Medicine Fingerprint Similarity Evaluation System," and then HPLC fingerprint spectra were obtained.

2.3. Inhibition activity assays of enzyme

2.3.1. The assessment of α-amylase inhibition activity

The assessment of α-amylase inhibition activity was conducted as described in previous studies, with necessary adjustments made.[13] Initially, 20 of samples at various concentrations were combined with 150 μL of α-amylase solution (0.58 U/mL) and incubated at 37°C for 5 min. Subsequently, 150 μL of 1% starch was added to the mixture, which was then incubated at 37°C for an additional 15 min. Finally, 250 μL of DNS reagent (dinitrosalicylic acid reagent, a reagent used for the determination of reducing sugar content, prepared by dissolving 18.2 g of potassium sodium tartrate in 50 mL of distilled water, heating, and sequentially adding 0.63 g of 3,5-dinitrosalicylic acid, 2.1 g of NaOH, and 0.5 g of phenol to the hot solution, stirring until dissolved, cooling, and diluting with distilled water to 100 mL, stored in a brown bottle at room temperature) was mixed, and the mixture was boiled for 5 min. After diluting the solution to an appropriate concentration, its absorbance was recorded at 540 nm using a microplate reader.

2.3.2. The assessment of α-glucosidase inhibition activity

The assessment of α-glucosidase inhibition activity was conducted as described in previous studies, with necessary adjustments made.[14] Initially, a mixture was prepared by adding 50 μL of α-glucosidase at a concentration of 2 U/mL, followed by 50 μL of the inhibitor at varying concentrations, and 80 μL of buffer solution. This mixture was incubated for 10 min at 37°C. Subsequently, 50 μL of pNPG (20 mM) was added, and the contents were mixed thoroughly and shaken. The reaction was allowed to continue at 37°C for 20 min, after which it was concluded by adding 100 μL of Na_2CO_3. The solution was then diluted to a suitable concentration, and the absorbance at 540 nm was measured with a microplate reader. The inhibition rate was calculated using the following equation:

$$\text{Inhibition rate}\% = 1 - [\frac{(A_3 - A_4)}{(A_1 - A_2)}] \times 100 \quad \text{(I)}$$

A^1 served as the blank group, consisting of enzyme and buffer, while A_2 acted as the blank control group, containing only the buffer. A_3 represented the sample group that included the enzyme and the inhibitor, and A_4 functions as the sample control group, which was made up of buffer and the inhibitor. The IC_{50} value indicated the concentration of the inhibitor needed to reduce the enzyme activity by half. Acarbose was established as a control substance.

2.4. Correlation analysis

The research focused on the concentration-effect relationships of various solvent extracts regarding their ability to inhibit the functions of α-amylase and α-glucosidase. Grey relation analysis (GRA) was performed using Statistical Package for the Social Sciences (SPSS) Pro, utilizing the peak areas obtained from the HPLC fingerprint profiles of the different extracts, in conjunction with the IC_{50} values associated with enzyme inhibition activities.[15,16] By examining the overlapping areas of the HPLC fingerprints and their corresponding IC_{50} values, CAADA was used to assess the peak areas of each constituent in relation to the IC_{50} values. This approach established a relationship between individual components and their enzyme inhibitory effects, while also proposing hypotheses about the compounds responsible for these inhibitory activities.

2.5. Kinetic assay for inhibition action

For the overall assessment of inhibition kinetics, the same procedures utilized for evaluating enzyme activity were applied. The Lineweaver–Burk plot was employed to analyze the inhibition type.[8] This plot was derived using specific equations:

$$\text{Competitive inhibition: } \frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad \text{(II)}$$

$$\text{Uncompetitive inhibition: } \frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{(III)}$$

Noncompetitive inhibition:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{(IV)}$$

$$\text{Mixed inhibition: } \frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right) \quad \text{(V)}$$

$$\text{Slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{(VI)}$$

$$Y_{intercept} = \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right) \quad \text{(VII)}$$

Where v is the reaction rate; K_m, K_i, and K_{is} represent the Michaelis-Menten, free and bound enzyme inhibition constants, respectively; [S] stands for substrate concentration; [I] represents the concentration of the inhibitor.

2.6. Characterization method

2.6.1. Fluorescence spectrum analysis

A fluorescence quenching test was conducted to study the interaction between flavonoids in FBSJ and α-amylase or α-glucosidase, with slightly modifications.[17] α-amylase (0.58 U/mL) and α-glucosidase (2 U/mL) were incubated with different concentrations of quercetin/kaempferol in the water bath at 298 K, 304 K, and 310 K for 5 min. Measurements were performed under an excitation wavelength of 280 nm and a scanning wavelength range of 300 – 500 nm, with both the excitation and emission slit widths set to 5.0 nm. The information about K_{SV}, K_q, and K_a and quenching type derived from the Stern-Volmer equation as follows:

$$F_0 / F = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \quad \text{(VIII)}$$

$$\lg\frac{F_0 - F}{F} = \lg K_a + n\lg[Q] \quad \text{(IX)}$$

Where F_0 and F represent the fluorescence intensities with or without flavonoids, respectively, and τ_0 is 10^{-8} s; K_{SV}, and K_q represent the quenching constants and diffusion collision quenching rate constants, respectively; [Q] represents the concentration of flavonoids.

2.6.2. Calculation of thermodynamic parameters

The temperature-dependent interactions were investigated by calculating the interactions between inhibitors and enzymes using the following equations:

$$\ln K_a = -\frac{1}{T}\left(\frac{\Delta H}{R}\right) + \frac{\Delta S}{R} \quad (X)$$

$$\Delta G° = \Delta H° - T\Delta S° = -RT\ln K_a \quad (XI)$$

Where K_a ($Lmol^{-1}$) is the binding constant, R (8.314 J mol^{-1} K^{-1}) is the gas constant, $\Delta S°$ and $\Delta H°$ represent the values used to evaluate the binding force, and $\Delta G°$ indicates the spontaneity of the interaction.

2.6.3. CD spectrum analysis

The α-amylase and α-glucosidase were mixed with flavonoid samples in different proportions and scanned by CD spectroscopy. The combined solutions were scanned across a wavelength range of 190 – 260 nm. The results of the measurements were then calculated using CDNN software (Aviv Biomedical Inc., Lakewood, USA).

2.7. Molecular docking

Molecular docking simulations were carried out using AutoDock 4.0. The structures of flavonoid compounds were downloaded from PubChem. The protein data for α-amylase (PDB ID: 1OSE) were obtained from the Protein Data Bank, and α-glucosidase was obtained from homology modeling. Before docking, the enzyme structures were prepared by removing all water molecules, adding hydrogen atoms, and distributing the Gasteiger charges to generate the PDBQT file. Finally, the docked complexes were visualized and analyzed by PyMOL.[18,19]

2.8. Molecular dynamics (MD)

MD simulations at 100 ns were performed using GROMACS 2020. The Amber99 SB force field was applied to the proteins in this study, and the topologies of a-amylase and α-glucosidase were prepared using the pdb2gmx module of GROMACS 2020. The topology and coordinate parameter files for quercetin and kaempferol were generated through simulation on the ACPYPE server (https://www.bio2byte.be/acpype/status/). The physiological environment of the proteins was simulated by adding the TIP3P aqueous model to the protein complex system, which was solvated using dodecahedral boxes, with sodium ions added to neutralize the overall charge. The simulations were performed using the conjugate gradient method combined with the most rapid descent method for 50,000 steps each to minimize energy. The V-rescale thermostat was used for temperature coupling to obtain a correct trajectory distribution of the canonical ensemble. The Berendsen pressure control method was used to slowly heat the system temperature to 300 K within 10 ns. After heating, the NPT ensemble (T = 300 K and P = 1 atm) was used for 100 ns simulation under periodic boundary conditions.[20]

2.9. Statistical analysis

All data were analyzed and presented as the mean ± standard deviation of the results. Three experiments were performed for each data set. All statistical analyses were carried out using SPSS 19.0 (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8 (GraphPad, San Diego, USA) to evaluate the significance of differences.

3. Results

3.1. Identification of flavonoids in FBSJ and establishment of HPLC fingerprint

Figure 1 shows the fingerprints of flavonoids in FBSJ extracted with different solvents. A total of six common peaks were calibrated from the six samples. Compared with the peak diagram of the reference substance, the six components were identified as rutin, kaempferol-3-O-rutinoside, narcissoside (isorhamnetin-3-O-rutoside), quercetin, kaempferol, and isorhamnetin according to the peak times, which was consistent with previous reports.[21,22] The flavonoids in FBSJ include three flavonols and their corresponding flavonol glycosides, which contributed to comparing the activities of flavonols and their corresponding glycosides. Combined with the fingerprints, it was evident that the main chromatographic peak was rutin in various ethanol solutions extract of FBSJ, while the main chromatographic peak was quercetin in ethyl acetate extract of FBSJ. It was shown that the extraction of FBSJ with different solvents resulted in varying enzyme inhibition rates (expressed by the half inhibition rate concentration, IC_{50}). The order of α-amylase inhibition rates was ethyl acetate >30% ethanol >90% ethanol >50% ethanol >70% ethanol >water. The order of α-glucosidase inhibition rate was ethyl acetate >70% ethanol >90% ethanol >50% ethanol >30% ethanol >water (Table 1). The inhibition effect of the ethyl acetate extract of FBSJ was significant compared to the other extracts, although acarbose showed the most obvious inhibition. Based on the data in Figure 1, quercetin was the main constituent in ethyl acetate, which suggests that quercetin may be the favorite natural compound responsible for the inhibitory activity.

3.2. Correlation analysis

The peak areas of six common peaks, represented by P1-P6, were established as the comparison sequence, with the IC_{50} values for enzyme inhibition as the reference sequence.[23] According to references,[24-26] using the enzyme inhibition rate as a pharmacodynamic index, the results are shown in Table 2. The correlation order with α-amylase was P5, P4, P3,

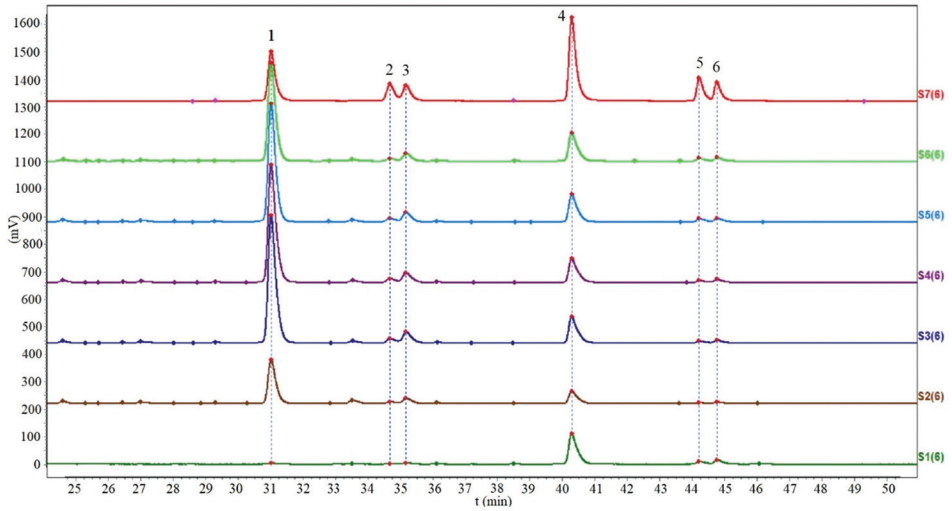

Figure 1. High-performance liquid chromatography fingerprint of extracts with different solvents. S1: Ethyl acetate extract, S2: Water extract, S3: 90% ethanol extract, S4: 70% ethanol extract, S5: 50% ethanol extract, S6: 30% ethanol extract, S7: Flavonoids standard mixed solution (Peak 1: Rutin, Peak 2: Kaempferol-3-rutinoside, Peak 3: Narcissoside, Peak 4: Quercetin, Peak 5: Kaempferol, Peak 6: Isorhamnetin).

Table 1. Inhibition effects of different extracts of FBSJ on enzymes

Samples	α-amylase inhibition IC_{50} (mg/mL)	α-glucosidase inhibition IC_{50} (mg/mL)
Acarbose	0.95±0.01	0.00033±0.0001
30% ethanol extract	13.59±0.03	0.63±0.02
50% ethanol extract	14.68±0.05	0.61±0.01
70% ethanol extract	20.09±0.12	0.29±0.02
90% ethanol extract	14.36±0.03	0.43±0.03
Ethyl acetate extract	10.16±0.01	0.09±0.01
Water extract	44.26±0.16	0.94±0.03

Abbreviation: FBSJ: Flower buds of *Sophora japonica* L.

P6, P2, and P1, while the correlation order with α-glucosidase was P5, P4, P6, P3, P2, and P1. Among the six characteristic peaks of the HPLC pattern, the correlations of peaks were all >0.6, indicating that the inhibition of both enzyme's activities by FBSJ was jointly exerted.[27] Peak P4 (quercetin), peak P5 (kaempferol), and peak P6 (isorhamnetin) were more closely related, indicating that quercetin, kaempferol and isorhamnetin might have a better inhibitory effect. The use of CAADA was an integrated method to assess the changes in the content of each substance in different solvent extracts and their corresponding enzyme inhibition activity. By comparing the changes in each substance across different solvent extracts and the total inhibition rate (up- or down-regulation), and according to the significant difference (*P*-value) of the peak areas of the fingerprint profiles of different solvent extracts, the results were evaluated. If the change of the substance and the change of the total inhibition rate were the same and $P < 0.01$ scored 2 points, and $P < 0.05$ scored 1 point, on the contrary, the opposite change scored −1 point. The results, shown in Tables 3-5, indicate that the highest scores were obtained for P4, P5, and P6, indicating that quercetin, kaempferol, and isorhamnetin had significant effects on the inhibition of both enzyme's activities. From the above results, the CAADA findings were in general agreement with the GRA and the IC_{50} values of each substance, indicating the reliability of CAADA and its relative simplicity and ease of operation.[28,29] Except for isorhamnetin, the result of the new method closely matched those of the GPA and IC_{50}, likely due to the lower concentration of isorhamnetin causing a system error. This analysis led to the conclusion that quercetin and kaempferol might be better inhibitors of both enzymes. From the IC_{50} values of each flavonoid, it was inferred that flavonols exhibited greater inhibitory activity than their corresponding flavonol glycosides against α-amylase and α-glucosidase under the same conditions, which is consistent with the structural requirements for enzyme activity reported in previous studies.[30]

3.3. Inhibition types of flavonoids

The inhibitory kinetics of kaempferol and quercetin against the enzymes α-amylase and α-glucosidase were investigated using Lineweaver–Burk plots to determine the type of inhibition.[31,32] As shown in Figure 2A-D, the Lineweaver–Burk plot for quercetin demonstrated several lines intersecting on the y-axis, showing that quercetin acts as a competitive inhibitor of α-amylase. Conversely, the plots for α-glucosidase revealed intersections in the second or third quadrants, indicating that quercetin inhibits α-glucosidase in a mixed inhibition manner.

Table 2. IC_{50} values and GRA analysis of flavonoids from FBSJ extracts

Peak	α-amylase		α-glucosidase	
	Correlation coefficient	IC_{50} (mg/mL)	Correlation coefficient	IC_{50} (mg/mL)
P5 (Kaempferol)	0.787	0.33±0.05	0.678	0.19±0.05
P4 (Quercetin)	0.783	0.29±0.03	0.669	0.06±0.01
P6 (Isorhamnetin)	0.777	1.1±0.10	0.664	0.21±0.06
P3 (Narcissoside)	0.781	>5	0.644	5.04±0.02
P2 (Kaempferol-3-rutinoside)	0.777	>5	0.64	3.00±0.03
P1 (Rutin)	0.772	ND	0.626	0.99±0.01

Abbreviations: ND: No activity detected; GRA: Grey relation analysis.

Table 3. Comparative index of different solvent extracts of various substances and inhibition of α- amylase activity

	30% ethanol	50% ethanol	70% ethanol	90% ethanol	Ethyl acetate	Water
30% ethanol	--	1↑2 ↑ 3↑4 ↓ 5↑6 ↑ I↓**	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↓**	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↓**	1↓2 ↓ 3↓4 ↑ 5↓6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
50% ethanol	--	--	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↓**	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↑**	1↓2 ↓ 3↓4 ↑ 5↓6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
70% ethanol	--	--	--	1↑2 ↑ 3↑4 ↑ 5↓6 ↓ I↑**	1↓2 ↓ 3↓4 ↑ 5↑6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
90% ethanol	--	--	--	--	1↓2 ↓ 3↓4 ↑ 5↑6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
ethyl acetate	--	--	--	--	--	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
water	--	--	--	--	--	--

Note: 1=Rutin; 2=Kaempferol-3-O-rutinoside; 3=Narcissoside; 4=Quercetin; 5=Kaempferol; 6=Isorhamnetin; I=inhibition; ↑/↓ = contrast peak area or inhibition activity rise or fall. *P<0.05, **P<0.01.

