DETERMINATION OF BIOACTIVITIES OF *Thesium aureum* Jaub.&Spach SPECIES AND ELUCIDATION OF ITS PHYTOCHEMICAL CONTENET BY LC-MS/MS

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FEBRUARY 2025

DIYARBAKIR

GRADUATE SCHOOL
OF NATURAL AND APPLIED SCIENCES
OF DİCLE UNIVERSITY

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BY
HELIN SULIMAN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE IN
CHEMISTRY

FEBRUARY 2025

DİYARBAKIR

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Dedicated to My Children...

I hereby declare that all information in this document has been obtained and presented following the Thesis Manual (prepared by the Dicle University Graduate School of Natural and Applied Sciences), academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work. I shall be solely responsible if any evidence is found against my declaration.

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ACKNOWLEDGEMENTS

First and foremost, I want to express my gratitude to my advisor, Assoc. Prof. Dr. Mustafa Abdullah YILMAZ, for his important guidance, support, and patience during the whole thesis completion process. He also helped me understand the analyses and make necessary revisions to the draft thesis. I also want to express my gratitude to my jury members for their critiques and recommendations on how to improve the quality of my thesis.

I express my gratitude to Assoc. Prof. Dr. Oğuz ÇAKIR for sharing his expertise and support throughout the fieldwork. Additionally, I am grateful to Prof. Dr. Göksel KIZIL, and Prof. Dr. Mehmet BOĞA for sharing their expertise and guiding me in overcoming the challenges.

I am especially grateful to my family and friends, whose unflinching believe in me and support have kept me going throughout this trip. I sincerely appreciate your understanding, love, and patience.

Furthermore, I would like to express my deep appreciation to all my professors in the Department of Chemistry at Dicle University who supported my academic journey and helped me in this thesis, either directly or indirectly.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Definition
°C	Celsius degree
g	Gram
kg	Kilogram
L	Liter
M	Molarity
m	Meter
min	Minute
mg	Milligram
mL	Milliliter
mM	Millimolar
mm	Millimeter
mmol	Millimole
µL	Microliter
nm	Nanometer

Abbreviation	Definition
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Acarbose equivalents
AChE	Acetylcholinesterase
AD	Alzheimer's disease
APCI	Atmospheric pressure chemical ionization.
APPI	Atmospheric pressure photoionization.
ATP	Adenosine triphosphate
BChE	Butyrylcholinesterase
CUPRAC	CUPric reducing antioxidant capacity
D3	deuterium-labeled internal standard

DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
Ir-Tr	Iran-Turkiye
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionisation
ET	Electron transfer
F-C	Folin-Ciocalteu
FCR	Folin-Ciocalteu reagent
FOX	Ferrous oxidation-xylenol
FRAP	Ferric reducing antioxidant power
FTC	Ferric thiocyanate
GAE	Gallic acid equivalent
GC	Gas chromatography
GLP-1	Glucagon-like peptide-1
GSH-Px	Glutathion peroxydase
HAT	Hydrogen atom transfer
KAEs	Kojic acid equivalents
kDa	Kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
PFRAP	potassium ferricyanide reducing power
pH	Potential of hydrogen
PRI	Partial reversible inhibitor
RE	Routine equivalent
REACH	Registration, evaluation, authorisation and restriction of chemicals
REP	Retinal epithelial pigment
ROS	Reactive oxygen species
SET	Single electron transfer
SOD	Superoxide dismutase
T.	*Thesium*

TE	Trolox equivalent
T2DM	Type 2 diabetes mellitus
TLC	Thin layer chromatography
TMC	Transition metal chelation
TPC	Total phenolic content
U-HPLC	Ultrahigh performance liquid chromatography
US	United States
UV/Vis	Ultraviolet/Visible

ABSTRACT

DETERMINATION OF BIOACTIVITIES OF *Thesium aureum* JAUP.&SPACH AND ELUCIDATION OF ITS PHYTOCHEMICAL CONTENT BY LC-MS/MS

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Master of Science in Department of Chemistry
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February 2025, 98 pages

