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Integrated Experimental, Molecular Docking, and Dynamics Simulations Studies of Propolis Extracts as Promising Anti-inflammatory and Anti-diabetic Agents

Presented by

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In front of the following jury

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Dedication

Praise be to God at the beginning and at the end. Whoever said "I am for her" has won her over. It has been a long road filled with difficulties and successes. We are proud of our struggle to achieve our dreams.

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To all of them, I dedicate this work. May God guide us all to goodness.

Assil Bia

Dedication

I would like to express my heartfelt gratitude and praise to Allah, the Almighty, for guiding me to complete this work.

This work is dedicated to my dearest ones: my father and mother, my small family my beloved husband and son, my sisters and their children and husbands, my brothers, and to my companion on this academic journey. I also dedicate it to all my professors and dear friends.

Elouahma Benzerrouk

Abstract

Propolis is a bee-created natural resinous material, commonly known to possess potential biological activity, especially anti-inflammatory and anti-diabetic action. The present work seeks to scientifically establish these actions through several extraction methods: maceration (ME), Soxhlet extraction (SE), and ultrasound-assisted extraction (UAE). Yields varied from 19.58% to 28%, with the best one achieved by the combination of maceration and UAE (UAE-M) after 24 hours.

Chemical composition was also assessed by LC-MS, showing the principal bioactive compounds such as quercetin, caffeic acid, curcumin, and oleanolic acid. Qualitative as well as electrochemical analysis identified polyphenols and flavonoids. Quantitative assessments via spectrophotometry and voltammetry revealed that the 24-hour UAE-M extract contained the highest levels of total phenolics (148.1 mg GAE/g) and flavonoids (270.3 mg QE/g).

In vitro assays revealed strong anti-inflammatory activity (IC_{50} = 0.187 mg/mL, comparable to diclofenac at 0.190 mg/mL) and strong anti-diabetic activity by inhibiting alpha-amylase (IC_{50} = 0.227 mg/mL against acarbose at 0.221 mg/mL). Molecular docking and dynamics simulation also confirmed the safe binding of lead compounds against inflammation and glucose metabolic target proteins. These findings point to propolis extracts—more specifically UAE-M extract—as potential leads to the discovery of natural therapeutic agents against inflammation and diabetes.

Key words: Propolis, Polyphenols, Flavonoids, Anti-inflammatory Activity, Antidiabetic Activity, Molecular Docking, Molecular Dynamic.

Résumé

La propolis est une substance résineuse naturelle produite par les abeilles, qui suscite un grand intérêt en raison de ses potentielles propriétés anti-inflammatoires et anti-diabétiques. Cette étude vise à vérifier scientifiquement les activités biologiques des extraits de propolis. Les échantillons ont été extraits en utilisant méthodes différentes : macération (ME), extraction Soxhlet(SE) et extraction assistée par ultrasons (UAE).

L'analyse a été réalisée en utilisant la chromatographie liquide couplée à la spectrométrie de masse (LC-MS). Il a pu identifier les principaux bicomposés actifs, tels que la quercétine, l'acide caféique, la curcumine et l'acide oléanolique. De plus, la valeur de l'étude réside dans la teneur en polyphénols et flavonoïdes dans la propolis de deux manières. L'analyse qualitative a montré que les flavonoïdes et les polyphénols étaient présents, et la voltamétrie cyclique a testé comment ils réagissent lors de l'oxydation et de la réduction. L'analyse quantitative utilisant la spectrophotométrie et la voltamétrie a montré que l'extrait UAE-M sur 24 heures présentait les niveaux les plus élevés de phénols totaux (148,1 mg GAE/g) et de flavonoïdes (270,3 mg QE/g). Les effets anti-inflammatoires et antidiabétiques ont également été testés en laboratoire. L'extrait a montré une activité anti-inflammatoire significative avec un IC50 de 0,187 mg/mL (comparé au diclofénac à 0,190 mg/mL) dans l'inhibition de l'albumine sérique bovine (BSA) et une activité anti-diabétique avec inhibition de l'alpha-amylase (IC50 = 0,227 mg/mL comparé à l'acarbose à 0,221 mg/mL). De plus, des études de docking moléculaire ont été menées pour évaluer l'affinité de liaison des composés sélectionnés avec des protéines clés impliquées dans l'inflammation et le métabolisme du glucose, suivies de simulations de dynamique moléculaire pour examiner la stabilité et les interactions des meilleurs complexes ligand-protéine. Dans l'ensemble, les résultats intégrés mettent en évidence le potentiel des extraits de propolis comme une source naturelle prometteuse pour le développement d'agents thérapeutiques contre l'inflammation et le diabète.

Mots clés : Propolis, Polyphénols, Flavonoïdes, Anti-inflammatoire Activité, Antidiabétique Activité, Docking moléculaire, Dynamique moléculaire.

الملخص

البروبوليس مادة راتنجية طبيعية ينتجها النحل، قد أصبح مصدر اهتماما كبيرا بسبب خصائصه المحتملة المضادة للالتهاب والمضادة للسكري. تهدف هذه الدراسة الى التحقق العلمي من الأنشطة البيولوجية لمستخلصات البروبوليس. تم استخلاص العينات باستخدام أربع طرق مختلفة: النقع، استخلاص سوكسليت، واستخلاص بمساعدة الموجات فوق الصوتية. بعد المقارنة تراوحت قيم المردود بين 19.58% و28% وأعطت طريقة النقع المقترنة بالموجات فوق الصوتية أعلى مردود بعد 24ساعة. وأجري التحليل باستخدام الكروماتوغرافيا السائلة بالاقتران مع مطيافية الكتلة(LC-MS) . تمكن من تحديد المركبات الحيوية النشطة الرئيسية مثل الكيرستين،حمض االكافيك،الكوركمين وحمض اولينيك. كذلك، قيمة الدراسة محتوى البروبوليس من البوليفينولات والفلافونويدات بطريقتين. التحليل النوعي أكد على وجود الفلافونويدات البوليفينولات وكذلك التحليل الكهروكيميائي على خاصيتها النشطة في الاكسدة والاختزال، وأظهر التحليل الكمي باستخدام المطيفية والفولطمترية ان مستخلص UAE-Mلمدة 24ساعة أظهر أعلى مستوى من الفينولات الكلية (148.1ملغ/GAE /غ) وفلافونويدات (270.3 ملغ/QE غ) .

كما تم اختبار التأثيرات المضادة للالتهاب والسكري في المختبر .اظهر المستخلص نشاطا مضادا للالتهاب ملحوظ مع (IC50 قدره 0.187ملغ/مل) مقارنة بالديكلوفيناك عند 0.190 ملغ/مل) في تثبيط بروتين مصل البقر BSA،ونشاطا مضادا للسكري مع تثبيط الفا -اميلاز (IC50=0.227ملغ/مل مقارنة بالاكاربوز عند 0.221 ملغ/مل).

بالإضافة الى ذلك، تم اجراء دراسات الربط الجزيئي لتقييم تقارب المركبات المرشحة مع البروتينات المستهدفة الرئيسية المشاركة في الالتهاب وعمليات التمثيل الغذائي للجلوكوز، تلتها محاكاة الديناميكا الجزيئية للتحقيق في استقرار وتفاعلات أفضل معقدات الربائط-البروتين. بشكل عام، تسلط النتائج المدمجة الضوء على إمكانيات مستخلصات البروبوليس كمصدر طبيعي واعد لتطوير عوامل علاجية ضد الالتهابات والسكري.

الكلمات المفتاحية: البروبوليس، بولفينول، فلافوندات، نشاط مضاد الالتهاب، نشاط مضاد السكري، الارساء الجزيئي، الديناميك الجزيئية.

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Abbreviation list

ΔG	Binding Free Energy
ADT	AutoDock Tools
BSA	Bovine serum albumin
BBB	Blood-Brain Barrier
DNS	Dinitrosalicylic
DFT	Density-Functional Theory
DLG	Docking log file
GAE	Galic Acid
GI	GastroIntestinal
GLG	Grid log file
GPF	Grid parameter file
IC50	The half maximal inhibitory concentration
LC-MS	Liquid chromatography-mass spectrometry
SE	Soxhlet
ME	Maceration
PBD	Protein data bank
PDBQT	Protein data bank Q for charges and T for torsion
QE	Quercetin
TFC	Total Flavonoid content
TPC	Total Polyphenol content
UAE	Ultrasound
UV-Vis	Ultraviolet visible

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Introduction

INTRODUCTION

Propolis is known by various names in different cultures, including bee glue in English, propolis in Latin, bee wax in Chinese, and akbar in Arabic. The term "propolis" is derived from the Latin "pro" meaning "before" and "separlis" meaning "a local defense." Therefore, propolis could be interpreted as a substance that bees use for defense before they start collecting pollen. Propolis, is also known under the name "bee glue"[1]. It is a peculiar, multifunctional, and complex material of biological origin that honey bees produce. Bees create it by collecting resinous exudate from various flower buds or parts of trees or leafy plants and combine it with beeswax. Before depositing the propolis into well-defined portions of cavities in their hives, bees mix it with metabolic secretions, pollen collected from the flowers of their choice, and volatiles emitted from their bodies, and modify it so that its curing occurs in the best possible way[2]. The curing process of the collected plant material in combination with bee-added substances causes a biotransformation that concerns the chemical composition and biological properties of propolis[3]. Accordingly, propolis is a complex mix of over 180 different natural substances, including resin, wax, volatiles, pollen, cinnamic acid and esters, flavonoids, terpenes, plant-derived compounds, and other substances[4].

Propolis, a sticky, resinous substance, known for thousands of years, has been used as a folk medicine to treat a wide range of diseases, especially due to its presence of flavonoids and polyphenols. Several studies have shown that propolis and its compounds possess a broad spectrum of pharmacological activities, including cytoprotection, immunomodulation, antioxidant, antitumor, antimicrobial, anti-inflammatory, antiulcer, antigenotoxic, antiviral, antidiabetic, wound healing, and tissue regenerative effects[5]. Given the many available sources of plant resin, propolis can be found worldwide, most commonly in Brazil, where researchers have found that propolis from the temperate zones contains higher amounts of flavonoids. One of the flavonoids found in propolis is isorhamnetin, a yellow pigment of the flavonol class and a 3′-methyl derivative of quercetin, and isorhamnetin 3-β-d-glucopyranoside[6]. Isorhamnetin possesses potentially interesting anticancer activities, such as apoptosis induction, cell cycle arrest, and reactive oxygen species enhancement, in addition to having the ability to inhibit pro-inflammatory responses and protect against diseases associated with systemic chronic inflammation. The study of isorhamnetin was carried out because there are few reports on the isolation of isorhamnetin in propolis, and even less data in the literature to explain its anti-inflammatory activity. After the isolation, the antioxidant and anti-inflammatory activities were investigated[7]. Such studies on possible molecular targets conducted by molecular docking and molecular dynamics simulation tools were carried out.

The data show that isorhamnetin has potential as an agent employed in diseases with systemic chronic inflammation[8].

It is widely accepted that inflammation is associated with oxidative stress, which is the underlying factor responsible for several chronic diseases, including diabetes mellitus. The current availability of anti-inflammatory and hypoglycemic agents in the market has several downsides, such as a relatively short half-life, adverse effects, and high cost. As such, researchers all over the world are attempting to explore potential therapeutically effective drugs extracted from plants[9]. One such source is propolis, a sticky, gummy substance collected by honeybees from exudates of plants and used for the construction, restoration, and protection of beehives. It is a complex matrix and mainly contains flavonoids, phenolic acid compounds, and diterpenes, as well as some minor constituents such as amino acids, sugars, fatty acids, vitamins, and microbiological substances. The biological activities of propolis are the best known of all natural products. It has antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, tumor inhibitory, and other biological activities, such as protecting against diabetes[10].

In the present work, two approaches, experimental and computational, were employed in order to investigate the anti-inflammatory and antidiabetic potential of propolis. Firstly, an experimental approach was used to extract, analyze, and evaluate the in vitro therapeutic potential of propolis extract. In vitro evaluation constitutes an assay for estimating the potential of propolis as a lead compound for the development of drugs for the treatment of inflammation and diabetes. Second, docking simulations have been performed for the propolis active compounds at tumor necrosis factor-alpha (TNF-α). The major extracted compounds from propolis have been docked with appropriate target receptors referenced for anti-inflammatories and diabetes as they possess both in vitro activities. The methods involved in this are scanning the database for known drug-binding proteins and performing a virtual screening for small molecules to bind to a protein of known three-dimensional structure. The molecules identified by molecular docking provide important information about simulation-based structural modifications and give a much faster indication of the potential action of a set of propolis compounds for the treatment of inflammation. For example, counting on the computational model of TNF-α bound to quercetin, important parameters for the transport of these molecules could be estimated. Full understanding of these structure-activity relationships contributes to the design of new and safer therapeutic compounds, increasing the chance of success in clinical trials.

Inflammation and Diabetes

Inflammation and diabetes are interconnected conditions that significantly impact global health, contributing to the development of chronic diseases and metabolic disorders. Both conditions are characterized by dysregulated immune responses and metabolic imbalances, creating a vicious cycle

that exacerbates their progression and severity. Chronic low-grade inflammation is a hallmark of type 2 diabetes, and it plays a crucial role in the onset of insulin resistance, a defining feature of the disease[11]. Inflammation in the context of diabetes is often initiated and sustained by metabolic stress, including the accumulation of free fatty acids, hyperglycemia, and adipose tissue dysfunction. These factors activate immune cells, such as macrophages and T-cells, in metabolic tissues, leading to the release of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP). These cytokines interfere with the insulin signaling pathway by inhibiting the phosphorylation of insulin receptors, thereby reducing glucose uptake by cells and perpetuating hyperglycemia[12].

Over time, the sustained inflammatory state contributes to the progression of diabetes-related complications, including cardiovascular diseases, neuropathy, nephropathy, and retinopathy. For instance, chronic inflammation damages blood vessel walls, leading to atherosclerosis, a major cause of cardiovascular morbidity and mortality in diabetic patients. Furthermore, inflammation-driven oxidative stress exacerbates tissue damage, disrupting the normal function of organs such as the pancreas, where beta cells responsible for insulin production are particularly vulnerable[13]

Several factors contribute to the interplay between inflammation and diabetes, including:

- **Obesity:** Excess adipose tissue secretes pro-inflammatory molecules, such as leptin and resistin, while reducing anti-inflammatory adipokines like adiponectin, creating a state of metabolic inflammation.
- **Unhealthy diets**: Diets rich in refined sugars, trans fats, and processed foods can trigger inflammation, worsening insulin resistance and glucose metabolism.
- **Sedentary lifestyles**: Physical inactivity contributes to weight gain, fat accumulation, and systemic inflammation, increasing the risk of insulin resistance.
- **Gut microbiota imbalance**: Dysbiosis, or an imbalance in gut bacteria, can lead to the release of inflammatory metabolites that affect systemic metabolic processes.
- **Genetic predisposition**: Individuals with a genetic susceptibility to inflammatory and metabolic disorders are at higher risk of developing type 2 diabetes.

Early detection and management of inflammation in individuals with diabetes are critical to preventing long-term complications and improving outcomes. Lifestyle modifications, such as adopting a healthy diet, increasing physical activity, and losing weight, are foundational strategies for reducing systemic inflammation and improving insulin sensitivity. Medications, including metformin, thiazolidinediones, and anti-inflammatory agents, can also help manage glucose levels and inflammation[14]

Emerging therapies focus on targeting inflammation directly to mitigate its impact on diabetes progression. For example, natural compounds like flavonoids and phenolic acids found in propolis have shown promise in reducing inflammatory cytokines and improving insulin sensitivity. Similarly, advanced research into molecular mechanisms, such as inhibiting the NF-kB and JNK signaling pathways, offers potential for novel anti-inflammatory and antidiabetic treatments.

By addressing the intricate relationship between inflammation and diabetes, researchers aim to develop more effective interventions to break the cycle of inflammation-driven metabolic dysfunction, ultimately improving the quality of life for individuals affected by these interconnected conditions[15]. All aspects of this research have been consolidated into a structured framework, divided into two main parts:

The first part is a literature review, which is consist of two chapter:

- **Chapter one** includes an overview of propolis and its therapeutic potentials.
- **Chapter two** deals with the overview of anti-inflammatory and antidiabetic activities .

The second part represents the experimental section, which includes two chapters:

- **Chapter one** discusses the materials and methods.
- **Chapter two** presents the results and their discussion.

The study concludes with a general conclusion that synthesizes the main findings and underscores the scientific contribution of this research work.

PART ONE

LITERATURE REVIEW

Part I: literature review

1.1. Propolis in history

Humans first kept bees by domesticating wasps in woven wicker baskets, wooden pots, and hollow logs. Simple wooden pots were used to store honey in ancient Egypt. Greek and Roman physicians employed propolis, a bee product, as an antiseptic and to cure wounds, and Egyptians used it to mummify their dead [16]. Again. in this period, propolis was used in various home-made recipes. It is estimated that the Egyptians learned the mummification feature of propolis from bees and used it in mummification processes [17]. So much so that honey bees were "mummies" with propolis [18] to prevent the spread of disease after killing foreign bees that occupy the hive.

The significance of beekeeping in Ancient Egypt reached a divine level about 3100 BC. The gods were linked to honey bees, which were converted into hieroglyphs. Pharaohs were called 'Bee King ', and bees were thought to be used for ointments, honey, and medicines[19].

The Incas suggested propolis, an antipyretic medication that was made legal in the 17th century. Its chemical makeup and biological characteristics are being investigated, and it is utilized in health and cosmetic product[20]. The Bible also mentions propolis in Hebrew (6).

In his work Aristo Historia Animalium, he mentions propolis as follows: "When the hive is delivered to honey bees cleanly and emptily, the bees build the wax cells, extract the sap of all kinds of flowers and the nectars of willow and elm trees. With this to protect against the attacks of other creatures. If the entrances of the bucket with the same material are very wide, they narrow it. This substance (propolis) is dark black and is a kind of wax slag or by-product; it has a pungent smell and is a curative for wounds" (6).

Nicolas Louis Vauquelin, a French pharmacist and chemist, explored propolis in the nineteenth century[22]. In 1814 or 1815, a French chemist, Michel Eugène Chevreul, and in 1864, Piccard identified several important flavones from this substance [23].

1.2. Chemical composition

Propolis has a complex chemical composition that varies depending on its botanical origin, geographic location, and the season it is collected. Propolis contains more than 300 components, including resins, waxes, essential oils, pollen, organic compounds, vitamins, and mineral salts. However, it generally consists of the following major components[24]:

1.2.1. Resin and Balsams (50–60%)

This is the major fraction of propolis, consisting of plant-derived bioactive compounds like flavonoids, phenolic acids, and their derivatives. Flavonoids include quercetin, galangin,

kaempferol, apigenin, chrysin, and pinocembrin. Phenolic acids and esters include caffeic acid, ferulic acid, p-coumaric acid, caffeic acid phenethyl ester.

The therapeutic potential of propolis related to flavonoids, contribute significantly to its therapeutic potential. Flavonoids such as quercetin, galangin, kaempferol, apigenin, chrysin, and pinocembrin are potent antioxidants, effectively protecting cells from oxidative stress, which can otherwise lead to cellular damage and contribute to the development of chronic diseases like cancer and cardiovascular disorders. These compounds also exhibit anti-inflammatory properties by inhibiting pro-inflammatory pathways, thereby reducing inflammation in various tissues, which is beneficial for treating conditions like arthritis, asthma, and inflammatory bowel diseases. Additionally, flavonoids in propolis display strong antimicrobial activity, demonstrating efficacy against a wide range of microorganisms, including bacteria, fungi, and viruses. Their antimicrobial action involves disrupting microbial cell membranes or inhibiting essential microbial enzymes. Propolis also contains antidiabetic flavonoids, such as quercetin, which have shown potential in lowering blood sugar levels, improving insulin sensitivity, and protecting pancreatic cells from oxidative damage, making it a valuable tool for managing diabetes. Lastly, the anticancer properties of flavonoids in propolis are notable, as they can modulate key cellular pathways involved in growth, apoptosis (programmed cell death), and angiogenesis (the formation of new blood vessels), helping to inhibit cancer cell proliferation and metastasis, and contributing to cancer prevention and therapy.

The therapeutic potentials of propolis related to phenolic acids, such as caffeic acid and ferulic acid, are integral to its therapeutic potential. These phenolic acids are powerful antioxidants that effectively neutralize free radicals, reducing oxidative stress, which is a key contributor to aging and the development of various diseases, including cancer and cardiovascular conditions. In addition to their antioxidant properties, phenolic acids also possess anti-inflammatory effects. They reduce the production of inflammatory cytokines and other mediators, thereby alleviating inflammation in tissues and offering relief in conditions like arthritis, asthma, and inflammatory bowel diseases. Phenolic acids in propolis further demonstrate notable antimicrobial properties, exhibiting activity against a broad range of microorganisms, including bacteria, fungi, and viruses. This antimicrobial action is achieved by interfering with microbial cell structures or inhibiting essential microbial functions. Furthermore, caffeic acid in particular has been shown to have anticancer properties, inhibiting cancer cell growth and inducing apoptosis (programmed cell death) in certain types of cancer cells, thus contributing to cancer prevention and therapy. Additionally, neuroprotective effects of phenolic acids, especially

caffeic acid, have been suggested in the context of neurodegenerative diseases. These compounds help protect brain cells by reducing oxidative stress and inflammation, making them potentially beneficial in managing conditions like Alzheimer's disease and Parkinson's disease[25].

1.2.2. Waxes (25–35%)

Waxes in propolis are primarily composed of fatty acids, esters, and alcohols. Examples of long-chain fatty acids is palmitic acid, esters, and hydrocarbons[24].

1.2.3. Essential Oils (10%)

Volatile organic compounds in propolis are primarily terpenoids and aromatic hydrocarbons. Examples of terpenoids found in propolis include pinene, limonene, and eucalyptol, while examples of aromatic hydrocarbons include vanillin and benzyl benzoate.

Essential oils in propolis contribute significantly to its diverse therapeutic potential, enhancing its therapeutic potential. These oils exhibit powerful antimicrobial properties, with strong activity against bacteria, fungi, and viruses, helping to prevent infections and support the body's defense mechanisms. In addition to their antimicrobial effects, essential oils in propolis also display anti-inflammatory properties. They help reduce inflammation by modulating various immune pathways, which can be beneficial for managing inflammatory conditions such as arthritis and respiratory diseases. Furthermore, essential oils in propolis act as antioxidants, protecting cells from oxidative damage caused by free radicals, which can contribute to aging and chronic diseases like cancer and cardiovascular disorders. Essential oils also play a role in wound healing, as some of them accelerate tissue regeneration, reduce the risk of infection, and promote faster healing of wounds, making propolis a valuable natural remedy for cuts, burns, and other skin injuries. These combined therapeutic potentials make essential oils in propolis a key factor in its medicinal properties.

Terpenoids found in propolis are known for their significant therapeutic potentials, contributing to its wide range of therapeutic effects. One of the primary actions of terpenes, such as borneol and cineole, is their antimicrobial activity. These compounds exhibit strong activity against a variety of microorganisms, including bacteria, fungi, and viruses, by disrupting microbial cell membranes or inhibiting microbial enzymes. Terpenes also possess notable anti-inflammatory effects, as they modulate the production of pro-inflammatory mediators like cytokines and prostaglandins, helping reduce inflammation in tissues and offering therapeutic potential for inflammatory conditions such as arthritis and respiratory disorders. Additionally, certain terpenes in propolis have been shown to have analgesic (pain-relieving) properties. They can act as natural pain relievers by reducing inflammation and blocking pain receptors, providing

relief in conditions characterized by pain and discomfort. Furthermore, terpenes also exhibit antioxidant properties, helping to protect cells from oxidative damage caused by free radicals, which is crucial for preventing cellular aging and the onset of chronic diseases like cancer and neurodegenerative disorders. These combined therapeutic potentials make terpenes in propolis valuable for various therapeutic applications.

Aromatic hydrocarbons in propolis play a significant role in its therapeutic potentials, contributing to its diverse therapeutic properties. For instance, compounds like vanillin and benzyl benzoate are known for their strong antimicrobial effects, particularly against bacteria and fungi. These compounds help inhibit microbial growth, making propolis a natural remedy for infections. Additionally, aromatic hydrocarbons exhibit potent antioxidant properties by acting as free radical scavengers, which protect cells from oxidative damage, reducing the risk of chronic diseases such as cancer and cardiovascular disorders. Certain aromatic hydrocarbons also demonstrate anticancer effects, with some studies indicating that they can inhibit cancer cell proliferation and induce apoptosis (programmed cell death), contributing to the prevention and treatment of cancer. Furthermore, anti-inflammatory properties are another key feature of aromatic hydrocarbons like vanillin, which help reduce inflammation by inhibiting key inflammatory enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), making them beneficial for conditions such as arthritis and other inflammatory diseases. Together, these therapeutic potentials underscore the therapeutic potential of aromatic hydrocarbons in propolis[24].

1.2.4. Pollen (5–10%)

Pollen particles are naturally incorporated into propolis during its collection by bees. Its components include proteins, amino acids, vitamins, and minerals.

Vitamins and minerals found in propolis play essential roles in supporting various therapeutic potentials that contribute to its therapeutic potential. Vitamins, particularly Vitamin C, act as powerful antioxidants, neutralizing free radicals and protecting cells from oxidative damage. This antioxidant action helps reduce the risk of chronic diseases and supports overall health. Additionally, vitamins in propolis also contribute to immune support, enhancing the body's defense mechanisms and improving the immune response to pathogens, which is crucial for maintaining good health and preventing infections. On the other hand, minerals such as calcium, magnesium, and potassium are vital for maintaining bone health, as they support bone density and strength. These minerals also play important roles in muscle function, nerve transmission, and various cellular processes essential for proper body functioning. Together, the vitamins and

minerals in propolis contribute significantly to immune function, bone health, and protection against oxidative stress, making it a valuable natural remedy for overall well-being[26].

1.2.5. Organic Compounds (5%)

Organic compounds make up approximately 5% of propolis and include a variety of essential substances. Among the vitamins present, Vitamin C, Vitamin E, and B-complex vitamins play key roles in promoting health. The amino acids found in propolis, such as proline, arginine, and cysteine, are crucial for protein synthesis and various metabolic functions. Additionally, sugars like glucose and fructose are present, providing energy. Organic acids, including succinic acid and malic acid, contribute to the overall chemical composition and potential biological activity of propolis. These organic compounds collectively enhance the nutritional and therapeutic value of propolis[27].

1.2.6. Inorganic Compounds (Minerals and Trace Elements)

Inorganic compounds, including minerals and trace elements, are also found in propolis and contribute to its nutritional and therapeutic properties. These include essential minerals such as magnesium, zinc, iron, potassium, and calcium. These elements play vital roles in various biological processes, such as enzyme activation, bone health, immune function, and cellular metabolism, further enhancing the beneficial effects of propolis.

Minerals and **trace elements** are essential nutrients that are present in **propolis** in small amounts but play vital roles in maintaining health. These components contribute significantly to various biological functions and overall well-being.

Minerals, such as calcium, magnesium, phosphorus, sodium, and potassium, support bone health, muscle function, and fluid balance. For instance, calcium and phosphorus are key components of bones and teeth, while magnesium plays a role in muscle relaxation and nerve function. Potassium helps regulate blood pressure and fluid balance in cells.