Table 4. Comparative index of different solvent extracts of various substances and inhibition of α-glucosidase activity

	30% ethanol	50% ethanol	70% ethanol	90% ethanol	Ethyl acetate	Water
30% ethanol	--	1↑2 ↑ 3↑4 ↓ 5↑6 ↑ I↑*	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↑**	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↑**	1↓2 ↓ 3↓4 ↑ 5↓6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
50% ethanol	--	--	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↓**	1↑2 ↑ 3↑4 ↓ 5↓6 ↓ I↑**	1↓2 ↓ 3↓4 ↑ 5↓6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
70% ethanol	--	--	--	1↑2 ↑ 3↑4 ↑ 5↓6 ↓ I↓**	1↓2 ↓ 3↓4 ↑ 5↑6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
90% ethanol	--	--	--	--	1↓2 ↓ 3↓4 ↑ 5↑6 ↑ I↑**	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
Ethyl acetate	--	--	--	--	--	1↓2 ↓ 3↓4 ↓ 5↓6 ↓ I↓**
Water	--	--	--	--	--	--

Note: 1=Rutin; 2=Kaempferol-3-O-rutinoside; 3=Narcissoside; 4=Quercetin; 5=Kaempferol; 6=Isorhamnetin; I=inhibition; ↑/↓ =contrast peak area or inhibition activity rise or fall. *P<0.05, **P<0.01.

Table 5. Integral method score table

Compound	α-amylase (score)	α-glucosidase (score)
P4 (Quercetin)	27	15
P5 (Kaempferol)	15	14
P6 (Isorhamnetin)	21	20
P4 (Rutin)	6	11
P2 (Kaempferol-3-O-rutinoside)	6	11
P3 (Narcissoside)	6	11

Similarly, kaempferol exhibited a mixed inhibition manner for both α-amylase and α-glucosidase.[33,34] The linear relationship between the slope and inhibitor concentration (as shown in the insets of Figure 2A-D) proving that these flavonoids bind to a single inhibition site on α-amylase and α-glucosidase.[35]

The K_i and K_{is} values for quercetin, kaempferol, and their interactions with the two enzymes were calculated according to equations (2) – (7).[36] The pertinent outcomes were displayed in Table 6. For kaempferol, the K_i value was lower than the K_{is} value, suggesting that kaempferol binds more tightly to α-amylase and α-glucosidase than the enzyme-substrate interaction. However, quercetin inhibited α-amylase competitively, indicating that quercetin binds directly to α-amylase and competes with the substrate. For

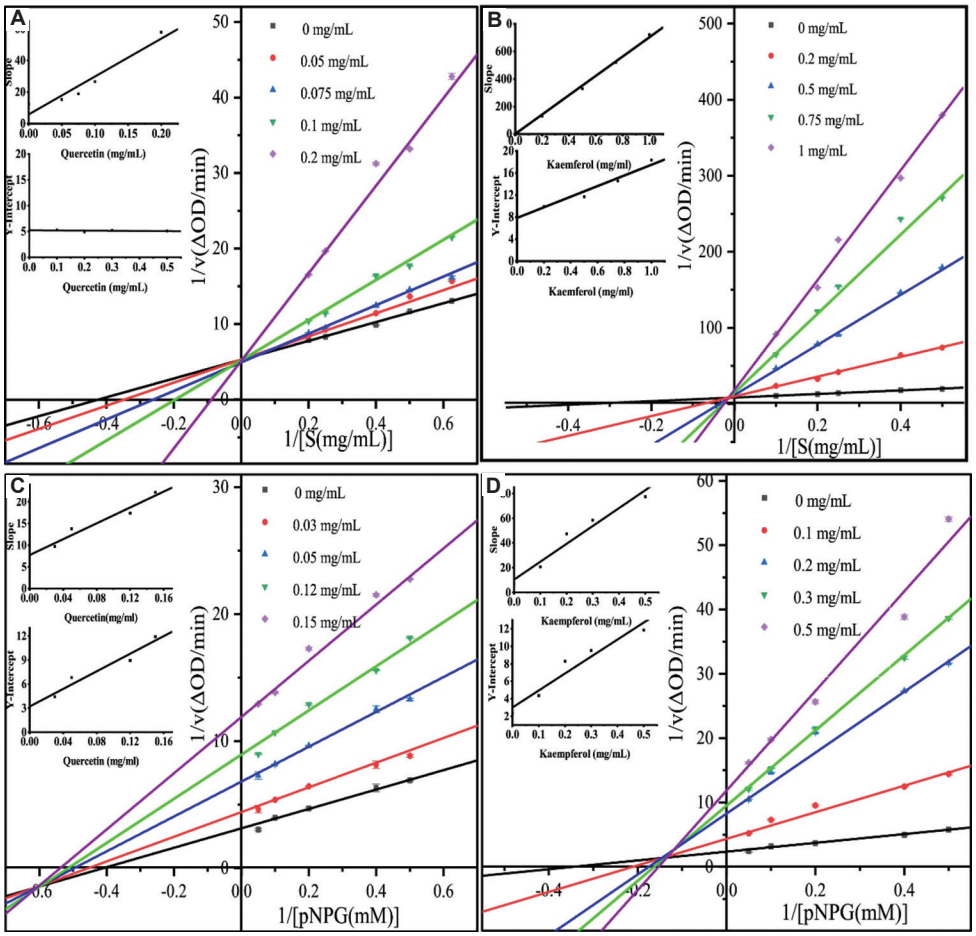

Figure 2. The inhibition kinetics of α-amylase by quercetin (A) and kaempferol (B), and α-glucosidase by quercetin (C) and kaempferol (D), as depicted by Lineweaver–Burk plots. The flavonoid concentrations used are indicated in the corresponding legend entries. The secondary plots (in the insets) show the linear relationship between the slope and y-intercept against the flavonoid concentration for each enzyme and inhibitor combination.
Abbreviation: pNPG: 4-Nitrophenyl-α-D-glucopyranoside.

quercetin, the K_i values (0.085 ± 0.002 mg/mL) were higher than the K_{is} values (0.058 ± 0.007 mg/mL), indicating that α-glucosidase binds stronger to the substrate complex than to the free enzyme.[37]

3.4. Fluorescence quenching

Figure 3 shows the fluorescence emission spectra of quercetin and kaempferol at different temperatures with α-amylase and α-glucosidase. The fluorescence intensity gradually reduced with increasing concentrations of quercetin and kaempferol, suggesting a noticeable impact of the inhibitors on both α-amylase and α-glucosidase.[38] The fluorescence intensity of the inhibitor's binding to the enzyme decreases with increasing inhibitor concentration, suggesting a more pronounced quenching effect on the enzymes.

Stern-Volmer analysis was performed to determine the quenching mechanism and binding constants. Quenching mainly occurs through two mechanisms between inhibitors and enzymes, including static and dynamic.[39] Using the equations (8) – (9), F_0/F pair [Q] plots were generated to calculate the values.[12,40] The K_q values were all $>2 \times 10^{10}$ L $mol^{-1}s^{-1}$, and these values gradually decreased with increasing temperature (Table 7). This suggested that the quenching mechanism of quercetin and kaempferol

Table 6. Detailed kinetic parameters of quercetin and kaempferol

Concentrations	K_i (mg/mL)	K_{is} (mg/mL)	Inhibition type
α-amylase			
Quercetin	0.024±0.003	-	Competitive
Kaempferol	0.036±0.0003	0.051±0.006	Mixed
α-glucosidase			
Quercetin	0.085±0.002	0.058±0.007	Mixed
Kaempferol	0.071±0.003	0.155±0.012	Mixed

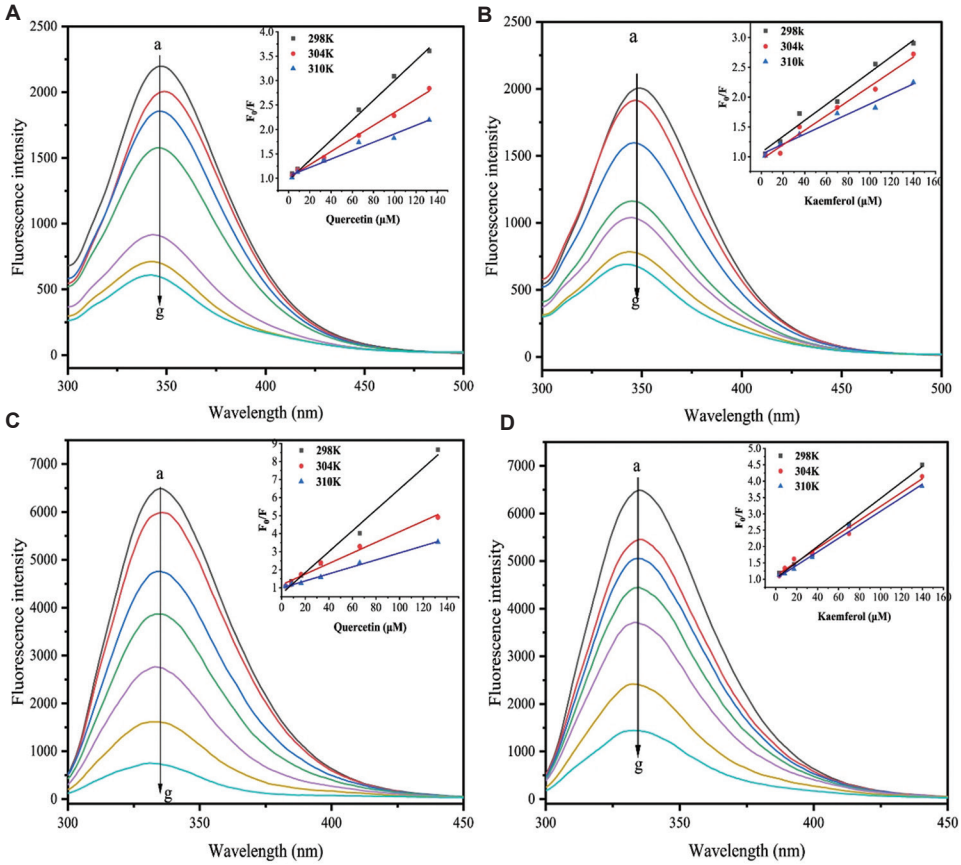

Figure 3. Fluorescence quenching of quercetin and kaempferol on α-amylase/α-glucosidase. (A) Fluorescence quenching spectra of α-amylase caused by quercetin at 298 K, with concentrations of 0, 3.31, 8.28, 16.56, 33.11, 66.22, 132.45 × 10^{-6} mol/L from (curves a to g). (B) Fluorescence quenching spectra of α-amylase caused by kaempferol at 298 K, with concentrations of 0, 3.49, 8.73, 17.47, 34.94, 69.87, 139.75 × 10^{-6} mol/L (curves a to g). (C) Fluorescence quenching spectra of α-glucosidase caused by quercetin at 298 K, with concentrations of 0, 3.31, 8.28, 33.11, 66.22, 99.34, 132.45 × 10^{-6} mol/L (curves a to g). (D) Fluorescence quenching spectra of α-glucosidase caused by kaempferol at 298 K, with concentrations of 0, 3.49, 17.47, 34.94, 69.87, 104.81, 139.75 × 10^{-6} mol/L (curves a to g). The secondary plot represents the Stern-Volmer plot (insets in each panel).

was static. The K_a values of quercetin and kaempferol considerably altered with rising temperature and were consistent with the change of K_{sv} values. This indicated that quercetin and kaempferol form complexes with enzymes, which decreased in stability as the temperature increased. This was probably due to the inhibition of endogenous fluorescence caused by the formation of these enzyme-inhibitor complexes. Both quercetin and kaempferol exhibited binding sites close to 1, suggesting a single type of binding site.[1] This observation was consistent with the kinetic results.

3.5. Thermodynamic parameters

Typically, small molecules interact with enzymes non-covalently, and these interactions can be calculated by thermodynamic parameters including electrostatic interactions, hydrogen bonding, van der Waals forces, and hydrophobic interactions. Negative ΔG^{o} values imply that the binding of quercetin and kaempferol to α-amylase and α-glucosidase was a spontaneous process. The calculated negative $\Delta H°$ value indicates the interaction between quercetin and kaempferol and both enzymes was an exothermic process (Table 7). In addition, $\Delta H° < 0$ and $\Delta S° < 0$ demonstrate that hydrogen binding and van der Waals forces are the principal forces driving the binding of quercetin and kaempferol to both enzymes. This aligns with the guidelines proposed by Ross and Subramanian.[41,42]

3.6. CD spectra analysis

CD is an effective tool for examining protein conformational changes induced by ligands. As shown in Figure 4, α-amylase and α-glucosidase exhibit two negative bands at approximately 207 and 228 nm, and 208 and 220 nm, respectively.[43] For α-amylase, the α-helix content increased from 14.25% to 14.72% (quercetin), 14.57% (kaempferol), 15.50% (isorhamnetin), 15.35% (rutin), 15.93% (kaempferol-3-O-rutinoside), and 15.90% (narcissoside), while the β-sheet content decreased from

Table 7. Binding constants, binding sites, thermodynamic parameters of quercetin and kaempferol with α-amylase and α-glucosidase at 298 K, 304 K, and 310 K

System	T (K)	K_q ($\times10^{12}$ $Lmol^{-1}$)	K_a ($\times10^{4}$ $Lmol^{-1}$)	*n*	ΔH^o (KJ mol^{-1})	ΔG^o (KJ mol^{-1})	ΔS^o (J $mol^{-1}k^{-1}$)
Quercetin-α-amylase	298 K	2.04±0.02	0.83±0.03	0.91	−60.39±2.52	−22.41±1.02	−127.45±3.52
	304 K	1.34±0.03	0.54±0.02	0.90		−21.65±2.54	
	310 K	0.86±0.02	0.32±0.01	0.88		−20.88±1.32	
Kaempferol-α-amylase	298 K	1.35±0.05	1.51±0.02	1.00	−126.08±4.57	−24.48±0.58	−340.96±5.76
	304 K	1.23±0.03	1.20±0.04	1.25		−22.43±1.24	
	310 K	0.85±0.03	0.21±0.01	1.20		−20.38±2.65	
Quercetin-α-glucosidase	298 K	5.81±0.02	25.12±0.22	1.17	−204.95±3.68	−30.71±3.02	−584.70±6.52
	304 K	2.94±0.01	4.37±0.04	1.02		−27.21±1.61	
	310 K	1.94±0.03	1.02±0.02	0.94		−23.69±4.32	
Kaempferol-α-glucosidase	298 K	2.45±0.04	0.33±0.03	0.80	−168.35±5.09	−19.75±2.32	−498.67±6.35
	304 K	2.12±0.04	0.05±0.04	0.85		−16.75±2.65	
	310 K	2.07±0.03	0.02±0.02	0.90		−13.76±1.53	

Table 8. The contents of secondary structures of α-amylase and α-glucosidase in *Sophora japonica* L

The ratio of enzymes to flavanoids	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
α-amylase	14.25	47.46	11.92	26.45
α-amylase: Quercetin=4:1	14.72	46.16	12.13	26.92
α-amylase: Kaempferol=4:1	14.57	46.16	11.84	27.42
α-amylase: Isorhamnetin=4:1	15.50	45.92	12.88	25.76
α-amylase: Rutin=4:1	15.35	42.81	11.81	29.25
α-amylase: Kaempferol-3-O-rutinoside=4:1	15.93	42.49	12.44	29.21
α-amylase: Narcissoside=4:1	15.9	41.97	12.23	30.05
α-glucosidase	24.54	24.30	15.11	37.05
α-glucosidase: Quercetin=3:1	23.47	27.00	14.89	36.19
α-glucosidase: Kaempferol=3:1	22.66	28.90	14.67	35.84
α-glucosidase: Isorhamnetin=3:1	23.13	28.10	14.79	35.77
α-glucosidase: Rutin=3:1	22.51	28.30	14.23	36.93
α-glucosidase: Kaempferol-3-O-rutinoside=3:1	23.16	27.00	14.41	37.10
α-glucosidase: Narcissoside=3:1	22.77	27.20	14.25	37.49

the initial content of 47.46 – 46.16% (quercetin), 46.16% (kaempferol), 45.92% (isorhamnetin), 42.81% (rutin), 42.49% (kaempferol-3-O-rutinoside), and 45.92% (narcissoside) (Table 8). For α-glucosidase, the α-helix content decreased from 24.54% to 23.47% (quercetin), 22.66% (kaempferol), 23.13% (isorhamnetin), 22.51% (rutin), 23.16% (kaempferol-3-O-rutinoside), and 22.77% (narcissoside). However, the β-sheet content increased

from the initial content of 24.30% to 27.00% (quercetin), 28.90% (kaempferol), 28.10% (isorhamnetin), 28.30% (rutin), 27.00% (kaempferol-3-O-rutinoside), and 27.20% (narcissoside), possibly due to alterations in enzyme activity and structure caused by the inhibitors.[44] These results suggest that flavonoid compounds induced changes in the secondary structure of enzymes, blocking the formation of active sites or hindering substrate binding, thereby affecting the activity of enzymes.[45,46] This is consistent with the fluorescence data, which showed that these substances impacted the enzyme's structure and suggested that they affected the enzyme activity.

3.7. Molecular docking analysis

The 3D structure of α-glucosidase derived from homology modeling was assessed using Verify-3D assay, which revealed that 95.35% of the amino acid residues had an average 3D/one-dimensional fraction ≥0.2. This indicated that 95.35% of the target amino acid sequence was compatible with other amino acid residues (Figure 5). The detection results of Ramachandran plot program showed that 99.6% of the residues in the model were in the optimal and permissive regions, with 89.7% in the optimal region and 9.9% were in the permissive region. These findings indicate the reasonable distribution of amino acid dihedral angles in the model, confirming its reliability and accuracy.