Human beings have been using natural herbal materials for various purposes, such as treating various diseases, since time immemorial. Medicinal plants are a vital source of modern and traditional drugs, pharmaceutical intermediates, nutrients, nutritional supplements and herbal medicines. Plants produce many different molecules that act on targeted constituents in the complicated cellular pathways by interacting separately or synergistically. Conducting scientific research on plants in terms of biological activity and identifying plant compounds is among the the most active research fields world wide, out of belief in the importance of plants in discovering new and safer treatments. Since it was important to investigate the phytoconstituents potentially accountable for the biological impacts, we have benefited from the liquid chromatography tandem mass spectrometry apparatus for examining the ethanolic extract's chemical composition of the *Thesium aureum* species (mainly phenolic acids and flavonoids). The component with the greatest concentration in the ethanolic extract of the pieces of *Thesium aureum* has been identified as quinic acid (199 mg analyte/kg extract). Also we determined the presence of naringenin (3 mg analyte/kg extract), luteolin (9 mg analyte/kg extract), apigenin (3 mg analyte/kg extract), chrysin (5 mg analyte/kg extract), acacetin (37 mg analyte/kg extract), isoquercitrin (9 mg analyte/kg extract), and hesperidin (49 mg analyte/kg extract). We also studied the efficacy of the *Thesium aureum* species' ethanolic extract by conducting bioactivity tests related to the inhibition of the butyrylcholinesterase (BChE), tyrosinase, and acetylcholinesterase (AChE). Also we assessed the antihyperglycemic effectiveness of the *Thesium aureum* species' ethanolic extract by evaluate the potential inhibitory impact on enzymes that break down carbohydrates, including α-glucosidase and also α-amylase. The biological activity was also assessed by using different tests for antioxidants capacity, such as the activity for scavenging free radicals utilizing the ABTS and also DPPH assays, metal chelation test, iron and copper reduction potency methods (FRAP and CUPRAC), as well as the total antioxidant capacity (phosphomolybdenum). We have conducted several methods to test antioxidant effectiveness because one test was not enough. The analytical techniques were carried out using the spectrophotometric concept.

Keywords: Alzheimer's disease, *Thesium aureum*, Secondary metabolites, Enzyme inhibition, Antioxidants

ÖZET

Thesium aureum JAUP.&SPACH'IN BİYOAKTİVİTESİNİN BELİRLENMESİ VE FİTOKİMYASAL İÇERİĞİNİN LC-MS/MS İLE AYDINLATILMASI

SULIMAN, Helin.
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Şubat 2025, 98 pages

İnsanlar, çok eski zamanlardan beri çeşitli hastalıkları tedavi etmek gibi çeşitli amaçlar için doğal bitkisel malzemeler kullanmaktadır. Tıbbi bitkiler, modern ve geleneksel ilaçların, farmasötik ara maddelerin, besinlerin, besin takviyelerinin ve bitkisel ilaçların hayati bir kaynağıdır. Bitkiler, karmaşık hücresel yollardaki hedeflenen bileşenler üzerinde ayrı ayrı veya sinerjik olarak etkileşime girerek hareket eden birçok farklı molekül üretir. Bitkiler üzerinde biyolojik aktivite açısından bilimsel araştırma yapmak ve bitki bileşiklerini tanımlamak, yeni ve daha güvenli tedaviler keşfetmede bitkilerin önemine olan inançtan dolayı tüm dünyada aktif araştırma alanlarından biridir. Biyolojik etkilerden potansiyel olarak sorumlu olan fitokonstitüentleri araştırmak önemli olduğundan, araştırmamızda *Thesium aureum* türlerinin etanolik ekstresinin kimyasal bileşimini (esas olarak fenolik asitler ve flavonoidler) incelemek için sıvı kromatografi tandem kütle spektrometrisi cihazı kullanılmıştır. *Thesium aureum* türünün etanolik ekstresinde en yüksek konsantrasyona sahip bileşen kinik asit (199 mg analit/kg ekstre) olarak tanımlanmıştır. Ayrıca naringenin (3 mg analit/kg ekstre), luteolin (9 mg analit/kg ekstre), apigenin (3 mg analit/kg ekstre), krisin (5 mg analit/kg ekstre), akasetin (37 mg analit/kg ekstre), izo kuersitrin (9 mg analit/kg ekstre) ve hesperidin (49 mg analit/kg ekstre) varlığını belirledik. Ayrıca, tirozinaz, asetilkolinesteraz (AChE) ve bütirilkolinesteraz (BChE) enzimlerinin inhibisyonuyla ilgili biyoaktivite testleri yürüterek bu türün etanolik özütünün etkinliğini de inceledik. Ayrıca, *Thesium aureum* türünün etanolik özütünün antihiperglisemik etkinliğini, özellikle α-amilaz ve α-glukozidazı hedef alarak karbonhidrat sindirim enzimleri üzerindeki potansiyel inhibitör etkisini değerlendirerek değerlendirdik. Biyolojik aktiviteler ayrıca, (2,2-difenil-1-pikrilhidrazil) DPPH testi ve ABTS testi, metal şelasyon testi, demir ve bakır indirgeme potens yöntemleri (CUPRAC ve FRAP) ve toplam antioksidan kapasitesi (fosfomolibden) gibi serbest radikal temizleme aktiviteleri gibi antioksidan kapasitesi için farklı testler kullanılarak değerlendirildi. Tek bir test yeterli olmadığından antioksidan etkinliğini test etmek için birkaç yöntem yürüttük. Analitik teknikler, spektrofotometrik konsept kullanılarak gerçekleştirildi.