Trace elements, though needed in much smaller amounts, are equally important for maintaining proper physiological functions. Zinc, for example, is essential for immune function, wound healing, and protein synthesis. Iron is crucial for oxygen transport in the blood, while copper supports the formation of red blood cells and the maintenance of a healthy cardiovascular system. Manganese plays a role in the formation of connective tissue, bones, and clotting factors.

Together, these minerals and trace elements in propolis not only support structural integrity, like bone and cell function, but also contribute to immune health, metabolism, and antioxidant defense, ensuring the proper functioning of numerous physiological systems. Their presence in

propolis enhances its overall therapeutic potential and makes it a valuable natural supplement for maintaining health[28].

1.2.7. Enzymes

Enzymes are introduced into propolis by bees during its production and play a significant role in its composition and functionality. Notable examples of these enzymes include glucose oxidase and catalase. Glucose oxidase is involved in the conversion of glucose into hydrogen peroxide, contributing to the antimicrobial properties of propolis, while catalase helps to break down hydrogen peroxide, protecting the propolis from oxidative damage. These enzymes enhance the overall bioactivity and therapeutic potential of propolis.

Enzymes present in propolis play crucial roles in various therapeutic potentials, contributing to its medicinal and therapeutic properties. These biological catalysts are proteins that speed up chemical reactions in the body and are essential for numerous physiological processes.

Antioxidant Enzymes: Some enzymes in propolis, such as superoxide dismutase and catalase, have antioxidant properties. They help neutralize harmful free radicals in the body, reducing oxidative stress and protecting cells from damage that can contribute to aging, cancer, and other chronic diseases.

Anti-inflammatory Enzymes: Certain enzymes found in propolis can modulate the production of pro-inflammatory mediators like cytokines and prostaglandins, helping to reduce inflammation. This makes propolis effective in managing inflammatory conditions such as arthritis and other diseases linked to chronic inflammation.

Digestive Enzymes: Enzymes in propolis also support digestion by assisting in the breakdown of food into nutrients the body can absorb. These enzymes help enhance gut health, improving nutrient absorption and overall digestive function.

Antimicrobial Enzymes: Propolis contains enzymes that contribute to its antimicrobial properties by disrupting microbial cell walls or inhibiting microbial growth. These enzymes help protect the body from infections caused by bacteria, fungi, and viruses.

Wound Healing Enzymes: Some enzymes in propolis promote tissue regeneration and wound healing by aiding in the breakdown of dead cells and facilitating the growth of new tissue[29].

1.2.8. Lipids

Lipids in propolis include sterols, fatty acids, and glycerides, which contribute to its biological activity and therapeutic benefits. Examples of lipids found in propolis are palmitic acid, a saturated fatty acid, linoleic acid, an essential polyunsaturated fatty acid, and sterols, which are involved in maintaining cellular structure and function. These lipids help improve the overall stability and efficacy of propolis, enhancing its potential health benefits.

Fatty acids found in propolis play an essential role in its diverse therapeutic potentials, contributing to its therapeutic properties. For instance, fatty acids such as palmitic acid and linoleic acid are known for their anti-inflammatory effects. These compounds help modulate the immune response and reduce the production of pro-inflammatory mediators, making them effective in managing conditions like arthritis and other inflammatory diseases. Additionally, fatty acids exhibit antibacterial and antifungal properties by disrupting the membranes of pathogens, including bacteria and fungi, thereby preventing infection and supporting the antimicrobial action of propolis. Fatty acids also play a crucial role in skin healing, as they promote skin regeneration and aid in wound healing, making propolis a valuable natural remedy for cuts, burns, and other skin injuries. Furthermore, these compounds possess antioxidant properties, helping to reduce oxidative damage to cells caused by free radicals, which is vital for protecting tissues and preventing conditions related to oxidative stress, such as aging and chronic diseases. Together, the therapeutic potentials of fatty acids in propolis enhance its therapeutic potential for inflammation, infection, skin care, and overall cellular protection[29].

1.2.9. Other Unique Compounds

Certain propolis samples may contain unique bioactive compounds that vary depending on their geographic or botanical origin. For instance, tropical propolis often contains benzophenones, which are known for their antioxidant and antimicrobial properties. Brazilian propolis, on the other hand, is rich in artepillin C, a compound with potent anti-inflammatory and anticancer activities. These unique compounds further enhance the therapeutic potential of propolis and contribute to its diverse medicinal properties.

Other Unique Compounds found in propolis contribute to its wide-ranging therapeutic potentials and therapeutic potential. The composition of propolis can vary significantly depending on its geographic or botanical origin, leading to the presence of distinct bioactive compounds that further enhance its medicinal properties.

For example, tropical propolis is often enriched with benzophenones, which are known for their antioxidant and antimicrobial effects. These compounds help protect cells from oxidative stress and enhance the ability of propolis to fight infections caused by bacteria, fungi, and viruses.

In contrast, Brazilian propolis is particularly rich in artepillin C, a bioactive compound with potent anti-inflammatory and anticancer properties. Artepillin C has been shown to reduce inflammation by modulating immune responses and inhibit the growth of cancer cells, making it a promising compound for the treatment of inflammatory diseases and certain types of cancer[30].

These unique compounds, along with others that may be present in propolis depending on its origin, contribute to the diverse range of therapeutic effects of propolis, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and immune-modulating activities. The variety of bioactive compounds further cements propolis as a valuable natural remedy with significant potential for healthcare applications across different regions and medical conditions.

Table 1 summarizes the key therapeutic potentials of various components found in propolis. Each component plays a distinct role in contributing to propolis's therapeutic potential.

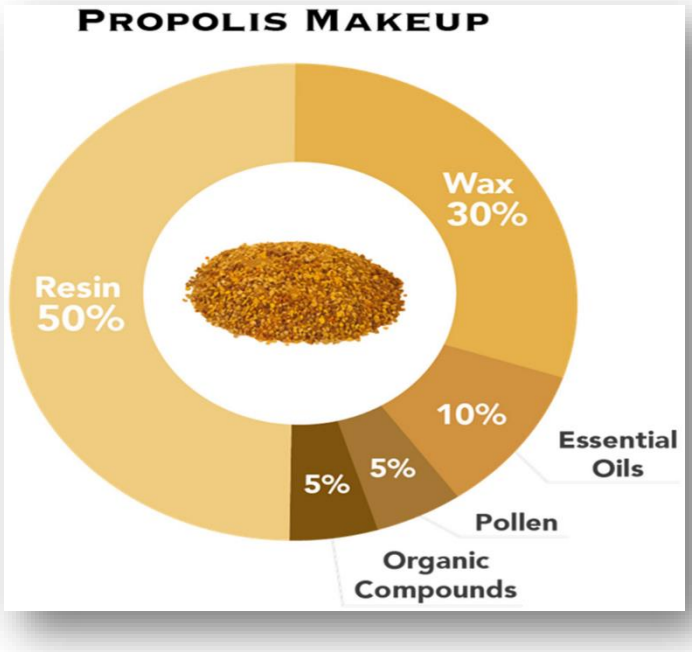
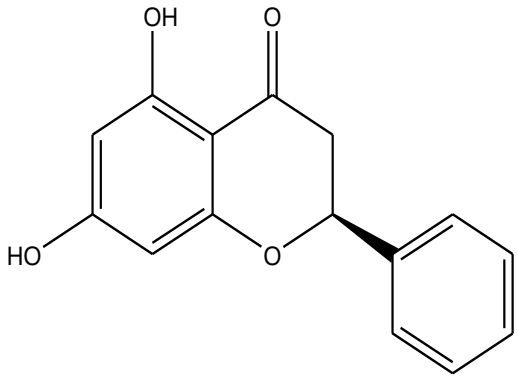


Figure I.1.1. chemical composition of propolis

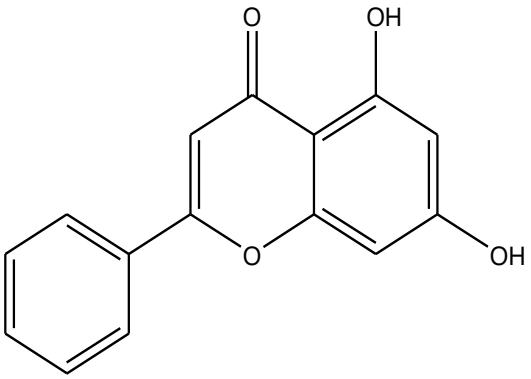
Table I.1.1. Summary of key therapeutic potential

Component	Therapeutic Potential
Flavonoids	Antioxidant, Anti-inflammatory, Antimicrobial, Anticancer
Phenolic Acids	Antioxidant, Antidiabetic, Neuroprotective, Anti-inflammatory
Terpenoids	Antimicrobial, Anti-inflammatory, Analgesic
Vitamins	Immune Support, Antioxidant, Cell Repair
Amino Acids	Wound-Healing, Metabolism Support
Essential Oils	Antimicrobial, Stress Reduction, Pain Relief
Minerals (e.g., Zinc)	Enzyme Cofactors, Immune Support

These diverse therapeutic potentials make propolis a valuable natural product with broad therapeutic applications, particularly in the fields of pharmacology, medicine, and cosmetology.



(a)



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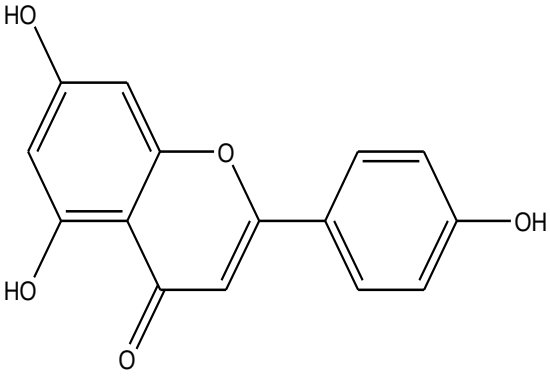

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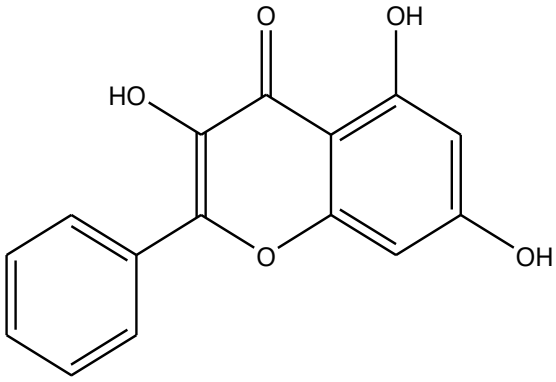

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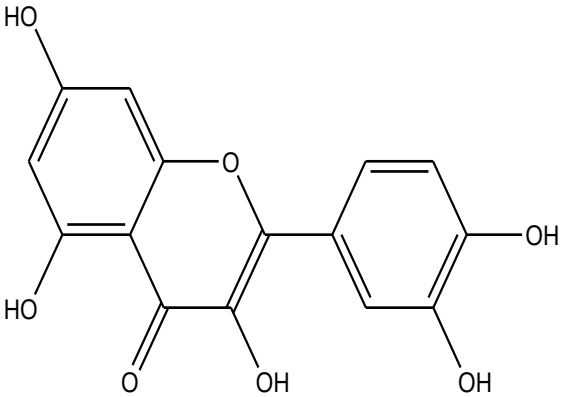

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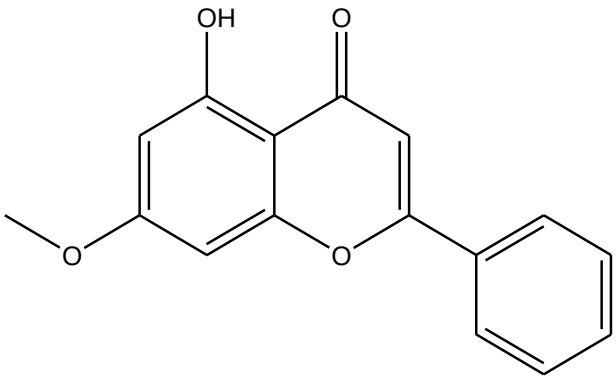


(f)

Figure I.1. 2. Flavonoids found in propolis: (**a**) chrysin; (**b**) pinocembrin; (**c**) apigenin; (**d**) galangin; (**e**) quercetin; (**f**) tectochrysin

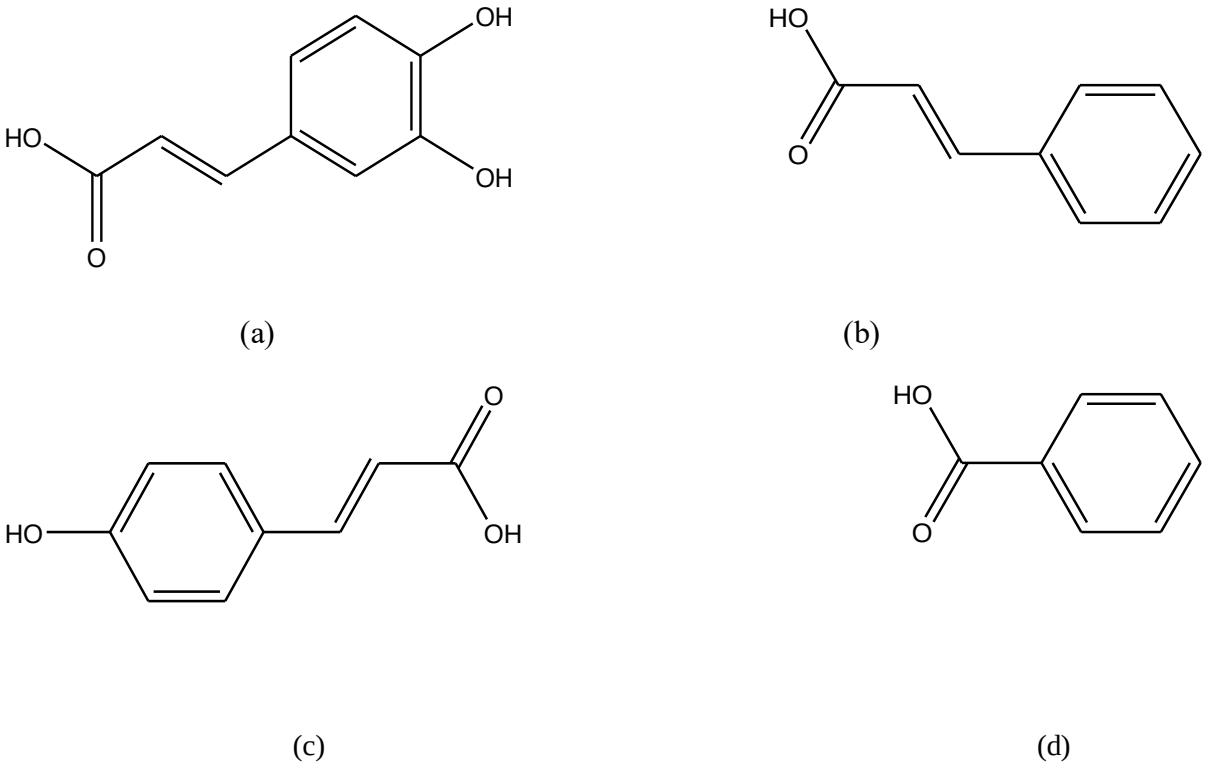


Figure I.1.3. Polyphenols found in propolis: (a) caffeic acid; (b) cinnamic acid; (c) p-coumaric acid; (d) benzoic acid

1.3. Propolis Effects and Therapeutic Properties

Propolis is predominantly constituted of flavonoids, phenols, terpenes, and other chemical components that contribute to its distinct medicinal qualities [31].

1.3.1. Antioxidant Properties

Propolis contains a high concentration of phenolic chemicals and flavonoids, which are potent antioxidants [32]. These chemicals minimize oxidative stress in cells and protect against chronic illnesses such as cancer and cardiovascular disease [33].

1.3.2. Anti-inflammatory Properties

Numerous investigations have shown that propolis has powerful anti-inflammatory properties by blocking certain enzymes and inflammatory mediators such as cytokines and prostaglandins (2). This aids in the treatment of chronic inflammatory conditions such as arthritis and respiratory infections (3).

1.3.3. Antimicrobial Effects

Propolis has significant antibacterial, antiviral, and antifungal activities [34]. Studies have shown that it is efficient against a wide variety of antibiotic-resistant bacterial strains, making it an attractive choice for alternative medicine and natural infection remedies [35]

1.3.4. Immune System Modulation

Propolis has been demonstrated to improve immune function by increasing antibody production and immune cell responses [31]. This makes it beneficial for disease prevention and overall health enhancement [34].

1.3.5. Role in Diabetes Management

According to studies, propolis may help control blood sugar levels by modulating glucose metabolism and boosting insulin sensitivity (3). Its active components are expected to reduce oxidative stress and inflammation, perhaps reducing diabetes complications [35].

Because of these distinguishing characteristics, propolis is regarded as a promising natural component in alternative medicine and nutritional supplements. Ongoing scientific research continues to look into its multiple health benefits (1, 5).

1.4. Previous Studies on Propolis and its Biological Activities

Propolis has been extensively researched for its biological qualities, including anti-inflammatory and anti-diabetic ones. This section provides a summary of the most significant research findings regarding its medicinal potential.

Summary Table of Previous Studies on Propolis

The table below summarizes and evaluates previous studies on the chemical composition of propolis, as well as its various biological effects.

Table I.1.2. Previous studies on propolis

No.	Study Title	Researcher	Year	Key Findings
1	Antidiabetic and Anti-Inflammatory Effects of Propolis	Hernández-Martínez et al.	**2024**	Inhibits α-amylase and α-glucosidase; reduces inflammation [36]
2	Antioxidant and Polyphenolic Profile	Ören et al.	**2024**	Strong antioxidant and anti-inflammatory effects [37]
3	Chemical Composition Analysis of Propolis	SAOUD et al.	**2024**	High content of phenols and flavonoids [38]
4	Antioxidant Activity of Bee Products	Rabii Abdelkarim et al	**2016**	Strong free radical scavenging ability [39]
5	Antimicrobial Effects of Algerian Propolis	Yasmina Mokhtaria Boufadi	**2016**	Strong activity against bacteria and fungi [40]

No.	Study Title	Researcher	Year	Key Findings

The graph shows the tremendous increase in the number of scholarly studies on propolis.

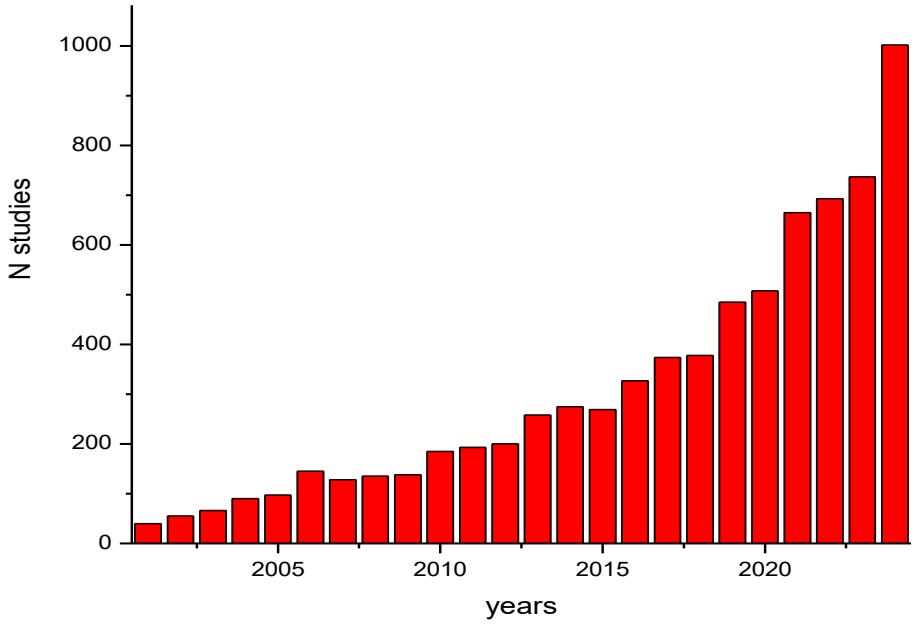


Figure I.1.4. Scientific studies on propolis

According to a recent study, propolis has antioxidant, antibacterial, and anti-inflammatory properties. Because of these characteristics, propolis is regarded as a promising substance for medical and therapeutic applications, paving the way for future research into pharmaceutical products based on it.

Chapter 2

Overview of anti-inflammatory and antidiabetic

Part I: literature review

Propolis has been used as a folk medicine for thousands of years to heal several types of diseases. The present chapter aims to summarize the anti-inflammatory mechanism and antidiabetic activities of propolis in various in vitro and in silico. The anti-inflammatory activity of propolis is attributed to the presence of flavonoids and phenolic acids, which inhibit the release and function of some cytokines and mediators important for inflammation. Several types of in vitro and in silico studies have been conducted and have shown that propolis has anti-inflammatory effects, and this anti-inflammatory effect of propolis is contributed to by its main components like caffeic acid derivative, acetyl-11-keto-β-boswellic acid, and galangin[41]. The antidiabetic properties of propolis may be through the increase of the level of insulin and the decrease of the level of blood glucose. Several clinical and preclinical studies have been carried out and have shown that propolis has antidiabetic effects. Therefore, these anti-inflammatory and antidiabetic activities of propolis should receive higher regard in medicinal treatment[42].

2.1. Inflammation

Inflammation is the complex biological response of affected tissue to harmful stimuli such as physical damage, intermediary metabolism, and infections caused by bacteria, viruses, fungi, or parasites. It is a defense mechanism that is initiated and controlled by tissues to neutralize and eliminate harmful stimuli[43]. The classical symptoms of inflammation are calor, rubor, tumor, and dolor, which mean heat, redness, swelling, and pain in Latin. Recent research illustrated that there are two kinds of inflammation processes: acute inflammation and chronic inflammation, and both inflammation processes result from various types of stimulatory responses from endocrine glands and immune cells. Inflammation classically endorses tissue repair. However, if it exists in the long term, inflammation collaborates with the weakening of bodily functions and is involved in several diseases or medical conditions such as cancer, atherosclerosis, and Alzheimer's disease. Anti-inflammatory agents are often used to prevent or remedy inflammatory diseases[44].

The development of inflammation is usually divided into two categories: local inflammation and systemic inflammation. Local inflammation is a protective response of the tissue towards injury or damage, which occurs within injured tissue or near damaged areas. The process ordinarily involves the release of mediators, which are called inflammatory mediators, such as hormonal substances from cells in the injured area, including platelets, endothelial cells, leukocytes, mast cells, and neurogenic cells5. These mediators lead the nearby blood vessels to increase blood flow to the tissue, with an adjacent enhancement in vascular permeability, which

allows native white blood cells to quickly reach the site of injury. The process is vital to help repair the tissue and assist the defensive response that protects against exogenous pathogens, infectious products, or damaged organic fragments from the surrounding area. Nonetheless, local inflammation can cause unwanted damage. Nonsteroidal anti-inflammatory drugs and steroidal anti-inflammatory drugs are commonly used for their anti-inflammatory effects.

2.1.1. Anti-inflammatory activity

Anti-inflammatory activity refers to the ability of a substance to reduce inflammation[45], a natural response by the body to injury, infection, or harmful stimuli. Inflammation is an essential and protective mechanism in living organisms that helps the body clear infections and repair tissue damage. However, uncontrolled inflammation is a major contributor to diseases such as asthma, rheumatoid arthritis, neurodegenerative conditions, and cancer, among others. Current anti-inflammatory therapies are often associated with side effects due to their activity against general targets. Natural compounds derived from dietary sources can modulate both the activation of inflammatory damage response programs and the resolution of inflammation, and these actions can be implemented to maintain or restore a healthier homeostasis[46]. This subsection highlights the current progress in the identification, preclinical and clinical development, and limitations of bioactive natural anti-inflammatory compounds. Given that the potential beneficial effects of the natural compounds discussed here may be best achieved only when used for the intended purpose, additional in-depth, evidence-based reviews should be explored in order to accelerate further efforts for future development as preventive or therapeutic agents against inflammation and closely related diseases in humans. Inflammation is a natural defense mechanism that is crucial for host defense, as it protects the body from infection and helps repair injured tissue[47]. The cardinal signs of inflammation are redness, swelling, heat, and pain. These signs are a visible expression of the vascular and tissue response to the accumulation of leukocytes in an area of inflammation. When damage occurs, inflammation is classically divided into two major groups: acute and chronic. Most of the current anti-inflammatory drugs have been designed to alleviate one or more of these cardinal signs in an attempt to control the chronic inflammation that occurs in long-term conditions. However, they frequently delay the healing process and might lead to chronic conditions, where the ongoing inflammation poses a risk to the surrounding normal tissue. Moreover, there is evidence that inflammation is a factor for cancer and involves chronic low-grade inflammation in which the cells are unaware of the injury. In an attempt to amplify the autocrine or paracrine production of inflammatory molecules, potential ways to fight chronic inflammation have been developed to allow return to a healthier homeostasis.

2.2. Diabetes

2.2.1. Antidiabetic activity

Antidiabetic activity refers to the ability of a substance, compound, or therapeutic intervention to prevent, manage[48], or alleviate the symptoms and complications associated with diabetes, primarily by regulating blood glucose levels and improving insulin function. Diabetes, particularly type 2 diabetes, is characterized by the body's inability to properly produce or respond to insulin, leading to high levels of glucose in the blood[49]. Antidiabetic activity encompasses several mechanisms by which substances can help regulate blood sugar. These include promoting insulin secretion from the pancreas, enhancing insulin sensitivity in cells, and reducing hepatic glucose production. Additionally, antidiabetic agents may improve the ability of muscle and fat cells to take up glucose from the bloodstream, thereby lowering blood sugar levels[50].

Beyond direct effects on glucose metabolism, many antidiabetic substances also address insulin resistance, a hallmark of type 2 diabetes, by improving the function of insulin receptors on cells. Some compounds may also modulate inflammatory pathways and reduce oxidative stress, both of which are commonly associated with the onset and progression of diabetes. Antidiabetic activity can also extend to improving lipid profiles, lowering blood pressure, and preventing complications such as cardiovascular diseases, kidney damage, and nerve damage, which are common in individuals with poorly controlled diabetes[51].

Natural compounds found in plants, such as flavonoids, phenolic acids, and terpenoids, as well as other bioactive substances like those in propolis, have shown significant antidiabetic activity in various studies[52]. These compounds can act through multiple pathways, including enhancing glucose uptake, increasing insulin secretion, modulating enzymes involved in glucose metabolism, and reducing chronic inflammation. Ultimately, the antidiabetic activity of various substances plays a crucial role in managing blood sugar levels, improving metabolic control, and reducing the risk of long-term complications associated with diabetes[53].

2.3. Assessment of anti-inflammatory activity

2.3.1. In vitro assessment

Cell Culture Models: These studies involve exposing cultured cells, such as macrophages, monocytes, or fibroblasts, to pro-inflammatory stimuli (e.g., lipopolysaccharides, cytokines) and then testing the ability of the substance to modulate inflammation. Common endpoints

measured include the production of pro-inflammatory cytokines (e.g., TNF-α, IL-6), prostaglandins, and nitric oxid [54].

Enzyme Inhibition Assays: The inhibition of key enzymes involved in the inflammatory process, such as cyclooxygenase-2 (COX-2) or lipoxygenase (LOX), can be assessed. These enzymes play critical roles in the synthesis of inflammatory mediators like prostaglandins and leukotrienes[55].