To further understand the binding pattern of the flavonoid components of FBSJ, we used molecular docking to mimic the interaction of the compounds with α-amylase and α-glucosidase. The optimal binding active component was determined based on the binding energy generated during the docking process. A higher binding energy indicates a more stable complex with tighter binding. The molecular docking results showed that quercetin and kaempferol had the highest binding energies for both enzymes. The binding energies for quercetin and kaempferol

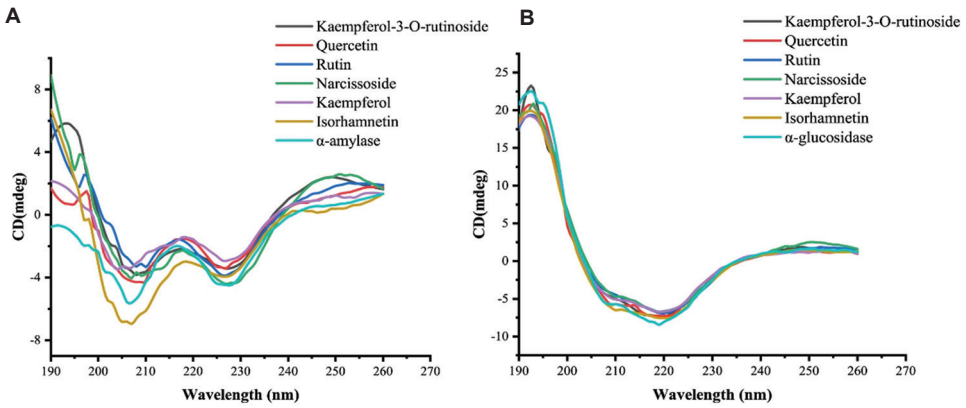

Figure 4. CD spectra of flavonoids-α-amylase/α-glucosidase systems. (A) Flavonoids-α-amylase; (B) Flavonoids-α-glucosidase. Abbreviations: CD: Circular dichroism; mdeg: Millidegree.

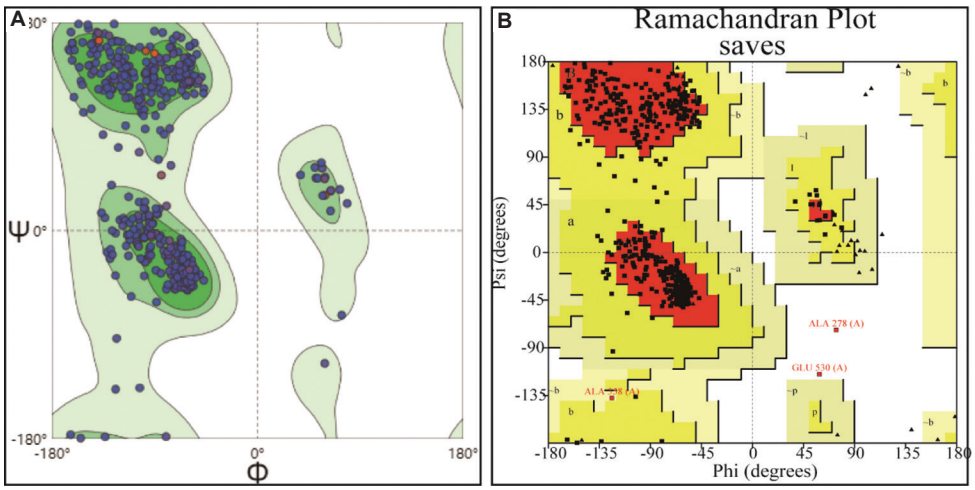

Figure 5. (A and B) Ramachandran plot model quality evaluation diagram

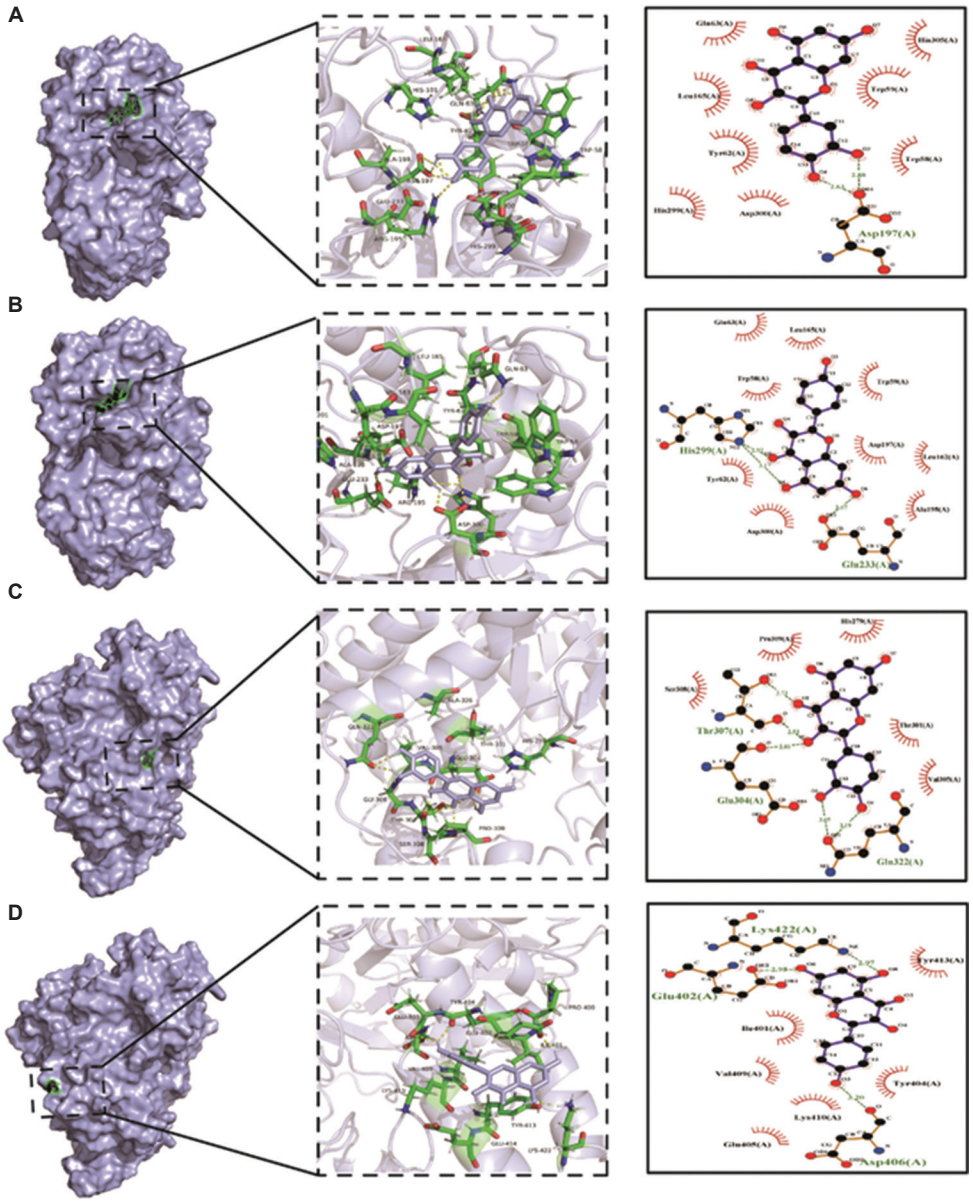

Figure 6. The molecular docking results between quercetin, and kaempferol, and α-amylase/α-glucosidase. The 3D binding diagram of quercetin-α-amylase (A), kaempferol-α-amylase (B), quercetin-α-glucosidase (C), and kaempferol-α-glucosidase (D) (left), and the corresponding 2D interaction diagrams (right).

were −5.7 kcal/mol and −4.63 kcal/mol for α-amylase, respectively, and 5.15 kcal/mol and −5.06 kcal/mol for α-glucosidase (Table 9).[47,48] Thus, from an energetic point of view, quercetin and kaempferol had better binding affinity, which were consistent with the previous correlation analysis and the IC_{50} results for each substance.

As shown in Figure 6, for α-amylase, quercetin formed hydrogen bonds with Asp197 and interacted hydrophobically with Asp300, Gln63, His305, Trp59, Leu165, Tyr62, Trp58, and His299 (Figure 6A). Kaempferol established hydrogen bonding interactions with Glu233 and His299, as well as hydrophobic interactions with residues Asp300, Asp197, Gln63, Trp59, Leu165, Tyr62, Leu16, and Aln198 (Figure 6B). When binding to α-amylase, quercetin and kaempferol impeded the entry of the substrate or occupied the starch binding site, thus further inhibiting α-amylase activity. Asp197, Glu233, and Asp300 were identified as the key active site residues of α-amylase in previous research, and are essential to the catalytic process. The hydrogen bonding interactions of quercetin and kaempferol with these key active site residues

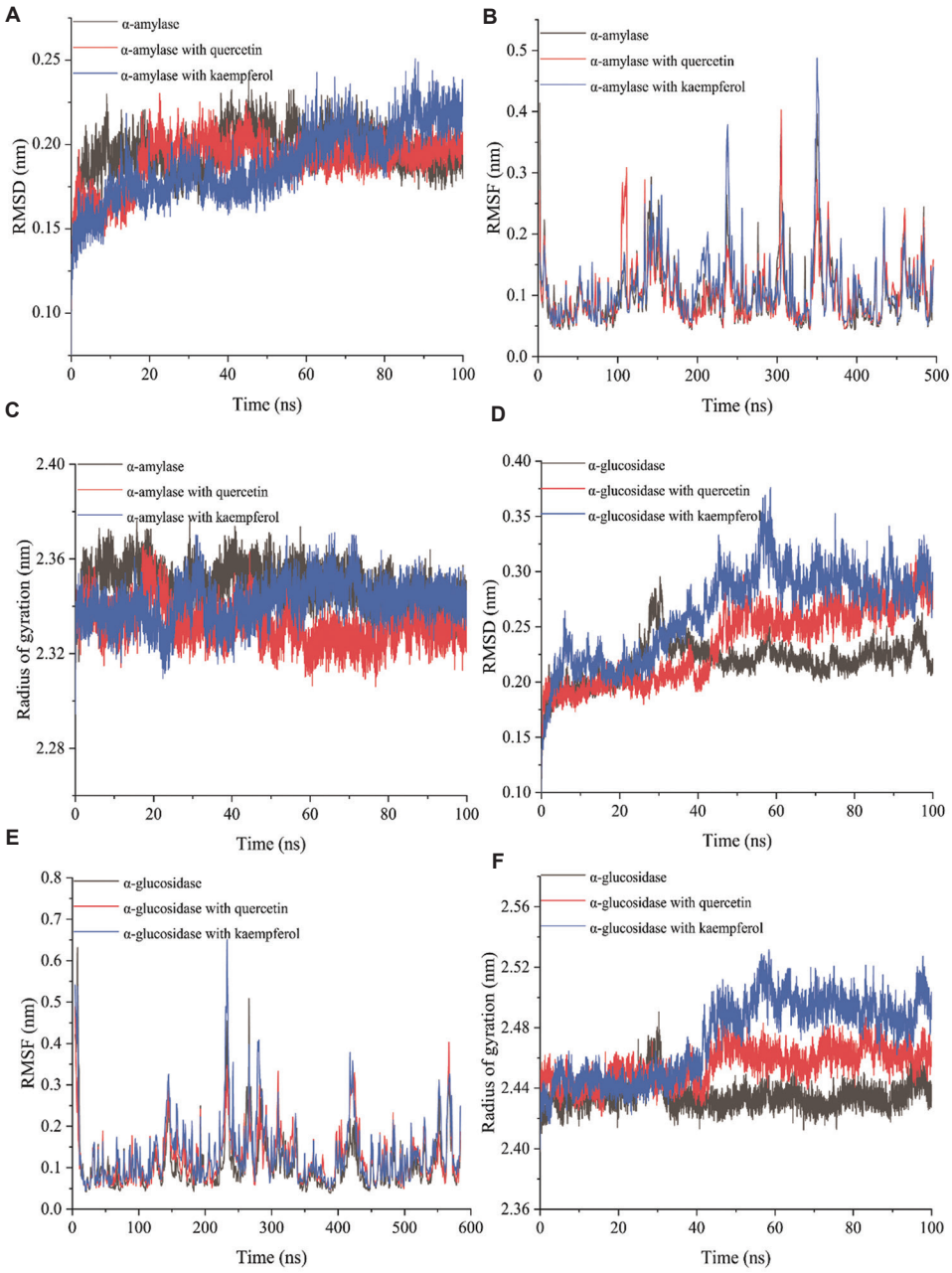

Figure 7. Molecular dynamics simulation of quercetin/kaempferol with α-amylase and α-glucosidase. Molecular dynamics simulations of quercetin and kaempferol with α-amylase (A-C) and α-glucosidase (D-F) for 100 ns.
Abbreviations: RMSD: Root-mean-square deviation; RMSF: Root-mean-square fluctuation.

suggest that they are located within the active cavity of α-amylase. For α-glucosidase, quercetin formed hydrogen bonds with Thr307, Glu304, and Gln322, while forming hydrophobic interactions with His279, Pro309, Ser308, Thr301, and Val305 (Figure 6C). In addition, kaempferol formed hydrogen bonds with Lys422, Glu402 and Asp406, and hydrophobic interactions with Tyr413, Ile401, Val409, Tyr404, Lys410, and Glu405 (Figure 6D). The main active site residues in α-glucosidase, namely Glu277, Asp352, and Glu411, are essential to the catalytic process.[49] However, neither quercetin nor kaempferol interacted directly with these key residues of α-glucosidase, most likely due to their binding around the active site. In conclusion, the flavonoids in FBSJ interact hydrophobically and through hydrogen bonds with both α-amylase and α-glucosidase, significantly influencing their inhibitory activities.[50]

Table 9. Flavonoids in *Sophora japonica* L. molecular docking evaluation table

Number	Compound	α-amylase binding energy (kcal/mol)	α-glucosidase binding energy (kcal/mol)
1	Quercetin	−5.70	−5.15
2	Rutin	−3.71	−3.27
3	Isorhamnetin	−4.54	−4.74
4	Kaempferol-3-O-rutinoside	−3.20	−2.51
5	Kaempferol	−4.63	−5.06
6	Narcissoside	−3.14	−2.19

3.8. MD simulation

The binding stability of quercetin/kaempferol-α-amylase and α-glucosidase complexes was evaluated using root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF) and radius of gyration (Rg). The results of RMSD were used to evaluate the dynamic stability of the system.[51] In Figure 7A, the free α-amylase stabilized around 10 ns, and the RMSD of the quercetin-α-amylase complex fluctuated in the same region as that of free α-amylase, indicating that quercetin binding only slightly changed the degree of freedom of protein movement, with stability comparable to that of free α-amylase. The RMSD value of the kaempferol-α-amylase complex was significantly higher than that of the quercetin-α-amylase complex, suggesting relatively poorer stability. The flexibility of amino acid is shown in Figure 7B, reflecting the RMSF. During the whole simulation process, the peptide chain from residues 105 – 111 in the quercetin-α-amylase complex fluctuated greatly. The combination of kaempferol and α-amylase caused residues 200 – 213 and 238 to fluctuate greatly. These differences in the protein-ligand complexes reflect the interactions between small molecules and proteins, which changes the protein conformation and may relate to the protein's functional role. Rg is an important parameter to examine the density of the protein structure; the larger the Rg value of the system, the looser the protein structure.[52,53] As shown in Figure 7C, the binding of quercetin reduced the Rg value of α-glucosidase, indicating that quercetin binds to α-glucosidase and forms a more stable system, thus showing significant strong inhibitory activity.

The RMSD value of free α-glucosidase in Figure 7D fluctuated around 0.22 nm, and the RMSD value of the α-glucosidase-kaempferol complex was significantly higher than that of α-glucosidase-quercetin complex. All ligand-α-glucosidase complexes in Figure 7E showed similar fluctuations. Among the Lys233, His 279, and Ser281 residues, the kaempferol-α-glucosidase complex showed higher fluctuations than the quercetin-α-glucosidase complex, indicating that the residue structure was more flexible and unstable. The Rg value of the complex stabilized after 50 ns, as shown in the Rg value in Figure 7F. The binding of both quercetin and kaempferol increased the Rg value of α-glucosidase. The Rg value of the α-glucosidase-kaempferol complex was significantly higher than that of the α-glucosidase-quercetin complex, which made the whole conformation loose and its structure unstable, resulting in a decrease in the catalytic activity of the α-glucosidase active site.