Anahtar Kelimeler: Alzheimer hastalığı, *Thesium aureum*, Sekonder metabolitler, Enzim inhibisyonu, Antioksidanlar

1. INTRODUCTION

Humans have used plants to heal from diseases since time immemorial, by using the entire plant or specific parts of it. Scientists have found, through the fossils, that people have been using plants to heal for about 60,000 years. Human societies have accumulated experience with natural products to produce various medicinal systems, such as Chinese medicine, European medicine, Indian medicine, Japanese kambo, and prophetic medicine. Medicinal plants represent cultural and civilizational symbols in different societies in our current era, and the use of them to confront and prevent various diseases is still at a high degree. Indeed, people's demand for them increases day after day to treat all their health problems, and this has increased the number of producers of natural medical medicines.

Natural products have proven their effectiveness as anti-inflammatories, anti-rheumatics, antimicrobials, antidiabetics, and anti agings and gained wide acceptance for treating liver cirrhosis, asthma, chronic fatigue, Alzheimer's disease, and a host of other illnesses. Also they are well known factors for relaxation, and improving memory. Official statistics say that 80 % of people currently turn to herbs and herbal formulations as their first choice in treating any health disorder they are exposed to. In response, the World Health Organization has established special guidelines for the quality of plant-based medicines and the safety of their use, following this growing interest among people in plant-based medicines, which they consider to be inexpensive, easy to access, and with very few or no side effects compared to modern manufactured medicines with toxicity and negative reactions, in addition to the difficulty of their availability in many countries. Sometimes, its cost is high. Modern medicine, in turn, is increasingly interested day after day in the chemical compounds that medicinal plants contain, as nearly 200,000 different chemicals have been identified and isolated by researchers. Phytochemical compounds may have therapeutic potential individually or have a synergistic effect when they are present alongside other compounds (Shrestha et al., 2024; Saggar et al., 2022).

The great change in human habits, diet, and physical activity in the past thirty years has led to a noticeable increase in diabetes, which is a chronic condition with severe

complications and requires enormous spending, since its treatment lasts a lifetime. The disease results either from insufficient insulin secretion, which is called type 1 diabetes, or from insulin resistance, which is called type 2 diabetes, and is distinguished by a consistently elevated blood glucose level. The number of diabetics in 2019 reached 463 million, as stated by the International Federation of Diabetes, with diabetes of the second type accounting for almost 90% of cases (Lam et al., 2024; Ge et al., 2023).

Many millions of individuals suffer from Alzheimer's disease around the world, which constitutes a major economic and social burden. Alzheimer's disease ranks seventh among the diseases that cause death globally, and the rate of this disease is still increasing, as it is expected to reach 113 million worldwide in 2050. Preventing the hydrolysis of acetylcholine - the neurotransmitter - by inhibiting the cholinesterase enzyme is based on postponing the aggravation of Alzheimer's symptoms (Chen et al., 2017). Neuron loss (shrinkage of brain tissue) due to Alzheimer's disease leads to dementia, gradual loss of motor abilities, and then death. Researchers are rushing to search for cholinesterase inhibitors from natural sources as more accurate, effective and less expensive treatments (Jain et al., 2025).

Tyrosine is a oxidative enzyme which is essential in the melanin formation process. Many skin diseases occur due to hyperpigmentation such as pregnancy pigmentation, fatal skin cancer, freckles, and age spots. the browning that affects fruits and plants as well, which affects the quality of agricultural products and their economic returns, results from the imposition of the activity of the tyrosine enzyme. We also have insects that depend on the tyrosine enzyme for their defense mechanisms against insecticides, which greatly affects the Agriculture sector. From a neurological health perspective, it is also found that increased activity of this oxidative enzyme causes Parkinson’s disease and other neurological diseases (Najafi et al., 2024). Melanin is responsible for protection from the ultraviolet rays of the sun in living organisms, and it has an important role as a scavenger of free radicals, metal chelating and as a redox. this pigment is a double-edged sword sometimes, so the need to inhibit melanin production is a necessary requirement in various cells, and it is an important

topic for researchers in various specialties, such as medicine, cosmetic, and agriculture (Solano, 2014).

Free radicals are produced in our bodies naturally as products of metabolic processes, or during the body's exposure to radioactive and chemical pollutants such as X-rays, industrial chemicals, polluted air, and cigarettes. The body can deal with these radicals in quantities within normal limits, but their abundance and accumulation in the body causes serious dangers, such as damage to DNA, proteins, and fats. You can imagine the endless number of diseases resulting from this, such as high blood pressure, asthma, cataracts, heart disease, Alzheimer's, diabetes, and cancer. Therefore, we need additional antioxidants in order to neutralize the excess free radicals and nullify their catastrophic effects on cell damage. It has been found that phenolic acids and flavonoids work as powerful antioxidants. Which can be obtained from herbs, vegetables and fruits (Liu et al., 2024; Shrestha et al., 2024).