NF-kB Pathway Assessment: The NF-kB pathway is a major regulator of inflammation. Methods such as Western blotting, ELISA, or luciferase reporter assays are used to evaluate whether a compound can inhibit the activation of NF-kB and the subsequent expression of pro-inflammatory genes[56].

2.3.2. In silico assessment

Assessment of anti-inflammatory activity in Silico refers to the use of computational methods to predict, model, and simulate the anti-inflammatory effects of compounds before they are tested experimentally. These in silico approaches are valuable tools for screening large numbers of compounds, understanding their mechanisms of action, and optimizing their structures for enhanced activity[57]. Common in silico methods used to assess anti-inflammatory activity include:

Molecular Docking Studies

Molecular docking is a widely used computational technique to predict how a compound (ligand) interacts with a target protein (receptor) involved in the inflammatory process. By simulating the binding of a compound to enzymes or receptors such as COX-2, TNF-α, or NF-kB, researchers can evaluate whether a compound might inhibit the activity of these key inflammatory mediators. The docking results provide insights into the binding affinity and potential interaction sites between the compound and the target, which can be used to assess anti-inflammatory potential[58].

Molecular Dynamics Simulations

After molecular docking, molecular dynamics simulations[59] can be used to further explore the stability of the compound-receptor complex over time. This technique simulates the movements of atoms and molecules in a given system, helping researchers observe the interactions and stability of the ligand-receptor binding in a dynamic environment. Molecular dynamics simulations provide detailed insights into the conformational changes of proteins and their interactions with compounds, which is useful in understanding how a compound might modulate inflammatory pathways.

Quantitative Structure-Activity Relationship (QSAR) Models

QSAR modeling is a computational method used to predict the anti-inflammatory activity of compounds based on their chemical structure. By analyzing the relationship between the molecular properties (such as hydrophobicity, charge distribution, and functional groups) and their observed biological activity[60], QSAR models can predict the potential efficacy of novel compounds. These models help in designing new anti-inflammatory agents with optimized chemical features for greater potency and specificity.

Virtual Screening

Virtual screening is the process of computationally testing a large library of compounds to identify those that have the highest potential for binding to specific inflammatory target proteins. By screening databases of existing compounds or newly synthesized molecules, researchers can prioritize candidates for further in vitro or in vivo testing. Virtual screening allows for a more efficient search for potential anti-inflammatory agents by narrowing down the list of compounds to those with the greatest likelihood of success[61].

Binding Free Energy Calculations

After identifying potential inhibitors through docking and virtual screening, binding free energy calculations can be used to estimate the strength and stability of the interaction between a compound and its target protein. Methods like MM-PBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) or MM-GBSA (Molecular Mechanics Generalized Born Surface Area) can predict the binding affinity, which correlates with the compound's potential to inhibit the target's activity in a biological system. Higher binding affinity generally indicates a more potent anti-inflammatory effect[62].

Target Prediction and Pathway Analysis

Target prediction tools like STITCH, DrugBank, or Tox21 are used to identify potential protein targets of a compound. By mapping these targets to known inflammatory pathways (e.g., NF-kB signaling, prostaglandin synthesis, cytokine production), researchers can predict how a compound might modulate various components of the inflammatory process. This allows for a better understanding of the compound's mechanism of action.

Pharmacokinetic and Toxicity Predictions

In silico methods can also be used to predict the pharmacokinetics (ADME: absorption, distribution, metabolism, and excretion) and toxicity of anti-inflammatory compounds. Tools such as Lipinski's Rule of Five can help assess the drug-likeness of a compound, while **toxicity** prediction models assess the safety profile of compounds, ensuring that the identified anti-inflammatory agents are not only effective but also safe for use.

These in silico methods significantly accelerate the drug discovery process, reduce the costs of experimental testing, and provide valuable insights into the potential anti-inflammatory effects of novel compounds. By simulating and predicting how compounds interact with targets involved in inflammation, these techniques play an essential role in identifying and optimizing candidates for further experimental and clinical testing[63].

2.4. Assessment of antidiabetic activity

In silico assessment of antidiabetic activity involves the use of computational tools and methods to predict and evaluate the potential antidiabetic effects of compounds before conducting experimental trials[64]. This approach leverages various molecular modeling techniques to understand the mechanisms by which compounds can modulate diabetes-related targets, such as insulin receptors, glucose transporters, enzymes involved in glucose metabolism, and inflammatory pathways. Below is the key in silico methods used for assessing antidiabetic activity:

Molecular Docking Studies

Molecular docking is a powerful computational technique used to simulate the binding of small molecules (ligands) to specific protein targets involved in diabetes regulation. For antidiabetic activity, docking can be performed against key enzymes or receptors such as insulin receptors, glucagon-like peptide-1 (GLP-1) receptors, alpha-glucosidase, and DPP-4 (dipeptidyl peptidase-4) inhibitors. By simulating how well a compound binds to these targets, researchers can predict whether a compound may act as an insulin sensitizer, stimulate insulin secretion, or inhibit glucose absorption[64]. The binding affinity and the interaction energies derived from docking studies can indicate the likelihood of a compound's efficacy in controlling blood glucose levels.

Molecular Dynamics (MD) Simulations

MD simulations provide more detailed insights into the dynamics of the ligand-receptor interactions obtained from docking studies. After docking, MD simulations can help evaluate the stability of the complex over time by simulating the movements of atoms and molecules. This helps assess whether the ligand-receptor interaction remains stable and if the compound can effectively influence the diabetes-related protein's activity. This method is particularly useful for evaluating insulin receptor and GLP-1 receptor binding, which plays a role in insulin secretion and glucose homeostasis[65].

Quantitative Structure-Activity Relationship (QSAR) Modeling

QSAR modeling is a computational technique used to predict the biological activity of compounds based on their chemical structure. By analyzing the relationship between the structural features of compounds and their observed antidiabetic activity, QSAR models can predict the effectiveness of new compounds[66]. QSAR models can be used to optimize the chemical structure of molecules for better glucose-lowering effects, insulin sensitivity, or enhanced inhibition of enzymes like alpha-glucosidase. These models can assist in designing molecules that maximize antidiabetic activity while minimizing undesirable side effects.

Virtual Screening

Virtual screening involves the computational analysis of large compound libraries to identify potential antidiabetic agents by simulating their interactions with known diabetes-related targets. The compounds that exhibit the best binding affinities to these targets can be prioritized for further in vitro or in vivo testing. For example, virtual screening can be performed against the glucagon receptor, insulin receptor, or SGLT2 (sodium-glucose co-transporter 2) to identify molecules that may block glucose reabsorption in the kidneys or enhance insulin action[67].

Target Prediction and Pathway Analysis

Target prediction tools (e.g., STITCH, Tox21, DrugBank) can help identify potential diabetes-related protein targets for a given compound. Once a target is identified, pathway analysis tools can map out the signaling pathways involved in glucose metabolism, insulin secretion, and other metabolic processes. This analysis helps determine whether a compound is likely to influence important pathways like insulin signaling, glucose uptake, or lipid metabolism. Understanding these pathways allows for a comprehensive evaluation of a compound's potential antidiabetic mechanisms[68].

Binding Free Energy Calculations

After identifying promising compounds through docking and virtual screening, binding free energy calculations (e.g., MM-PBSA or MM-GBSA) are used to estimate the binding affinity of a compound to its target protein. This helps predict the strength and stability of the compound-target interaction, which correlates with the potential for inhibiting the target protein's activity(29). Compounds with high binding affinity to key targets involved in glucose metabolism are more likely to exhibit strong antidiabetic activity.

Pharmacokinetic (ADME) Predictions

ADME (Absorption, Distribution, Metabolism, and Excretion) predictions help assess the drug-likeness of potential antidiabetic compounds. Tools like Lipinski’s Rule of Five or ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) predictions can be used to determine whether the compound has favorable pharmacokinetic properties, ensuring that it is

well absorbed, distributed in the body, metabolized effectively, and excreted safely. This is critical for assessing the likelihood of a compound's success in clinical development.

Toxicity Prediction

Toxicity prediction models, such as Toxtree or Tox21, can help assess the safety profile of potential antidiabetic compounds by predicting their potential to cause adverse effects. These models evaluate factors like genotoxicity, carcinogenicity, and organ toxicity, which are crucial for identifying compounds that can be safely used in the treatment of diabetes[70].

Metabolic Pathway Modeling

Metabolic pathway modeling using computational tools can simulate the complex metabolic processes in the human body, including glucose metabolism, insulin secretion, and lipid metabolism. By incorporating antidiabetic compounds into these models, researchers can predict how the compound might alter glucose homeostasis and influence insulin sensitivity, thus helping to prioritize compounds with the most promising therapeutic effects[71].

Multi-Target Approaches

Diabetes is a multifactorial disease, and multi-target approaches involve screening compounds for activity against multiple targets involved in diabetes regulation. In silico methods can be used to identify compounds that simultaneously affect different aspects of diabetes, such as enhancing insulin sensitivity, inhibiting glucose production, and reducing inflammation. By targeting multiple pathways, these compounds may have synergistic effects, making them more effective in managing diabetes[72].

In silico methods for assessing antidiabetic activity provide a fast, cost-effective, and efficient way to screen large numbers of compounds, optimize lead compounds, and gain a deeper understanding of their mechanisms of action. These computational tools can significantly accelerate the drug discovery process, reducing the need for extensive experimental testing and providing insights into how compounds interact with diabetes-related targets.

PART TWO

EXPREMENTAL

Chapter 1
Materials and Methods

Part II: Experimental

1.1. Chemical reagents and equipment

All chemicals used in this study are of analytical grade and they are as follows:

Ethanol 96% from France,

KH_2PO_4 / K_2HPO_4 buffer solution (pH =6.5),

Folin-Ciocalteu reagent from Biochem chemopharma Co (Canada),

Na_2CO_3 sodium carbonate solution (99%),

Gallic acid 99% from CE EMB

Quercetin 97%

NaOH 97%

HCl (1M)

1.2. Collection and Preparation of Propolis Extracts

1.2.1. Collection of propolis

The propolis samples utilized in this investigation were from *Apis mellifera* hives in Tipaza, northern Algeria, Figure II.1.1 which is situated near the Mediterranean Sea. They were bought from local shops. The climate of the area is Mediterranean, with moderate, rainy winters and scorching, dry summers. Because of the diversified ecosystem this climate fosters, the region is rich in both ecology and history. The proximity of the Mediterranean Sea lowers the temperature, contributing to a relatively humid environment compared to the arid areas of southern Algeria. The region has a combination of Mediterranean scrubland and forests, including species such as junipers, pines, and olive trees. The region is also known for its aromatic and resin-producing plants, such as mastic trees (*Pistacia lentiscus*), which are common in Mediterranean ecosystems. The territory includes rolling hills, fertile plains, and rugged coastal cliffs, providing a scenic and diverse habitat. In addition to mastic trees, plants such as rosemary (*Rosmarinus officinalis*), Aleppo pine (*Pinus halepensis*) and other coniferous species thrive in this region. The fertile areas near the coast are home to crops such as olives, vines and citrus fruits, which are an integral part of the local economy[73], [74].

Figure II.1.1. Photograph of the raw propolis sample

1.2.2. Preparation of propolis

The fresh raw collected propolis was cleaned by rinsing it with cold water to remove contaminants like dirt and bee parts, then was allowed to dry and left in a freezer until become frozen hard solid. Then the dried frozen propolis was ground into small pieces using a mortar and pestle, this increases its surface area which improve the extraction yield, the final product is presented in Figure II.1.2.

Figure II.1.2. Photograph of the prepared propolis sample

1.2.3. Extraction procedure

1.2.3.1. Maceration extraction

The initial mass used for maceration extraction was 5g of cleaned powdered propolis. It was transferred into a 250 milliliters glass conical flask, and 150 mL of a 70:30 ethanol/water

mixture was added to it. The obtained suspension was shaken to ensure that the whole mass of propolis was fully submerged in the extracting solvent, then the flask was sealed with plastic wrap to avoid evaporation and contamination. The flask was then put on a shaker at 50°C for 24 hours in a dark place to allow the solvent to extract the active molecules[75].

After the extraction period, the mixture was filtered, using filter paper, to separate the liquid extract from the solid propolis residue. The residue was subjected to a second round of maceration extraction to maximize the yield of extraction.

The solvent was removed from the extract using a rotary evaporator under reduced pressure, with the temperature bath set to 50°C. This procedure aided to preserve the active molecules while efficiently eliminating the majority of the solvent. The resulting residue was thoroughly dried and then kept in a freezer for 24 hours, finally it was transferred to a freeze dryer to obtain the final product as dried powder. The final powdered product was transferred to an airtight amber glass bottle and stored at low temperatures (4°C)[76], [77].

1.2.3.2. Soxhlet extraction

5 g of the cleaned powdered propolis is put inside a cellulose thimble paper and inserted in the Soxhlet extractor. 250 mL of a 70:30 ethanol/water mixture is poured into the attached round-bottom flask. The Soxhlet apparatus is assembled with the extractor, condenser, and round-bottom flask securely in place. The whole system is then placed over a heating mantle, then the solvent is gently heated until the boiling point (approximately 78°C for ethanol). The extraction cycle is repeated 5 hours until the solvent in the flask becomes clear, indicating that maximum extraction has been achieved. Once the extraction was complete, the solvent mixture was removed, and the resulting residue underwent the same procedure as outlined for the maceration extraction[78].

1.2.3.3. Ultrasonic-assisted extraction

To begin, 5 g of cleaned powdered propolis is placed in a beaker, and 200 mL of a 70:30 ethanol/water mixture is added to fully submerge the propolis. The beaker is then positioned in an ultrasonic bath filled with distilled water. before starting the extraction, the water in the ultrasonic bath is aired by functioning the bath without the propolis sample for ten minutes to remove any air bubbles that could affect the procedure of extraction. Once degassed, the beaker is placed into the bath, and the temperature is set at 40°C. The frequency of the ultrasonic waves of 50 kHz was applied for 40 minutes. At the end of extraction, the continent of the beaker was filtered to isolated the extract from the solid remainder. The obtained extract solution is then evaporated using rotary evaporation to remove the solvent, leaving behind the polyphenol-rich

residue, which underwent the same procedure as outlined for the maceration and Soxhlet extraction[79].

1.2.3.4. Ultrasonic-assisted extraction combined with maceration

Ultrasonic-assisted extraction combined with maceration (UAE-M) is an efficient hybrid technique that enhances the extraction of bioactive compounds by integrating ultrasound-induced cavitation with passive solvent diffusion. In the UAE stage, high-frequency ultrasound waves (20–100 kHz) generate microscopic bubbles that collapse violently, breaking sample cell walls and improving solvent penetration. This procedure shortens the extraction time and uses less solvent while facilitating the quick release of intracellular chemicals. After UAE, maceration ensures maximal chemical recovery by allowing bioactive compounds to continue diffusing into the solvent over time. By combining the extended solvent contact of maceration with the mechanical disruption of ultrasound, this combination increases the yields of phenolics, flavonoids, and other important phytochemicals while maintaining their structural integrity[80].

1.3. Screening test for phenolic and flavonoid compounds

Screening test for phenolic and flavonoid compounds are qualitative screening tests and are defined as tests that result in a "yes" or "no" response at a concentration of phenolic and flavonoid compounds present in a test sample. These tests are used as an initial screen for the presence of phenolic and flavonoid compounds in a sample. they are very simple to perform and require a minimum of laboratory equipment or specially trained personnel. Screening for phenolic and flavonoid compounds tests detect the presence of phenolics and flavonoids based on color changes or precipitation reactions[81], [82].

1.3.1. Ferric chloride test for phenolic compounds

Ferric chloride test is usually used to detect the presence of hydroxyls groups attached to aromatic rings (phenolic hydroxyls) in a chemical sample; the test is based on the ability of the phenolic hydroxyls to form colored complexes with ferric cations (Fe^{3+}). These complexes show distinguishing colors like red, purple, green, or blue depending on the type of structure of the phenolic compound. The test is performed by addition of a few drops of a brownish-yellow solution of ferric chloride ($FeCl_3$) to a test tube containing the test solution[83], [84].

- Blue or green shades typically indicate the presence of monophenolic structures (e.g., simple phenolic acids).
- Purple to black hues suggest polyphenolic systems (e.g., tannins or highly conjugated flavonoids like quercetin), which form more stable, multi-dentate complexes with Fe^{3+}.

1.3.2. Sodium hydroxide test

Sodium hydroxide test is based on the aptitude of flavonoids to form colored complexes in a basic solution due to their phenolic structure. It is a qualitative test used to detect the presence of flavonoids in plant extracts or chemical samples.

When a sodium hydroxide solution is added to a sample containing flavonoid, the latter get deprotonated, forming a flavonoid anion that exhibits a color change. The color varies depending on the flavonoid subclass (flavones, flavonols, flavanones, etc.). When acid (HCl or acetic acid) is added, the color disappears or fades, confirming the presence of flavonoids.

1.3.3. Electrochemical test for phenolic and flavonoid compounds

Electrochemical techniques involve the study of electroactive species through competition of species for an electron. Electrochemical methods are useful tools in the investigation of phenolic and flavonoid compounds, since they offer fast, cost-effective, and, in many cases, simple means for this purpose.

The technique is based on cyclic voltammetry which record the anodic current produced by the electroactive compounds which are oxidized on the surface of the working electrode, while the potential of a working electrode is linearly scanned from an initial value to a final value and back [62]. The current produced is proportional to the combined concentration of the electroactive compounds [63,64]. The current produced is plotted versus the applied potential to give a cyclic voltammogram. Cyclic voltammograms of propolis extract and quercetin in a phosphate buffer solution at pH 6.5 were recorded using a three-electrode electrochemical cell with a total capacity of 25 mL. The working electrode consisted of a glassy carbon disk electrode with an active surface area of 0.07 cm^2, while a platinum wire served as the counter electrode. A saturated calomel electrode (SCE) was used as the reference electrode to ensure stable and reproducible potential measurements. The applied potential window varied depending on the type of compounds analyzed: for polyphenolic compounds, the potential ranged from 0 to 1400 mV, whereas for flavonoid compounds, the scan was performed within a -300 to 400 mV window. This selection of potential ranges ensures the effective detection of oxidation and reduction processes characteristic of each class of compounds.

1.3.3.1. Electrochemical test for phenolic compounds

The cyclic voltammogram of a 1.0 mg/mL phosphate buffered solution of gallic acid at pH = 6.5 on a glassy carbon working electrode at potential scan rate of 100 mV/s, is illustrated in Figure II.1.3. The voltammogram exhibits a well-defined anodic peak potential at 553.08 mV, with a corresponding peak current density of 74.36 $\mu A/cm^2$. Samples containing polyphenolic compounds is expected to exhibit a similar voltametric profile, characterized by a well-defined anodic peak around 553 mV. This anodic peak potential remains independent of the sample

concentration. However, the anodic peak current density is directly proportional to the concentration of the polyphenolic compounds in the sample, indicating a quantitative relationship between the electrochemical response and analyte concentration.

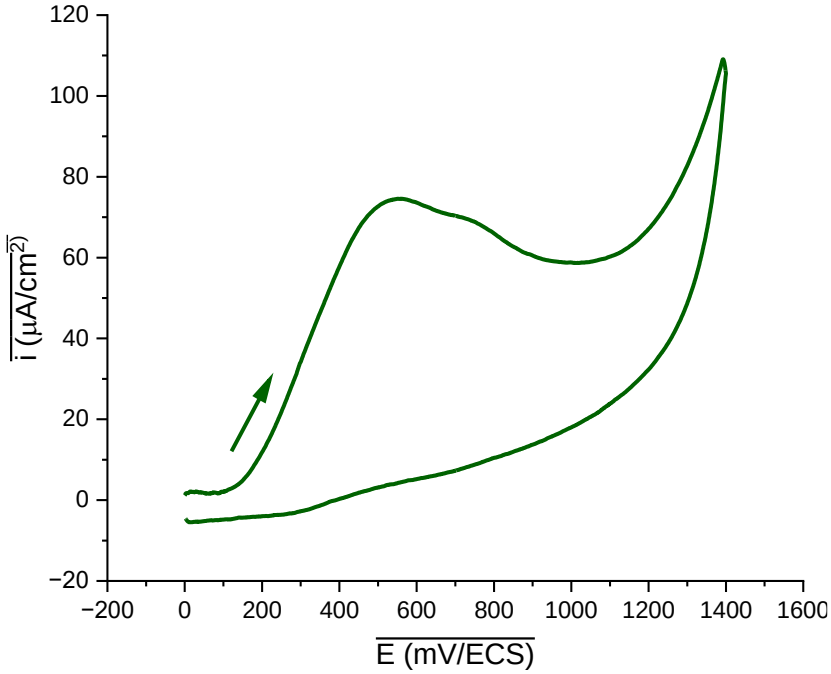


Figure II.1.3. Cyclic voltammogram of gallic acid (1.0 mg/mL), in phosphate buffer solution at pH = 6.5 on a glassy carbon working electrode at potential scan rate equal 100 mV/s

1.3.3.2. Electrochemical test for flavonoid compounds

The cyclic electrochemical behavior of quercetin (1.0 mg/mL) in a phosphate buffer solution at pH 6.5, recorded using a glassy carbon working electrode at a scan rate of 100 mV/s, is presented in Figure II.1.4. The voltammogram reveals a well-defined anodic peak at 158.7 mV, with a corresponding peak current density of 4.90 µA/cm². Additionally, a cathodic peak appears at 52.26 mV, with a peak current density of -4.56 µA/cm². These values yield a half-wave potential of 105.48 mV and an anodic-to-cathodic peak current ratio of 1.07, which is close to the theoretical value of 1, indicating a reversible electrochemical process.

Any sample containing electroactive flavonoid compounds similar to quercetin is expected to exhibit a comparable voltametric profile, characterized by distinct anodic and cathodic peaks within the same potential range. While the anodic peak potential remains independent of concentration, the anodic peak current density varies proportionally with the analyte concentration, making cyclic voltammetry a valuable tool for quantitative analysis.

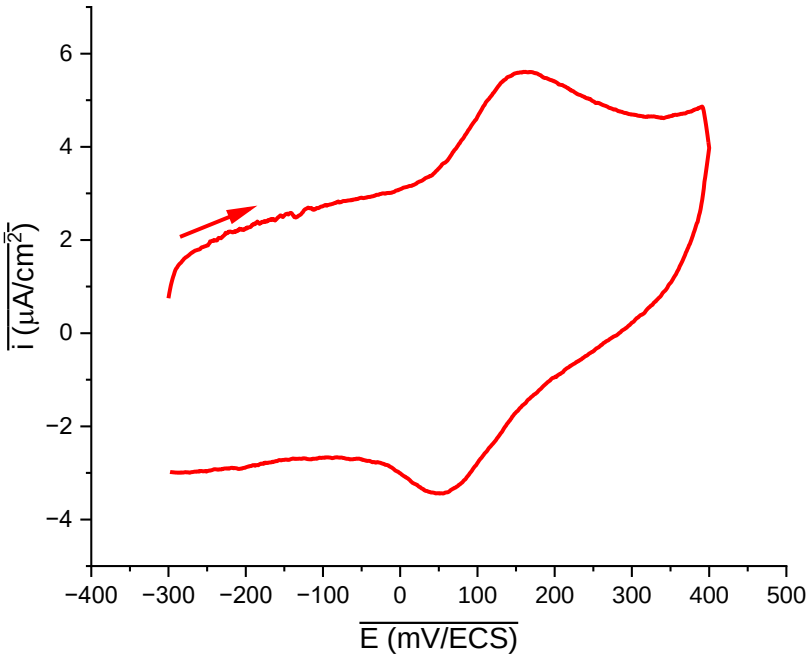


Figure II.1.4. Cyclic voltammogram of quercetin (0.1 mg/mL), in phosphate buffer solution at pH = 6.5 on a glassy carbon working electrode at potential scan rate equal 100 mV/s

1.4. Determination of total flavonoid and phenolic content

1.4.1. Determination of Polyphenol Content Using Folin-Ciocalteu Assay

The reagents for this test are prepared as follows. A donated volume of the Folin-Ciocalteu reagent is diluted ten times with distilled water before use. This reagent is essential for reacting with the phenolic compounds present in the samples. A 20% sodium carbonate solution is prepared by dissolving 20 g of sodium carbonate in 100 mL of distilled water.

A standard curve is obtained to quantify the polyphenol content in the sample. A stock solution of a reference phenolic compounds example gallic acid is prepared at a concentration of 1 mg/mL in distilled water. Starting from this stock solution, a series of dilutions is made to obtain standards with different concentrations, better ranging from 0 to 100 µg/mL. For each standard, 0.5 mL of the gallic acid solution is pipetted into a test tube. 2.5 mL of the diluted Folin-Ciocalteu reagent is added to the test tube, and the reaction is allowed to begin for 5 minutes. After this, 2 mL of the sodium carbonate solution (20% w/v) is added. The obtained mixture is mixed well and then is incubated in the dark at room temperature for at least half an hour. After incubation, the absorbance of the solution is measured at 765 nm. The standard curve is plotted by tracing the absorbance versus the gallic acid concentration (µg/mL), creating a reference for polyphenol content.

For sample analysis, the extract is diluted with distilled water so that its concentration falls within the range of the standard curve. 0.5 mL of the diluted sample is transferred into a test tube and 2.5 mL of the diluted Folin-Ciocalteu reagent is added to it, and the reaction is allowed to continue for at least five minutes. Then, 2.0 mL of sodium carbonate solution is added to the mixture mixed thoroughly. The test tube is then incubated in the dark at room temperature for half an hour. After the incubation, the absorbance of the sample is measured at 765 nm. The polyphenol content of the sample is quantified by comparing the absorbance with the standard curve.

To calculate the Total Polyphenol Content (TPC) of the sample, the concentration of gallic acid equivalents (GAE) is first determined using the standard curve. The absorbance of the sample is compared to the values from the standard curve to find the corresponding concentration of polyphenols in the sample. This concentration is expressed as mg GAE/g of sample using the following formula:

$$TPC(mg\ GAE / g\ sample) = \frac{C(mg/mL) \times V(mL)}{W(g)} \qquad \text{(II.1.1)}$$

Where:

C is the concentration of polyphenols, obtained from the calibration curve, in mg/mL.

V is the total volume of the sample extract used in the assay, in mL.

W is the weight of the sample used for extraction, in g.

This calculation allows for the determination of the TPC in the sample, expressed as milligrams of gallic acid equivalents per gram of the sample.

1.4.2. Determination of the flavonoid content using aluminum chloride assay

The reagents used in this test for flavonoid content determination are prepared as follows: A 2% aluminum chloride alcoholic solution is prepared by dissolving two gramme of aluminum chloride in one hundred milliliters of ethanol. A 1 mg/mL stock solution of quercetin is prepared by dissolving one milligram of quercetin in one milliliter of ethanol. A 5% sodium acetate solution is prepared by dissolving five grams g of sodium acetate in one hundred milliliters of ethanol.

The standard curve is prepared by a series of dilutions made from the quercetin stock solution to obtain standards with different concentrations in the range 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/mL. For each standard, one milliliter of the quercetin solution is pipetted into a test tube and one millimeter of the 2% aluminum chloride solution in ethanol is added to it, followed by the addition of one millimeter of the 5% sodium acetate solution in ethanol. The solution is mixed thoroughly and then incubated for half an hour at room temperature, after

incubation the absorbance of the obtained yellow complex formed between the flavonoids and aluminum chloride is measured at 415 nm. A standard curve is plotted by graphing the absorbance against the quercetin concentration (µg/mL).