4. Discussion

In this study, we separated and identified six flavonoids from FBSJ and then developed a method to screen their inhibitory effects on α-amylase and α-glucosidase activity *in vitro*. The significance of the inhibition was evaluated through various analytical methods. In addition, we investigated the inhibitory mechanisms through enzyme inhibition kinetics, fluorescence spectrometry, CD, and molecular docking. Our analysis indicated that quercetin and kaempferol were the most effective inhibitors of α-amylase and α-glucosidase, consistent with the IC_{50} and molecular docking results. Both quercetin and kaempferol were found to act as competitive inhibitors against α-amylase and α-glucosidase enzymes. The study revealed that quercetin exhibited a mixed inhibition pattern against α-amylase, while kaempferol demonstrated a mixed inhibition pattern against both α-amylase and α-glucosidase. Fluorescence and CD spectra revealed that flavonoids in FBSJ caused a reduction in fluorescence intensity and structural changes in the enzymes. Molecular docking analysis revealed that quercetin and kaempferol exhibited higher binding energies and tighter interactions with the enzymes. MDs simulations provided further insights into the structure and characteristics of the α-amylase-quercetin/kaempferol complexes and α-glucosidase-quercetin/kaempferol complexes. Notably, the α-amylase/α-glucosidase-quercetin complexes were more stable compared to the α-amylase/α-glucosidase-kaempferol complexes.

5. Conclusion

Based on scoring for significance, the chromatographic analysis CAADA employed in this study proved to be a simple and reliable method. Overall, the findings suggest that quercetin and kaempferol exhibit potential as inhibitors of α-amylase and α-glucosidase in FBSJ.

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Conflict of interest

The authors declare that they have no competing interests.

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Writing–original draft: Ranying Zhang
Writing–review & editing: Yunfeng Ma

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author on reasonable request.

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ORIGINAL RESEARCH ARTICLE

Exploring the dynamic impact of lemon essential oil on anthropometric measurements in a 6-week study

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Abstract

Bioactive compounds in lemon essential oil possess antioxidant and antimicrobial properties. Although traditional uses of lemon essential oil are well-documented, scientific research evaluating its effects on anthropometric parameters remains limited. This study examines the impact of lemon essential oil on anthropometric measurements, specifically focused on skinfold thickness and body composition. Participants (n = 26) were divided into treatment and control groups using a randomized and double-blinded design. The treatment group received a 5-min abdomen exfoliation and a 30-min modeling massage using sweet almond and lemon essential oils, while the control group underwent an identical procedure without the lemon essential oil. The study spanned 6 weeks, and anthropometric variables, including skinfold thickness, abdominal perimeter, weight, and body mass index (BMI), were assessed before and after each session. Statistical analyses revealed a 3.8% reduction in abdominal perimeter (P = 0.041) and significant improvements in the treatment group's tricep skinfold thickness (P = 0.046). However, no statistically significant differences were observed in weight or BMI. Notable changes were session-specific, particularly in sessions 3, 5, and 6, suggesting variability in the effects of lemon essential oil. These findings demonstrate that lemon essential oil may enhance the effectiveness of massage-based interventions aimed at body contouring or skin condition improvement in weight management and wellness programs. However, limitations should be considered when interpreting these findings, including the small sample size and focus on female participants. Overall, these findings highlight the potential of lemon essential oil to modulate anthropometric measurements and advance the understanding of essential oils in healthcare and wellness applications.

Keywords: Lemon essential oil; Abdomen; Massage; Fat reduction; Aromatherapy

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1. Introduction

In recent years, natural remedies and alternative therapies have gained increasing attention for their potential health benefits and therapeutic applications.[1] Among the vast array of natural substances, essential oils derived from aromatic plants have shown promise in aromatherapy, skincare, and wellness.[2-5] Essential oils consist of volatile

organic compounds extracted from plant material, often through steam distillation or cold-press methods.[6] These oils are known for their distinctive fragrances and have been traditionally employed for their potential curative and therapeutic properties.[7]

One such essential oil that has garnered interest for its potential health effects is lemon essential oil. Lemon (*Citrus limon*) is renowned for its zesty scent and is a prominent source of the essential oil used in various applications. It contains multiple bioactive compounds, including limonene and β-pinene, contributing to its antioxidant and antimicrobial properties.[8,9] Lemon essential oil's invigorating fragrance and versatile nature make it suitable for applications in skincare, aromatherapy, and culinary practices.

While the traditional uses of lemon essential oil are well-documented, scientific research evaluating its effects on anthropometric parameters, such as skinfold measurements and body composition, is limited. As the demand for natural alternatives to support well-being increases, the potential influence of lemon essential oil on human physiology warrants investigation.

This study focuses on skinfold thickness and body composition as primary variables due to their established significance in assessing changes in adipose tissue distribution and overall physical health.[10] Skinfold measurements, in particular, are non-invasive, widely used, and cost-effective methods for evaluating subcutaneous fat, which is a key component of body composition.[11] These metrics are clinically relevant in wellness and obesity management contexts, where targeted fat reduction and improvements in body esthetics are prioritized outcomes.[12]

In the broader context of wellness, interventions aimed at enhancing physical appearance and self-esteem often focus on reducing localized fat deposits and improving body contours.[13] Skinfold measurements provide quantifiable data on these changes, making them ideal for evaluating the effects of lemon essential oil, which is hypothesized to have properties that may modulate fat metabolism.[14]

From an obesity management perspective, body composition analysis provides insights into the effectiveness of interventions targeting fat mass while preserving or enhancing lean mass.[15] By focusing on skinfold thickness and body composition as primary variables, this study aims to integrate traditional aromatherapy applications with evidence-based approaches to wellness and obesity management. These measures enable a detailed assessment of intervention outcomes and align with broader trends in personalized and integrative health practices.

Lemon essential oil's bioactive compounds, including limonene and β-pinene, have demonstrated potential anti-inflammatory and antioxidant properties, which could influence adipose tissue dynamics and support weight management strategies.[16] Limonene, the predominant monoterpene in lemon essential oil, has been extensively studied for its effects on lipid metabolism and fat oxidation.[17] Limonene exhibits strong free radical-scavenging properties, which may mitigate oxidative stress – a key factor in adipose tissue dysfunction.[18] Limonene may promote healthier adipocyte function and support lipid mobilization by reducing oxidative damage, leading to localized reductions in subcutaneous fat.[19] Chronic low-grade inflammation is a hallmark of adipose tissue expansion and obesity. Limonene has been shown to suppress pro-inflammatory cytokine production (e.g., TNF-α and IL-6) and modulate inflammatory pathways, potentially improving adipose tissue health and reducing skinfold thickness.[20,21] In addition, limonene may enhance the breakdown of triglycerides into free fatty acids and glycerol, a process known as lipolysis. When combined with mechanical stimulation from massage, this effect may amplify the mobilization and reduction of subcutaneous fat in targeted areas.[19] Lemon essential oil's invigorating properties may also improve blood flow and lymphatic drainage when applied topically.[22] Enhanced circulation can facilitate the removal of metabolic waste products and support fat metabolism, potentially contributing to reductions in skinfold thickness.

By leveraging these mechanisms, the lemon essential oil may offer a natural and integrative approach to localized fat reduction and body contouring, aligning with its traditional uses in wellness and esthetics. This study investigates these potential effects by evaluating changes in anthropometric parameters over a structured intervention period, contributing to a deeper understanding of lemon essential oil's therapeutic applications.

While the therapeutic properties of lemon essential oil, such as its antioxidant, anti-inflammatory, and antimicrobial effects, are well-documented, its direct impact on anthropometric parameters remains underexplored. Most existing studies on lemon essential oil have focused on its applications in skincare, aromatherapy, and antimicrobial treatments, with limited evidence regarding its role in modulating body composition or skinfold thickness.

Furthermore, existing research on essential oils and body composition often focuses on broader categories of essential oils, such as blends or other citrus oils, rather than explicitly isolating the effects of lemon essential oil. These studies suggest that essential oils may influence adipose tissue dynamics indirectly through mechanisms such as oxidative stress reduction and anti-inflammatory action;

however, they rarely quantify these effects in a controlled, clinical setting.[22,23]

At present, there is no consensus or substantial body of evidence detailing the extent to which lemon essential oil impacts anthropometric measures, such as skinfold thickness or abdominal perimeter. This study represents a novel contribution to the field by employing a randomized double-blinded trial to evaluate these effects rigorously. It aims to address a critical gap in the literature by providing data on the targeted effects of lemon essential oil, particularly in the context of body contouring and wellness interventions.

While essential oils, including lemon essential oil, have shown significant potential in therapeutic and wellness applications, their use is not without limitations. These challenges must be considered when interpreting their efficacy and designing interventions. Essential oils primarily exert their effects through topical application and olfactory stimulation. However, their penetration through the skin is limited due to the barrier properties of the stratum corneum.[24] Although some compounds, such as limonene, are small and lipophilic enough to penetrate the epidermis, their bioavailability in deeper tissues or systemic circulation remains low. This limitation may affect the extent of their therapeutic impact on adipose tissue or subcutaneous fat.

The chemical composition of essential oils can vary significantly depending on factors such as plant variety, geographic origin, extraction method, and storage conditions.[25] These variations may result in inconsistent therapeutic outcomes and make it challenging to standardize treatments. Essential oils can cause allergic or irritant reactions, particularly in sensitive skin.[26] Lemon essential oil, in particular, contains citral and limonene, which are known allergens that may trigger contact dermatitis if misused or in excessive concentrations.[27] Proper dilution and patch testing are essential to minimize these risks. Some citrus oils, including lemon essential oil, are phototoxic due to compounds like furanocoumarins.[28] When applied to the skin and exposed to sunlight or ultraviolet radiation, they may cause photosensitivity, leading to burns or pigmentation changes. Although steam-distilled lemon oil has lower phototoxicity than cold-pressed oil, careful application guidelines are necessary to ensure safe use.

Despite growing interest in essential oils, the evidence supporting their therapeutic efficacy is often anecdotal or based on small-scale studies.[29] This highlights the need for rigorous, well-controlled trials to substantiate claims and understand the mechanisms underlying their effects. Prolonged exposure to essential oils can lead to sensitization, even when used at safe concentrations. In addition, the systemic effects of repeated exposure, mainly through inhalation or dermal absorption, are not fully understood and warrant further investigation.

This study explores the impact of lemon essential oil on skinfold measurements and various anthropometric variables. It seeks to assess whether lemon essential oil, when administered over multiple sessions, can induce significant changes in these parameters, thereby providing valuable insights into the potential therapeutic applications of this natural remedy.

The exploration of lemon essential oil's impact on anthropometric measurements served as a bridge between traditional practices and contemporary scientific inquiry. Furthermore, it contributes to the broader discussion on the utility of essential oils in healthcare and wellness, adding to the growing body of knowledge on natural remedies and their potential applications.

2. Materials and methods

2.1. Study design

This study utilized a randomized double-blinded study design to assess the effects of a specific intervention on abdominal fat reduction. The trial encompassed two groups: a treatment group and a control group. Participants were randomly assigned to the treatment group (TG) or the control group (CG) using a computer-generated randomization sequence to minimize selection bias. Randomization was conducted after all participants met the inclusion criteria and completed the baseline assessment. To ensure balance between the groups, stratified randomization was employed based on two key variables: age and body mass index (BMI). Participants (n = 26) were first categorized into strata according to age ranges (30 – 39 years and 40 – 49 years) and BMI categories (e.g., normal weight, overweight, and obese). Within each stratum, participants were randomly allocated to the TG or CG, ensuring an even distribution of age and BMI between the groups.

This stratification process was implemented to reduce the potential confounding effects of these variables on the study's outcomes, as both age and BMI can influence anthropometric parameters, such as skinfold thickness and abdominal perimeter.

In addition, to maintain the double-blinded nature of the trial, the randomization sequence and group assignments were concealed from participants and therapists using sequentially numbered, opaque, and sealed envelopes.

The treatment group underwent a comprehensive procedure consisting of a 5-min exfoliation and a 30-min

modeling massage, during which a carefully blended oil of sweet almond and lemon essential oil was applied. In contrast, the control group received an identical procedure, except the massage oil did not include lemon essential oil. In each session, objective measurements were recorded, encompassing body weight, abdominal perimeter, and skinfold measurements at various sites, including the abdominal area, tricep, supra iliac, and thigh. These assessments were conducted consistently across 6-weekly sessions, ensuring a comprehensive evaluation of the intervention's impact over time. To provide a robust scientific basis for investigating the impact of lemon essential oil, this study selected anthropometric parameters – such as skinfold thickness, BMI, and abdominal perimeter – as primary outcomes. These measures are widely used in clinical and wellness settings to assess changes in body composition, which are particularly relevant to interventions involving topical applications.

The 6-week intervention period was selected based on a review of prior studies and practical considerations for observing changes in anthropometric parameters. Previous research on body composition and therapeutic massage has demonstrated that significant, measurable effects often emerge within a 4 – 8 week timeframe, particularly in interventions targeting localized fat reduction or improvements in skinfold thickness.[30] For example, studies investigating the impact of essential oils or massage therapies on body contouring frequently adopt similar durations to balance the need for observable results with participant retention and compliance.[31]

In addition, the 6-week duration was deemed feasible for participants, minimizing the risk of dropouts while ensuring consistent exposure to the intervention. Weekly sessions allowed standardized data collection and gradual changes to be assessed without overburdening participants.

2.2. Site identification and access

The research was conducted at Clínica Áurea – Esthetic Biomedicine Clinic in Vila Real, Portugal, between September 2021 and December 2021. Access to the research site was facilitated through collaboration with the clinical facility, which operates as a commercial venue specializing in biomedical esthetics. Prior approval was obtained from the facility management to conduct the study on-site, and participant recruitment was facilitated through established communication channels with clinic staff.

2.3. Recruitment process

Participants were recruited through a systematic process involving the distribution of questionnaires to potential volunteers attending Clínica Áurea. Clear selection criteria were established to ensure the inclusion of eligible individuals meeting specific criteria related to BMI, physical activity levels, medical history, and lifestyle habits. Recruitment procedures were conducted transparently, with an emphasis on obtaining voluntary participation and informed consent from all eligible individuals.

2.4. Participants

The controlled trial sample consisted of 26 female volunteers, selected through a questionnaire and divided randomly into treatment (TG, n = 13) and control (CG, n = 13) groups. Volunteers were chosen with a BMI ranging from 18.5 to 34.9, corresponding to the normal weight and obese categories. Exclusion criteria included regular physical activity, disease, or risk factors that may influence lipid metabolism, contraindications for the treatments, and regular smoking or alcohol consumption.

2.5. Instruments

Height and perimeters were measured using a non-stretchable measuring tape (COMED, France). Bioelectrical impedance Tanita UM-076 (Tanita, Japan) was used to register weight. Skinfolds were determined using a Digital Skinfold Analyzer (COMED, France).

2.6. Preparation of exfoliant

The exfoliant used in this research was prepared by combining 28 mL of sweet almond oil (Plena Natura, Portugal), 80 g of dead sea salt (AromaZone, France), ten drops of lemon (citrus lemon peel oil distilled), and lavender (Aromazone, Paris, France) essential oils. The essential oil used in this study was derived from the *Citrus limon* variety sourced from AromaZone, France, known for its high limonene content. The lemon essential oil was obtained using a cold-press extraction method, a standard practice for citrus oils. This technique preserves volatile compounds, such as limonene and β-pinene, critical to the oil's therapeutic potential. Including this detail ensures reproducibility and offers insights into the quality of the oil used in this intervention.

2.7. Preparation of massage oil

The massage oil utilized in this study was meticulously prepared by combining 20 mL of sweet almond oil with 12 drops of lemon essential oil, adhering to precise measurements. Sweet almond oil, a widely recognized carrier oil in aromatherapy, was chosen for its gentle and nourishing properties, making it a suitable base for massage oil.[32] In contrast, lemon essential oil, with its distinctive citrus aroma and potential therapeutic benefits, was incorporated to provide both olfactory delight and possible physiological effects.[33] This specific ratio of carrier

to essential oil was selected to achieve optimal dilution, thereby ensuring the safety of topical application while preserving the characteristic fragrance and the potential therapeutic efficacy associated with lemon essential oil. The chosen ratio falls within the recommended range for crucial oil dilution in massage oils, typically between 1% and 3%, aligning with established safety guidelines. The meticulous preparation of the massage oil aimed to create a harmonious blend that balances both safety and the desired aromatic and therapeutic outcomes.[34]

2.8. Intervention

Before participating, all individuals provided informed consent following the principles of the Declaration of Helsinki. Subsequently, a baseline interview was conducted to gather essential participant information, encompassing age, weight, height, level of physical activity, and presence of any underlying medical conditions or risk factors that could potentially impact lipid metabolism, and habits related to smoking and alcohol consumption.

The treatment group received a 5-min abdomen exfoliation by a Clínica Áurea therapist, followed by a 30-min modeling massage once a week for 6 weeks. The massage utilized lemon essential oil diluted in sweet almond oil with a ratio of 1:2 and a final concentration of 3%.

The control group received the same intervention as the aromatherapy group but with sweet almond oil only. Both the essential oils and sweet almond oil were packed in similar bottles with the same color, shape, and size. The researcher was the only one aware of the group assignments according to the number on each bottle.

2.9. Preparation of massage oil

Assessments were done before and after each of the six sessions. Measurements included height, weight, and the perimeter measured for the abdomen.[35,36] Skinfold measurements were taken at the triceps, suprailia, and thigh areas.[35,37]

The percentage of body fat was estimated using skinfold measurements' according to Equations I–II:

$$\text{Body density} = 1.0994921 - (0.0009929 \times X1) + (0.0000023 \times X1^2) - (0.0001392 \times \text{age}) \quad \text{(I)}$$

$$\text{Percentage of body fat} = \frac{495}{\text{Body density}} - 450 \quad \text{(II)}$$

2.10. Statistical analysis

The collected data were subjected to statistical analysis using IBM Statistical Package for the Social Sciences (SPSS) Statistics software (SPSS, United States), version 20. The t-student independent sample *t*-test was used to assess differences in the means of various anthropometric measurements between groups. A Mann–Whitney test was applied to compare groups, and a Wilcoxon test allowed comparison between initial and final measures in each group. Statistical significance was set at $P<0.05$.