Phenols are very diverse and important chemical compounds produced by plants as secondary metabolites to protect them from harmful sunlight and to fight pathogens in plants, in addition to their role in plant growth and reproduction. They are also responsible for giving the plant its distinctive colour. Phenols have also proven to be of great importance to humans through their action as antioxidants that protect us from all diseases resulting from the oxidative stress in the body. Of the phenolic chemicals, flavonoids are the most worthwhile category and the most diverse. Researches have proven their antibacterial, anti-cancer, anti-viral, and also anti-inflammatory properties, beside their contribution to liver protection. The expanded medicinal ability of flavonoids and their great role in promoting health comes from the great diversity of their chemical structure (Shrestha et al., 2024).

Analytical techniques that identify the naturally occurring compounds in therapeutic as well as fragrant plant species should satisfy the standards for separation efficiency, sensitivity, and selectivity. Given that matrix systems are usually quite complicated and that the chemicals of curiosity could exist in trace quantities, the researchers must look for much more trustworthy technologies compared to UV/Vis spectrophotometry or thin-layer chromatography (TLC). Furthermore, capillary

electrophoresis can occasionally have repeatability issues, and GC is basically restricted to substances with volatile characteristics. MS/MS tandem mass spectrometry paired with U-HPLC -ultrahigh performance liquid chromatography- is the latest and greatest technique has been used for identification and quantification of different kinds of phenolic compounds in plant matrices (Yilmaz, 2020).

Thesium is the biggest genus that belongs to the Santalaceae family, containing approximately 350 species. *Thesium* is distributed on all continents except Antarctica, and it is worth noting that almost half of the species are found in South Africa, specifically in the Cape Florist region. There are various uses of many types of *Thesium* in the cultures of African and Asian peoples, and researches have reinforced the importance of these plants in the modern era (Miguel et al., 2024; Lombard et al., 2022).

Türkiye, because it is affected by different climates, represents the intersection of three different phytogeographic regions, and also has different topographic structures and soil groups, is also a genetic center for some plant species; All of this made it a country with a very large plant diversity, and also made it unique in the presence of some plants (Davis and Hedge, 1975). Among these endemic plants is *Thesium aureum* species. A review of the literature revealed that no research has been carried out concerning the chemical makeup or the biological efficacy of the *Thesium aureum* species.

There is no research by scientists has been done regarding *Thesium aureum* species at all. Therefore, in this study and for the first time at all, we revealed the phytochemical content of the aerial along with root portions of *Thesium aureum* spices, particularly the phenolics in its ethanol extract. We also studiedthe *Thesium aureum* species's biochemical effectiveness in treating incurable maladies such as diabetes, hyperpigmentation, and Alzheimer's disease. We are also tested its effectiveness as an antioxidant. This study will constitute a practical starting point for other studies on *Thesium aureum* species, which is a member of the significant medicinal genus *Thesium*, and will enable the identification of other new sources of raw materials in line with the data obtained.

2. LITERATURE REVIEW

2.1 Enzymes

Enzymes are proteins that work as catalysts in biological systems. The activity of an enzyme is understood to be especially dependent on its shape. Among biological compounds, enzymes are most likely the most researched. They are the tools that nature uses to create and decompose the chemicals that cells need for repair, maintenance, growth, and death. Enzymes are needed for almost all biological processes at some point. Even the most accomplished organic chemists seldomly accomplish the complicated transformations that enzymes may perform in water solution at physiological pH and temperature in a stereospecific and regiospecific way (Yurt et al., 2024).

The natural catalysts, enzymes facilitate numerous kinds of chemical reactions in biological systems. Enzyme kinetic studies, as they are collectively called, are concerned with identifying the catalysis rate, substrate affinity, and inhibitor effects in order to better understand how enzymes work. When researchers called for an explanation to better understand how substrate and enzyme quantities linked to substrate turnover rates (catalysis), the study of enzyme catalysis was initiated in the last decade of the nineteenth century. The formation of the fundamental enzyme kinetic mechanism, depending on some logical assumptions, made it possible to conduct the first research of the kinetics of enzymes.

$$E + S \rightleftharpoons ES \rightarrow E + P$$

(S: substrate), (E: enzyme), (P: product) (Mak, 2024).

Enzymes are also used as an additive to detergents to make them effective with the least possible energy, which gives them great economic importance and also an effective role in preserving the environment (Al-Ghanayem and Joseph, 2020). Several facets of the industrial uses of the enzymes are including the cleaning products and textile industry, the bioremediation and ecological applications, the food and fermenting sector, in addition to the beauty and pharmaceutical industries (Kumar et al., 2021; Hamid et al., 2022). Currently existing world market magnitude for enzymes is estimated to be 12.27 billions of dollars in 2022. In the period between 2023 and 2030, it is anticipated to increase at a yearly growth rate of 6.5 %.

More importantly, it is anticipated that up to 40 % of industrial chemical processes involving hazardous solvents would be replaced by enzymatic reactions (Xu et al., 2024).