For sample analysis, the sample extract is prepared in an appropriate volume of ethanol, the volume of ethanol depends on the expected concentration of flavonoids in the sample. One millimeter of the sample is pipetted into a test tube. Then, one millimeter of the 2% aluminum chloride solution in ethanol is added, followed by one millimeter of the 5% sodium acetate solution in ethanol. The obtained solution is then mixed thoroughly to ensure good interaction of the reagents with the flavonoids. The mixture is incubated for half an hour at room temperature. After the incubation, the absorbance of the sample is measured at 415 nm. The flavonoid content of the sample is quantified by comparing the absorbance with the standard curve.

To calculate the Total Flavonoid Content (TFC) of the sample, the flavonoid concentration is determined from the absorbance by comparing it to the calibration curve. The TFC is expressed as mg QE/g of sample using the following formula:

$$TFC(mg\ QE/g\ sample) = \frac{C(mg/mL) \times V(mL)}{W(g)} \qquad \text{(II.1.2)}$$

Where:

C is the concentration of flavonoids, determined from the calibration curve, in mg/mL.

V is the total volume of the sample extract used for the assay, in mL.

W is the weight of the sample used for extraction, in g.

This formula allows the total flavonoid content to be quantified in terms of milligrams of quercetin equivalents per gram of sample.

1.5. Chromatographic Analysis for Compound Identification (LC-MS)

A chromatographic system consists of a sample injection device, a separation column, a mobile phase delivery system, a detection device, and a data display or analysis system. The mobile phase flows through a column in which the separation takes place and is separated based on differences in chemical and physical properties. The flowing mobile phase is a solvent mixture and includes gaseous solvents, such as in gas chromatography, and liquid solvents, such as in liquid chromatography. The solvent system used in liquid chromatography is called the mobile phase, and the solvent system used in gas chromatography is called the carrier gas. As the mobile phase moves through the column, the individual solutes that comprise the injected sample travel through the column at different rates.

The chromatographic process includes retention time, peak shape, and peak area. Retention time is the time it takes a compound to travel from the sample injection valve to the detection system. The quality of a separation is frequently evaluated by determining plate numbers and peak symmetry. Plate numbers of a column are used to evaluate the performance of a separating column. The smaller the plate number, the poorer the column packing, and the broader the peaks. The larger the plate number, the better the column packing, and the narrower the peaks. The identification of molecules present in the propolis extract is achieved by using liquid chromatography-mass spectrometry (LC-MS). Chromatographic analysis for compound identification allows the detection and identification of target compounds from complex mixtures. Massive amounts of data with corresponding irrelevant and complex information make sensitive and automated data processing and evaluation mandatory. The analysis is performed as follows: Firstly, a small quantity of the propolis extract is prepared for analysis by diluting it with methanol, a reverse-phase C18 column, is employed, and a gradient elution with a mobile phase consisting of a mixture of Solvent A (water with 0.1% formic acid) and Solvent B (methanol) is used to achieve optimal separation.

The resulting data can also be used to quantify the concentration of the identified compounds, which is crucial for understanding the potential bioactivity and therapeutic properties of propolis.

1.6. Anti-inflammatory Activity Evaluation

Denaturation refers to the loss of native shape in proteins due to changes in pH, temperature, and solubility. The main causes of denaturation are the application of heat, strong acids, bases, high concentrations of inorganic salts, and non-ionic detergents. The assay of denatured products under test conditions is called the denaturation assay. Since denaturation frequently involves the unfolding process, the physicochemical characteristics of proteins currently serve the ultimate purpose of describing the unfolding. Nowadays, there are methods available to describe the unfolding; some of them include colorimetry, spectroscopy, and differential scanning calorimetry. Colorimetric methods are frequently used in denaturation assays. The number of different measures depends on the specific denaturation environment. Ultraviolet and visible absorption spectra may provide methods for the determination of proteins and peptides exposed to different stresses. Denaturation of proteins under certain conditions can be monitored not only by color change but also by changes in secondary structures, disulfide compounds breaking, or uncoiling within protein fragments. Generally, these methods must be verified in parallel with reference methods.

1.6.1. In vitro assays

Protein denaturation assay was used to evaluate the anti-inflammatory activity according to the method described by Ashwini et al. [85]. The reaction mixture (5 mL) consisted of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL of propolis extract, and the mixture was mixed, and was incubated in a water bath (37 °C) for 15 min, and then the reaction mixture was heated at 70 °C for 5 min. After cooling, the turbidity was measured at 660 nm. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula,

$$\%\ \text{inhibition of denaturation} = \left(\frac{A - A_0}{A}\right) \times 100 \qquad \text{II.1.3}$$

where A is the absorption of the test sample, and A_0 is the absorption of the control sample

1.6.2. In silico assays

The purpose of the in silico assays is to assess the potential of propolis extract to bind robustly to a bimolecular target that affects the inflammatory response. One of the advantages of running in silico assays is the possibility of predicting the binding energy, the binding constant, reacting residues and the types of interactions.

The macromolecule targets used in this study are BSA (PDB ID: 6QS9) and cyclooxygenase-2 (PDB ID: 1PXX) retrieved from www.rcsb.org. The macromolecules are then prepared using Autodock Tools suits software [86]. The molecular structure of the ligands used were optimized using Gaussian 12 software.

Table I.1.3 Docking parameters are given in details in the results and discussion section

	Center coordinates			**Box size**		
	X	**Y**	**Z**	**X**	**Y**	**Z**
6QS9	-11.134	-8.424	14.203	46	31	24
1PXX	27.131	24.348	14.747	42	42	42

1.7. Antidiabetic Activity Evaluation

There are several in vitro, in vivo, and in silico methods used to evaluate antidiabetic activity of potentially antidiabetic compounds among which is the in vitro assays which is laboratory-based assays**.** These assays assess the potential of compounds to inhibit key enzymes involved in diabetes or to enhance glucose uptake. The in vivo assays which are animal-based assays, these assays involve testing potential antidiabetic agents in animal models to evaluate their effects on blood glucose levels and insulin sensitivity. The in silico assays (Computational-based assays) are assays use molecular modeling to predict interactions between bioactive compounds and diabetes-related targets.

1.7.1. In vitro assays

The α-amylase inhibition assay was used to evaluate the antidiabetic activity of propolis extract according to the method described by Ashwini et al. The reaction mixture (5 mL) consisted of 0.2 mL of α-amylase enzyme solution (1% w/v), 4.78 mL of phosphate-buffered saline (PBS, pH 6.4), and 0.02 mL of propolis extract. The mixture was thoroughly mixed and incubated in a water bath at 37 °C for 15 min. After incubation, 1 mL of starch solution (1% w/v) was added, and the reaction was further incubated at 37 °C for 5 min. The reaction was then terminated by adding 1 mL of dinitrosalicylic acid (DNS) reagent, followed by heating at 100 °C for 5 min. After cooling, the absorbance was measured at 540 nm. Phosphate-buffered solution without the test sample served as the control.

The percentage inhibition of α-amylase activity was calculated using the following formula:

$$\%\ \text{inhibition of}\ \alpha-\text{amylase} = \left(\frac{A - A_0}{A}\right) \times 100 \qquad \text{II.1.4}$$

where A is the absorbance of the test sample, and A_0 is the absorbance of the control sample.

1.7.2. In silico assays

The in silico assay was performed to evaluate the antidiabetic potential of propolis bioactive compounds using the method described by Ashwini et al. The three-dimensional structure of target proteins associated with diabetes, such as α-amylase (PDB ID: 2QV4) and α-glucosidase (PDB ID: 3A4A) were retrieved from the protein data bank (www.rcsb.org). The 3D structures of active compounds from propolis were fully optimized by gaussian 12 software using energy minimization techniques.

Molecular docking simulations were performed using AutoDock Vina, where the ligand-protein binding interactions were evaluated based on binding energy and tupe of interactions. The docking site was defined based on the active site residues of each enzyme. The docking results were analyzed using PyMOL.

Table I.1.4 Docking parameters are given in details in the results and discussion section

	Center coordinates			**Box size**		
	X	**Y**	**Z**	**X**	**Y**	**Z**
2QV4	12.942	47.169	26.200	50	40	40
3A4A	8.652	15.386	40.189	52	42	42

1.8. Molecular Dynamics Simulations

Molecular dynamics simulations were carried out using the GROMACS 2024 software package to assess the stability of protein–ligand complexes over a 100-nanosecond timescale. Ligand topology files were generated via the SwissParam server, ensuring appropriate structural

definitions and force field parameters [87]. The CHARMM36 all-atom force field was applied to the protein structures for accurate representation of enzymatic features [88] .

Each complex was placed in a cubic simulation box filled with TIP3P water molecules, and counterions (Na^+ and Cl^-) were added to neutralize the system, mimicking physiological ionic conditions [89]. Energy minimization was performed using the steepest descent algorithm until the system's maximum force was reduced below 10.0 kJ/mol, ensuring stability before equilibration [90].

System equilibration consisted of two stages: an initial NVT ensemble run at 300 K for 100 ps using the velocity-rescale thermostat (coupling constant: 0.1 ps) to stabilize temperature, followed by NPT

equilibration for another 100 ps using the Berendsen barostat (coupling constant: 2.0 ps) to stabilize pressure [91].

The dynamic behavior and structural integrity of the complexes were evaluated using several key parameters, including root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and solvent-accessible surface area (SASA). These metrics provided detailed insights into the molecular interactions and conformational stability of the complexes throughout the simulation period.

Chapter 2
Results and Discussion

Part II: Experimental

II.1. Yield of extraction

The yield of extraction, a critical parameter for evaluating the efficacy of the extraction procedure, is calculated using equation II.2.1, this represents the yield percentage of an extraction process, commonly used to evaluate the efficiency of extracting bioactive compounds from raw materials such as propolis.

$$\text{Yield}(\%) = \frac{W_{\text{extracted}}}{W_{\text{sample}}} \times 100 \qquad \text{II.2.1}$$

Where $W_{extracted}$ represent the dry weight of the extract in grams, and W_{sample} donates the initial dry weight of the sample in grams, which is five grams in this work. Thus, equation II.2.1 provides the yield representing the constituents extracted relative to the starting material.

Here, $W_{extracted}$ represents the dry weight of the extracted constituents in grams, obtained after solvent evaporation and subsequent drying of the obtained extract, while W_{sample} denotes the initial dry weight of the raw propolis sample in grams prior to extraction. In this work, the value of W_{sample} was standardized at five grams for all experimental trials to ensure consistency and comparability across replicates. This equation quantifies the percentage of bioactive compounds successfully isolated from the starting material (dry raw propolis), thus reflecting the efficiency of the extraction procedure under the applied conditions (e.g., solvent type, temperature, and duration).

The results of the extraction yield calculations, as tabulated in Table II.2.1, reveal important differences in efficacy depending on the extraction technique employed. For example, both UAE and UAE-ME-2h or UAE-ME-24h demonstrated superior yields compared to ME and SE, likely due to their enhanced aptitude to dissolve a wider range of propolis constituents, including polar phenolic acids and nonpolar terpenoids. The results accentuate the importance of extraction technique selection in maximizing the retrieval of target compounds while minimizing the loss of valuable phytochemicals. The obtained yields, ranging from 19.58% to 28%, align with literature reports for propolis extracts and highlight the material's complex matrix, which necessitates optimized protocols to achieve both high yield and compositional fidelity[92], [93].

Table II.2.1. Extraction yield of propolis constituents

Extraction method	$W_{extracted}$ (g)	Yield (%)
ME	0.98	19.58
SE	1.30	26.09
UAE	1.10	22.04
UAE-ME-2h	1.35	27.03
UAE-ME-24h	1.40	28

Recent studies have explored various extraction methods to optimize the yield of bioactive compounds from propolis. A preliminary study compared UAE-ME and ME. The findings indicated that UAE achieved the highest extraction yield of phenolics and flavonoids, significantly reducing extraction time compared to ME. Notably, extending sonication beyond 30 minutes did not substantially increase yields, suggesting this duration is sufficient for effective extraction [92].

Another comprehensive review evaluated classical and modern extraction techniques, including ME, SE, UAE, and UAE-ME extraction methods. The authors concluded that UAE offers an optimal balance between extraction time and yield. Regarding solvents, mixtures of water and ethanol were found to be the most effective for extracting a wide range of bioactive compounds from propolis[93].

2.2. Qualitative Analysis of Flavonoid and Phenolic Constituents

The qualitative tests of flavonoid and phenolic contents of propolis extract were performed to identify the existence and variety of these bioactive phytochemicals, which are integral to propolis's anti-inflammatory and antidiabetic properties. Following the extraction procedure, a suite of established phytochemical screening assays and electrochemical techniques were employed to detect and characterize the flavonoid and phenolic profiles, adhering to standardized protocols adapted from the methodology detailed by [94], [95], [96], [97].

Initial qualitative screening for phenolic compounds was performed using the ferric chloride test, wherein the formation of a purple coloration upon reaction with the extract indicated the presence of hydroxylated aromatic rings characteristic of phenolics. Similarly, flavonoids were preliminarily identified via the aluminum chloride test, where the development of a yellow coloration under ultraviolet light confirmed flavonoid glycosides or aglycones. These colorimetric assays were complemented by electrochemical assays.

2.2.1. Screening test for phenolic compounds

To confirm the presence of phenolic compounds in the aqueous ethanol propolis extract, a standardized ferric chloride colorimetric assay was performed, following established protocols for phytochemical screening. The procedure was conducted as follows:

Approximately 2 mL of 70% aqueous ethanol propolis extract (prepared as outlined in Section 1.3.1) was transferred into a clean, dry glass test tube using a calibrated micropipette to ensure volumetric precision. The extract, which contained solubilized phenolic acids, flavonoids, and other polar constituents, was then treated with 2–3 drops of freshly prepared 5% (w/v) aqueous ferric chloride solution, added dropwise via a disposable Pasteur pipette to avoid cross-contamination. The mixture was gently agitated by swirling the test tube in a circular motion for 10–15 seconds to ensure homogeneous interaction between the ferric chloride reagent and the extract, while avoiding vigorous shaking that could introduce air bubbles or splashing[98], [99].

Within 1–2 minutes of reagent addition, a distinct purple, coloration developed in the reaction medium. This chromatic shift arises from the formation of coordinate complexes between ferric ions and the deprotonated hydroxyl groups of phenolic compounds and flavonoid. The positive result from this test confirms the abundance of phenolic derivatives in the propolis extract, aligning with prior literature on the phytochemical profile of propolis. Phenolic compounds are critical to the biological activities of propolis, including its antioxidant, antimicrobial, and anti-inflammatory properties. The observed reaction not only validates the efficacy of the extraction protocol in isolating these bioactive constituents but also provides preliminary insight into the complexity of the phenolic pool[99].

Figure II.2.1. Ferric chloride test for phenolic compounds in propolis extract: (left) before reagent addition, (right) after reagent addition showing a positive color change

2.2.2. Screening test for flavonoid compounds

The sodium hydroxide alkalinity test was performed to verify the presence of flavonoid compounds in the aqueous ethanol propolis extract, leveraging the pH-dependent chromogenic behavior of flavonoid. This test capitalizes on structural rearrangements in flavonoids under alkaline conditions, leading to distinct colorimetric responses. The procedure was executed as follows: 2 mL of 70% aqueous ethanol propolis extract was pipetted into a clean dry test tube and was allowed to equilibrate to room temperature to avoid temperature-induced artifacts in color development. Then 1 mL of 10% (w/v) aqueous sodium hydroxide solution was added dropwise to the extract, with gentle swirling to ensure thorough mixing. The reaction mixture was observed immediately for instantaneous color changes (yellow) upon alkalization. After 2 minutes, the mixture was neutralized by adding 1 mL of 10% (v/v) hydrochloric acid dropwise. The solution was again observed for reversibility of the color change, a key diagnostic feature for flavonoids[100].

The results presented in Table II.2.2 demonstrate that the aqueous ethanol propolis extract contains abundant phenolic and flavonoid compounds, as evidenced by the "strongly positive" outcomes in both qualitative assays. These findings align with the chromogenic responses observed during the ferric chloride test for phenolics and the NaOH tests for flavonoids (Sections 2.2.1 and –2.2.2), where intense colorimetric shifts confirmed the high concentration of these bioactive constituents.

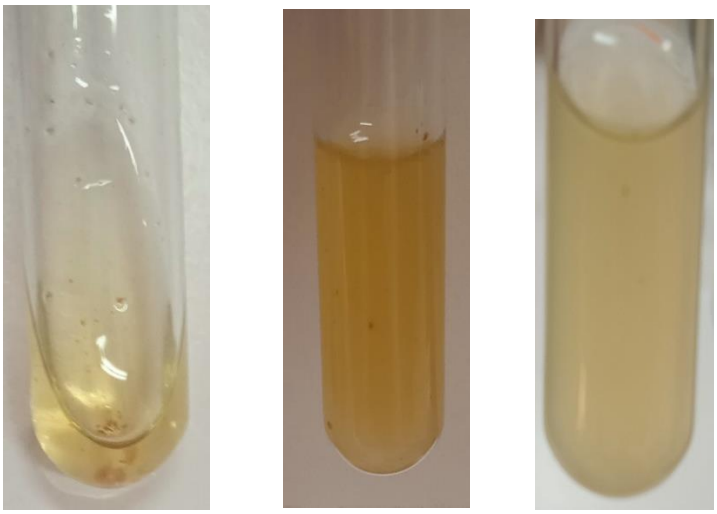


Figure II.2.2 Summarizes the presence of phenolic and flavonoid compounds in propolis extracts, indicating "strongly positive" results for both. However, specifying the exact qualitative tests performed (e.g., ferric chloride for phenolics, NaOH for flavonoids) would enhance clarity.

Table II.2.2. Phenolic and flavonoid contents test of propolis extracts

Bioactive compounds	Intensity of test
Phenolic compounds	Test strongly positive
Flavonoid compounds	Test strongly positive

The robust reactivity with ferric chloride suggests a high density of hydroxylated aromatic structures, such as phenolic acids (e.g., caffeic acid, ferulic acid) which form stable complexes with ferric ions.

The pronounced color changes in the NaOH tests indicate a rich diversity of flavonoid subclasses (e.g., flavones, flavonols, flavanones), which are critical to propolis's anti-inflammatory properties.

2.2.3. Electrochemical detection of phenolic compounds

Although, electronic spectroscopy technics are well developed and successfully applied to the analysis of phenolic compounds, there are some disadvantages such as the need of long sampling times. Therefore, the development of a more reliable and fast technique for the analysis of phenolic compounds has become an important research topic. Electrochemical technics such as cyclic voltammetry are a field of interest in determining phenolic compounds. They possess high sensitivity, selectivity, and rapid response, as well as requiring fewer sample sizes. The method relies on the oxidation of phenolic hydroxyl groups, which generates well-defined anodic peaks, allowing for qualitative and quantitative assessments. Compared to conventional spectroscopic techniques, electrochemical detection offers advantages such as rapid analysis, low cost, minimal sample preparation, and the ability to perform real-time monitoring. This makes it particularly useful for the qualitative and quantitative analysis of phenolic-rich substances.

In this section, the phenolic constituents of the propolis extract were qualitatively analyzed using cyclic voltammetry. A 0.33 mg/mL solution of propolis extract in a phosphate buffer at pH 6.5 was subjected to a potential sweep ranging from 0 to 1400 mV at a scan rate of 100 mV/s, utilizing a three-electrode electrochemical system. The resulting voltammogram is depicted in Figure II.2.3 (red line). For comparison, a reference voltammogram was recorded under identical experimental conditions using a 0.1 mg/mL solution of gallic acid as a phenolic standard, as shown in Figure II.2.3 (green line).

The similarity between the voltammograms of the propolis extract and the gallic acid standard indicates the presence of phenolic compounds in the extract. Both voltammograms exhibit well-defined anodic peaks within the same potential range at around 540 mV, suggesting that the electroactive species in propolis share similar redox behavior with gallic acid. This result

confirms that phenolic constituents contribute significantly to the electrochemical profile of the extract. Furthermore, the comparable peak shapes and potentials reinforce the reliability of cyclic voltammetry as an effective tool for the qualitative analysis of phenolic compounds in natural products.

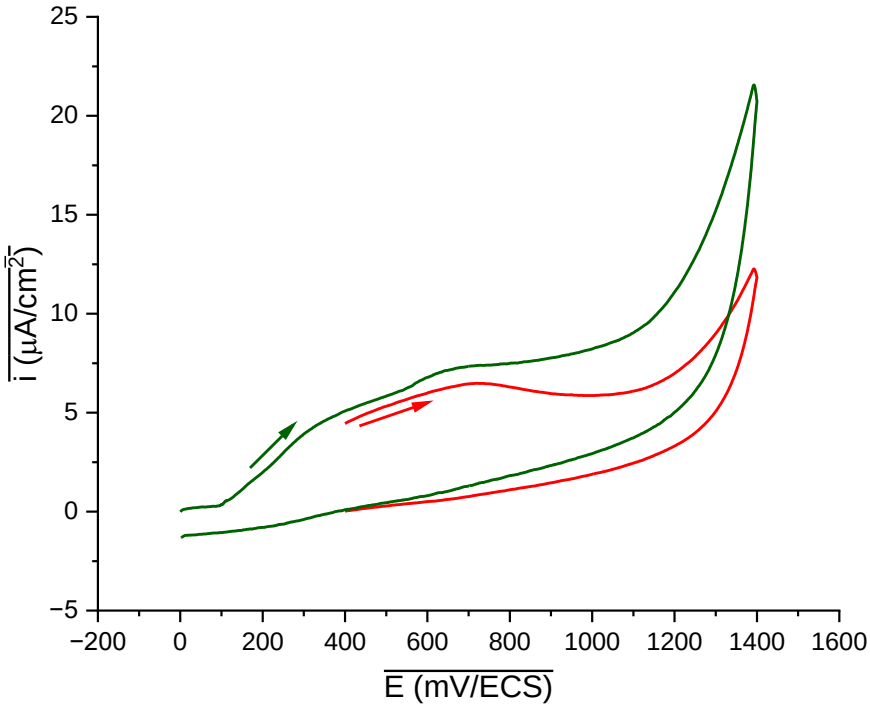


Figure II.2.3 Cyclic voltammograms of gallic acid (0.1 mg/mL, green line) and propolis extract (0.33 mg/mL, red line) in a phosphate buffer solution at pH 6.5, recorded on a glassy carbon working electrode at a scan rate of 100 mV/s

2.2.4. Electrochemical detection of flavonoid compounds

Electrochemical techniques were used to confirm the presence of flavonoid compounds in the aqueous ethanol propolis extract, a standardized cyclic voltammetry assay was performed, following established protocols for phytochemical screening. The procedure was conducted as follows:

Cyclic voltammograms of 0.33 mg/mL of propolis extract and 0.1 mg/mL of quercetin in a phosphate buffer solution at pH 6.5 were obtained, Figure II.2.4. The obtained voltammograms exhibit a high degree of similarity, suggesting the presence of flavonoid compounds in the propolis extract that share electrochemical characteristics with quercetin. This similarity indicates that the flavonoids in the propolis sample undergo oxidation and reduction processes

within the same potential range, confirming their structural and electrochemical resemblance to quercetin.

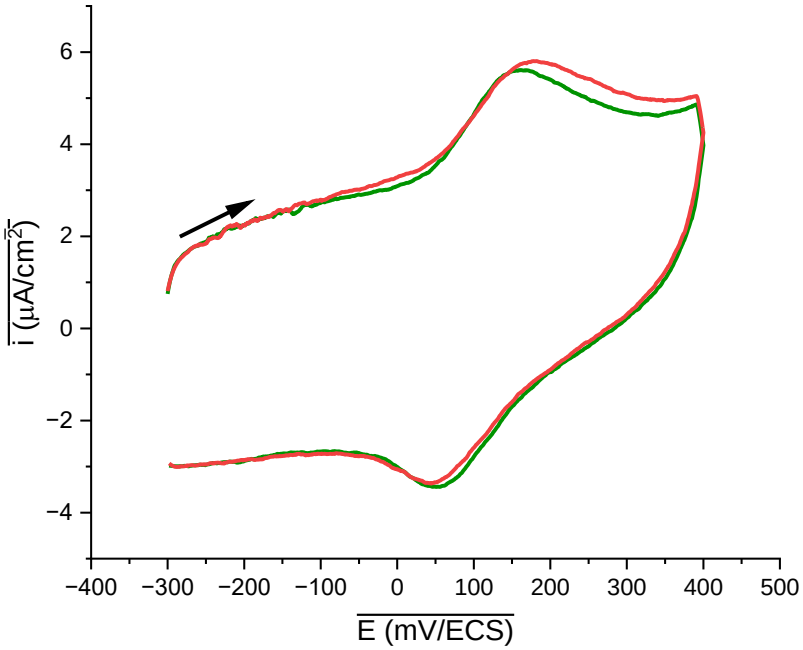


Figure II.2.4. Cyclic voltammograms of quercetin (0.1 mg/mL, green line) and propolis extract (0.33 mg/mL, red line) in a phosphate buffer solution at pH 6.5, recorded using a glassy carbon working electrode at a scan rate of 100 mV/s

2.3. Quantitative Analysis of Total Phenolic and Flavonoid Contents

Several methods based on the individual reactivity of polyphenols and flavonoids have been developed for their quantitative analysis.

Total phenolic and flavonoid contents can be measured by spectrophotometric determination in the presence of a colorimetric reagent. The colorimetric reagent reacts with phenolic compounds to form a blue complex that absorbs at 725 nm, and aluminum chloride, which reacts with flavonoid compounds to cause a color change at 510–550 nm.

Electrochemical techniques, such as cyclic voltammetry and differential pulse voltammetry, provide an alternative and highly sensitive means for phenolic and flavonoid quantification. These methods rely on the redox properties of phenolic hydroxyl groups, enabling rapid detection and analysis without extensive sample preparation. The electrochemical signals, including peak currents and potentials, correlate with the total phenolic and flavonoid contents, offering valuable insights into their bioactive compound contents.

By integrating these complementary techniques, a comprehensive assessment of total phenolic and flavonoid contents can be achieved, facilitating the standardization and quality control of propolis extracts.

2.3.1. Spectrophotometric methods

The total phenolic and flavonoid contents of the aqueous ethanol propolis extract were quantified using standardized spectrophotometric methods, with results expressed in terms of gallic acid equivalents (GAE) for phenolics and quercetin equivalents (QE) for flavonoids. These metrics provide a comparative measure of the extract's phytochemical richness relative to well-characterized reference compounds, enabling cross-study comparisons and insights into bioactivity potential.

2.3.1.1. Total Phenolic Content

100 µL of propolis extract (1 mg/mL in 30%-70% aqueous ethanol) was mixed with 500 µL of 10% (v/v) Folin-Ciocalteu reagent. After 5 minutes, 400 µL of 7.5% (w/v) sodium carbonate solution was added.

The mixture was incubated in the dark at 25°C for 30 minutes, and absorbance was measured at 765 nm using a UV-Vis spectrophotometer. A standard curve was constructed with gallic acid (0–30 mg/mL) obtained absorbance are summarized in Table II.2.3.