3. Results

3.1. Sample characterization and initial group comparisons

The sample, consisting of 26 individuals, was meticulously characterized, with findings presented in Table 1. The participants were randomly divided into two groups: the treatment group (TG, n = 13) and the control group (CG, n = 13). The table provides medians, minimum and maximum values for age, height, weight, and BMI, along with corresponding Mann–Whitney U-test results. These results confirm the comparability of the two groups, as no significant differences were observed in any of the examined variables.

3.2. Abdominal skinfold measurements analysis

In the subsequent analysis of abdominal skinfold thickness measurements (Table 2), the study sought to determine differences between the treatment and control groups through independent *t*-tests. The results reveal several intriguing findings. Notably, for most sessions (1, 2, 4), the *t*-tests indicate no statistically significant differences in the means of skinfold thickness between the groups, irrespective of whether equal variances are assumed. This suggests that the treatment did not significantly affect abdominal skinfold thickness in these specific sessions compared to the control group.

However, in sessions 3, 5, and 6, the results indicate a significant difference in the means of skinfold thickness,

Table 1. Sample characterization in the treatment and control groups at baseline

Variable	Group	Median	Minimum	Maximum	U	*P*
Age (y)	TG	35.67	32	42	1.00	0.127
	CG	44.67	37	53		
Height (m)	TG	1.59	1.54	1.64	2.50	0.376
	CG	1.65	1.60	1.71		
Weight (kg)	TG	64.1	60.0	73.0	4.00	0.827
	CG	59.20	64.93	69.20		
BMI (kg/m^2)	TG	25.79	23.16	30.78	4.00	0.827
	CG	23.96	22.28	25.94		

Note: U refers to the Mann–Whitney test scores.
Abbreviations: BMI: Body mass index; CG: Control group; TG: Treatment group.

although this difference is highly dependent on the assumption of equal variances. This underscores the importance of considering the assumption of equal variances when interpreting the results. In session 3, the mean reduction in the TG was 7.1 mm (±1.2) compared to the CG, which showed a negligible change of 0.8 mm (±0.5). The difference between groups was statistically significant (P = 0.004). In session 5, the TG demonstrated a reduction of 6.9 mm (±1.0), whereas the CG showed a change of 1.1 mm (±0.6). The observed difference was significant (P = 0.001). TG showed a decrease of 3.5 cm (±0.9), while the CG displayed a minimal change of 0.5 cm (±0.3) (P = 0.041). In session 6, the TG experienced a mean reduction of 7.2 mm (±1.1), contrasting with the CG's reduction of 1.0 mm (±0.4) (P = 0.001). TG showed a mean decrease of 2.3 mm (±0.8) compared to the CG's 0.6 mm (±0.3) (P = 0.046).

3.3. Trends in anthropometric changes across sessions

The analysis of anthropometric measurements across sessions revealed distinct trends, shedding light on the dynamics of the intervention's effects over time. Regarding progressive trends, in the TG, reductions in abdominal skinfold thickness were generally progressive, with minor improvements observed in earlier sessions (e.g., session 1: 1.5 mm, session 2: 2.8 mm) and more substantial changes in later sessions (e.g., session 5: 6.9 mm, session 6: 7.2 mm). This trend suggests a cumulative effect of the intervention, where repeated sessions enhanced the impact of lemon essential oil and massage.

Regarding session-specific variability, sessions 3, 5, and 6 showed statistically significant improvements in the TG compared to the CG, suggesting possible inflection points where the intervention's effects were amplified. Factors contributing to this variability may include physiological adaptation, enhanced circulation, and fat mobilization with repeated treatments.

Stable or minimal changes in weight and BMI were observed, as no significant changes were noted in weight or BMI across sessions, indicating that the effects of the intervention were localized and did not substantially alter overall body composition metrics.

The CG exhibited minimal changes throughout all sessions, reinforcing the hypothesis that the observed trends in TG were explicitly associated with the intervention involving lemon essential oil.

Table 2. Statistical analysis of the abdominal skinfold measurements using independent student t-test

Variable	Session	Z	*P*
Abdominal skinfold measurements	1	1.928	0.259
	2	0.073	0.805
	3	7.109	0.004*
	4	0.547	0.513
	5	250.605	0.001*
	6	229.955	0.001*

Note: * indicates significance level at P<0.05.

3.4. Wilcoxon test results

The results of Wilcoxon signed-rank tests for paired skinfold measurements and various anthropometric variables are presented in Table 3. Z-scores and corresponding P-values are reported for each measurement, helping to determine significant differences between paired samples. Notably, there are considerable differences in tricep skinfold measurements and abdominal perimeter, as indicated by P < 0.05. No statistically significant differences were observed between the paired samples for the other measurements.

4. Discussion

This study aimed to rigorously evaluate the effects of lemon essential oil, denoted as the TG, on various anthropometric measurements, mainly focusing on skinfold thickness and related variables. The results of this investigation bring forth several vital insights and invite a considered reflection on the efficacy and implications of lemon essential oil as an intervention.

4.1. Initial group comparisons

The initial step entailed characterizing the sample and ascertaining potential differences between the TG and the CG. This initial step is critical to ensure that the two groups are comparable, minimizing bias. As presented in Table 1, the age, height, weight, and BMI of participants in TG and CG demonstrate no statistically significant

Table 3. Wilcoxon test results (Z value) and comparison values between initial and final moments after six intervention sessions

Variable	U	*P*
Skinfold measurements (mm)		
Abdominal	−1.572	0.166
Triceps	−1.992	0.046*
Supra iliac	−0.314	0.753
Thigh	−0.734	0.173
Abdominal perimeter	−2.04	0.041*
Weight	−0.631	0.528
BMI	−0.314	0.753

Note: *indicates significance level at P<0.05.
Abbreviation: BMI: Body mass index.

differences. These findings affirm the initial homogeneity of the TG and CG, which is vital to minimize the impact of confounding variables in the ensuing analyses.

4.2. Abdominal skinfold measurements

The core focus of the study revolved around assessing the impact of lemon essential oil, the TG, on abdominal skinfold measurements. The results from independent *t*-tests comparing the TG and CG for skinfold thickness (Table 2) offer valuable insights into this investigation.

Sessions 1, 2, and 4 consistently show no statistically significant differences between the groups, regardless of whether equal variances are assumed. This implies that, in these specific sessions, lemon essential oil does not have a statistically significant effect on abdominal skinfold thickness compared to the CG.

However, a different picture emerges in sessions 3, 5, and 6. The results in these sessions reveal statistically significant differences in skinfold thickness means. Regarding abdominal skinfold thickness, a reduction of approximately 6.9 – 7.2 mm in abdominal skinfold thickness was observed in the TG during sessions 5 and 6. Abdominal skinfold measurements serve as a proxy for subcutaneous fat levels in the abdominal region, which is closely linked to overall body fat distribution.[38] Reducing abdominal fat is associated with improved metabolic health, as excess abdominal fat is a known risk factor for conditions such as insulin resistance, cardiovascular disease, and type 2 diabetes.[39] While the changes observed in this study are localized and relatively modest, they may indicate early benefits of targeted interventions, particularly for individuals looking to improve body contours or reduce fat deposits for esthetic or health reasons.

Regarding abdominal perimeter, a reduction of 3.5 cm in abdominal perimeter was recorded in the TG during session 5. Waist circumference is an established marker of central adiposity, and reductions in this measure are linked to decreased cardiovascular and metabolic risks.[40] Even small changes in waist circumference can contribute to better health outcomes, especially when sustained over time.

The reductions observed in abdominal and tricep skinfold thickness highlight the potential for localized fat loss, often a goal in body contouring and wellness treatments. However, it is important to note that these changes do not necessarily translate to systemic improvements in overall fat mass or BMI, which remained unchanged.

The results demonstrate that non-invasive, affordable interventions, such as aromatherapy massage with lemon essential oil, may provide meaningful improvements in body contour and appearance. This aligns with wellness objectives focused on esthetic enhancement and self-esteem. For health outcomes, these findings suggest a potential complementary role for such interventions as part of broader lifestyle strategies targeting body composition and fat reduction.

The observed changes, while modest, underscore the feasibility of integrating affordable, non-invasive techniques into wellness programs. Further studies are warranted to explore whether such interventions can produce sustained and clinically meaningful outcomes when combined with other approaches, such as diet and exercise.

The study observed statistically significant improvements in abdominal skinfold thickness and other parameters during these sessions, indicating that the intervention's effects were not uniform across the 6 weeks. This variability could be attributed to differences in individual responses regarding physiological adaptation. The body may require repeated exposure to the intervention before showing measurable changes,[41] which could explain why significant differences emerged later in the study, such as in sessions 5 and 6. The cumulative effects of the essential oil's bioactive compounds, such as limonene, may amplify over time, enhancing fat mobilization or improving circulation after multiple applications.[16,18] Baseline variability is another potential factor. Participants' initial fat distribution, skin characteristics, or metabolic rates could influence how quickly, and significantly, they respond to the treatment.[42] Genetic factors affecting fat metabolism and inflammatory responses might also contribute to the observed variations.[43]

Consistency of procedures is another consideration. While the study aimed to standardize the exfoliation and massage procedures, slight differences in technique (e.g., pressure, duration, or area covered) between sessions or therapists could have influenced outcomes. Therapist fatigue or variability in skill might have introduced inconsistencies. In addition, variations in participants' hydration levels, food intake, or stress before measurement could have influenced skinfold thickness or abdominal perimeter readings, introducing session-specific noise. Environmental conditions, such as room temperature or humidity, could have affected the penetration and effectiveness of the essential oil.

Synergistic effects may also play a role. Lemon essential oil's bioactive compounds, such as limonene and β-pinene, may exhibit cumulative effects when applied consistently over time.[44] This might explain why later sessions showed more pronounced differences. Repeated massage sessions could progressively improve circulation and lymphatic

drainage, making the effects more noticeable in later sessions.

Future recommendations for research include conducting more detailed analyses of individual responses to identify characteristics associated with greater effectiveness. Ensuring stricter standardization of procedures, potentially through automated massage devices or detailed therapist training, is also crucial. Investigating whether external factors (e.g., hydration, diet) significantly influence session-specific results and controlling for these variables in future studies would be beneficial. For wellness centers, these findings suggest that clients may need multiple sessions to achieve noticeable results aligning with practical guidance for long-term intervention planning.

The lack of statistically significant reductions in skinfold thickness and abdominal perimeter during sessions 1, 2, and 4 suggests important considerations about the efficacy of lemon essential oil when combined with massage. These findings may reflect the need for specific conditions or cumulative exposure for the observed effects to manifest consistently.

The significant changes observed in later sessions (3, 5, and 6) suggest that lemon essential oil may require a particular duration of repeated application to exert measurable effects. This delay could be attributed to the time needed for the bioactive compounds, such as limonene, to modulate lipid metabolism, improve circulation, and reduce oxidative stress in adipose tissues.

It is also possible that the 3% concentration of lemon essential oil used in this study, while safe and within recommended topical application guidelines, may need to be optimized for greater efficacy. Higher concentrations might enhance the penetration and activity of key compounds, provided safety and tolerability are maintained.

Early sessions may represent a period of physiological adjustment where the effects of the massage and essential oil are not yet pronounced. Over time, repeated stimulation could amplify circulation, lymphatic drainage, and lipolysis, contributing to the significant outcomes observed in later sessions.

Differences in skinfold thickness and fat distribution among participants may also influence the timeline and magnitude of response. Those with higher baseline adiposity or slower metabolic rates might require more prolonged exposure or additional sessions to experience significant changes.

Variability in non-significant sessions may also highlight limitations in study protocols, such as the sensitivity of anthropometric measurements or the standardization of massage techniques across sessions. Addressing these factors in future research could reduce variability and clarify the conditions for consistent results.

These findings suggest that lemon essential oil interventions may benefit from extended durations or optimized application protocols to achieve consistent results. Future studies could explore varying concentrations, session frequencies, or durations to identify the most effective regimen. In addition, integrating adjunctive strategies, such as dietary adjustments or physical activity, may enhance the outcomes of such interventions.

4.3. Wilcoxon test results

To further explore the effects of lemon essential oil, Wilcoxon signed-rank tests were applied to paired skinfold measurements and various anthropometric variables (Table 3). The analysis identifies significant differences between paired samples for tricep skinfold measurements and abdominal perimeter, with $P<0.05$. These findings provide additional evidence of the influence of lemon essential oil on specific anthropometric parameters.

4.4. Comparison with other studies

Our findings align with previous research indicating the potential of essential oils to influence body composition. For instance, some studies demonstrated citrus essential oils' antimicrobial and antioxidant activities, including lemon, which may contribute to their overall health benefits.[33,45] In addition, research on the anti-inflammatory properties of essential oils highlights the potential mechanisms through which lemon essential oil might influence adipose tissue and skinfold thickness.[46,47]

Another study reviewed the therapeutic properties of lemon essential oil and found it beneficial in various health contexts, supporting its application in aromatherapy and skincare.[9] These studies, combined with our findings, suggest a broader applicability of lemon essential oil in wellness practices.

4.5. Limitations

This study has several limitations. First, the sample size was relatively small, with only 26 participants, which may restrict the findings' generalizability. A larger sample size would provide more robust statistical power and allow for a broader application of the results. In addition, the study was conducted over a short duration of 6 weeks. While this period is sufficient to observe some changes, longer-term studies are necessary to evaluate lemon essential oil's sustainability and long-term effects on anthropometric measurements.

Another limitation is that the study included only female participants, restricting the generalization of the findings to males or other demographic groups. Including a more diverse participant pool in future research would enhance the applicability of the results. The control group received a massage with sweet almond oil but did not receive a placebo essential oil, which could introduce bias if participants perceived differences between the interventions. This represents a limitation in the study design, as sweet almond oil has beneficial skin properties. To mitigate this in future studies, it is recommended that a neutral carrier oil, such as jojoba oil, be used for the control group.

Measurement variability is another concern, as skinfold measurements and other anthropometric assessments can be subject to inter- and intra-observer variability. Despite efforts to standardize the measurements, some degree of variability is inevitable. Several strategies can be implemented in future research to mitigate this issue, such as ensuring all observers undergo rigorous and standardized training in anthropometric measurement techniques. This includes repeated practice sessions using standardized protocols and cross-validation exercises to minimize individual variability. Furthermore, advanced and automated tools, such as digital calipers with integrated software for skinfold analysis or three-dimensional body scanning technologies, can be integrated into the analysis. These methods reduce human error, enhance precision, and provide more reliable data across sessions and participants. Regular audits and recalibration of manual measurement devices can be conducted to ensure accuracy.

In addition, periodic inter-observer reliability tests can help identify and correct inconsistencies during the study. Multiple measurements at each site can be taken, and the average can be calculated to minimize the impact of outliers or observer inconsistencies. This approach is particularly important for variables with high inherent variability, such as skinfold thickness. Employing blinded measurement protocols where the observer is unaware of the participant's group assignment reduces the potential for unconscious bias in data collection. Detailed logs of environmental conditions, participant hydration levels, and other variables that could influence measurements can be maintained, as adjusting for these factors in the analysis could provide a clearer picture of the intervention effects.

Furthermore, potential confounding variables, such as diet, physical activity, and lifestyle habits, were not controlled or monitored throughout the study. These factors could influence the outcomes and introduce confounding effects. While this study did not actively control or monitor these factors, several strategies can be implemented to minimize their impact in future research. One way is by requiring participants to maintain daily food diaries during the study period, documenting all meals, snacks, and beverages consumed. This provides insights into caloric intake and dietary composition, which could affect fat metabolism. Participants can be provided with a standardized meal plan to ensure uniform caloric intake and macronutrient distribution across the study cohort. In addition, participants can be asked to log their daily physical activities, including exercise, work-related activities, and leisure-time movements, to estimate overall energy expenditure. Participants can be equipped with wearable activity trackers to objectively measure physical activity levels, such as step counts, energy expenditure, and sedentary time. Standardized questionnaires to assess lifestyle factors, such as sleep patterns, stress levels, and smoking or alcohol consumption, that could influence results can be administered, while baseline data on lifestyle habits can be collected to include these variables as covariates in statistical analyses, adjusting for their potential confounding effects. Moreover, randomization can be enhanced by stratifying participants based on key confounders, such as baseline activity levels or dietary habits, ensuring balanced distribution across intervention groups. Other measures include conducting subgroup analyses to assess whether specific baseline characteristics influence the effectiveness of the intervention, scheduling weekly or bi-weekly check-ins to review adherence to dietary and activity guidelines, as well as addressing any deviations or concerns. These measures would help improve participant compliance and data accuracy. Blinded outcome assessments can be implemented to minimize observer bias and standardizing pre-assessment conditions, such as fasting status, hydration, and timing of measurements, could reduce variability introduced by uncontrolled factors.