2.1.1 Classification of enzymes

a) *Oxidoreductases:* The enzymes in this class are responsible for catalyzing oxidation processes. They are categorized as oxidoreductases because the oxidation of one group necessitates the reduction of another.

b) *Transferases:* Based on the overall process, these enzymes transport a group from one substrate to another one (from the donor to the acceptor).

$$X–Y + Z = X + Y–Z$$

c) *Hydrolases:* These enzymes catalyze the hydrolytic dissociation of different bonds, such as phosphoric anhydride bonds, as well as bonds like C–O, C–C, and C–N. It is challenging to set up broad guidelines that apply to every single member of this class due to the overlapping specificities of many of these enzymes.

d) *Lyases:* Other bonds beyond those created by oxidation or hydrolysis are broken by these enzymes, such as C–N, C–O, as well as C–C. Unlike the rest of the enzymes, they can include two substrates in a particular direction of the process but only one in the opposite direction One way to conceptualize the reaction is as an internal transfer where a molecule is taken out, leaving double bonds or rings behind, when they operate on a single substrate.

e) *Isomerases:* The structure or geometry of molecules can be altered by these enzymes. Some names for them include cycloisomerases, racemases, isomerases, epimerases, tautomerases, (cis-trans) isomerases, and mutases, depending on the kind of isomerism that is involved.

f) *Ligases:* The ligature of two molecules is catalyzed by these sort of enzymes. while also hydrolyzing a diphosphate bond in ATP or another similar triphosphate.

g) *Translocases:* These types of enzymes facilitate the movement of ions as well as molecules across membranes and their separation inside them (McDonald et al., 2015).

2.1.2 Enzyme inhibition

Compounds known as modifiers alter the pace at which catalytic enzyme reactions occur. Typically, "inhibition" is used to express an impact that lowers the rate. One of the most important methods of regulation for live cells and a crucial diagnostic procedure for biologists studying enzymology is the suppression of enzyme activity. The circumstance that the inhibitor, substrate, and enzyme interact is what controls this. For pharmacological research, this topic is of great interest. The process of enzyme inhibition is categorized into two main groups: irreversible and reversible inhibition. For a simpler explanation, each group is further subdivided into smaller groups. There are four types of reversible inhibition: mixed, uncompetitive, non-competitive, and competitive inhibition (Hajizadeh et al., 2022) (Figure 1).

Competitive inhibition is an inhibition form, when an inhibitor molecule -that resembles an enzyme's typical substrate in structure- becomes attached to the active site reversibly, decreasing the period of enzyme that is accessible. Only the substrate-enzyme combination is bound by the inhibitor in uncompetitive inhibition (Zhao et al., 2012).

When there is noncompetitive inhibition, the inhibitor has the same level of affinity for both the enzyme as well as the complex of the substrate and enzyme, because the allosteric site is where it binds, independent of substrate binding. Once an inhibitor attaches to the enzyme or the complex of enzyme and substrate, it stops the enzyme from producing its product. Since the inhibitor and substrate do not compete with one another for binding to the active site, the following decline in enzyme activity is unaffected by the concentration of the substrate. Noncompetitive inhibition reduces the maximum rate of an enzyme-catalyzed process while leaving the enzyme's affinity for its substrate unchanged (Delaune and Alsayouri, 2019).