Table II.2.3. Gallic acid and propolis extracts absorbance values

Gallic acid (mg/mL)	Absorbance (u.a.)
0.03	0.128
0.06	0.245
0.09	0.37
0.12	0.463
0.15	0.598
0.18	0.662
0.21	0.816
0.24	0.874
0.27	0.973
0.3	1.104
Sample ME	0.112
Sample SE	0.100
Sample UAE	0.115
Sample UAE-M 2 h	0.171
UAE-M 24 h	0.214

The obtained absorbance at 765 nm was then plotted versus gallic acid concentrations, Figure II.2.5

The regression curve presented in the graph illustrates the linear relationship between the concentration of gallic acid (x-axis, in mg/mL) and the corresponding absorbance (y-axis, in arbitrary units). The equation of the calibration curve is given as: $y = 0.0392 + 3.54x$

where: the slope (3.54) represents the sensitivity of the assay, indicating the change in absorbance per unit increase in gallic acid concentration. The intercept (0.0392) suggests a minor baseline absorbance, possibly due to solvent or reagent background.

The coefficient of determination ($R^2 = 0.996$) indicates an excellent linear fit, suggesting that the model effectively describes the relationship between concentration and absorbance with minimal deviation. The high R^2 value confirms the reliability of the calibration curve for quantitative analysis of gallic acid in unknown samples using this spectrophotometric method.

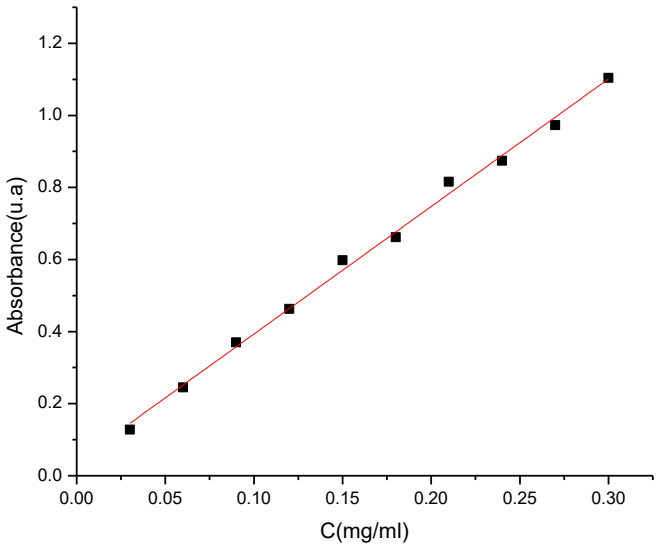


Figure II.2.5. Calibration curve of gallic acid showing the linear relationship between concentration (mg/mL) and absorbance (a.u)

The total phenolic content (TPC) was calculated using the following equation II.2.2,

$$TPC\ (mg\ GA/\ g\ sample) = \frac{C(mg/mL) \times V(mL)}{W(g)} \qquad \text{II.2.2}$$

where: C (mg/mL) represents the concentration of gallic acid equivalents (GAE) obtained from the calibration curve, V (mL): The total volume of the extract used for the assay, and W (g): The weight of the sample used for extraction.

TPC expressing total phenolic content as mg gallic acid equivalent per gram of the dry propolis extract (GAE/g).

This method allows for a standardized quantification of total phenolics in the sample, ensuring comparability across different extracts and studies.

Data presented in Table II.2.4 highlights significant variations in TPC across different extraction methods, emphasizing the critical role of technique and processing time in phenolic compound recovery from propolis.

Table II.2.4. TPC of propolis extracts obtained using different extraction methods

Extraction type	TPC (mg/g)
ME	61.7
SE	85.9
UAE	64.2
UAE-M 2 h	111.7
UAE-M 24 h	148.1

The lowest TPC (61.7 mg/g) obtained by ME suggests that passive solvent extraction at ambient conditions is less efficient, possibly due to limited cell wall disruption and incomplete compound solubilization.

Higher TPC (85.9 mg/g) reflects the efficiency of continuous solvent cycling and elevated temperatures in breaking down the propolis matrix, though prolonged heat exposure may degrade thermolabile phenolics.

Standard UAE: TPC (64.2 mg/g) marginally exceeds maceration but underperforms compared to SE, likely due to suboptimal sonication parameters (e.g., time, power, or solvent ratio).

UAE with Maceration (UAE-M):

UAE-M 2 h: A sharp increase to 111.7 mg/g demonstrates the synergistic effect of ultrasonication (mechanical disruption) and short-term maceration (diffusion enhancement).

UAE-M 24 h: The highest TPC (148.1 mg/g) underscores the importance of extended maceration time post-sonication, enabling maximal release of bound phenolics from the propolis matrix.

2.3.1.2. Total flavonoid content

500 µL of extract (1 mg/mL) was mixed with 150 µL of 5% (w/v) $NaNO_2$. After 5 minutes, 150 µL of 10% (w/v) $AlCl_3$ was added, followed by 1 mL of 1 M NaOH at the 6-minute mark.

Absorbance was measured at 510 nm after 15 minutes. A standard curve was generated using quercetin (10–110 mg/mL). Results were expressed as mg QE/g dry extract.

Table II.2.5 presents calibration data correlating propolis extract concentrations with absorbance values, essential for establishing a quantitative relationship in spectrophotometric analysis.

Table II.2.5. Calibration data for quercetin and absorbance values of propolis extracts

Quercetin (mg/mL)	Absorbance (u.a.)
0.0001095	0.26
0.0002190	0.4
0.0003284	0.651
0.0004379	0.902
0.0005474	1.135
0.0006569	1.363
0.0007664	1.567
0.0008759	1.747
0.0009853	1.943
0.0010948	2.088
Sample ME	0.112
Sample SE	0.1
Sample UAE	0.115
Sample UAE-M 2 h	0.171
UAE-M 24 h	0.214

Figure II.2.6 shows the plotting of the measured absorbance at 510 nm against the amounts of quercetin. The graph's regression curve shows the linear connection between the quercetin concentration (x-axis, in mg/mL) and the associated absorbance (y-axis, in arbitrary units). The calibration curve's equation is as follows: $y = 0.0404 + 1935x$, with the slope (1935) signifying the assay's sensitivity and the change in absorbance for every unit rise in quercetin concentration. A small baseline absorbance is suggested by the intercept (0.0404), which might be the result of reagent or solvent background.

An excellent linear fit is indicated by the coefficient of determination (R^2 = 0.995), indicating that the model reflects the connection between concentration and absorbance with little variance. The strong R^2 value attests to the calibration's dependability.

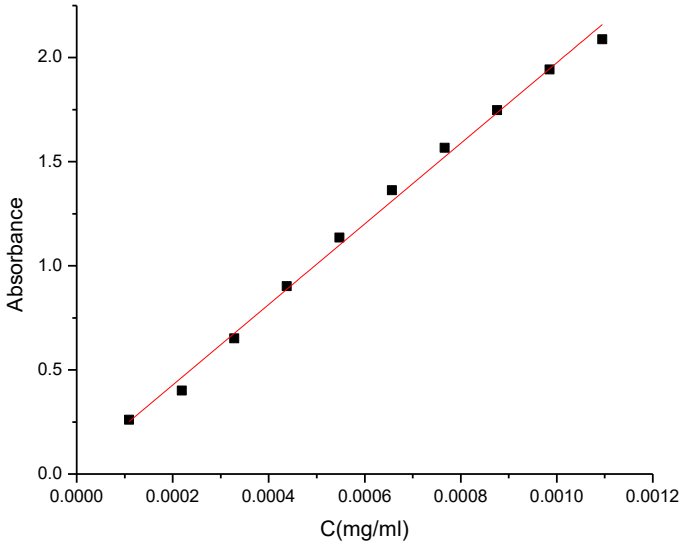


Figure II.2.6. linear regression calibration curve of quercetin

The following equation, II.2.2, was used to determine the total phenolic content (TPC),

$$TFC(mg\ QE/g\ sample) = \frac{C(mg/mL) \times V(mL)}{W(g)} \qquad \text{II.2.3}$$

where: V (mL) is the total volume of the extract used for the test, W (g) is the weight of the sample used for extraction, and C (mg/mL) is the concentration of quercetin equivalents (QE) derived from the calibration curve presented in Figure II.2.6.

TPC is expressed as milligrams of quercetin equivalent per gram of dry propolis extract.

This technique ensures comparability across various extracts and research by enabling a standardized measurement of the sample's total flavonoids.

Significant differences in TFC across various extraction techniques are shown by the data in Table II.2.6, highlighting the crucial impact that technique and processing time play in the recovery of flavonoid compounds from propolis.

Table II.2.6. TFC of propolis extracts obtained via various extraction methods

Extraction type	TFC (mg/g)
ME	111.3
SE	154.4
UAE	115.9
UAE-M 2 h	203.0
UAE-M 24 h	269.8

2.3.2. Electrochemical methods

2.3.2.1. Total Phenolic Content

A potential swap running from 0 mV to 1400 mV and then reversed from 1400 to 0 mV was imposed on a series of gallic acid solutions in a phosphate buffer solution at pH 6.5, the scan rate of the potential was 100 mV/s.

The obtained cyclic voltammograms exhibit a well-defined anodic peak, which increases proportionally with concentration, indicating a diffusion-controlled electrochemical process. The potential range extends from approximately 0 mV to 1400 mV, covering the oxidation region of phenolic compounds, with the anodic peak potential remaining relatively stable, reinforcing the electrochemical stability of gallic acid oxidation. The presence of a less prominent cathodic peak suggests some degree of electrochemical reversibility, although the peak separation and shape indicate that the process may not be fully reversible. All obtained voltammograms confirm the electroactive nature of gallic acid, with an oxidation response directly dependent on its concentration, demonstrating the suitability of cyclic voltammetry for the quantitative analysis of phenolic compounds in propolis extracts.

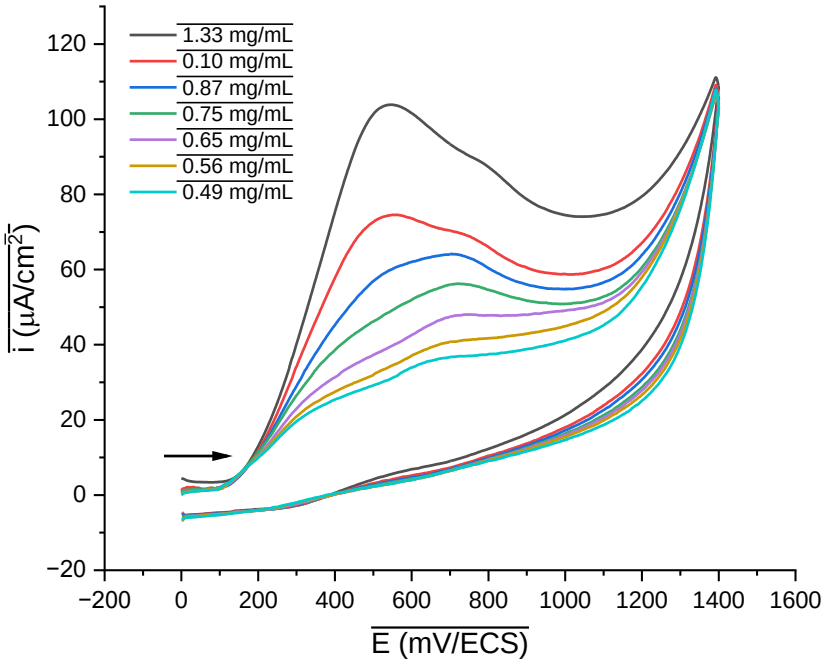


Figure II.2.7. Succession of cyclic voltammograms of gallic acid at different concentrations in phosphate buffer solution (pH 6.5), scan rate 100 mV/s

The cyclic voltammograms presented in Figure II.2.7 illustrate the electrochemical behavior of gallic acid at different concentrations in a phosphate buffer solution (pH 6.5). The data indicate a direct correlation between the concentration of gallic acid and the peak anodic current density, confirming the proportionality between polyphenol content and electrochemical response. The highest concentration (1.33 mg/mL) exhibits the most significant peak current density (104.04 µA/cm²), whereas the lowest concentration (0.49 mg/mL) shows a reduced response (36.61 µA/cm²), demonstrating the sensitivity of the method to analyte concentration. Additionally, the table includes various anodic peak current density of propolis extract samples (ME, SE, UAE, UAE-M-2h, and UAE-M-24h). Their comparison with standard gallic acid solutions can help assess the TPC of the extracts.

Table II.2.7. Anodic peak current density of gallic acid versus concentrations

Gallic acid (mg/mL)	Current density (µA.cm^{-2})
1.33	104.04
1.00	74.14
0.87	64.17
0.75	56.38
0.65	47.45
0.56	40.81
0.49	36.61

Figure II.2.8 represents a calibration curve for gallic acid, where the x-axis (C) denotes the concentration of gallic acid (mg/mL), and the y-axis (|Ip|) corresponds to the anodic peak current density (µA/cm²). The data points, marked in green, exhibit a strong linear correlation, as evidenced by the regression equation $y = 4.189 + 80.2x$ and the coefficient of determination $R^2 = 0.996$. This high R^2 value indicates an excellent linear fit, suggesting that the anodic peak current density is directly proportional to the concentration of gallic acid in the tested range. The linear regression model can be effectively used to determine unknown gallic acid concentrations in propolis samples based on their electrochemical response.

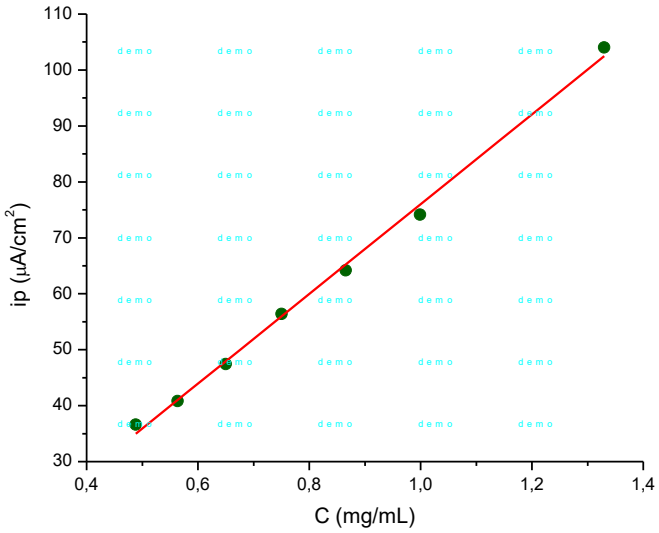


Figure II.2.8. Relationship between concentration (mg/mL) and anodic peak current density (µA/cm²) in phosphate buffer solution

To determine the TPC of propolis extracts, a set of cyclic voltammograms of propolis extracts obtained using different extraction methods were firstly obtained under the same conditions as described for gallic acid, Figure II.2.9. The similarities of the obtained voltammograms to the that of gallic acid indicate the presence of the same electroactive compounds extracted. The overall electrochemical response suggests the presence of phenolic constituents. The variations in current intensity among the extraction methods suggest differences in extraction efficiency. Methods that yield higher peak currents likely extract a greater concentration of electroactive phenolic compounds.

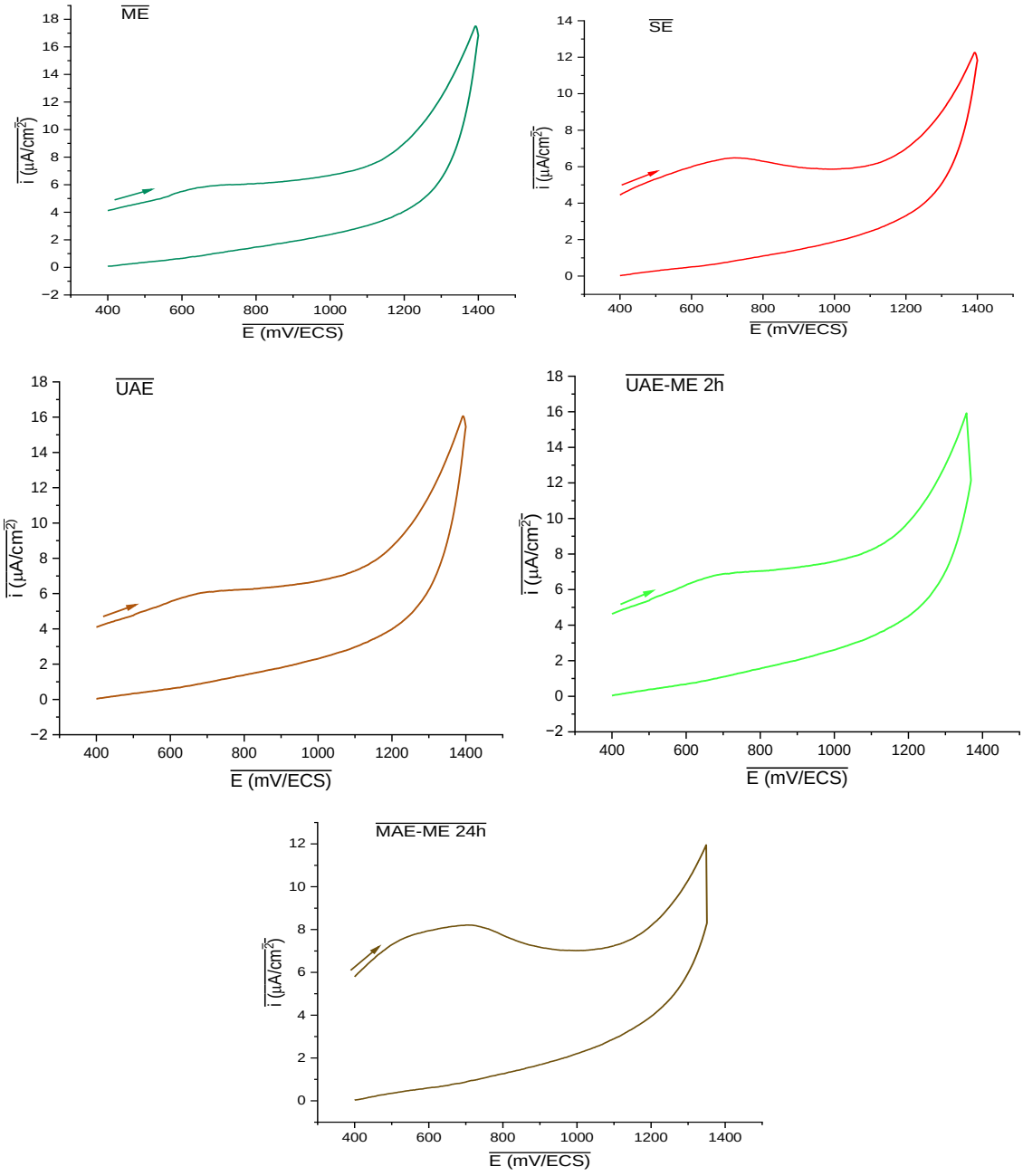


Figure II.2.9. Cyclic voltammograms of propolis extracts obtained using different extraction methods

Table II.2.8 presents the current density values obtained for propolis extracts (at a fixed concentration of 0.33 mg/mL) using different extraction methods. The variations in current density indicate differences in the electrochemical response of the extracts, which are likely influenced by the phenolic content extracted by each method. The ME sample exhibits the lowest current density (5.978 µA.cm^{-2}), suggesting a lower concentration of electroactive phenolic compounds. The SE sample shows a higher response (6.481 µA.cm^{-2}), indicating

improved extraction efficiency. The UAE-M method yields an even greater current density, highlighting its potential for enhancing phenolic compound recovery. The highest values are observed for UAE-M 24h extraction (8.211 $\mu A.cm^{-2}$), suggesting that combining ultrasound with maceration maximizes phenolic extraction. These results reinforce the effectiveness of ultrasound-based techniques in extracting electroactive compounds from propolis, making them preferable for extraction of bioactive compounds.

Table II.2.8. Anodic peak current density of propolis extract samples processed using different extraction method

Propolis extracts (mg/mL)	Extraction method	Current density ($\mu A.cm^{-2}$)
0.33	ME	5.978
0.33	SE	6.481
0.33	UAE	6.102
0.33	UAE-M 2h	6.899
0.33	UAE-M 24h	8.211

Equation II.2.2 was used to determine the TPC in the propolis extracts, expressed as mg of gallic acid equivalent per gram of sample. The calculation is based on the concentration of phenolic compounds in mg/mL obtained from a calibration curve of Figure II.2.8, the volume of the extract solution in mL, and the mass of the sample used for extraction in gram. This approach allows for a standardized quantification of phenolic content by relating the measured anodic peak current density to a gallic acid concentrations. The accuracy of TPC determination depends on precise measurement of these parameters and the proper construction of the calibration curve.

The results in Table II.2.9 illustrate the TPC of propolis extracts obtained through different extraction methods, as determined by cyclic voltammetry assays. The values reveal significant differences in polyphenolic extraction efficiency among the methods. ME yielded the lowest TPC (66.9 mg/g), indicating a limited ability to extract polyphenols efficiently under mild conditions. SE resulted in a higher TPC (85.6 mg/g), suggesting improved polyphenolic recovery due to continuous solvent circulation and heat application. UAE produced a moderate TPC of 71.6 mg/g, which, while slightly higher than ME, did not surpass SE, possibly due to insufficient sonication time or power. The most effective methods were UAE-M, particularly with extended treatment durations. The 2-hour UAE-M process yielded 101.4 mg/g, while the 24-hour UAE-M treatment provided the highest TPC (150.4 mg/g), demonstrating the combined impact of ultrasound and prolonged maceration on enhancing polyphenols extraction. These results confirm that UAE-M, especially for extended durations, significantly improves

polyphenolic compounds recovery, likely due to enhanced cell wall disruption and improved solvent penetration, making it the most efficient method for extracting polyphenols-rich propolis extracts.

Table II.2.9. TPC of propolis extracts obtained for various extraction methods using cyclic voltammetry assays

Extraction method	TPC (mg/g)
ME	66.9
SE	85.6
UAE	71.6
UAE-M-2h	101.4
UAE-M-24h	150.4

2.3.2.2. Total flavonoid content

A potential sweep ranging from -300 mV to 500 mV was applied to a series of quercetin solutions in a phosphate buffer solution at pH 6.5, with a scan rate of 100 mV/s. The cyclic voltammograms obtained exhibit a well-defined anodic peak, which increases proportionally with concentration, indicating a diffusion-controlled electrochemical process, Figure II.2.10. The oxidation process of quercetin is evident in the anodic peak, with the peak current rising as the concentration increases, confirming a linear relationship between the electrochemical response and analyte concentration. The relatively stable anodic peak potential suggests the robustness of quercetin oxidation under these experimental conditions. No corresponding cathodic peak is observed indicating no reversibility in the redox reaction. The voltammograms confirm the electroactive nature of quercetin, demonstrating the effectiveness of cyclic voltammetry in assessing its oxidation behavior and supporting its application in the electrochemical quantification of flavonoid compounds in complex matrices such as propolis extracts.

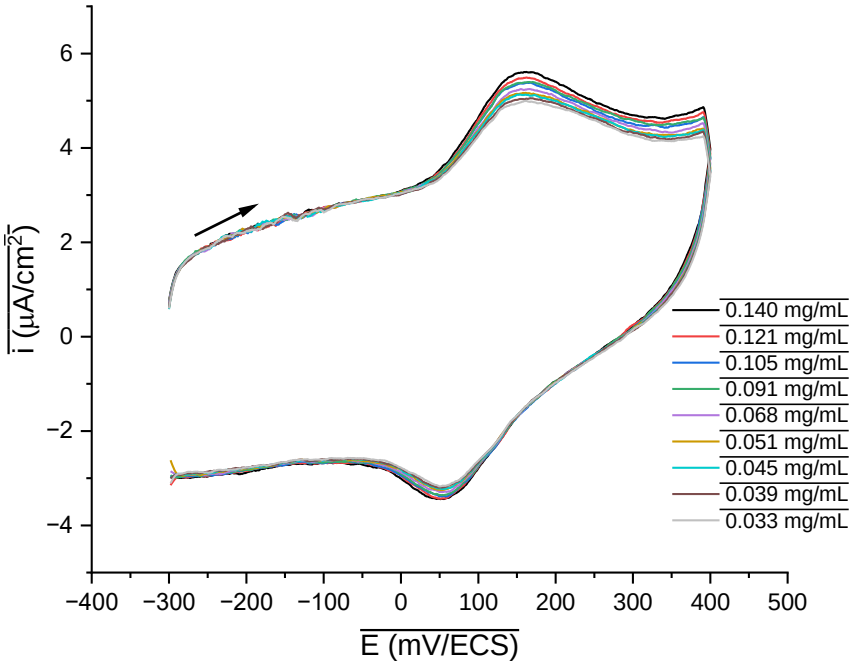


Figure II.2.10. Cyclic voltammograms of quercetin at different concentrations in a phosphate buffer solution (pH 6.5), recorded at a scan rate of 100 mV/s

Table II.2.10 presents the calibration data for quercetin, showing the relationship between quercetin concentration and the corresponding anodic current density obtained through cyclic voltammetry. The data reveal a general trend where the current density decreases slightly as the quercetin concentration decreases, indicating a proportional electrochemical response. However, a slight inconsistency is observed at the lowest concentration (0.03343 mg/mL), where the current density increases unexpectedly compared to neighboring values. This deviation could be attributed to experimental variations, instrumental noise, or slight inconsistencies in the measurement process. Despite this, the overall trend suggests a stable and reproducible electrochemical response of quercetin, confirming its oxidation behavior under the applied conditions. The observed data can be used to construct a calibration curve, which is essential for quantifying quercetin in propolis extract samples, demonstrating the applicability of cyclic voltammetry in the electrochemical detection and analysis of flavonoids.

Table II.2.10. Calibration Data for quercetin

Quercetin (mg/mL)	Current density (µA.cm^{-2})
0.13984	5.815
0.12119	5.682
0.10503	5.608
0.09103	5.475

0.07889	5.38
0.05136	5.239
0.04451	5.202
0.03857	5.154
0.03343	5.777

Figure II.2.11 represents the calibration curve for quercetin, illustrating the relationship between its concentration in mg/mL and the measured anodic current density in µA/cm². The data points, marked in red, exhibit a strong linear correlation, as evidenced by the high coefficient of determination ($R^2 = 0.995$). The linear regression equation $y = 4.903 + 6.468x$ suggests that the current density increases proportionally with quercetin concentration, confirming a direct electrochemical response. The excellent linearity indicates the reliability of cyclic voltammetry for the quantification of quercetin, making it a suitable analytical method for determining flavonoid content in propolis extract samples. The minor deviations from the fitted line could be attributed to experimental variability, but overall, the results validate the method's accuracy and precision in electrochemical analysis.