Self-reporting bias is also a consideration, as some data, including lifestyle habits and physical activity levels, may have been self-reported by participants, potentially leading to inaccuracies. The scope of the anthropometric measurements was limited, focusing primarily on skinfold thickness and related parameters. Incorporating more advanced techniques, such as dual-energy X-ray absorptiometry scans, could provide a more comprehensive body composition assessment.

The study did not investigate the underlying biological mechanisms by which lemon essential oil might affect anthropometric measurements. Future studies

should explore these mechanisms to provide a deeper understanding of the observed effects. In addition, while the study was designed to be double-blinded, ensuring that neither participants nor therapists were aware of group assignments, there might still be subtle cues that could inadvertently reveal group assignments and potentially affect the outcomes.

Finally, the study was conducted in a specific clinical setting, which may not reflect more general, everyday environments, potentially limiting the external validity of the findings. Addressing these limitations in future research will help validate and extend the findings of this study, providing a more comprehensive understanding of the effects of lemon essential oil on anthropometric measurements.

4.6. Broader applications in wellness and weight management programs

The findings of this study suggest that lemon essential oil, combined with massage, can play a complementary role in wellness and weight management programs. The reductions in skinfold thickness and abdominal perimeter observed in the TG align with goals commonly pursued in these contexts, such as body contouring and esthetic enhancement. While the changes were modest and localized, they may offer meaningful improvements for individuals seeking non-invasive and natural methods to enhance their physical appearance and boost self-esteem.

The observed benefits of lemon essential oil could be leveraged as part of holistic wellness interventions that combine aromatherapy with physical relaxation techniques. Massage therapies incorporating lemon essential oil may promote relaxation and provide tangible benefits in body contour improvement, making them appealing in spa and clinical settings focused on esthetic outcomes.

While systemic effects on weight and BMI were not observed, the localized reductions in fat thickness and abdominal perimeter could support broader weight management goals by enhancing motivation and adherence. For individuals already engaged in dietary and exercise programs, such complementary therapies may provide an additional boost, particularly in addressing areas resistant to fat loss.

The increasing interest in natural and non-invasive health solutions makes lemon essential oil attractive in personalized wellness care. Its dual role as a functional and aromatic agent adds value to interventions designed for stress reduction, improved body image, and targeted fat reduction.

The cumulative effects observed in this study highlight the importance of sustained and repeated interventions. When incorporated into long-term wellness plans, lemon essential oil-based therapies may yield gradual but meaningful improvements in body esthetics and localized fat reduction.

The findings encourage the development of targeted wellness protocols that integrate lemon essential oil into existing practices, such as lymphatic drainage massage, reflexology, or aromatherapy-based body wraps. These programs could be particularly beneficial for individuals with moderate body shaping or esthetic enhancement goals.

4.7. Mechanistic insights and future directions

The findings of this study provide a foundation for further research to validate and expand on the observed effects of lemon essential oil on anthropometric parameters. One promising area of investigation involves exploring the impact of other essential oils with similar bioactive properties, such as grapefruit (*Citrus paradisi*) or orange (*Citrus sinensis*). Comparative studies could clarify whether the effects seen with lemon essential oil are unique or part of a broader class of citrus oils with analogous benefits.

Another avenue for future research is the combination of lemon essential oil with other health interventions, such as dietary modifications or structured physical activity programs. These synergistic approaches could enhance outcomes by leveraging the bioactive compounds in lemon essential oil to support lipid metabolism, reduce oxidative stress, and improve circulation, thereby amplifying the effects of complementary strategies.

Mechanistic studies are also needed to explore the biochemical pathways through which lemon essential oil influences adipose tissue and anthropometric changes. Techniques, such as gene expression profiling, lipidomics, or metabolic pathway analyses, could provide deeper insights into how compounds, such as limonene and β-pinene, exert their effects. In addition, studies with longer intervention periods could help determine the sustainability of observed changes and whether the benefits plateau or continue to improve over time.

Expanding participant demographics is another critical area for exploration. Future studies should include a more diverse population, encompassing a broader range of ages, genders, and baseline health conditions, to enhance the generalizability of the findings. Investigating dose-response relationships could also provide valuable information about the optimal concentration of lemon essential oil and its application methods, balancing efficacy with safety.

Finally, researchers could assess alternative topical formulations, such as creams, gels, or transdermal patches, to optimize the delivery of essential oils, and enhance therapeutic outcomes. Psychological and behavioral dimensions, such as the impact of lemon essential oil on mood and motivation, could also be examined, particularly regarding adherence to wellness programs.

These avenues of research hold the potential to deepen understanding of the role of essential oils in wellness and healthcare, paving the way for innovative and integrative therapeutic applications.

5. Conclusion

This study demonstrates the potential of lemon essential oil to modulate anthropometric parameters, particularly skinfold thickness, and abdominal perimeter, over a 6-week intervention. These findings highlight the value of lemon essential oil as a natural adjunct in wellness and body contouring practices.

One of the key strengths of lemon essential oil is its accessibility and cost-effectiveness. As a widely available, plant-derived product, it offers a sustainable and affordable option for individuals seeking natural alternatives to support physical health and appearance. Its integration into routine wellness practices, such as massage therapies or skincare regimens, presents opportunities for broader application in clinical and non-clinical settings.

To optimize its therapeutic potential, lemon essential oil could be integrated into comprehensive health interventions that include dietary modifications and exercise programs. For instance, combining lemon essential oil massages with a calorie-controlled diet or resistance training regimen may enhance localized fat reduction by stimulating lipolysis and improving circulation. The bioactive compounds in lemon essential oil, such as limonene, may also complement the metabolic benefits of physical activity, potentially amplifying fat oxidation and reducing oxidative stress.

In addition, the uplifting aroma of lemon essential oil could improve mood and motivation, thereby encouraging adherence to diet and exercise programs. Incorporating aromatherapy into structured health plans may create a more holistic and engaging experience for individuals seeking to improve their body composition and overall well-being. These outcomes underscore the therapeutic potential of lemon essential oil in clinical and wellness settings, such as body contouring treatments or adjuncts to esthetic therapies.

By elucidating these effects, this research bridges the gap between traditional uses of essential oils and contemporary scientific inquiry, paving the way for future studies to explore their underlying mechanisms. In addition, the study highlights the importance of essential oil composition, application protocols, and individual variability in determining outcomes, contributing to a more nuanced understanding of aromatherapy's role in body composition management.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Ethical approval was obtained from Clínica Áurea (Doc1-CE-CA-2021). The study was conducted following the Declaration of Helsinki. Voluntary informed consent was obtained from participants to participate in this study.

Consent for publication

All individuals provided voluntary and informed consent for publication.

Availability of data

Data supporting the findings and conclusions are available on request from the corresponding author.

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ACCSCIENCE PUBLISHING

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SHORT COMMUNICATION

A rapid, efficient, and cost-effective method for titering third-generation lentiviral vectors

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Abstract

Lentiviral vectors are useful vectors for stable transduction and permanent expression in dividing and non-dividing cells. In particular, third-generation lentiviral vectors have been engineered to be significantly safer than their second-generation counterparts, incorporating several safety features not present in earlier versions. For example, the *tat* gene, which is essential for the replication of wild-type human immunodeficiency virus type 1, has been deleted, and vector packaging functions have been distributed across three separate plasmids, further enhancing safety. In both research and clinical settings, having a reliable and accurate method for titering lentiviral vectors is critical. We have developed a method using the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element as a template for a real-time quantitative polymerase chain reaction, coupled with TRIzol lysis buffer for ribonucleic acid isolation. This method yielded results comparable to those from a commonly used commercial kit, offering advantages of speed, cost-effectiveness, and accuracy. It presents a viable, economical alternative for both research and clinical laboratories.

Keywords: Titer; Third-generation lentiviral vector; Molecular biology; Ribonucleic acid; TRIzol

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1. Introduction

Lentiviral vectors are derived from ribonucleic acid (RNA) viruses belonging to the *Retroviridae* family. Unlike other retroviruses, lentiviruses can transduce both dividing and non-dividing cells, making lentiviral vectors derived from them valuable for a wide range of applications, including gene therapy. Second-generation lentiviral vectors utilize a single packaging plasmid that encodes the *pol*, *gag*, *rev*, and *tat* genes, whereas other virulence factors have been removed.[1-3] Although these vectors are significantly safer than the original lentiviral vectors, the possibility of generating recombinant viruses has not been entirely eliminated. In addition, given that they are almost exclusively derived from human immunodeficiency virus (HIV), safety concerns persist.[4] In contrast, third-generation lentiviral vectors mitigate this risk using three separate packaging plasmids: one for the *gag* and *pol* genes, and two for the *rev* and *env* genes. Further deletions in the 3' long terminal repeat of the vector plasmid make the vectors self-inactivating, providing an additional safety feature.[4,5]

Over the past four decades, the number of clinical trials using viral vectors for gene therapy has grown significantly. Throughout this time, numerous breakthroughs have

been made, alongside some setbacks. Despite these early challenges, intensive research efforts have continued, leading to the approval of several viral vector-based therapies, with many others currently in late-stage clinical trials.[6]

Lentiviral vectors are generated by transfecting human embryonic kidney 293 (HEK293T) cells with the transfer plasmid and packaging plasmids. A critical step in the lentiviral vector production process is calculating an accurate and reproducible vector titer, which can be time-consuming. Several methods for measuring viral vector titer have been described, including enzyme-linked immunosorbent assays to measure the p24 antigen (a protein component of HIV), reverse transcriptase activity, dot blotting, fluorescence-activated cell sorting (FACS), and quantitative polymerase chain reaction (qPCR), which are among the most accurate methods.[7,8] FACS analysis is not suitable for vectors lacking fluorescent reporter genes,[7] making qPCR the most universally reliable method for quantifying lentiviral titer.[6,9] For commercial viability, large-scale production of the vector at high titers is essential. However, many commercially available qPCR-based kits for titering lentiviral vectors are prohibitively expensive. Here, we present the development of a rapid, cost-effective, efficient, and accurate method of lentiviral vector titering. We utilized the third-generation lentiviral vector pRRL.sin.cPPT.LSP.IRES.mVenus.WPRE and compared the results from our TRIzol-based qPCR method with those obtained using the commonly used commercial qPCR Lentivirus Titer Kit (catalog number LV900; Applied Biological Materials [ABM] Inc., Canada). The TRIzol-based method described is not only rapid and cost-effective but also provides titer calculations that are as accurate as those generated by the ABM kit.

2. Materials and methods

2.1. Materials

2.1.1. Plasmids

pRRLSIN.cPPT.PGK-GFP.WPRE, pMDLg/pRRE, pRSV/REV, and pMD. 2/VSV.G were purchased from Addgene, United States.

2.1.2. Commercial kit

Applied Biological Materials Inc qPCR Lentivirus Titer Kit (catalog number LV900, Canada) was utilized in this study.

2.1.3. Chemicals

All chemicals and solutions, such as ethanol, were used in their pure form and were purchased from Merck Life Science Pty Ltd, Australia.

TRIzol reagent (catalog number 15596026) was purchased from Thermo Fisher Scientific, Australia.

2.1.4. Cells and cell culture

Human embryonic kidney 293 cells, 0.45 μm Nalgene filters, and OptiMEM 1 Reduced Serum Medium were purchased from Merck Life Science Pty Ltd, Australia.

Fetal calf serum (FCS), Dulbecco's Modification of Eagles Medium (DMEM), and penicillin/streptomycin (pen/strep) were purchased from Thermo Fisher Scientific, Australia.

CF2 CellSTACK 2 chambers (catalog number CLS3310) were purchased from Merck Life Science Pty Ltd, Australia.

2.1.5. Molecular reagents

PowerUp SYBR Green Master Mix was purchased from Thermo Fisher Scientific, Australia.

DNase/RNase free water, Micro Amp Fast Optical 96-well reaction plates, Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) forward primer (AGCTCCTTTCCGGGACTTTC), and WPRE reverse primer (AGCCATGGAAAGGACGTCAG) were purchased from Thermo Fisher, United States.

2.1.6. Equipment

Tangential Flow System (TFF; catalog number OS100T12) was purchased from Pall Corporation, Australia.

Centrifuge (catalog number Eppendorf 5424R) was purchased from Eppendorf South Pacific Pty Ltd, Australia.

Nanodrop One and QuantStudio 6 Flex Real-Time PCR systems were purchased from Thermo Fisher Scientific, Australia.

2.2. Lentiviral vector production

The third-generation lentiviral vector, pRRLSIN.cPPT.PGK-GFP.WPRE, was produced as follows:

The calcium phosphate ($CaPO_4$) precipitation method was used for the transient transfection and packaging of the four lentiviral plasmids in 293T cells.

(i) Day 1 – Preparation for transfection: 293T cells were split in DMEM (180 mL) containing 10% FCS and 1% pen/strep at a density of 6.0×10^7 cells per CF2 CellSTACK two chambers

(ii) Day 2 – Transfection: The medium on the 293T cells was changed to OptiMEM 1 (180 mL) containing 4% FCS. The transfection reagents (tubes 1 and 2) were prepared as shown in Table 1. The contents of tube 2 were slowly mixed into tube 1, and the mixture was left at room temperature for 30 min to

Table 1. Transfection reagents for lentiviral vector production. (A) tube 1, and (B) tube 2

(A) Tube 1		
Transfection reagents	**Amount/volume in CF2**	**Size (kb)**
pRRL.sin.cPPT.LSP. IRES.mVenus.WPRE	600 µg	8.512
pMDLg/pRRE	600 µg	8.89
pRSV/REV	320 µg	4.174
pMD2/VSV.G	400 µg	5.924
2M $CaCl_2$	1.2 mL	-
dH_2O	To – 8 mL	-
Total	8 mL	-
(B) Tube 2		
Reagents	**Volume in CF2**	
1M HEPES	0.4 mL	
2M NaCl	1 mL	
150mM Na_2HPO_4	120 µL	
dH_2O	6.508 mL	
Total	8 mL	

Abbreviations: $CaCl_2$: Calcium chloride; CF2: CF2 CellSTACK 2 chambers; dH_2O: Distilled water; CF2: CF2 CellSTACK 2 chambers; dH_2O: Distilled water; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaCl: Sodium chloride; Na_2HPO_4: Disodium phosphate.

allow complete precipitation. The mixture was then added to OptiMEM 1 medium without FCS; then it was transferred onto the 293T cells. The cells were incubated in a humidified chamber at 37°C with 5% carbon dioxide for 4 h. After incubation, the OptiMEM 1 medium (without FCS) was replaced with fresh OptiMEM 1 medium, and the cells were then incubated overnight at 37°C with 5% carbon dioxide

(iii) Day 3 – 5 – Virus harvesting: The virus-containing medium was harvested on days 3 – 5, replenished with fresh OptiMEM 1 medium (without FCS) after each harvest. The harvested medium may be stored at −80°C until ready for filtration through 0.45 µm Nalgene filters to remove cell debris.

2.2.1. Vector concentration and purification

The TFF system was used for filtration and concentration of the vector.

(i) Before starting the vector concentration, the flow rate of the TFF apparatus was ensured to be between 700 and 900 mL/min

(ii) The TFF was washed with 1,000 mL phosphate-buffered saline (PBS), followed by 500 mL OptiMEM 1 medium

(iii) The tank was filled with 7 L of medium collected from the transfected 293T cells. Once the medium was filtered, additional medium was added until 100 mL remained in the tank, after which the remaining medium was collected

(iv) 100 mL of OptiMEM 1 was added, and filtration was continued for another 15 min. This step was repeated twice

(v) The filtered medium was centrifuged at 50,000× g for 2 h at 4°C

(vi) The virus pellet was resuspended in 4 – 6 mL of OptiMEM1 medium, and the filtered viral supernatant was stored at −80°C

(vii) The TFF apparatus was cleaned with 1 L of 0.5 M sodium hydroxide for 30 min, and then the process was repeated once. Next, the apparatus was washed with 2 L of 0.1 M sodium hydroxide for 1 h, followed by two washes (1 h each) with 2 L of PBS each time. Finally, the TFF was run.

2.3. Methods for titering the lentiviral vector

2.3.1. qPCR lentivirus titer kit method

This methodology took approximately 2.5 h to complete, at a cost of Australian dollars (AUD) $1,958.00 per 96-well plate. The cost included the kit, quantitative standards, and associated freight charges (both international and local).

Following the manufacturer's instructions for titering lentiviral vectors (ABM kit; LV900; Canada), the supplied standards were diluted at 10^9, 10^8, 10^7, and 10^6 copies/mL for qPCR analysis and the production of a quantitative standard curve. Lentiviral vectors (1 µL) were diluted 1:1,000. Viral vector samples that produced large pellets after centrifugation were diluted 10,000 times. Quantitative analysis was performed when the R^2 value of the standard curve was >0.99, with an R^2 of 0.998 being typical for the standard curve.