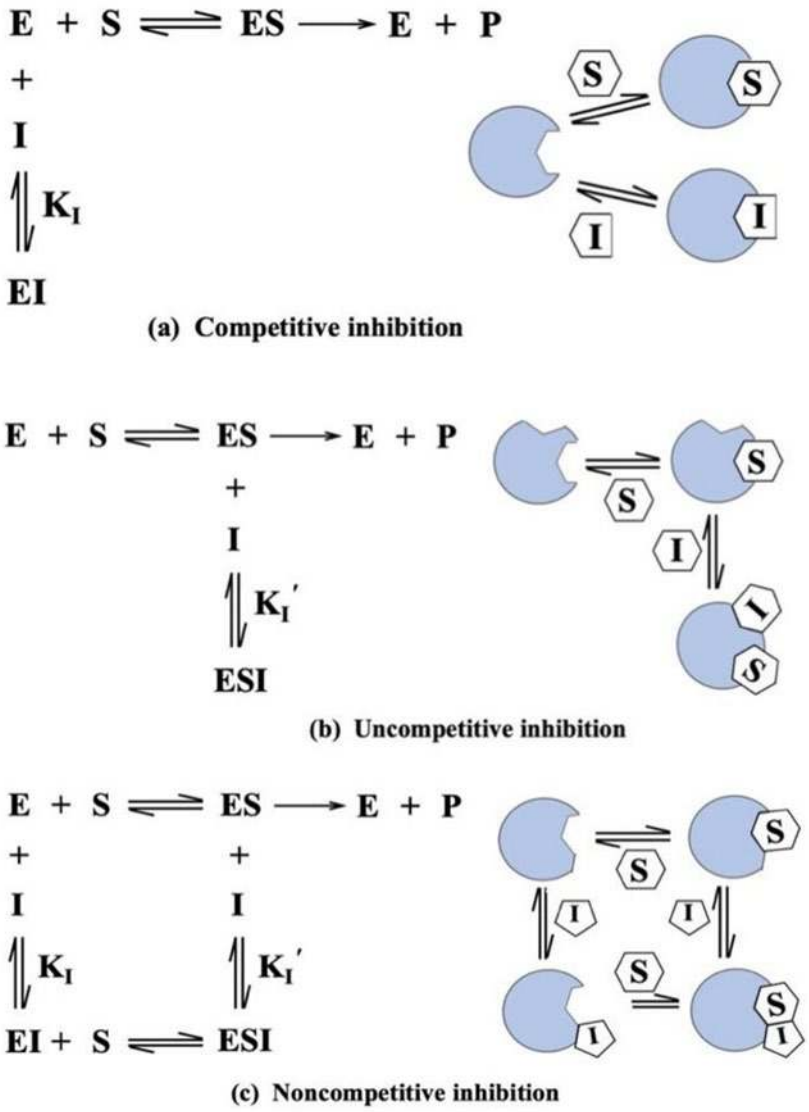


Figure 2.1 Different types of reversible inhibition (Zhao et al., 2012)

The inhibition caused by irreversible inhibitors is typically irreversible because they covalently alter the enzyme. Irreversible inhibition happens when a substance attaches to an enzyme so firmly that it is unable to dissociate. Irreversible inhibition of an enzyme often comes with kinetics that are first- order (Hajizadeh et al., 2022). The process of reversible inhibition of enzymes happens quite rapidly. The formation of the enzyme–inhibitor combination happens in just a couple of microseconds. Producing the enzyme-inhibitor's steady-state takes a lot longer and might take minutes, when using slow-binding inhibitors. Enzyme inhibitors that function as partial reversible inhibitors (PRIs) have significant therapeutic benefits over

traditional enzyme inhibitors used in medical treatments. PRIs are a rare kind of reversible inhibition that arises from an efficient inhibitor-enzyme complex. This is especially true with uncompetitive, partly reversible inhibitors. Partial reversible inhibition, or PRI, of target enzymes in toxicology can help defend against harmful natural and synthetic xenobiotics by dampening the acute consequences of toxicants (Masson and Mukhametgalieva, 2023).

A wide variety of enzyme inhibitors are naturally generated by plants, and they function by sticking to a particular enzyme's active site and hindering the natural substrate from approaching. Similar to substrate binding, inhibitor-enzyme binding necessitates certain structural and molecular complementarities (Hrmova and Fincher, 2001). Evaluation of enzyme inhibitory activity may aid in the identification of possible lead compounds for disorders associated with enzymes, which are key targets for therapeutic development (Zhao et al., 2024).

2.1.3 Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)

butyrylcholinesterase (BChE) along with Acetylcholinesterase (AChE) are two key enzymes within peripheral and central cholinergic system. They have positively charged substrates (Masson and Mukhametgalieva, 2023). AChE and BChE collaborate in order to regulate the neurotransmitter acetylcholine quantities (Bursal et al., 2021). Acetylcholine which discovered in 1914 by Sir Henry Dale and coworkersis is a monoamine neurotransmitter found naturally within both the peripheral as well as central nerve systems. Following synthesis at the terminals of cholinergic nerve cells, acetylcholine is retained in vesicles until the nerve is triggered by the passage of calcium into the nerve terminal. Acetylcholine is released by the vesicles into the synapses between the pre- and postsynaptic nerve fibers in response to stimulation (Brandt and Flurie, 2020).

Acetylcholine is a crucial neurotransmitter in the parasympathetic branch attached to the autonomic nervous system, that regulates digestion and resting processes. Acetylcholine neurotransmitter involved in many physiological functions such as memory, muscular contraction, and cognition. Also it can cause pancreatic beta cells to secrete more insulin via acting on muscarinic receptors. Insulin production or

function failure is the main characteristic of type 2 diabetes. Insulin is a hormone that's crucial in blood sugar regulation (Gajendra et al., 2024).

Acetylcholinesterase (AChE) is the enzyme, which its primary function is to hydrolyse the acetylcholine into acetic acid and choline (Figure 2). The majority of AChE is found in the brain, however it is also present in muscles, nerve cells, and red blood cells. It is engaged in both cholinergic and non-cholinergic activities within the neurological systems. In 1938 Oakeshott and coworkers identified the three-dimensional structure of AChE, which was first isolated from the tissue of Torpedo marmoreta, the electric fish. The structure of AChE comprises β layers and an α-helix. The active core contains the catalytic collective amino acids glutamate, histidine, and serine. This active center is surrounded by Fourteen aromatic residues. The estimated mass of acetylcholinesterase, an enzyme that features a glycoprotein structure, has a value of 70–80 kDa (Gajendra et al., 2024).