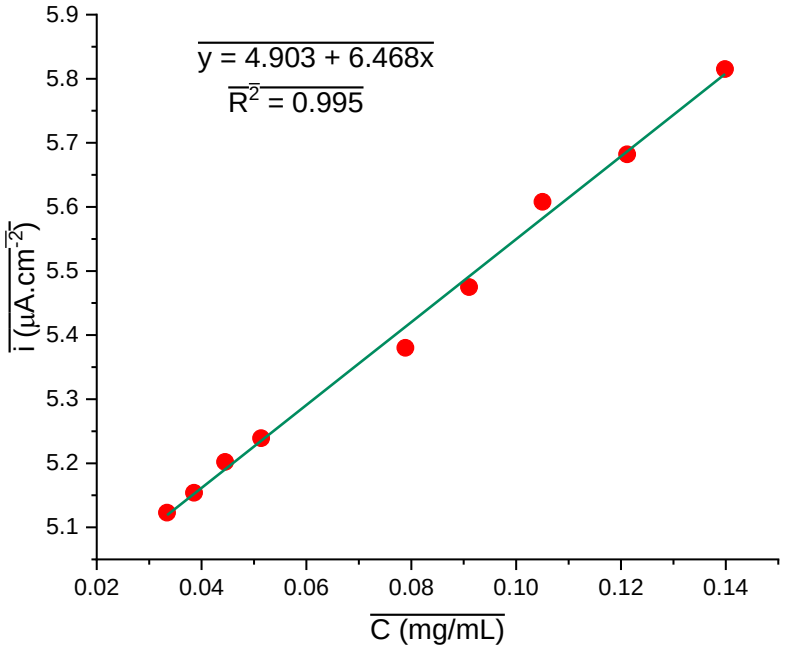
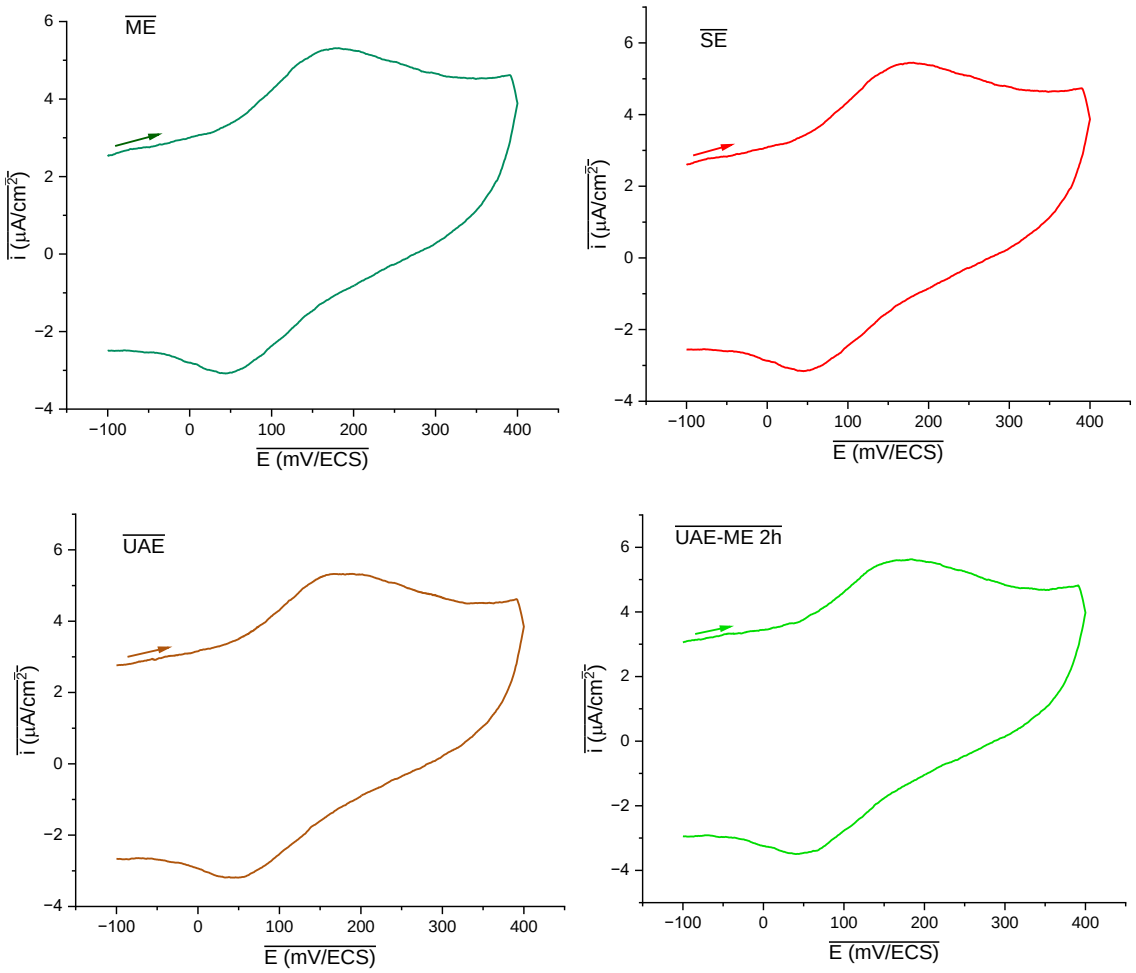


Figure II.2.11. Calibration curve for quercetin, showing the relationship between concentration (mg/mL) and current density (µA/cm²)

The figure presents cyclic voltammograms of different propolis extract samples, highlighting their electrochemical behavior. The distinct profiles suggest variations in antioxidant activity,

likely due to differences in phenolic composition resulting from the extraction methods used. Each voltammogram exhibits a characteristic oxidation peak, indicative of the redox-active compounds presents in the extracts. The shape and intensity of the peaks suggest diffusion-controlled electrochemical processes, with potential shifts reflecting variations in the phenolic constituents of each sample. These findings confirm the suitability of cyclic voltammetry for assessing the antioxidant potential of propolis extracts.



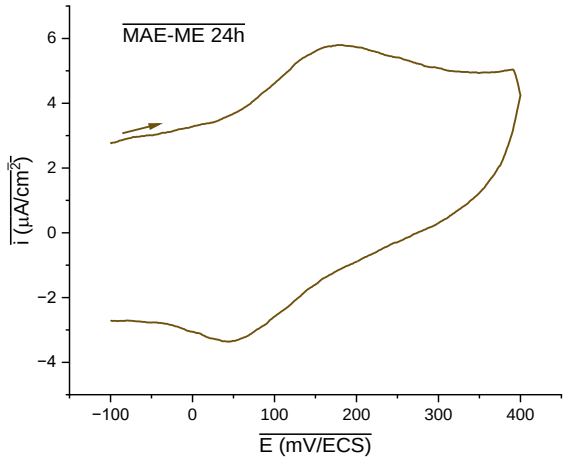


Figure II.2.12. Cyclic voltammograms of different propolis extract samples obtained using various extraction methods

The anodic peak current densities presented in Table II.2.11 correspond to the cyclic voltammograms displayed in Figure II.2.12, reflecting the electrochemical response of different propolis extracts. The results indicate that the extraction method significantly influences the electrochemical activity of the samples. The UAE-M methods, particularly at 24 h, exhibit the highest current densities, suggesting a higher concentration of electroactive compounds, likely flavonoids. In contrast, the ME method shows the lowest response, indicating a lower extraction efficiency for electroactive components.

Table II.2.11. Summary of anodic peak current densities for propolis extracts obtained using different extraction methods

Propolis extracts (mg/mL)	Extraction method	Current density (µA.cm^{-2})
0.33	ME	5.31
0.33	SE	5.428
0.33	UAE	5.313
0.33	UAE-M 2 h	5.602
0.33	UAE-M 24 h	5.777

TFC of propolis extracts obtained using different extraction methods are presented in Table II.2.12. The results reveal significant variations in flavonoid yield depending on the extraction technique. The UAE-M methods demonstrate the highest flavonoid content, with UAE-M 24 h yielding the highest value (270.3 mg/g), followed by UAE-M 2 h (216.1 mg/g). This suggests that combining ultrasound-assisted extraction with maceration enhances the recovery of flavonoids from propolis. Conversely, the ME method shows the lowest TFC (125.9 mg/g),

indicating its lower efficiency in extracting flavonoid compounds compared to SE and ultrasound-assisted techniques.

Table II.2.12. TFC of propolis extracts obtained using various extraction methods. recovery

Extraction type	TFC (mg/g)
ME	125.9
SE	162.3
UAE	126.8
UAE-M 2 h	216.1
UAE-M 24 h	270.3

2.3.3. LC-MS data identifying major compounds

The LC-MS/MS analysis revealed a diverse chemical profile across the five samples, The five samples (S1 to S5) were analyzed for 34 compounds, primarily flavonoids, phenolic acids, and other phytochemicals. The compounds were detected using positive or negative ESI modes, with specific mass transitions and collision energies. Table II.2.13 summarizes the detection frequency and average peak areas for key compounds across the samples. The five samples show variation in the identification of compounds:

- **S1**: Detected fewer compounds (13/34), with high areas for Caffeic Acid (1,247,374), Salicylic Acid (877,130), and Riboflavin (305,486). Quercetin, Curcumin, and Apigenin were not detected, suggesting a distinct chemical profile.
- **S2**: Detected 27/34 compounds, with high areas for Curcumin (1,358,400), Salicylic Acid (3,410,461), and Caffeic Acid (1,093,162). Quercetin (111,901) and Myricetin (135,764) were prominent.
- **S3**: Detected 27/34 compounds, with the highest areas for Curcumin (3,700,175), Salicylic Acid (3,025,561), and Caffeic Acid (2,298,596). Myricetin (193,475) and Quercetin (54,947) were well-represented.
- **S4**: Detected 24/34 compounds, with high areas for Curcumin (905,715), Oleanolic Acid (654,571), and Salicylic Acid (1,286,507). Quercetin (20,101) and Myricetin (180,434) were detected.
- **S5**: Detected 24/34 compounds, with high areas for Salicylic Acid (3,273,907), Caffeic Acid (1,205,532), and Myricetin (306,001). Quercetin (80,668) was detected, but Curcumin was absent.

Table II.2.13. Detection Frequency and Average Peak Areas of Key Compounds

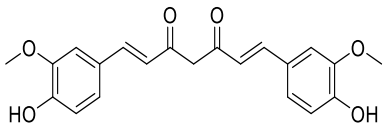
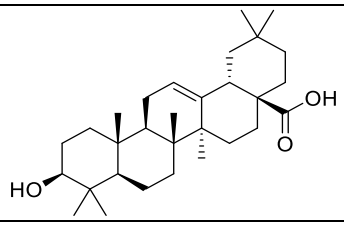
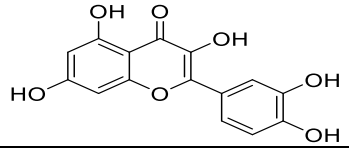
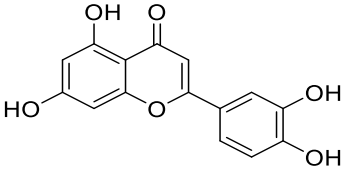
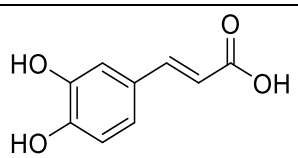
Compound	Formula	Detection (Samples)	Avg. Peak Area	Bioactivity (Anti-inflammatory/Antidiabetic)
Catechin	$C_{15}H_{14}O_6$	5/5	37,677	Moderate/Moderate
Myricetin	$C_{15}H_{10}O_8$	5/5	197,376	Strong/Strong
Orxyline A	$C_{16}H_{12}O_5$	5/5	167,255	Moderate/Weak
Chrysin	$C_{15}H_{10}O_4$	5/5	546,505	Strong/Weak
Luteolin	$C_{15}H_{10}O_6$	5/5	127,057	Strong/Moderate
Oleanolic Acid	$C_{30}H_{48}O_3$	5/5	418,824	Strong/Strong
Kojic Acid	$C_6H_6O_4$	5/5	10,426	Weak/Weak
Vanillin	$C_8H_8O_3$	5/5	77,315	Weak/Weak
Caffeic Acid	$C_9H_8O_4$	5/5	1,269,558	Strong/Strong
Salicylic Acid	$C_7H_6O_3$	5/5	2,174,673	Strong/Weak
Quercetin	$C_{15}H_{10}O_7$	4/5	59,829	Strong/Strong
Curcumin	$C_{21}H_{20}O_6$	3/5	1,987,763	Strong/Strong
Apigenin	$C_{15}H_{10}O_5$	4/5	80,771	Strong/Moderate
Naringenin	$C_{15}H_{12}O_5$	4/5	74,838	Moderate/Moderate
Resveratrol	$C_{14}H_{12}O_3$	4/5	67,690	Strong/Moderate
Epicatechin	$C_{15}H_{14}O_6$	3/5	63,340	Moderate/Moderate
Ferulic Acid	$C_{10}H_{10}O_4$	3/5	288,024	Strong/Moderate

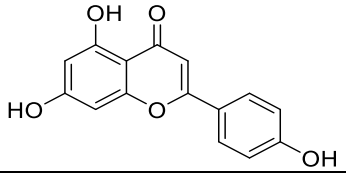
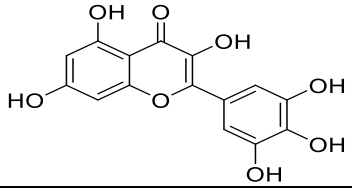
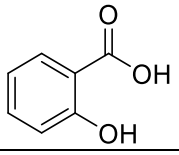
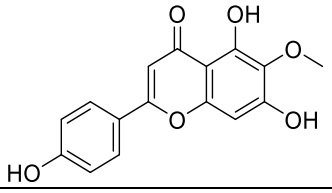
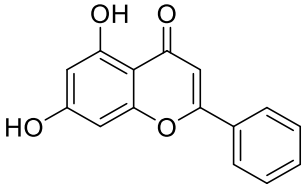
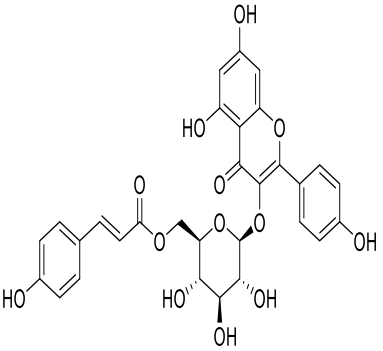
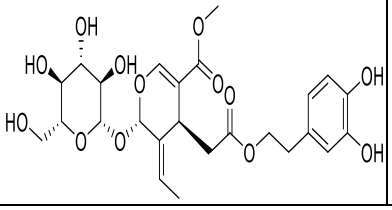
The consistent detection of compounds like Myricetin, Caffeic Acid, and Oleanolic Acid across all samples suggests their prominence in the propolis extracts, likely reflecting the botanical source's phytochemical richness. The variability in detection (e.g., Quercetin is absent in S1, Curcumin is absent in S1 and S5) may indicate differences in extraction conditions, sample preparation, or propolis material heterogeneity. The high peak areas of Salicylic Acid (up to 3,410,461) and Caffeic Acid (up to 2,298,596) underscore their abundance, making them practical candidates for further studies.

The compounds identified in Table II.2.14 exhibit promising anti-inflammatory and antidiabetic properties, aligning with the therapeutic goals of this study:

- **Quercetin** and **Myricetin** are standout flavonoids due to their potent inhibition of COX-2 and α-glucosidase, supported by their consistent detection and significant peak areas (e.g., Myricetin: 306,001 in S5). Their structural similarity (hydroxylated flavone backbone) suggests shared binding mechanisms, ideal for docking studies.
- **Caffeic Acid** is a leading phenolic acid, with exceptional abundance and dual bioactivity, making it a practical choice for pharmacological development.
- **Curcumin** shows remarkable potential where detected (e.g., 3,700,175 in S3), but its inconsistent presence limits its prioritization.
- **Oleanolic Acid**, a triterpenoid, offers strong antidiabetic activity and consistent detection, complementing the flavonoid-centric profile.

Table II.2.14. The major compounds exhibit promising anti-inflammatory and antidiabetic properties

Entry	**ID #**	Compound	Formula	Detected In	Area	D. Formula	Chemical class (Subgroup) / bioactivity
1	14	**Curcumin**	**$C_{21}H_{20}O_6$**	S2, S3, S4	3,700,175 in S3		Polyphenol, anti-inflammatory, antidiabetic
2	18	**Oleanolic Acid**	**$C_{30}H_{48}O_3$**	S2, S3, S4, S5	654,571 in S4		Triterpenoid anti-inflammatory, antidiabetic
3	20	**Quercetin**	**$C_{15}H_{10}O_7$**	S2, S3, S5	111,901 in S2		Flavonoid anti-inflammatory, antidiabetic
4	17	**Luteolin**	**$C_{15}H_{10}O_6$**	S1, S2, S3, S4	223,934 in S3		Flavonoid anti-inflammatory, antidiabetic
5	30	**Caffeic Acid**	**$C_9H_8O_4$**	All samples	2,298,596 in S3		Polyphenol anti-inflammatory, antidiabetic

6	10	**Apigenin**	**$C_{15}H_{10}O_5$**	S2, S3, S5	179,825 in S3		Flavonoid anti-inflammatory, antidiabetic
7	4	**Myricetin**	**$C_{15}H_{10}O_8$**	All	306,001 in S5		Flavonoid anti-inflammatory, antidiabetic
8	34	**Salicylic Acid**	**$C_7H_6O_3$**	All			Phenolic acid anti-inflammatory
9	11	**Hispidulin**	**$C_{16}H_{12}O_6$**	S1, S3			Flavonoid anti-inflammatory
10	13	**Chrysin**	**$C_{15}H_{10}O_4$**	S3, S5			Flavonoid anti-inflammatory
11	9	**Tiliroside**	**$C_{30}H_{26}O_{13}$**	S2, S3, S5	55,540 in S3		Glycosylated flavonoid anti-inflammatory
12	19	**Oleuropein**	**$C_{25}H_{32}O_{13}$**	S2, S3	Low abundance		Polyphenol antidiabetic

2.4. In vitro anti-inflammatory assays

The Table II.2.15 presents the absorbance values obtained from the BSA denaturation assay, comparing the effects of propolis extract and diclofenac as a reference anti-inflammatory drug. The results indicate a concentration-dependent response, where increasing concentrations of both compounds lead to higher absorbance values, signifying greater protein denaturation inhibition.

For propolis extract, the absorbance values increase significantly with concentration, with the highest recorded value at 4.000 u.a. for 0.67 mg/mL and 0.36 mg/mL, suggesting a strong interaction with BSA. Similarly, diclofenac shows an increasing trend in absorbance, with the highest value of 2.868 u.a. at 1.00 mg/mL. This indicates that both compounds effectively inhibit BSA denaturation, though propolis extract exhibits a stronger response at lower concentrations compared to diclofenac, potentially due to its rich composition of flavonoids and phenolic compounds known for their stabilizing and antioxidant properties.

Table II.2.15. Absorbance values for the effect of propolis extract and diclofenac on BSA denaturation, indicating their potential anti-inflammatory activity

Propolis extract (mg/mL)	Absorbance (u.a.)	Diclofenac (mg/mL)	Absorbance (u.a.)
0.00	0.920	0.00	0.523
0.75	2.658	1.00	2.868
0.67	4.000	0.50	1.752
0.36	4.000	0.25	1.153
0.32	1.640	0.12	0.925
0.23	1.481	0.06	0.832
0.21	1.174	0.03	0.774
0.19	1.095	0.01	0.71

In Table II.2.16, the inhibition percentage of BSA denaturation is summarized for both propolis extract and the reference drug diclofenac. The results suggest that both compounds exhibit inhibition effects that increase with concentration, eventually plateauing at higher values. This finding indicates that propolis extract has a comparable effect to diclofenac in reducing BSA denaturation, which is an important marker of anti-inflammatory potential.

Table II.2.16. Inhibition percentage of BSA denaturation by propolis extract and the reference drug diclofenac

Propolis extract (mg/mL)	Inhibition %	Diclofenac (mg/mL)	Inhibition %
0.75	77	1.00	81.75
0.67	77	0.50	70.14
0.36	43.90	0.25	54.64
0.32	39.47	0.12	43.45
0.23	21.63	0.06	37.13
0.21	15.98	0.03	32.42
0.19	9.45	0.01	26.34

The following Equation II.2.3 was used for the calculation of the percentage inhibition of BSA denaturation

$$\text{Inhibition}(\%) = \left(\frac{A_s - A_0}{A_s}\right) \times 100 \qquad \text{II.2.3}$$

where: A_s represents the absorbance of the sample (propolis extract or diclofenac) and A_0 represents the absorbance of the control (without inhibitors).

Plotting the percentage inhibition against the concentration of the sample, a **dose-response curve** is constructed. From this curve, the IC_{50} value (the concentration required to inhibit 50% of BSA denaturation) is determined. This equation is a crucial step in anti-inflammatory studies, providing insights into the potency of propolis extract compared to the reference drug, diclofenac.

The Figure II.2.13 presents exponential regression curves illustrating the relationship between inhibition percentage and concentration. The high R^2 values of 0.995 suggest a strong correlation, confirming the suitability of the exponential model for describing this behavior. The negative exponent indicates a saturation effect, meaning that increasing the concentration beyond a certain point does not lead to significant additional inhibition. It would be beneficial to justify the choice of an exponential model over other potential fits, such as linear or sigmoidal models.

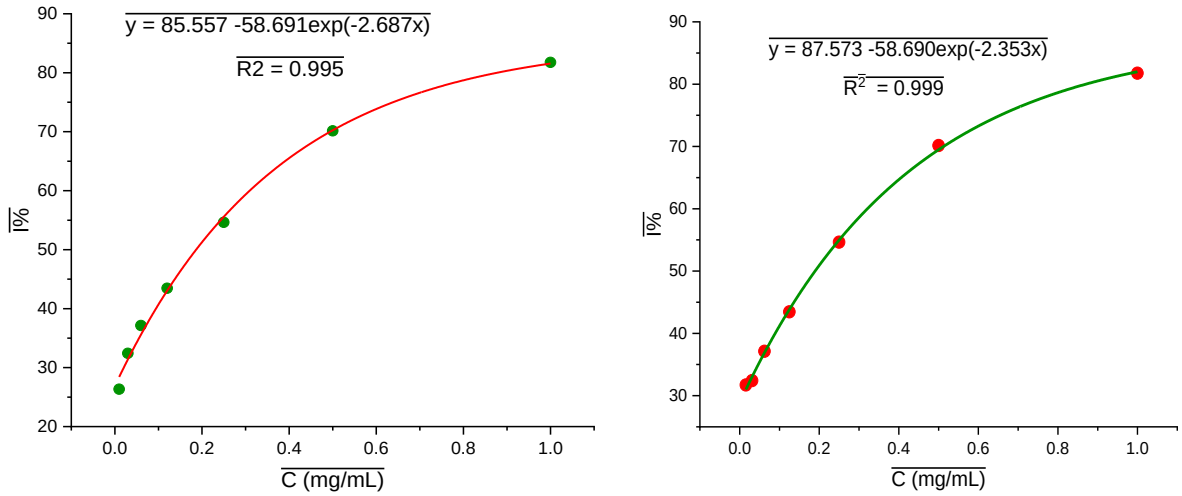


Figure II.2.13. Exponential regression curves showing the percentage inhibition of BSA denaturation as a function of concentration for propolis extract on the left and diclofenac on the right

Table II.2.17summarizes the IC_{50} values for propolis extract (0.187 mg/mL) and diclofenac (0.190 mg/mL), demonstrating their comparable effectiveness in inhibiting BSA denaturation. This similarity suggests that propolis extract exhibits anti-inflammatory properties nearly equivalent to diclofenac, which is a widely used pharmaceutical agent.

Table II.2.17. IC_{50} values for BSA inhibition by the propolis extract and the reference drug diclofenac

Compound	Equation	R^2	IC_{50} mg/mL
Propolis extract	y = 85.557 – -58.691exp-2.687x)	0.995	0.187
Diclofenac	y = 85.81 - 58.65exp(-2.639x)	0.995	0.190

2.5. In vitro antidiabetic assays

Table II.2.18 presents the absorbance values for different concentrations of propolis extract and the reference drug acarbose in an anti-diabetic in vitro assay, where higher absorbance indicates greater inhibitory activity. The results show that the absorbance increases significantly with the concentration of propolis extract, suggesting a dose-dependent inhibitory effect, which implies potential anti-diabetic activity. Similarly, acarbose follows the same trend, but at equivalent or lower concentrations, it exhibits higher absorbance values than propolis extract, indicating a stronger inhibitory effect at lower doses. This suggests that while acarbose is more potent, propolis extract still demonstrates considerable activity, making it a promising natural

alternative for anti-diabetic applications. The inclusion of a control (0.000 mg/mL) with the same absorbance for both samples confirm the validity of the assay, ensuring that the observed effects are due to the tested compounds rather than background interference.

Table II.2.18. Absorbance values of propolis extract and the reference drug acarbose used for anti-diabetic test

Propolis extract (mg/mL)	Absorbance (u.a.)	Acarbose (mg/mL)	Absorbance (u.a.)
0.000	0.093	0.000	0.093
0.500	1.836	0.647	2.180
0.450	1.833	0.323	1.660
0.405	1.808	0.161	1.247
0.364	1.780	0.080	0.734
0.328	1.757	0.040	0.377
0.239	1.674	0.020	0.278
0.215	1.631	0.010	0.199

Data in Table II.2.19presents the inhibition percentages of α-amylase by propolis extract and the reference drug acarbose at various concentrations, illustrating their potential anti-diabetic effects. The results indicate that both propolis extract and acarbose exhibit a concentration-dependent inhibition, with higher concentrations leading to greater enzyme inhibition. At the highest tested concentrations (0.5 mg/mL for propolis extract and 0.647 mg/mL for acarbose), both samples achieve nearly complete inhibition, with values of 96.05% and 95.73%, respectively, suggesting comparable efficacy. However, as the concentration decreases, the inhibition percentages also decline, but at different rates. Propolis extract maintains a higher inhibition percentage than acarbose at mid-range concentrations, notably at 0.328 mg/mL (87.33%) compared to acarbose at 0.04 mg/mL (75.33%). However, at lower concentrations, acarbose exhibits superior inhibition, with 66.54% at 0.02 mg/mL, whereas propolis extract at a similar concentration (0.239 mg/mL) shows only 53.27%. At the lowest tested concentration, 0.01 mg/mL, acarbose still retains significant inhibition (53.26%), while propolis extract at 0.215 mg/mL displays a lower inhibition of 43.64%. These results suggest that while propolis extract is effective, acarbose remains more potent at lower concentrations, highlighting its stronger inhibitory action against α-amylase. Nevertheless, the significant inhibition observed for propolis extract supports its potential as a natural alternative for managing postprandial hyperglycemia, warranting further investigation into its mechanism of action and possible synergistic effects when combined with standard treatments.

Table II.2.19. Inhibition percentage of α-amylase by propolis extract and the reference drug acarbose

Propolis extract (mg/ml)	Inhibition %	Acarbose (mg/ml)	Inhibition %
0.5	96.0476	0.647	95.73
0.45	95.73394	0.323	94.39
0.405	94.39759	0.161	92.54
0.3645	92.5421	0.08	87.32
0.32805	87.3297	0.04	75.33
0.23915	53.26633	0.02	66.54
0.21523	43.63636	0.01	53.26

The Figure II.2.14 presents the inhibition of α-amylase as a function of the concentration of propolis extract (left) and the reference drug acarbose (right). Both inhibition curves follow an exponential regression model, demonstrating a strong correlation (R^2 = 0.987) between concentration and percentage inhibition. The propolis extract curve exhibits a sigmoidal shape, indicating a gradual increase in inhibition as concentration increases, reaching a plateau close to 100% inhibition. On the other hand, the acarbose curve shows a sharp initial rise in inhibition, followed by an early saturation point, suggesting a high potency at lower concentrations. The regression equations Table II.2.20confirm the effectiveness of both inhibitors, with propolis extract reaching near-complete inhibition at moderate concentrations. The results reveal that propolis extract exhibits antidiabetic activity making it a potential natural alternative for α-amylase inhibition in the management of diabetes.