2.3.2. TRIzol method

The following methodology required 1 h for RNA isolation, 2 h 30 min for qPCR, at a cost of AUD$115.20 per 96-well plate. The TRIzol reagent was priced at $AUD296.00, and the primers cost approximately AUD$30.00. The total cost was approximately AUD$300.00 for 500 samples or $AUD58.00 for 96 samples. This was approximately 34 times less costly than using the ABM kit, which also measures 96 samples. The method is illustrated graphically in Figure 1.

i. The vector (1 µL) was diluted 1:1000 with PBS and mixed. Depending on the size of the pellet, the vector may have needed to be diluted between 1:100 and 1:10,000

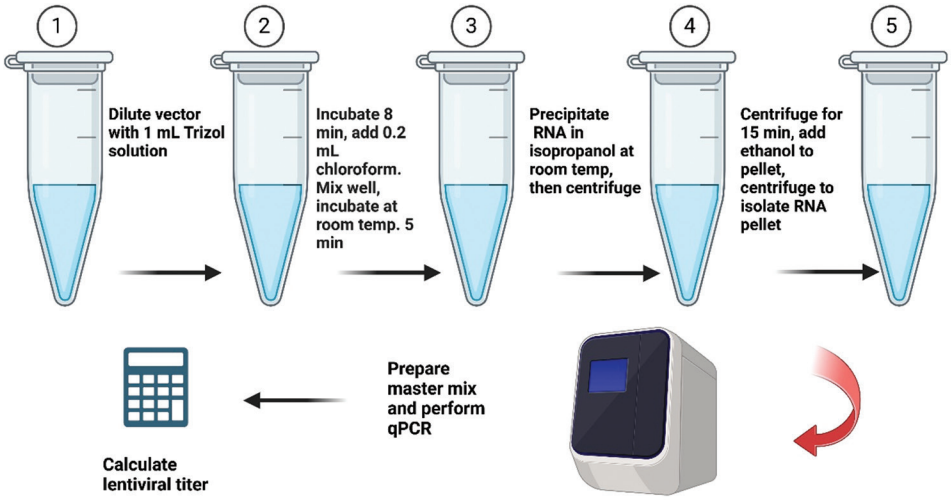

Figure 1. Graphical representation of the TRIzol method of lentiviral vector titration. Created in BioRender by Ann M. Simpson (2024). https://BioRender.com/t86k558.

ii. 1 µL of the diluted vector was added to 0.5 mL of TRIzol reagent. The mixture was shaken vigorously for 30 s to ensure complete lysis of the cells. The TRIzol solution, used in place of the ABM lysis solution, was superior for the lysis and extraction of viral RNA
iii. The sample was incubated at room temperature for 8 min, with intermittent mixing, to allow complete dissociation of the nucleoprotein complex
iv. 0.2 mL chloroform was added to the tube, mixed thoroughly by inverting several times, and incubated at room temperature for 5 min
v. The sample was centrifuged at 21,100 × g for 15 min at 4°C
vi. The colorless upper aqueous phase was transferred into a new 2 mL microcentrifuge tube
vii. The RNA was precipitated by adding 0.5 mL of isopropanol, mixing well by inversion, and the tube was incubated at room temperature for 10 min
viii. The sample was centrifuged at 21,100 × g for 15 min at 4°C
ix. If the pellet was small and not visible, the aqueous phase was carefully removed, leaving approximately 200 µL of liquid in the tube without disturbing the pellet
x. 1 mL of 75% (volume/volume) ethanol was added to the tube, and the mixture was inverted 3 – 5 times
xi. The sample was centrifuged at 211,309 × g for 10 min at 4°C
xii. The liquid was carefully aspirated and discarded, and the sample was allowed to dry for 5 min at room temperature
xiii. The RNA pellet was resuspended in 20 µL of ultrapure DNase/RNase-free water
xiv. 1 µL was used to test the RNA quantity and quality through Nanodrop spectroscopy or another appropriate method
xv. The RNA was stored at −20°C or −80°C.

The vectors titered in this study included clinical-grade samples that had been produced commercially for use in animal models at the Viral Vector Manufacturing Facility, Westmead Health, New South Wales, Australia.

2.3.3. qPCR analysis

i. Using the WPRE-containing plasmid pRRLSIN.cPPT.PGKGFP.WPRE of known concentration and serial dilutions of the plasmid were prepared in the range of 10^5 – 10^9 copies/mL
ii. Standards were amplified using the WPRE forward and WPRE reverses primers to generate a standard curve
iii. qPCR reactions were carried out using the Power Up SYBR Green Master Mix (2×) after reverse transcription, following the manufacturer's instructions
iv. Reactions were prepared by pipetting from a master mix, which was made for the appropriate number of reactions to be analyzed (standards and viral vector samples). Table 2 lists the components of one well.

2.3.4. Calculating lentiviral titer

All standards and samples were analyzed by qPCR in either duplicate or triplicate reactions. After qPCR, the mean cycle threshold (Ct) values for the standards were plotted against the deoxyribonucleic acid (DNA) copies

(copies/mL) to generate a standard curve. The mean Ct value for each sample was then interpolated on the standard curve to determine the copy number and titer (copies/mL). If the samples had been diluted before PCR, the final concentration and/or titer were adjusted according to the appropriate dilution factor.

2.4. Statistical analysis

Data (n = 9 for each method) were subjected to statistical analysis to determine whether there was a significant difference in the viral titers obtained by the two methods. A Mann–Whitney U test was applied to the data to identify significant differences. The standard curves were constructed using Graph Pad Prism7 software (United States) and were used for interpolation and titer calculations only if the R^2 value was >0.99.

Table 2. Real-time PCR components for one well and qPCR amplification parameters. (A) PCR components for one well, and (B) standard cycling conditions (primer temperature ≥60°C)

(A) PCR components for one well		
PCR component	**Volume**	
	Standards	**Samples**
PowerUp SYBR Green Master Mix (2×)	10 µL	10 µL
Forward primer	0.25 µL	0.25 µL
Reverse primer	0.25 µL	0.25 µL
Template	5 µL	1 µL
Nuclease free water	9.5 µL	13.5 µL
Total	25 µL	25 µL

(B) Standard cycling conditions (primer temperature ≥60°C)			
Step	**Temperature**	**Time**	**Number of cycles**
Reverse transcription	42°C	20 min	1
Enzyme activation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing/extension	60°C	1 min	

Abbreviation: PCR: Polymerase chain reaction.

3. Results

3.1. Performance comparison of two lysis buffer formulations for RNA preparations

The titers of vector stocks produced using either method were not significantly different. The titer obtained from the ABM kit was 32.6 ± 8.9 × 10^8 transduction units (TU)/mL, whereas the titer from the TRIzol method was 32.1 ± 4.6 × 10^8 TU/mL (n = 9). Table 3 shows the concentration and purity of RNA isolated using the ABM kit, following the manufacturer's instructions, while Table 4 presents the same data for the five samples processed using the TRIzol method. Statistically, the A260/A280 and A260/A230 ratios were significantly lower for the samples processed with the ABM kit compared to those extracted using the TRIzol method (P < 0.0001). The expected A260/230 ratios for "pure" nucleic acid are typically in the range of 2.0 – 2.2. A ratio lower than this may indicate the presence of contaminants. The higher A260/A230 ratios obtained with the TRIzol method were not significantly different from those of the ABM kit method but were closer to the expected values for "pure" nucleic acid. These ratios did not impact the PCR process in the titering assay. Moreover, the commercial lentiviral preparations used in this study have been successfully used in *in vitro* (cell culture) and *in vivo* transduction experiments without issue.

3.2. Comparison of lentiviral vector titration using the TRIzol method and the commercial kit

Figures 2 and 3 show the qPCR results for the ABM kit and the TRIzol method, respectively, applied to nine samples for lentiviral titer determination. Following the manufacturer's instructions for the Lentivirus Titer Kit (ABM; LV900; Canada), standards (supplied with the kit) were diluted in the range of 10^9, 10^8, 10^7, and 10^6 copies/mL, followed by qPCR. Samples producing small pellets were diluted 1:1,000 times, whereas those producing large pellets were diluted 1:10,000. An R^2 of 0.998 was obtained for the standard curve. For the TRIzol

Table 3. Summary of the concentration and purity of isolated viral ribonucleic acid using the quantitative polymerase chain reaction lentivirus titer kit

Sample	Lysis buffer (µL)	Vector volume (µL)	Vector RNA concentration (ng/µL)	A260/280 ratio	A260/230 ratio	Total volume (µL)
1	18	2	30.8	0.39	0.08	20
2	18	2	48.2	0.53	0.11	20
3	18	2	30.1	0.38	0.08	20
4	18	2	43.3	0.51	0.11	20
5	18	2	29.5	0.36	0.08	20

Abbreviation: RNA: Ribonucleic acid.

Table 4. Summary of the concentration and purity of isolated viral ribonucleic acid using the TRIzol method

Sample	Vector volume (μL)	Vector RNA concentration (ng/μL)	A260/280 ratio	A260/230 ratio	Total volume (μL)
1	1	478.5	2.03	0.45	20
2	1	382.8	1.95	0.35	20
3	1	749.1	1.67	0.27	20
4	1	548.4	1.61	0.24	20
5	1	655.8	1.61	0.24	20

Abbreviation: RNA: Ribonucleic acid.

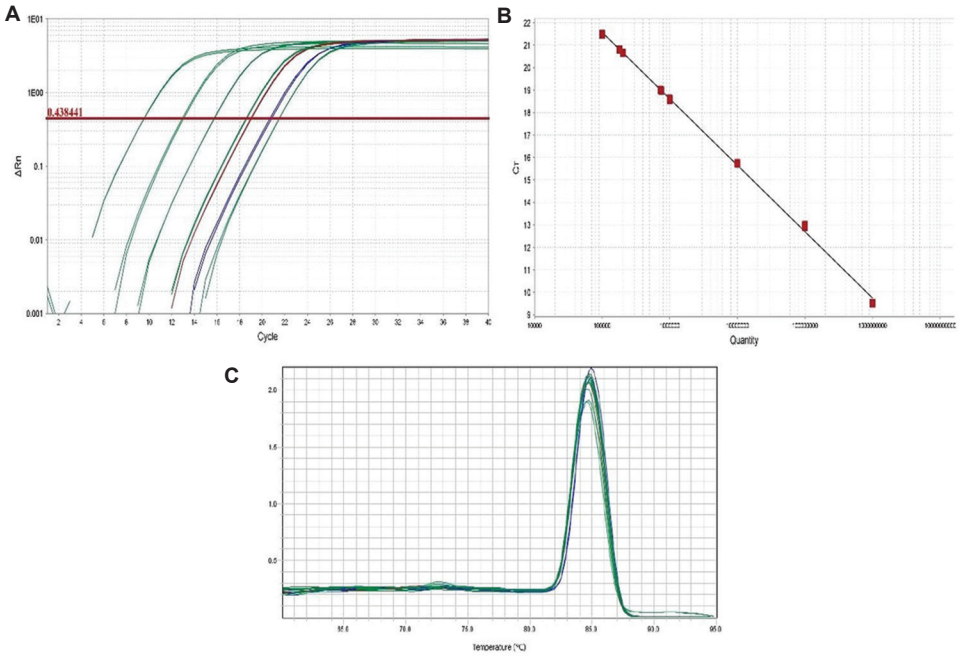

Figure 2. Quantitative polymerase chain reaction results from the Applied Biological Materials kit. (A) Amplification plots for standards and lentiviral vector samples. The amplification plots in green correspond to the standards provided in the kit (from left to right: 10^9, 10^8, 10^7, 10^6, and 10^5 copies/mL). The curves in other colors correspond to the viral samples. (B) Standard curve generated from the amplification plots of the provided deoxyribonucleic acid standards. (C) Melt curves of amplified products. R^2=0.998.

method (Figure 3), the qPCR standards were prepared using plasmid DNA containing the WPRE element, along with the corresponding WPRE-specific primers (forward and reverse). qPCR was carried out using the Power Up SYBR Green Master Mix, and an R^2 value of 0.998 was also obtained for the standard curve.

Both methods worked and produced similar results. The ABM kit calculated a titer of $6.2 \times 10^7 \pm 2.3 \times 10^7$ copies/mL, whereas the TRIzol method calculated $6.4 \times 10^7 \pm 2.3 \times 10^7$ copies/mL ($n = 5$). Analysis of the Ct values from the amplification curves indicated that the results from the two methods did not differ significantly.

4. Discussion

While a large number of methods for lentiviral titration has been described in the literature, including quantification of integrated proviral DNA, titration based on the expression of proteins linked to fluorescent marker genes, and other methods using SYBR Green-based real-time qPCR with the WPRE template[9] – similar to the approach used in this study – this paper highlights several unique aspects.

In this work, we describe the development of a rapid, efficient, and economical method for titering third-generation lentiviral vectors. Although the method itself is not entirely novel, the use of TRIzol reagent in this context is. Some traditional methods of lentiviral titration can take several days to complete,[6,7] whereas commercial kits, such as the one tested here, are quick but very expensive (AUD$1,958 for 96 samples). In addition, these kits provide reagents in specific, limited amounts and volumes. For example, the ABM

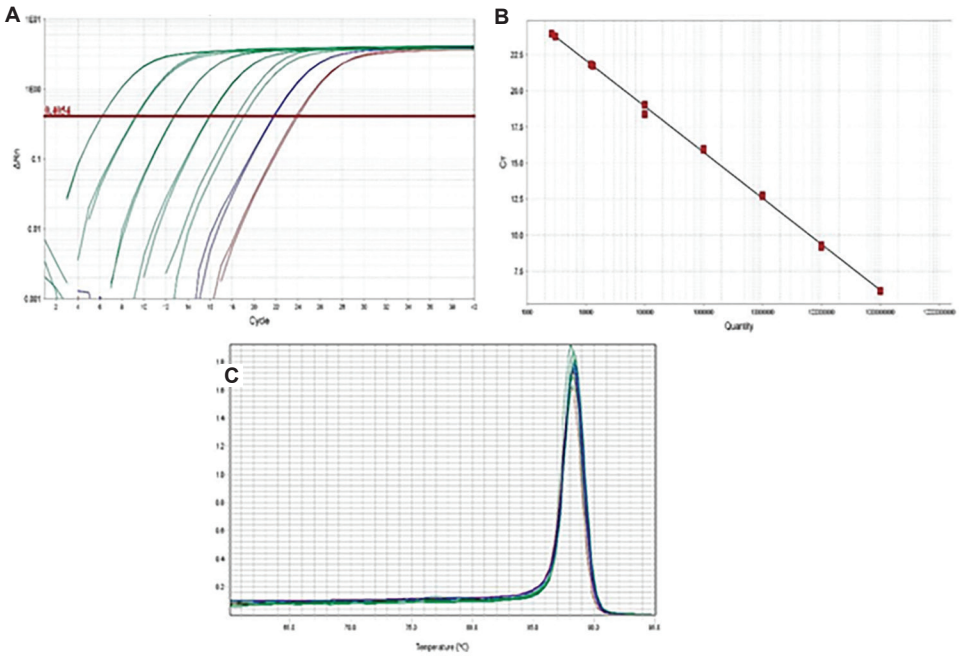

Figure 3. Quantitative polymerase chain reaction results for standards and lentiviral vector samples using the TRIzol method. (A) Amplification plots for in-house standards (green), with concentrations from left to right: 10^9, 10^8, 10^7, 10^6, and 10^5 copies/mL. The curves in other colors represent viral samples. (B) Standard curve generated from the amplification plots of the prepared deoxyribonucleic acid standards. (C) Melt curves of amplified products. R^2=0.998.

kit used in this study provides a limited volume of master mix (1.25 mL), primer mix (200 µL), standard DNA (50 uL), and virus lysis buffer (800 uL). To achieve accurate results, a viral range of 10^7 TU/mL is required, which often requires multiple dilutions of the samples (e.g., ×100, ×1,000, or ×10,000), consuming a significant amount of the sample. In contrast, although our technique also required sample dilutions to obtain the appropriate range, the use of TRIzol reagent is both more affordable and widely available (the procedure costs approximately AUD$115.00 for 96 samples). The lysis buffer used to extract RNA was also significantly better than that provided by the ABM kit. qPCR analysis using TRIzol yielded results that were accurate and not significantly different from those obtained with the ABM kit. The significantly lower A260/A280 ratios observed with the ABM kit may suggest the presence of contaminating proteins, phenol, or other impurities in those samples. Similarly, the lower A260/A230 ratios in the ABM samples may indicate contamination with guanidine hydrochloride or guanidine thiocyanate. While it was beyond the scope of this study to investigate these contaminants further, it would be of interest to test this methodology on other retroviral and lentiviral vector systems in future studies. However, we anticipate that this would not present any major issues, as the technique is simple and efficient for titering these vector systems.

5. Conclusion

We have developed a sensitive and accurate method for measuring viral titers based on the use of WPRE as the template for SYBR Green-based real-time qPCR. This method provides a reliable assessment of lentiviral copy number that is not significantly different from that obtained using a commonly used commercial kit. The use of TRIzol to isolate lentiviral vector RNA for qPCR-based titration is a novel approach. Our method is comparable in time to the commercial kit but is significantly more cost-effective, utilizing commonly available reagents found in most molecular biology laboratories. This benefit makes it an attractive alternative to more expensive commercial kits for many research laboratories. Although the technique was initially designed for small research and clinical laboratories, future studies could explore its scalability for larger-scale processing, potentially incorporating robotics or other forms of automation.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: Binhai Ren
Investigation: Binhai Ren
Methodology, Binhai Ren, Najah T Nassif
Visualization: Ann M. Simpson
Writing – original draft: Binhai Ren
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data can be obtained from the corresponding author.