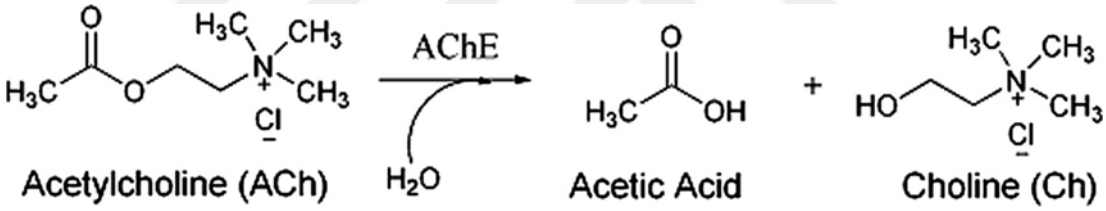


Figure 2.2 The enzyme acetylcholinesterase hydrolyzes acetylcholine to asetic acid and choline

Butyrylcholinesterase acts as a catalyst in the hydrolysing of butyrylcholine directly into butyrate and choline. It is combined with blood after being produced in the liver. It is present in the tissues of fat, the small intestine, the heart, the brain, and the muscles. The structure of butyrylcholinesterase is tetrameric glycoprotein, with a mass of around 342 kDa. Additionally, it has the ability to detoxify hazardous chemicals and aid in the hydrolysis of acetylcholine, according to reports.

Furthermore, excess acetylcholine, a neurotransmitter that operates in the cholinergic system, inhibits AChE, whereas excess substrate activates the sister enzyme BChE. This characteristic may act as a first line of defense to safeguard the cholinergic system in adaptation to a sudden rise in the acetylcholine level, such as in the event of acute inhibition of AChE by a toxicant, in central cholinergic synapses and at

neuromuscular junctions where both of those enzymes exist alongside one another. As a result of this, BChE is thought of as an AChE backup or substitute (Masson and Mukhametgalieva, 2023).

2.1.4 Alzheimer's disease (AD)

A neurodegenerative brain illness that worsens over time, Alzheimer's disease is characterized by a gradual loss of memory, strange behavior, personality changes, and ultimately mortality. Its anatomical symptoms are brain tissue shrinkage mainly in the basal forebrain together with hippocampal regions, and a decline in the amounts of the essential cholinergic neurotransmitter acetylcholine (Jain et al., 2025). Tens of millions of individuals throughout the globe are impacted by Alzheimer's disease, which is the dementia's most prevalent kind, and the number is sharply increasing. Alzheimer's disease has a significant financial and societal cost (Chen et al., 2017).

AD is a complicated illness that has an enormous variety of clinical symptoms and several genetic and environmental triggers, indicating that it is more like a syndrome than a conventional disease (Korczyn and Grinberg, 2024). AD can be classified as mild or severe if it has progressed to the dementia stage. When AD progresses to a severe level, voluntary functions such as walking, urinating, swallowing, and more are lost. At this stage of the disease, life-threatening complications are common, and full-time care is advised. Moderate AD causes trouble recognizing family and friends, more memory loss, and notable behavioral adjustments. Alzheimer's patients typically have a life expectancy of between three and ten years at the time of diagnosis, with progressive neurodegeneration predicted to continue until death. However, this depends on when the disease is diagnosed (Madnani, 2023).

It is well known that AChE along with BchE are the two main enzymes involved in controlling acetylcholine levels, which have been shown to have an impact on Alzheimer's disease phases in several investigations. Early Alzheimer's disease is marked by a noticeable rise in AChE activity, but later stages of the illness show an increase in BChE activity. The aforementioned factors make AChE and BChE

enzymes acceptable therapeutic targets for the recovery of cholinergic deficiency (Bursal et al., 2021).

Actually treatments are recommended to help with the illness's symptoms. AChE inhibitors from a variety of wide chemical families, including galantamine, tacrine, physostigmine, and heptyl-physostigmine, have been assessed for the treatment of the symptoms of AD. But it's still essential to explore the effectiveness of alternative drugs for the prevention and management of various mental health problems due to the poor therapeutic efficacy of the available medications that can be attributed to a number of issues, including non-selectivity, low absorption, hepatotoxicity, peripheral cholinergic side effects, and narrow therapeutic ranges. Studies assessing natural sources have been initiated in the hope to find a different, highly precise, hopefully less expensive treatment. Herbal treatment offers several techniques to reduce the symptoms and delay the initiation of AD. The use of medicinal plants to make and market drugs has grown, and these plants appear to have increasing scientific and commercial value in areas connected with health. These carefully standardized plant-derived chemicals have been demonstrated via tests to be safe and effective for a certain use (Çiçek Kaya et al., 2023; Jain et al., 2025).