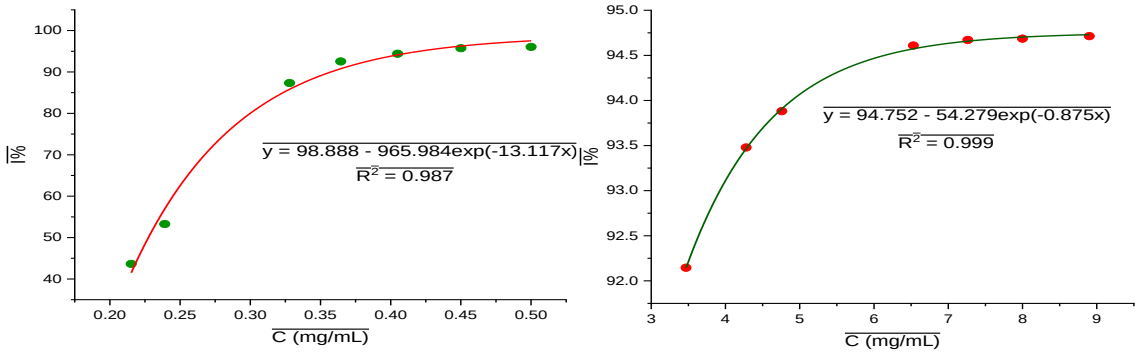


Figure II.2.14. Exponential regression curves of α-amylase inhibition as a function of the concentration of propolis extract (left) and acarbose (right)

The following Equation II.2.4 was used for the calculation of the percentage of α-amylase inhibition

$$\text{Inhibition(\%) of } \alpha\text{-amylase} = \left(\frac{A_s - A_0}{A_s}\right) \times 100 \qquad \text{II.2.4}$$

where As represents the absorbance of the sample with the enzyme, and A_0 corresponds to the absorbance of the control (without the inhibitor). This equation effectively quantifies the inhibitory effect of different concentrations of propolis extract and acarbose on α-amylase activity.

Table II.2.20The table presents the equations, correlation coefficients (R^2), and IC_{50} values for propolis extract and acarbose which represent the concentration required to inhibit 50% of α-amylase activity, illustrating their dose-response relationships in an antidiabetic study, where *x* represents the concentration and *y* the absorbance. The equations describe how absorbance changes with increasing concentration, and the high R^2 values (0.954 for propolis extract and 0.999 for acarbose) indicate a strong fit, confirming the reliability of the models. The IC_{50} values, 0.227 mg/mL for propolis extract and 0.221 mg/mL for acarbose, are very close, suggesting that propolis extract exhibits comparable α-amylase inhibitory activity to acarbose, a standard antidiabetic drug. This similarity highlights the potential of propolis extract as a natural alternative for diabetes management.

Table II.2.20. IC_{50} values for α-amylase inhibition by propolis extract and the reference drug acarbose, determined from exponential regression equations

Compound	Equation	R^2	IC_{50} mg/mL
Propolis extract	y = 98.888 – 965.984exp(-13.117x)	0.954	0.227
Acarbose	y = 94.752 – 54.279exp(-8.75x)	0.999	0.221

2.6. Docking analysis

In silico molecular docking studies were carried out on selected compounds identified via UPLC-ESI-MS-MS analysis across samples S1 to S5. The compounds prioritized for docking were chosen based on three main criteria: consistent detection across multiple samples, high relative abundance, and well-documented biological activities, particularly anti-inflammatory and antidiabetic effects.

The primary candidates included curcumin (CUR), oleanolic acid (OLA), quercetin (QUE), luteolin (LUT), caffeic acid (CAF), apigenin (API), myricetin (MYR), salicylic acid (SAL), hispidulin (HIS), and chrysin (CHR). These molecules mainly belong to the polyphenol and flavonoid classes, known for their strong interaction potential with various biological targets. Additionally, two secondary candidates such as tiliroside (TIL) and oleuropein (OLP), were included despite their lower abundance, due to their promising bioactivities reported in the

literature [71]. The molecular formulas, detection profiles, and associated pharmacological classes of these compounds are summarized in Table II.2.21.

Table II.2.21. Selected Compounds for Molecular Docking Studies

Entry	**ID#**	Compound	Detected In	Chemical class (Subgroup) / bioactivity
1	14	CUR	S2, S3, S4	**Polyphenol** anti-inflammatory, antidiabetic
2	18	OLA	S2, S3, S4, S5	**Triterpenoid** anti-inflammatory, antidiabetic
3	20	QUE	S2, S3, S5	**Flavonoid** anti-inflammatory, antidiabetic
4	17	LUT	S1, S2, S3, S4	**Flavonoid** anti-inflammatory, antidiabetic
5	30	CAF	All samples	**Polyphenol** anti-inflammatory, antidiabetic
6	10	API	S2, S3, S5	**Flavonoid** anti-inflammatory, antidiabetic
7	4	MYR	All	**Flavonoid** anti-inflammatory, antidiabetic
8	34	SAL	All	**Phenolic acid** anti-inflammatory
9	11	HIS	S1, S3	**Flavonoid** anti-inflammatory
10	13	CHR	S3, S5	**Flavonoid** anti-inflammatory
11	9	TIL	S2, S3, S5	**Glycosylated flavonoid** anti-inflammatory
12	19	OLP	S2, S3	**Polyphenol** antidiabetic

Based on the results in Table II.2.22, OLA exhibited the strongest binding affinity toward both α-amylase (−9.28 kcal/mol) and α-glucosidase (−9.22 kcal/mol), surpassing the reference drug acarbose (−4.79 and −3.86 kcal/mol, respectively). This suggests that OLA may serve as a potent inhibitor of carbohydrate-digesting enzymes and holds promise as a natural antidiabetic agent.

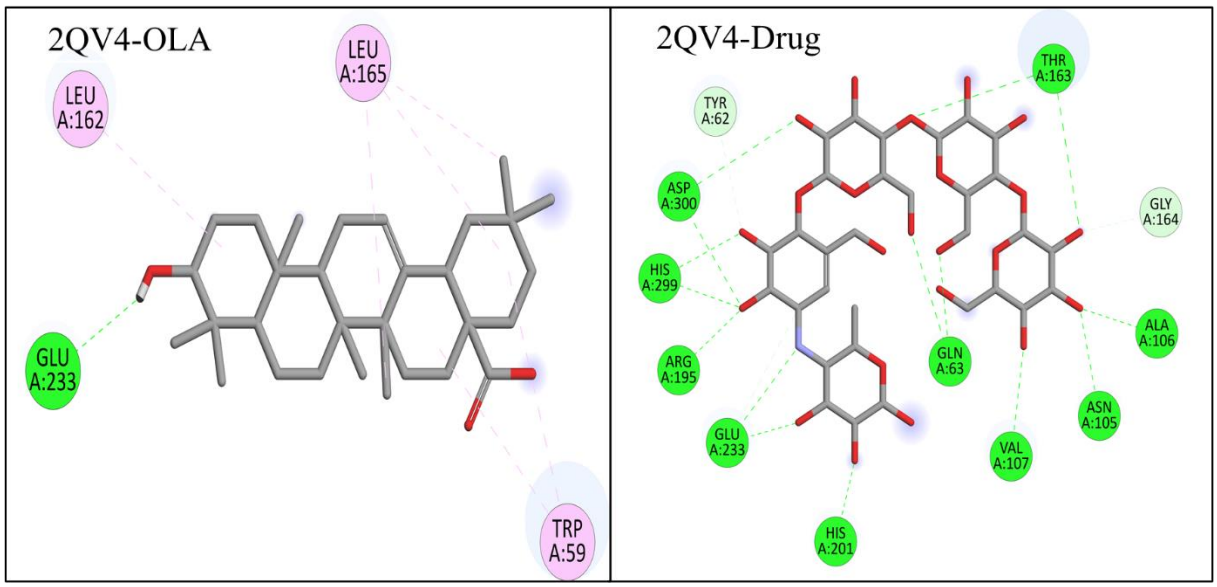
TIL showed remarkable affinity for cyclooxygenase-2 (−10.59 kcal/mol), significantly stronger than that of diclofenac (−7.70 kcal/mol), indicating high potential as an anti-inflammatory compound. Additionally, CUR displayed consistently strong binding across all targets, particularly with COX-2 (−9.38 kcal/mol) and BSA (−8.10 kcal/mol), reinforcing its reputation as a versatile bioactive with both anti-inflammatory and bioavailability-enhancing properties.

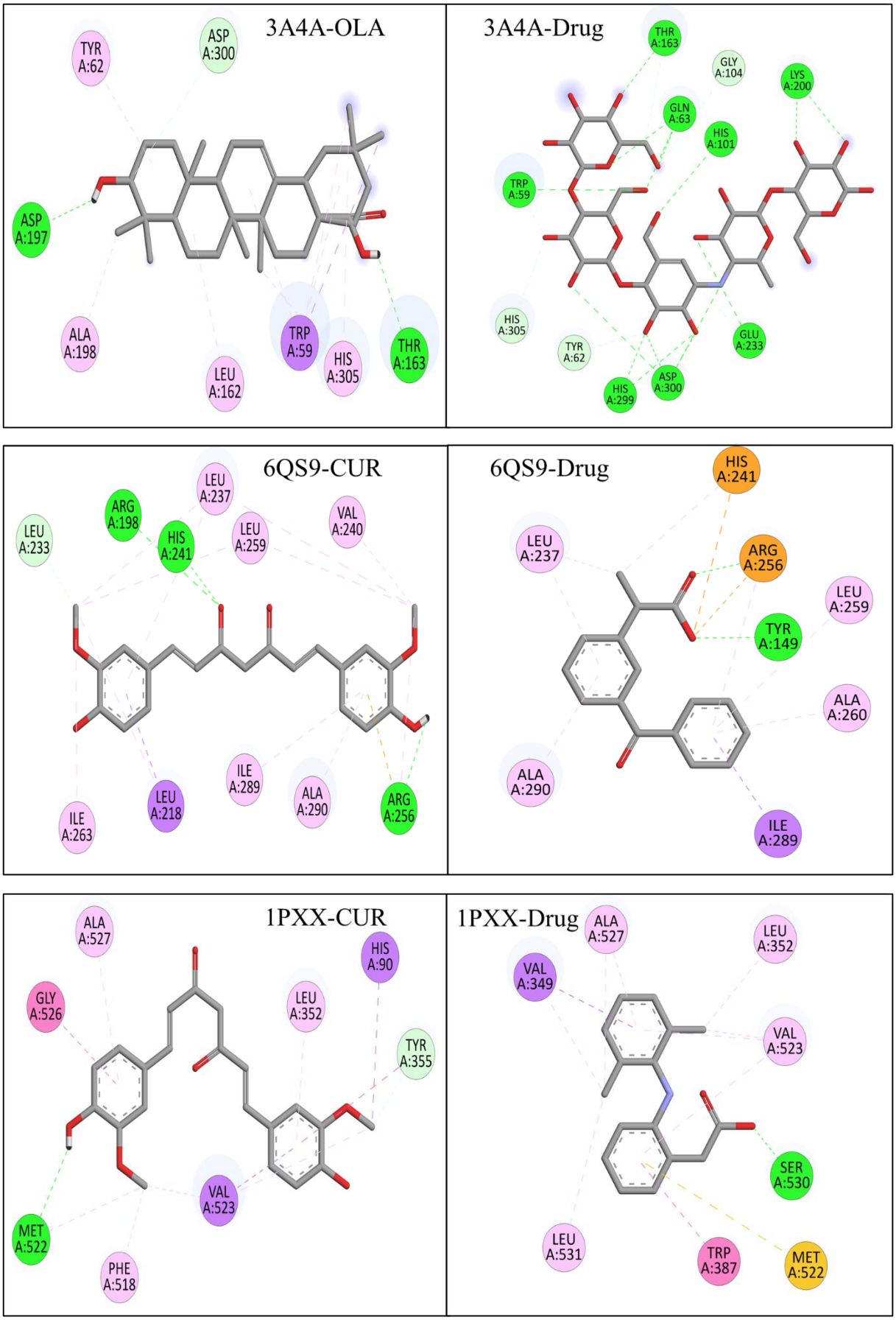
Flavonoids such as LUT, API, CHR, and HIS also demonstrated favorable binding profiles, generally ranging between −7.1 and −8.3 kcal/mol across the targets. These compounds are

known for their multifunctional roles in inflammation and metabolic regulation and may exert synergistic effects when present together in complex natural extracts like propolis.

Table II.2.22. Binding energy (kcal/mol) of Propolis Compounds with Target Proteins

	2QV4	**3A4A**	**1PXX**	**6QS9**
RMSD	0.759	1.971	1.56	1.42
Native ligand	-11.65	-11.85	-7.91	-7.87
Drug	-4.79	-3.86	-7.70	-7.79
CUR	-7.59	-7.66	**-9.38**	**-8.10**
OLA	**-9.28**	**-9.22**	-0.49	----
QUE	-6.63	-6.67	-7.85	-6.51
LUT	-7.18	-7.24	-8.28	-6.48
CAF	-4.38	-4.47	-5.05	-5.17
API	-7.20	-7.10	-8.24	-6.39
MYR	-6.65	-6.97	-7.78	-6.47
SAL	---	---	-4.19	-5.27
HIS	---	---	-8.31	-5.26
CHR	---	---	-8.28	-6.30
TIL	---	---	**-10.59**	-6.20
OLP	-6.54	-7.02	---	---




3A4A-OLA
TYR A:62
ASP A:300
ASP A:197
ALA A:198
TRP A:59
HIS A:305
THR A:163
LEU A:162
3A4A-Drug
THR A:163
GLY A:104
LYS A:200
GLN A:63
HIS A:101
TRP A:59
HIS A:305
TYR A:62
GLU A:233
HIS A:299
ASP A:300
6QS9-CUR
LEU A:237
ARG A:198
LEU A:233
HIS A:241
LEU A:259
VAL A:240
ILE A:289
LEU A:218
ALA A:290
ARG A:256
ILE A:263
6QS9-Drug
HIS A:241
LEU A:237
ARG A:256
LEU A:259
TYR A:149
ALA A:260
ALA A:290
ILE A:289
1PXX-CUR
ALA A:527
HIS A:90
GLY A:526
LEU A:352
TYR A:355
VAL A:523
MET A:522
PHE A:518
1PXX-Drug
ALA A:527
LEU A:352
VAL A:349
VAL A:523
SER A:530
LEU A:531
TRP A:387
MET A:522

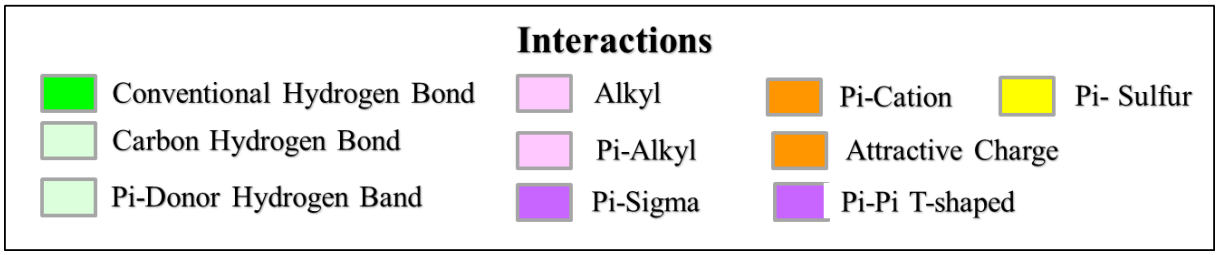


Figure II.2.15. 2D Binding interactions of top complexes

2.7. MD analysis

The selected protein–ligand complexes for molecular dynamics simulations were chosen based on their strong docking scores and the high abundance of the corresponding compounds in the samples. Specifically, OLA and CUR, which demonstrated favorable binding energies and well-documented bioactivities, were further evaluated in complex with their best-performing target proteins.

As shown in Table II.2.23, all complexes maintained low RMSD values (≤ 0.295 nm), indicating good structural stability throughout the simulation period (Figure II.2.15). Among them, OLA–2QV4 and OLA–3A4A complexes exhibited the lowest RMSD (0.156 nm and 0.170 nm, respectively), indicating the highest structural stability. The CUR–1PXX and CUR–6QS9 complexes also showed acceptable stability, with RMSD values of 0.190 nm and 0.295 nm, respectively. Nevertheless, all systems maintained overall stability, with no significant conformational changes during the simulation.

RMSF analysis supported these observations, demonstrating comparable residue-level flexibility across all complexes (Figure II.2.15). The values ranged from 0.109 nm (OLA–2QV4) to 0.159 nm (CUR–6QS9), indicating that ligand binding did not significantly disrupt protein dynamics.

The SASA analysis (Figure II.2.16) revealed the degree of solvent exposure of the protein structures during binding. The CUR–6QS9 complex had the highest SASA value (289.254 nm^2), indicating greater surface exposure, potentially due to differences in ligand size, binding orientation, or pocket depth. In contrast, OLA–2QV4 had the lowest SASA (201.205 nm^2), suggesting more compact and buried ligand binding. These differences reflect the nature of interactions between each ligand and the protein pocket.

The radius of gyration (Rg) results (Figure II.2.16) indicates the compactness and folding behavior of the protein–ligand complexes. Lower Rg values correspond to more compact structures. The OLA–2QV4 and OLA–3A4A complexes showed relatively lower Rg values (2.349 nm and 2.358 nm, respectively), indicating tighter protein packing during simulation.

The CUR–6QS9 complex, with an Rg of 2.760 nm, appeared more expanded, consistent with its higher SASA.

In summary, the MD simulation parameters suggest that OLA forms more compact, stable complexes with α-amylase and α-glucosidase, while CUR binds with slightly more flexibility and solvent exposure. Both compounds demonstrate stable interactions, reinforcing their therapeutic potential as antidiabetic and anti-inflammatory agents.

Table II.2.23. MD Parameters of Selected Compound Target Complexes

	OLA-2QV4	OLA-3A4A	CUR-6QS9	CUR-1PXX
RMSD	0.156	0.170	0.295	0.190
RMSF	0.109	0.124	0.159	0.143
SASA	201.205	205.604	289.254	253.478
Rg	2.349	2.358	2.760	2.450

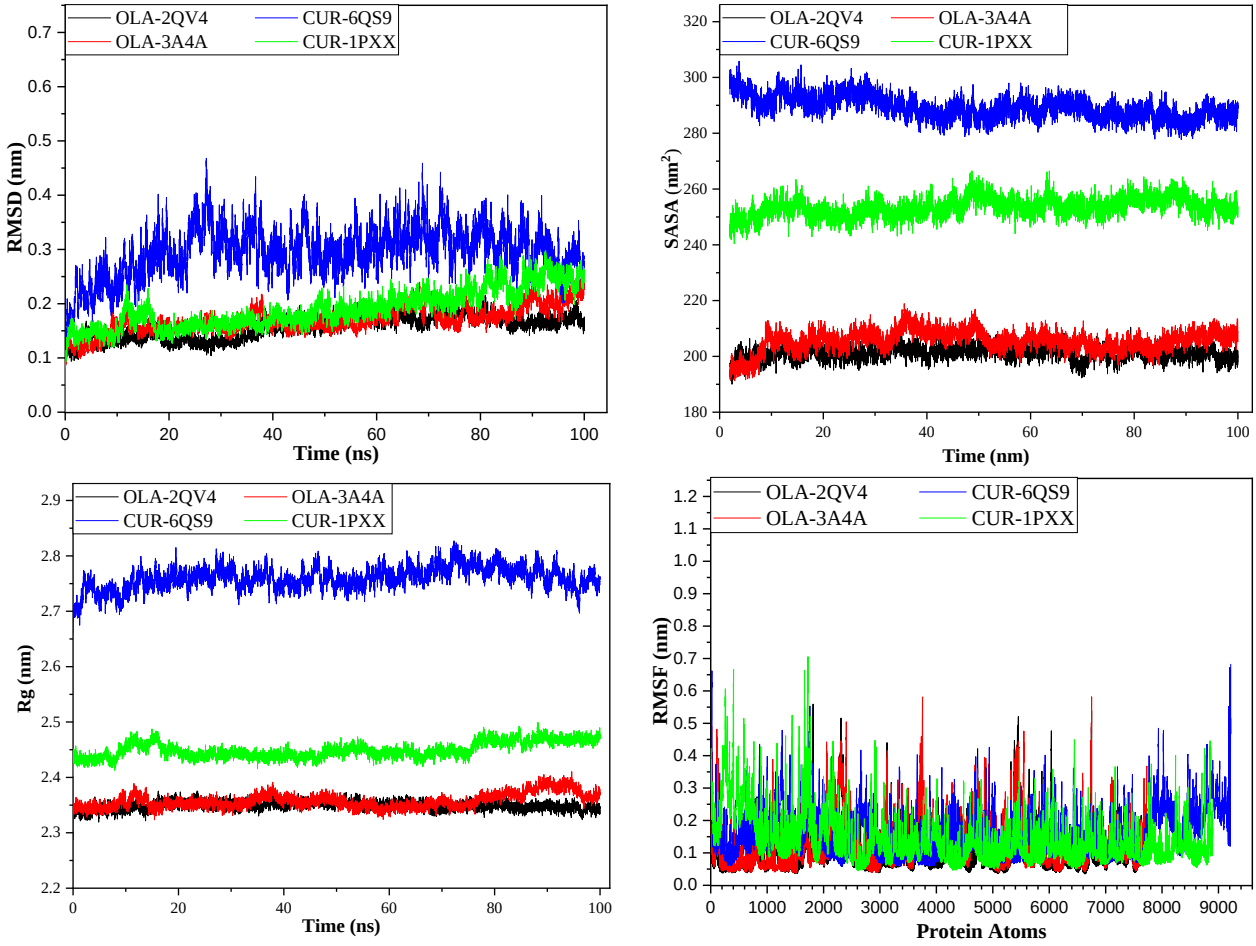


Figure II.2.16. Molecular stability of Selected Compound Target Complexes

Conclusion

In this study, a comprehensive analysis of the properties of propolis was conducted using different extraction methods. The results showed that the combined method of ultrasound and soaking achieved the highest extraction rate of active compounds.

Qualitative tests confirmed the presence of phenolic and flavonoid compounds at high concentrations in all extracts, with electrochemical results matching the spectral results, reflecting the accuracy of the measurements and the reliability of the findings. Furthermore, the propolis extract demonstrated anti-inflammatory activity comparable to that of the commonly used drug diclofenac, as well as significant activity in combating diabetes by inhibiting the alpha-amylase enzyme.

These findings were further supported by computational studies, including molecular dynamics simulations, which helped predict the potential biological mechanisms of the active compounds in propolis. The simulations revealed that these compounds could interact strongly with biological targets associated with inflammation and diabetes.

Future Perspectives

1. **Exploring the Effects of Propolis on Other Diseases:** The study could be expanded to investigate the effects of propolis extracts on other diseases, such as cancer or neurological disorders, by assessing its impact on cells and tissues in more detailed studies.
2. **Improving Extraction Techniques:** With the advancement of technology, future research could focus on developing new, more efficient extraction methods to increase the yield of active compounds from propolis. For example, exploring techniques such as green solvent extraction or using artificial intelligence to optimize the results could be valuable.
3. **Clinical Studies:** These studies should be followed by clinical trials to evaluate the efficacy of propolis in the treatment or prevention of human diseases. Its effect on a broader range of patients could be studied, and optimal dosages determined.
4. **Studying Interaction with Other Drugs:** It would be beneficial to explore how propolis extracts interact with other drugs used to treat inflammation or diabetes. These studies could contribute to the development of combination therapies that combine propolis with pharmaceutical drugs for enhanced effectiveness.
5. **Expanding Molecular Dynamics Simulations:** The use of molecular dynamics simulations could be intensified to study molecular interactions in greater depth between propolis compounds and biological targets. These studies could help in understanding the biological mechanisms more precisely and open new avenues for discovering potent compounds.
6. **Exploring Propolis from Diverse Sources:** The study could be expanded to include different types of propolis from various geographical regions, as the chemical composition of propolis varies depending on the plants that bees use for gathering it. These studies could reveal differences in biological activity.