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ACCSCIENCE PUBLISHING

Innovative Medicines & Omics

CASE REPORT

Rare subdural hematoma in a patient treated with avapritinib for gastrointestinal stromal tumor: A case report

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal malignancy of the digestive tract, accounting for 1% of all digestive tract malignancies. GISTs occur most frequently in the stomach, followed by the small intestine and colorectum, and rarely in the mesentery, omentum, and retroperitoneum. Avapritinib's approval for GISTs in 2020 marks a milestone in precision oncology, showing its significant antitumor activity against the resistant platelet-derived growth factor receptor-alpha D842V mutation. It has a manageable safety profile, with dose adjustments for mitigating side effects without reducing efficacy. Avapritinib-induced subdural hematomas are rare but potentially lethal complications. Our patient had a metastatic small bowel GIST and liver metastases and began avapritinib treatment in January 2024. He had a stable condition until early May. He presented with acute encephalopathy, altered mentation, and left subdural hematoma, which was suspected to be caused by avapritinib, resulting in its discontinuation. Despite receiving diligent care, the patient's condition did not improve, and he eventually died.

Keywords: Gastrointestinal stromal tumors; Chemotherapeutics; Novel targets; Side effects; Subdural hematomas

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1. Introduction

Metastatic small bowel gastrointestinal stromal tumors (GISTs) are rare (5 – 20 cases/million) but highly aggressive malignancies originating from the soft tissues supporting the digestive system, particularly the small intestine. They originate from the interstitial cells of Cajal, which regulate gut peristalsis.[1] Until recently, metastatic small bowel GISTs were usually diagnosed at a very advanced disease stage, when the cancer had spread to other organs such as the liver, lungs, or peritoneum. Traditional treatments

for GISTs include surgery, radiation therapy, and targeted drug therapy with imatinib and sunitinib. Surgery might be performed to remove the primary tumor and any associated regional lymph nodes. Chemotherapy and targeted therapy aim to slow tumor growth and shrink the tumor by applying drugs targeting cancerous cells.[2] However, some patients are resistant to these treatments, whereas others experience severe side effects. This has led to the exploration of new treatment options, including a highly selective inhibitor of mutated forms of two proteins-receptor tyrosine kinase type III (KIT) and platelet-derived growth factor receptor-alpha (PDGFRA) commonly found in GISTs.[3]

A recent clinical trial showed promising results for avapritinib in treating GISTs (Figure 1).[4] Avapritinib demonstrated a high response rate and prolonged progression-free survival in a phase 1 trial involving patients with advanced GIST who had undergone unsuccessful treatments.[5] The drug revealed a good safety profile with manageable side effects. These findings have translated into the FDA approval of avapritinib to treat GISTs, presenting new hope for patients with minimal treatment options.[6] The overall prognosis for patients with metastatic small bowel GISTs is generally poor, with a 5-year survival rate of 15% – 20%. The emergence of various treatment modalities has thus far improved outcomes in selected subsets of patients and includes novel targeted therapies, immunotherapies, and combination therapies. Ongoing studies into new treatment strategies and methods for early detection are being pursued to improve the survival and quality of life of patients with metastatic small bowel GISTs.[7] A significant safety concern associated with avapritinib is the risk of intracranial hemorrhage (ICH), including subdural hematomas (SDH). Although these events are uncommon in clinical trials, they affect approximately 2.4% of patients receiving a starting dose of 300 mg. Patients with severe thrombocytopenia (platelet counts $<50 \times 10^9$/L) at baseline are at an increased risk of complications linked to mast cell infiltration in the bone marrow. This association was observed during clinical trials of avapritinib in individuals with advanced systemic mastocytosis.[8] All cases of intracranial bleeding (ICB) were directly attributed to the treatment; however, the risk is significant enough to warrant caution, particularly in patients predisposed to bleeding because of thrombocytopenia, anticoagulant use, or previous vascular events.[9] Thus, close monitoring of the platelet count is essential before and during treatment. ICH symptoms, such as headache, nausea, vision changes, or altered mental status, require immediate medical attention. Dose adjustments or treatment discontinuation may be necessary in patients with severe adverse effects.[10]

2. Case presentation

A 66-year-old man with a history of GIST presented (in early May 2024) to the emergency room along with his wife with complaints of worsening altered mental status, confusion, gait changes, and global weakness. They denied any occurrence of recent trauma or falls. However, the patient has had multiple "near falls." In early 2019, he was diagnosed with a small bowel GIST with metastases to the liver – grade 1, pT3 pNX pM1 (liver), stage IV. Imatinib was initiated at diagnosis in February 2019. Disease progression was monitored closely. In December 2020, next-generation sequencing revealed two KIT mutations, one in exon 17 and the other in exon 9, and <1% programmed death ligand 1 mutations. In February 2021, regorafenib was initiated as a second-line treatment. Unfortunately, the disease progressed further, and various therapies were used. Sutent was initiated in April 2021, ripretinib in January 2022, Y90 therapy to a part of the liver in July 2022, Cabometyx in November 2022, and Avapritinib at the end of January 2024. While being treated with avapritinib (300 mg), the patient presented with periorbital edema (known side effect of Avapritinib) and some new papules on his bilateral lower extremities, particularly along the feet. Thus, in April 2024, the avapritinib dose was reduced to 200 mg.

The wife reported that the patient had worsening altered mental status in the past few days and believed that it was caused by his medication, which the patient had weaned and subsequently stopped earlier this week. His mental status has still not improved, and he has had an unsteady gait. At present, although he is fully oriented, his wife reported episodes of the patient getting dressed at home and not knowing where he was going. The patient believed that the reason for the visit was abdominal pain (right upper quadrant). He had a fever and vomiting (non-bloody and non-bilious). The patient denied the presence of skin infections, acute changes in hearing or vision, unilateral weakness or paresthesia or vision loss, chest pain, shortness of breath, cough, falls, seizures, drug or alcohol use, dysuria, and changes in urinary frequency.

The patient reported imbalance and confusion. Differential diagnosis included medication side effects; however, even after stopping the drug, his symptoms persisted. He did not exhibit focal symptoms; however, given his history, the plan was to obtain a computed tomography (CT) of the head to detect ICH or mass. The laboratory results were inconsistent with toxic metabolic etiologies such as electrolyte disturbances, hypoglycemia, uremia, acidosis states, and infection (i.e., sepsis). History examinations were performed, and toxidromes of intoxication or withdrawal, hypoxemia or hypercarbia, liver disease or liver failure causing

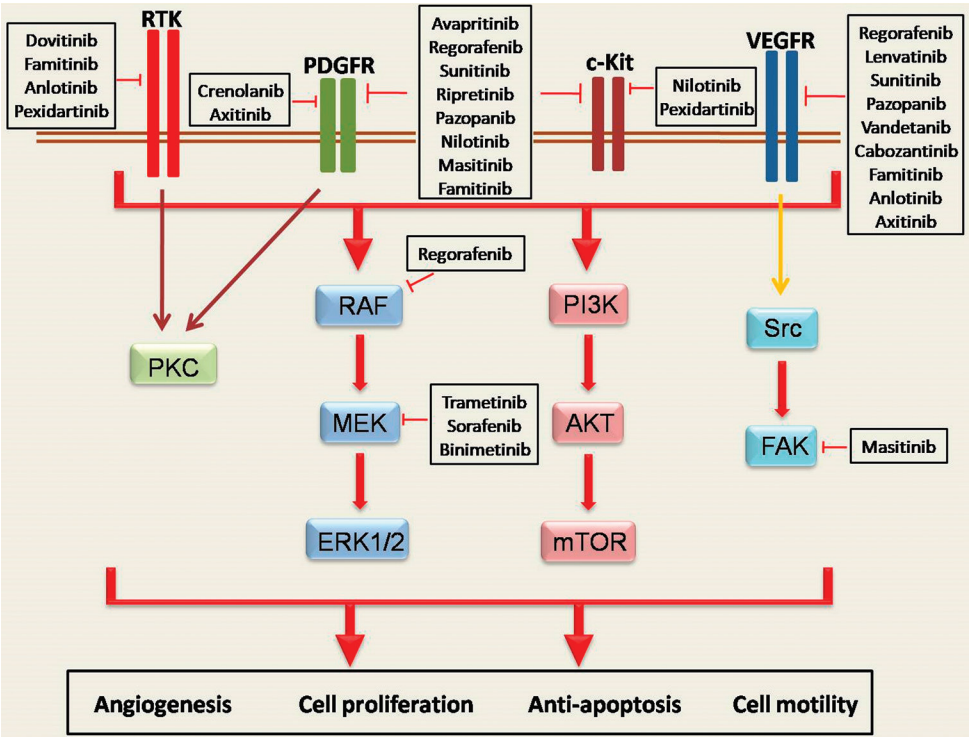

Figure 1. Role of receptors in the activation of signaling pathways in gastrointestinal stromal tumors. Reproduced from Vallilas *et al.*[4]
Abbreviations: RTK: Receptor tyrosine kinase; PDGFRA: Platelet-derived growth factor receptor a; c-Kit: Cellular Kit; VEGFR: Vascular endothelial growth factor receptor; RAF: Rapidly accelerated fibrosarcoma; MEK: Mitogen-activated protein kinase kinase; ERK ½: Extracellular signal-regulated kinase 1 and 2; PI3K: Phosphoinositide 3-kinase; AKT: Protein kinase b; mTOR: Mechanistic target of Rapamycin; Src: Sarcoma; FAK: Focal adhesion kinase.

hepatic encephalopathy, endocrine emergencies (hyper/hypothyroidism and adrenal insufficiency), seizures, and trauma were considered less likely.

Head CT (Figure 2) revealed a large SDH (up to 18 mm thick) overlying the left cerebral convexity, resulting in a 15-mm left-to-right shunt of midline structures and a trapped/dilated right lateral ventricle. Global sulcal effacement and uncal herniation with mild mass effect on the brainstem were noted. No evidence of a large transcortical infarct or acute bony abnormality was found. In the abdominal/pelvic examination, similar-appearing liver metastases and small nodules or lymph nodes in the lower mesentery were detected. No bowel obstruction, abscess, free gas, diverticulitis, or appendicitis was noted. Scattered sclerotic skeletal lesions indicated metastasis.

After CT, the neurosurgeon was consulted, and following a detailed discussion, the patient expressed verbal understanding, consented, and wished to proceed with a left burr hole washout (Figure 3). After the removal of the left scalp JP drain, the patient was cleared for discharge by neurosurgery to follow-up with them in 2 weeks with head CT. Acute metabolic encephalopathy secondary to SDH significantly improved, and the patient

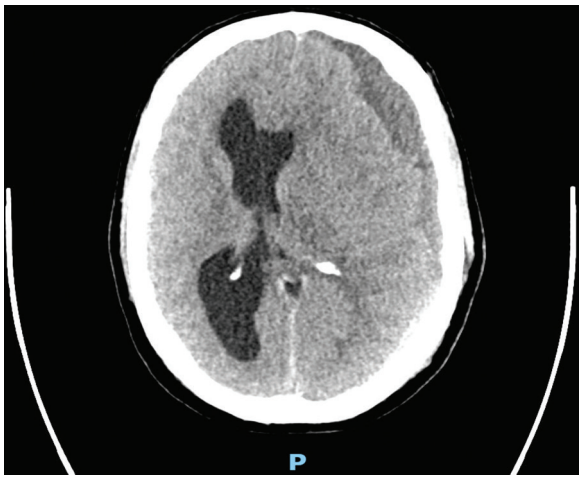

Figure 2. Non-contrasted computed tomography of the head showing left subdural hematoma, with rightward midline shift and enlargement of the right ventricular system, and effacement of ambient cisterns with uncal herniation.

was discharged in a stable clinical condition. Oncology recommended discontinuing the current chemotherapy (Avapritinib) because of the recent SDH development. This is reported as a severe and infrequent side effect of avapritnib. Avapritinib was not resumed, and the patient

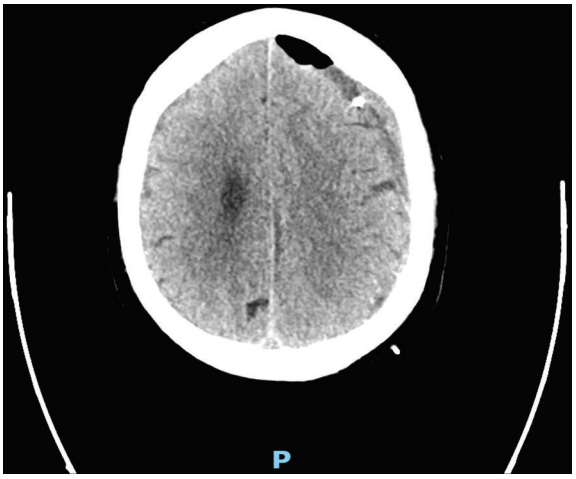

Figure 3. Repeat computed tomography post-surgery showing an overall decrease in the subdural collection volume, midline shift, and prominence of the ventricular system.

was planned for follow-up in the clinic in 2 – 4 weeks to discuss future management of metastatic GISTs.

The patient came back to the emergency department a few days later because he had not been eating and drinking and was progressively weak with a fever. A CT image of the head showed an increase in the low-attenuation component of the left SDH with an increase in sulcal effacement, left lateral ventricle effacement, and midline shift. The hyperdense hemorrhagic component appeared unchanged. The patient was admitted to the intensive care unit, with stable hemodynamics; however, he was increasingly somnolent. His encephalopathy continued to worsen. A component of metabolic encephalopathy was not suspected, although toxic encephalopathy/underlying bacterial infection could play a role. No hypoxia, uremia, or significant glucose abnormalities were noted. The patient's condition continued to decline despite repeat washout and antibiotic therapy. According to the patient and his wife's desire, the decision was to pursue comfort measures only. He was transferred to a palliative care unit and died a few days later.

3. Discussion

Avapritinib is a targeted therapy primarily used to treat GISTs. It is extremely effective for patients with PDGFRA exon 18 mutation, including D842V mutation, which is resistant to other treatments.[11] Avapritinib is also used to treat advanced systemic mastocytosis, a rare disorder that involves abnormal mast cell accumulation.[12] Avapritinib, a tyrosine kinase inhibitor, blocks proteins driving cancer growth, particularly in PDGFRA- and KIT-mutant tumors. Common side effects include fatigue, nausea, diarrhea, swelling around the eyes, cognitive impairment, and decreased appetite. ICH, anemia, liver toxicity, and memory issues are some of the rare but severe side effects. Avapritinib is a vital treatment option for patients with specific mutations but requires careful monitoring because of its potentially serious side effects.[13]

ICH is a severe side effect of avapritinib. ICH signs include severe headaches, dizziness, nausea, vomiting, neurological issues, and altered consciousness. Patients with hypertension and blood clotting disorders or those on anticoagulants may have a higher risk of ICH. Immediate interventions, including stopping avapritinib and providing emergency care, are necessary. Regular brain imaging and blood tests are recommended for patients on this treatment, especially if they have higher bleeding risks.[14]

4. Conclusion

This case highlights the rare but severe potential drug-related complication of SDH occurring in a patient treated with avapritnib for GISTs, without other known contributing factors. Such an adverse event underscores the importance of careful monitoring of neurological symptoms in patients receiving targeted therapies. Clinicians should remain vigilant regarding potential bleeding complications, particularly in patients with pre-existing risk factors. This case reinforces the need for further research to enhance the understanding of the relationship between avapritnib and hematological risks, improve patient outcomes, and determine the most optimal treatment protocols.

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Conflict of interest

Syed A. A. Rizvi is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

Conceptualization: Syed M. Imam
Investigation: Syed M. Imam
Methodology: Syed M. Imam, Faryal Haider
Writing – original draft: All authors
Writing – review & editing: All authors

Ethics approval and consent to participate

Patient gave consent to participate in this study.

Consent for publication

Verbal consent has been obtained from the patient to publish his data.

Availability of data

Not applicable.

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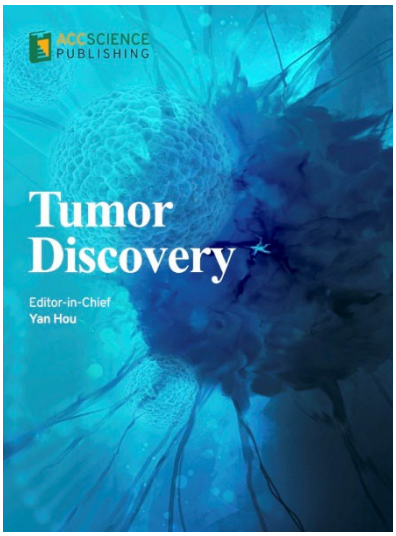

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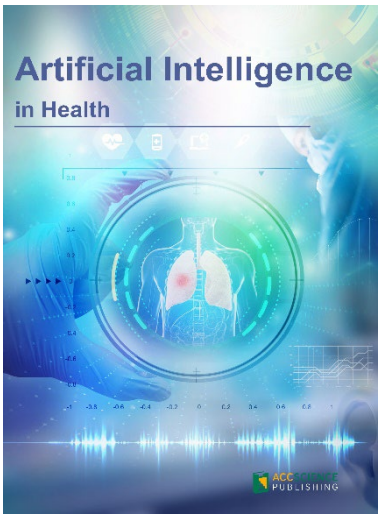

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