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Supplementary materials

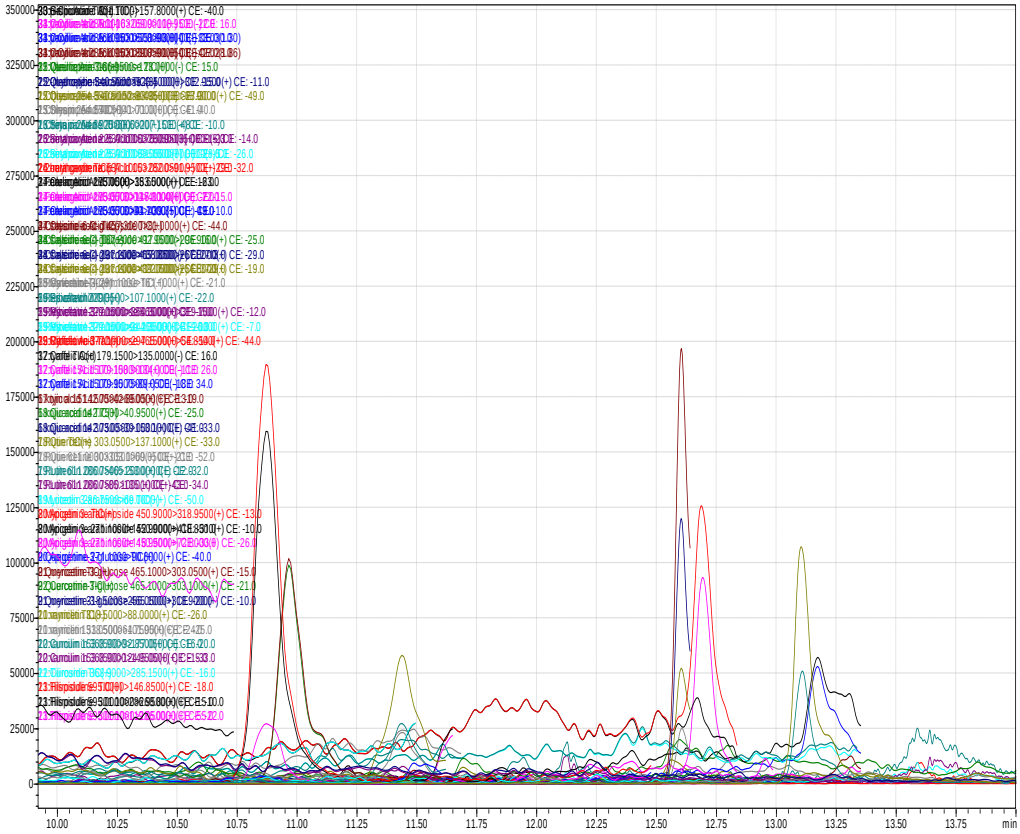

Figure1. Chromatogram of sample 1

Table1. Detection of compounds in sample1

ID#	Name	**Formula**	**ESI charge**	**CE (v)**	**Transition m/z**	**Ret. Time**	**Area**	**Height**
1	Catéchine (-)	C15H14O6	+	-13	291.1000>138.8500	12.602	85215	6349
4	myricetin	C15H10O8	+	-20	318.5000>256.0500	12.604	171208	75050
6	Orxyline A	C16H12O5	+	-22	285.1000>269.9000	12.955	51786	11415
8	Quercetine-3-glucoside	C21H19O12	+	-18	465.1000>303.0500	11.491	24412	4938
11	Hispidulin	C16H12O6	+	-10	301.1000>268.8000	12.672	226068	24143
13	Chrysin	C15H10O4	+	-27	254.8500>152.8000	2.222	813	467
17	Luteolin	C15H10O6	+	-32	286.7500>153.0000	11.946	87976	11745
18	Oleanolic Acid	C30H48O3	+	-15	457.3000>411.4000	12.397	115311	8769
22	Riboflavin	C27H30O16	+	-7	377.1000>360.3000	13.642	305486	11749
26	kojic acid	C6H6O4	+	-25	142.7500>69.0500	10.661	20294	2110
29	vanillin	C8H8O3	-	-15	153.0500>64.7500	11.441	159904	21637
30	Caffeic Acid	C9H8O4	-	34	179.1500>135.0000	10.875	1247374	158780
33	p-Coumaric Acid	C8H8O4	-	28	163.0500>118.9500	11.588	15065	8957
34	salicylic acid	C7H6O3	-	37	137.2000>92.9500	10.968	877130	98848

S2

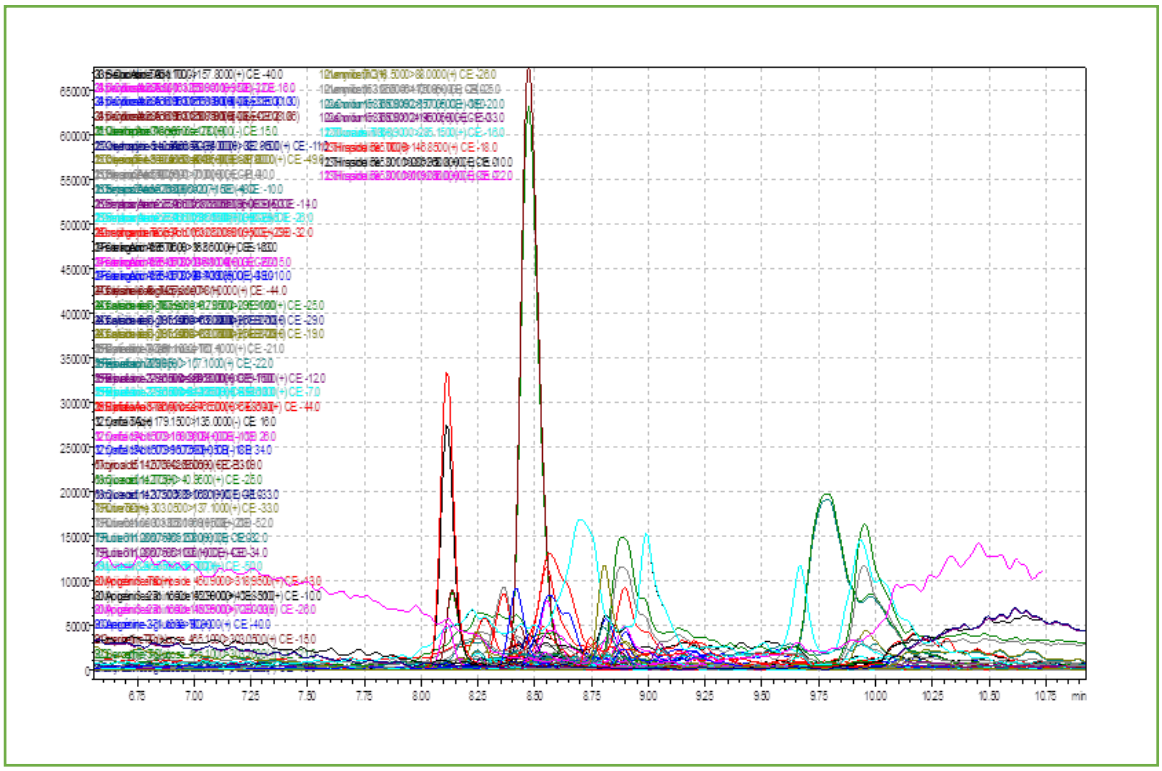

Figure 2. Chromatogram of sample 2

Table 2. Detection of compounds in sample 2

ID#	Name	Formula	ESI charge	CE (v)	Transition m/z	Ret. Time	Area	Height
1	Catéchine (-)	C15H14O6	+	-13	291.1000>138.8500	8.509	75347	5348
2	Chrysine-6-C-glucoside	C21H20O9	+	-25	417.0000>296.9000	8.607	34901	2567
4	myricetin	C15H10O8	+	-20	318.5000>256.0500	8.430	135764	31530
5	Myricetine-3-rhamnose	C27H30O17	+	-44	465.0000>319.1000	8.516	35685	2389
6	Orxyline A	C16H12O5	+	-22	285.1000>269.9000	8.822	128610	25364
7	Quercetine-3-arabinose	C20H18O11	+	-15	435.0000>302.9500	8.254	23360	3680
8	Quercetine-3-glucoside	C21H19O12	+	-18	465.1000>303.0500	8.894	29578	7073
9	Tiliroside	C30H26O13	+	-18	595.0000>146.8500	8.352	36386	7130
10	Apigenin	C15H10O5	+	-33	271.1000>152.9000	8.539	57112	14164
13	Chrysin	C15H10O4	+	-27	254.8500>152.8000	8.806	712410	115916
14	Curcumin	C21H20O6	+	-20	368.9000>177.0500	9.790	1358400	146728
15	Epicatechin	C15H14O6	+	-15	290.8000>139.1500	8.543	72011	4237
17	Luteolin	C15H10O6	+	-32	286.7500>153.0000	8.642	159317	10541
18	Oleanolic Acid	C30H48O3	+	-15	457.3000>411.4000	8.723	404795	7425
19	Oleuropein	C25H32O13	+	-15	540.5000>523.4000	9.708	108064	4582
20	Quercetin	C15H10O7	+	-52	303.0500>153.1000	8.397	111901	6346
21	Resveratrol	C14H12O3	+	-22	229.0500>107.1000	8.261	134671	3839
22	Riboflavin	C27H30O16	+	-7	377.1000>360.3000	8.808	293662	14352

23	Rutin	C27H30O16	+	-10	611.0000>303.1000	8.570	71279	3769
24	Sinapic Acid	C11H12O5	+	-23	225.0000>207.1500	8.217	761157	28777
25	beta carotene	C40H56	+	-19	537.1000>280.9500	10.000	145847	5053
26	kojic acid	C6H6O4	+	-25	142.7500>69.0500	8.036	4654	1467
27	naringenin	C15H12O5	+	-43	273.0500>153.0000	8.427	113881	6179
28	tymol	C10H14O	+	-18	151.1500>108.9000	8.547	314193	19633
29	vannilin	C8H8O3	-	-15	153.0500>64.7500	8.245	59207	14110
30	Caffeic Acid	C9H8O4	-	34	179.1500>135.0000	8.113	1093162	267112
33	p-Coumaric Acid	C8H8O4	-	28	163.0500>118.9500	8.265	54538	11968
34	salicylic acid	C7H6O3	-	37	137.2000>92.9500	8.473	3410461	619654

S3

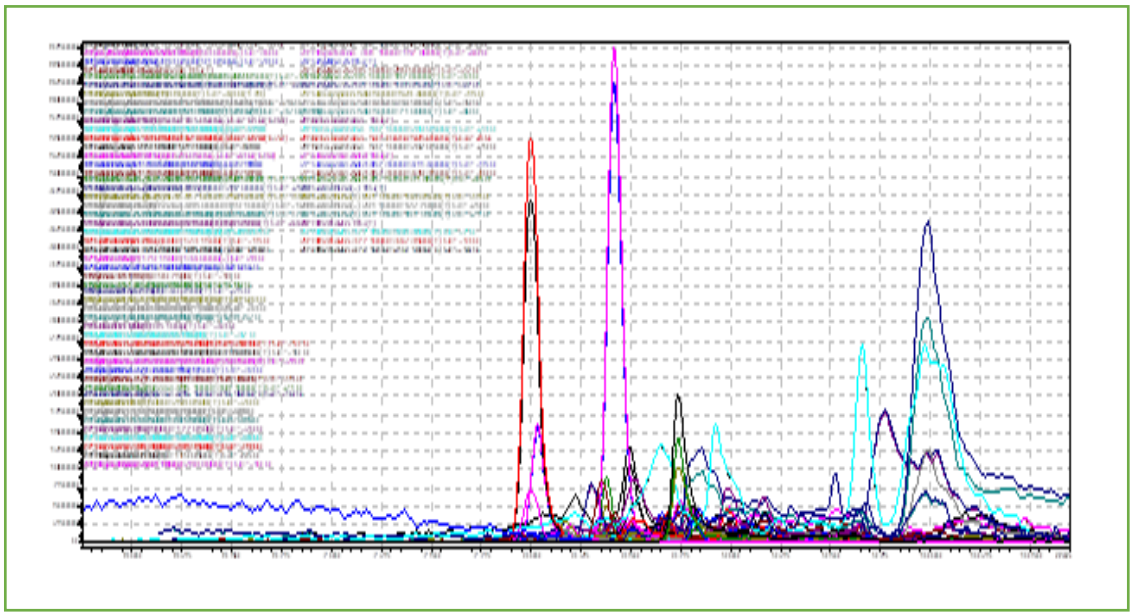

Figure 3. Chromatogram of sample 3

Table 3. Detection of compounds in sample 3

ID#	Name	Formula	ESI charge	CE (v)	Transition m/z	Ret. Time	Area	Height
1	Catéchine (-)	C15H14O6	+	-13	291.1000>138.8500	8.509	75347	5348
2	Chrysine-6-C-glucoside	C21H20O9	+	-25	417.0000>296.9000	8.607	34901	2567
4	myricetin	C15H10O8	+	-20	318.5000>256.0500	8.379	193475	51035
5	Myricetine-3-rhamnose	C27H30O17	+	-44	465.0000>319.1000	8.450	11147	3166
6	Orxyline A	C16H12O5	+	-22	285.1000>269.9000	8.752	582378	40073
7	Quercetine-3-arabinose	C20H18O11	+	-15	435.0000>302.9500	8.185	41355	7063
8	Quercetine-3-glucoside	C21H19O12	+	-18	465.1000>303.0500	8.821	64988	9978
9	Tiliroside	C30H26O13	+	-18	595.0000>146.8500	8.279	55540	10571
10	Apigénine	C15H10O5	+	-33	271.1000>152.9000	8.486	179825	27656
11	Hispiduline	C16H12O6	+	-10	301.1000>268.8000	8.449	528954	31842
13	Chrysin	C15H10O4	+	-27	254.8500>152.8000	8.737	1116800	144126

14	Curcumin	C21H20O6	+	-20	368.9000>177.0500	9.775	3700175	172212
15	Epicatechin	C15H14O6	+	-15	290.8000>139.1500	8.488	83495	6953
16	Ferulic Acid	C10H10O4	+	-16	195.0000>44.7000	8.355	386940	81830
17	Luteolin	C15H10O6	+	-32	286.7500>153.0000	8.588	223934	13195
18	Oleanolic Acid	C30H48O3	+	-15	457.3000>411.4000	8.995	386837	16448
19	Oleuropein	C25H32O13	+	-15	540.5000>523.4000	9.289	6014	2054
20	Quercetine	C15H10O7	+	-52	303.0500>153.1000	8.344	54947	9918
21	Resveratol	C14H12O3	+	-22	229.0500>107.1000	8.224	86333	4212
22	Riboflavin	C27H30O16	+	-7	377.1000>360.3000	9.083	48043	6320
23	Rutin	C27H30O16	+	-10	611.0000>303.1000	8.453	114250	6888
25	beta carotene	C40H56	+	-19	537.1000>280.9500	8.797	22914	7344
26	kojic acid	C6H6O4	+	-25	142.7500>69.0500	7.940	4344	2132
27	naringenin	C15H12O5	+	-43	273.0500>153.0000	8.414	23581	6679
28	tymol	C10H14O	+	-18	151.1500>108.9000	8.473	102077	17080
29	vannilin	C8H8O3	-	-15	153.0500>64.7500	8.171	72196	15612
30	Caffeic Acid	C9H8O4	-	34	179.1500>135.0000	7.998	2298596	435381
33	p-Coumaric Acid	C8H8O4	-	28	163.0500>118.9500	8.197	96825	20714
34	salicilic acid	C7H6O3	-	37	137.2000>92.9500	8.416	3025561	603959

S4

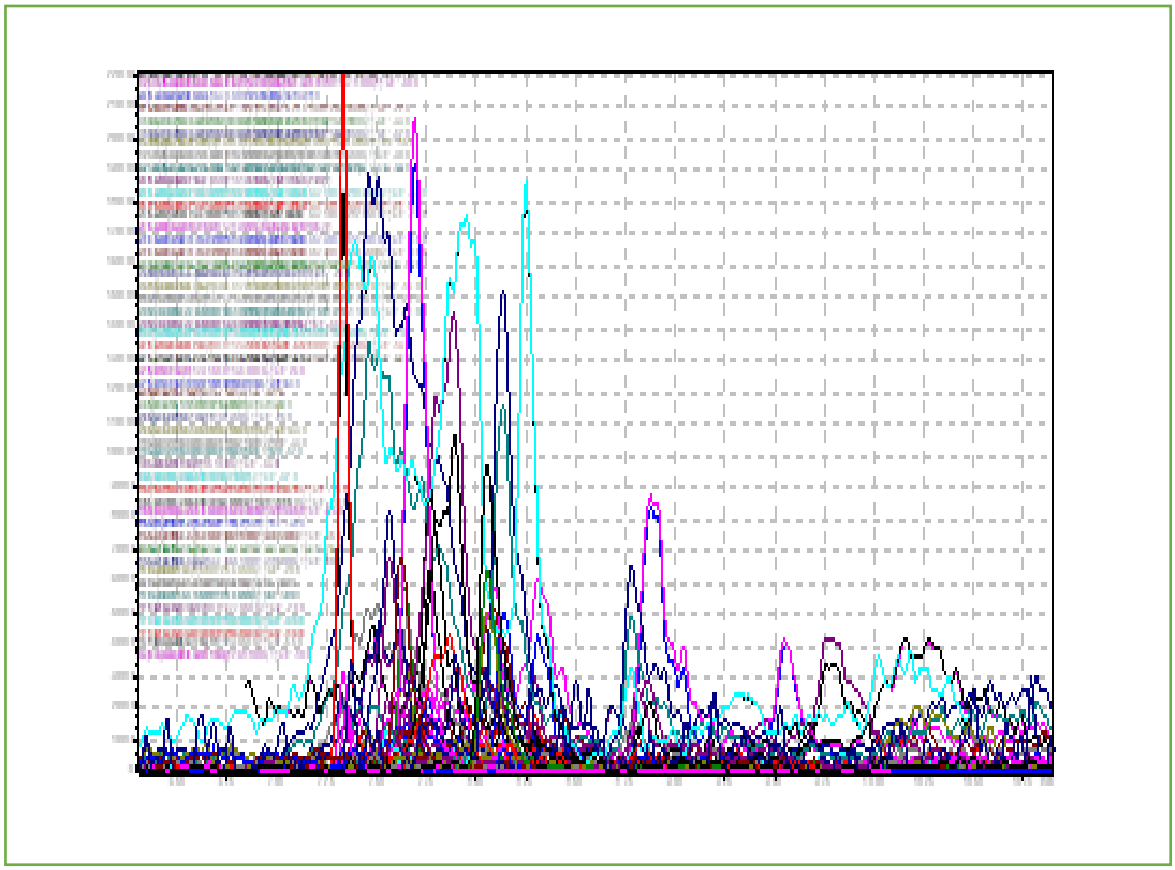

Figure 4. Chromatogram of sample 4

Table 4. Detection of compounds in sample 4

ID#	Name	Formula	ESI charge	CE (v)	Transition m/z	Ret. Time	Area	Height
1	Catéchine (-)	C15H14O6	+	-13	291.1000>138.8500	11.017	1051	635
3	Myricetin 3-arabinoside	C20H18O12	+	-13	450.9000>318.9500	8.373	10725	2866
4	myricetin	C15H10O8	+	-20	318.5000>256.0500	7.639	180434	36522
5	Myricetine-3-rhamnose	C27H30O17	+	-44	465.0000>319.1000	7.398	4277	2112
6	Orxyline A	C16H12O5	+	-22	285.1000>269.9000	8.074	55852	14939
7	Quercetine-3-arabinose	C20H18O11	+	-15	435.0000>302.9500	7.439	24337	4540
8	Quercetine-3-glucoside	C21H19O12	+	-18	465.1000>303.0500	7.433	11021	4642
9	Tiliroside	C30H26O13	+	-18	595.0000>146.8500	7.549	22086	8181
10	Apigénine	C15H10O5	+	-33	271.1000>152.9000	7.775	40886	9415
11	Hispiduline	C16H12O6	+	-10	301.1000>268.8000	7.479	121218	24656
13	Chrysin	C15H10O4	+	-27	254.8500>152.8000	8.055	518637	64394
14	Curcumin	C21H20O6	+	-20	368.9000>177.0500	7.515	905715	43746
16	Ferulic Acid	C10H10O4	+	-16	195.0000>44.7000	7.622	398683	66109
17	Luteolin	C15H10O6	+	-32	286.7500>153.0000	7.880	94456	7444
18	Oleanolic Acid	C30H48O3	+	-15	457.3000>411.4000	8.877	654571	77870
20	Quercetine	C15H10O7	+	-52	303.0500>153.1000	7.656	20101	3946
21	Resveratol	C14H12O3	+	-22	229.0500>107.1000	7.404	3817	1953
22	Riboflavin	C27H30O16	+	-7	377.1000>360.3000	10.925	5783	4221
25	beta carotene	C40H56	+	-19	537.1000>280.9500	8.802	218839	9393
26	kojic acid	C6H6O4	+	-25	142.7500>69.0500	6.694	10521	866
27	naringenin	C15H12O5	+	-43	273.0500>153.0000	7.648	85132	6108
28	tymol	C10H14O	+	-18	151.1500>108.9000	7.767	38799	8334
29	vannilin	C8H8O3	-	-15	153.0500>64.7500	7.462	35199	9435
30	Caffeic Acid	C9H8O4	-	34	179.1500>135.0000	7.332	603126	178458
34	salicilic acid	C7H6O3	-	37	137.2000>92.9500	7.692	1286507	187539

S5

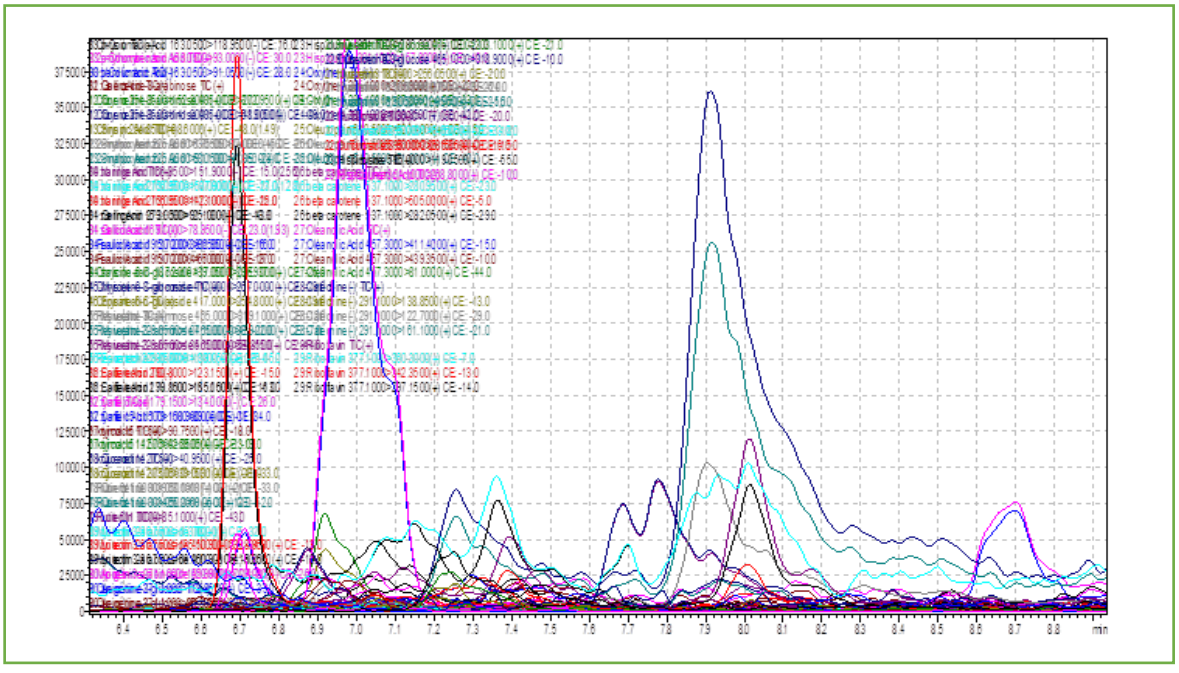

Figure 5. Chromatogram of sample5

Table 5. Detection of compounds in sample 5

ID#	Name	Formula	ESI charge	CE (v)	Transition m/z	Ret. Time	Area	Height
1	Catéchine (-)	C15H14O6	+	-13	291.1000>138.8500	10.965	877	528
2	Chrysine-6-C-glucoside	C21H20O9	+	-25	417.0000>296.9000	7.074	3342	1257
3	Myricetin 3-arabinoside	C20H18O12	+	-13	450.9000>318.9500	7.324	250558	23482
4	myricetin	C15H10O8	+	-20	318.5000>256.0500	6.919	306001	43031
6	Orxyline A	C16H12O5	+	-22	285.1000>269.9000	7.198	19667	4864
7	Quercetine-3-arabinose	C20H18O11	+	-15	435.0000>302.9500	6.827	12151	3073
8	Quercetine-3-glucoside	C21H19O12	+	-18	465.1000>303.0500	6.947	42705	4511
9	Tiliroside	C30H26O13	+	-18	595.0000>146.8500	6.900	17588	3643
10	Apigénine	C15H10O5	+	-33	271.1000>152.9000	7.124	45259	4365
11	Hispiduline	C16H12O6	+	-10	301.1000>268.8000	9.539	20084	8592
13	Chrysin	C15H10O4	+	-27	254.8500>152.8000	7.242	284867	26884
15	Epicatechin	C15H14O6	+	-15	290.8000>139.1500	7.078	36515	2129
16	Ferulic Acid	C10H10O4	+	-16	195.0000>44.7000	6.919	78450	15002
17	Luteolin	C15H10O6	+	-32	286.7500>153.0000	7.073	69623	4723
18	Oleanolic Acid	C30H48O3	+	-15	457.3000>411.4000	8.697	532619	62302
20	Quercetine	C15H10O7	+	-52	303.0500>153.1000	7.910	80668	4255
21	Resveratol	C14H12O3	+	-22	229.0500>107.1000	6.848	48639	3194
25	beta carotene	C40H56	+	-19	537.1000>280.9500	9.748	61547	6067
26	kojic acid	C6H6O4	+	-25	142.7500>69.0500	6.630	14719	2063
27	naringenin	C15H12O5	+	-43	273.0500>153.0000	6.991	68658	6780
28	tymol	C10H14O	+	-18	151.1500>108.9000	7.043	123259	9221
29	vannilin	C8H8O3	-	-15	153.0500>64.7500	6.799	45068	7442
30	Caffeic Acid	C9H8O4	-	34	179.1500>135.0000	6.690	1205532	319468
33	p-Coumaric Acid	C8H8O4	-	28	163.0500>118.9500	6.796	46080	9465
34	salicilic acid	C7H6O3	-	37	137.2000>92.9500	6.981	3273907	384939

LC-MS/MS Analysis of Bioactive Compounds in propolis Extracts for Anti-Inflammatory and

Antidiabetic Potential

Materials and Methods

Instrumentation

The analyses were performed using a Shimadzu 8040 UPLC-ESI-MS-MS system equipped with Ultra-Fast Mass Spectrometry (UFMS) technology and a binary pump Nexera XR LC-20AD. The system was configured with a Restek Ultra C18 column (3 µm, 150 x 4.6 mm) for separation.

LC-MS/MS Conditions

- Mobile Phase: Solvent A (water with 0.1% formic acid) and Solvent B (methanol).
- Gradient Elution:
  - ✓ 0–0.2 min: 98% A
  - ✓ 0.2–2.5 min: 25% A
  - ✓ 2.5–4 min: 0% A
  - ✓ 4–7 min: 0% A
  - ✓ 7–7.1 min: 98% A
  - ✓ 7.1–12 min: 98% A
- Flow Rate: 0.2 mL/min
- Injection Volume: 5 µL
- ESI Conditions:
  - ✓ CID gas: 230 kPa
  - ✓ Conversion dynode: −6.00 kV
  - ✓ Desolvation line (DL) temperature: 250°C
  - ✓ Nebulizing gas flow: 3.00 L/min
  - ✓ Heat block temperature: 400°C
  - ✓ Drying gas flow: 10 L/min

Data Analysis

The LC-MS/MS data included compound names, chemical formulas, ESI charge, collision energy (CE), mass transitions (m/z), retention times, peak areas, and peak heights. Compounds were identified based on their mass transitions and retention times. Peak areas were used to assess relative abundance, with non-detected compounds marked by zero retention time and area. The data were analyzed to identify consistently detected compounds with high abundance and known bioactivity for anti-inflammatory and antidiabetic effects.

Bioactivity Assessment

The compounds were evaluated for their anti-inflammatory and antidiabetic potential based on **literature evidence:**

- **Flavonoids:**
  - ✓ Quercetin: Inhibits COX-2 (IC50 ~10 µM), NF-κB, and α-glucosidase (IC50 ~4 µM), activates AMPK and GLUT4.

- ✓ Myricetin: Inhibits COX-2 (IC50 ~15 μM), LOX, and α-glucosidase (IC50 ~2 μM), activates PPAR-γ.
- ✓ Luteolin: Inhibits NF-κB, MAPK, and α-glucosidase, enhances insulin signaling.
- ✓ Apigenin: Inhibits COX-2, NF-κB, and α-glucosidase, activates PPAR-γ.
- ✓ Naringenin: Inhibits NF-κB, TNF-α, and α-glucosidase, improves glucose uptake.
- ✓ Catechin/Epicatechin: Inhibits COX-2, NF-κB, and α-glucosidase, enhances insulin sensitivity.
- ✓ Chrysin: Inhibits COX-2 and NF-κB, but limited antidiabetic activity.

- **Phenolic Acids:**
  - ✓ Caffeic Acid: Inhibits COX-2, LOX (IC50 ~50 μM), and α-glucosidase (IC50 ~20 μM), enhances glucose uptake.
  - ✓ Ferulic Acid: Inhibits COX-2, NF-κB, and α-glucosidase, improves insulin sensitivity.
  - ✓ Salicylic Acid: Inhibits COX-1/2, but limited antidiabetic evidence.
- **Other Compounds:**
  - ✓ Curcumin: Inhibits NF-κB, COX-2 (IC50 ~20 μM), and α-glucosidase, activates PPAR-γ.
  - ✓ Oleanolic Acid: Inhibits NF-κB, α-glucosidase (IC50 ~10 μM), enhances insulin signaling.
  - ✓ Resveratrol: Inhibits COX-2, NF-κB, and α-glucosidase, activates SIRT1.