Numerous medical disorders are frequently coexisting with Alzheimer's disease, the most prevalent ones including depression, osteoarthritis, diabetes, hypertension, and cerebrovascular disease. A great deal of these disorders have a large inflammatory response. Many studies have been conducted to examine the function of insulin dysregulation in AD, depending on the crucial role that inflammation plays in the development of Type 2 Diabetes Mellitus as well insulin resistance. Insulin plays crucial roles in the central nervous system, such as maintaining glucose homeostasis, promoting the development and survival of neurons, and synthesizing neurotransmitters. Whilst this area of research is still developing, currently available information indicate that managing insulin resistance should be a part of AD methodologies for therapy, whether pharmaceutical or lifestyle-based. It is extremely important that the scientific community keeps researching AD and works on finding novel therapies that might hinders the progression of AD and neurodegeneration since as life expectancy rises worldwide, consequently will the prevalence of

Alzheimer's disease. Although AD cannot be cured, there are several therapy options that can halt the progression of the illness and improve the quality of life for sufferers. Sustaining cardiovascular health, engaging in continual education, and consuming plant-based diets can all help to improve cognitive function and lower the risk of AD (Madnani, 2023).

Most AD instances can be prevented by adopting positive lifestyle and behavioural modifications, such having regular physical activities. A greater frequency of physical exercise appear to be linked to a decrease in the potential of cognitive impairments, Alzheimer's disorder, and dementia as well as grown hippocampus volume, according to human epidemiological and experimental investigations. Further, it has been determined that an important controllable risky variable for the growth of AD is midlife lack of physical activity. With exercise counteracting several elements of AD pathology, the advantages of exercise have become increasingly apparent within animal experiments (Kim et al., 2025).

2.1.5 Alpha-glucosidase and alpha-amylase

α-1,4-glucan-4-glucanohydrolase is the scientific term used for α-amylase, and its EC is 3.2.1.1. The molecular weight of α-Amylase is 57.6 kDa and it is composed of just 1 oligosaccharide chain with 512 amino acids. The molecular structure of α-Amylase is three-dimensional, which facilitates substrate binding and increases its specificity. The three recognized domains of α-amylase are A, B, and C. The biggest domain among them is A, characterized by the barrel-shaped super structure of (β/α). Its second domain, B, is situated in between the C and A domains. The C domain's function is yet unclear, and disulfide bridges connect domain A to domain C. α-amylase is produced by microorganisms , plants, and higher animals. Starch operates as α-amylase's substrate. An endo-amylase that breaks down the α-d-(1,4) glycosidic linkage and prevents the hydrolysis of glucose and α-1, 6-linkages at the end. As a metalloenzyme, calcium metal is implemented as a co-factor that is essential to the enzyme's correct functionality. The activities of enzymes are significantly impacted by temperature and other factors. Within pH 7.0, the α-amylase functions at its peak performance (Farooq et al., 2021).

The most often utilized source for α-amylase in the α-amylase involvement test is swine pancreatic α-amylase, which is then followed by human-derived sources such human pancreatic and also salivary α-amylase. Of particular note, commercial human α-amylase is readily accessible, which makes inhibition experiments more successful and economical (Lam et al., 2024). The critical metalloenzyme α-amylase has a key role in the hydrolysis of carbohydrates, and subsequently regulating postprandial hyperglycemia. Alpha-amylase, which is present in both saliva and pancreatic juice, increases blood sugar levels by hydrolyzing α-1,4 glycosidic bonds throughout the starch into smaller units called oligosaccharides or disaccharides and by selectively targeting random locations uses an endo-type capacity. Other than that, the process of softening crops like fruits and vegetables depends heavily on α-amylase. It is a ripening chemical that is applied during the manufacturing process of food (Iqbal at al., 2024; Kim et al., 2024).

The expression "α-glucosidases" defines the group of enzymes that hydrolyze oligosaccharides and glycosides, which includes isomaltase, glucoamylase, sucrase, and maltase. The last stage of digesting carbohydrates is catalyzed by alpha-glucosidase, which converts ingested starch and oligosaccharides into monosaccharides that can pass through the circulation and become utilized by cells. Alpha-glucosidase operates as an exoenzyme which breaks down glucose molecules connected by α-1,4 bonds at their non-reducing ends. It is located on the brush edge of small intestinal epithelial cells (Yang et al., 2017; Lin et al., 2016). The most prevalent source of α-glucosidase that has been used the works of literature is yeast, particularly that which is obtained from Saccharomyces cerevisiae, accounting for approximately 70% of the total (Lam et al., 2024).

Taking into account the biochemical processes performed by these two enzymes, α-amylase plays a vital role in the hydrolysis of α-1,4-glycosidic bonds in polysaccharides, resulting in lower molecular weight molecules that α-glucosidase then further breaks down to produce glucose as one of the main end products (Figure 3) (Neagu, E et al., 2023).

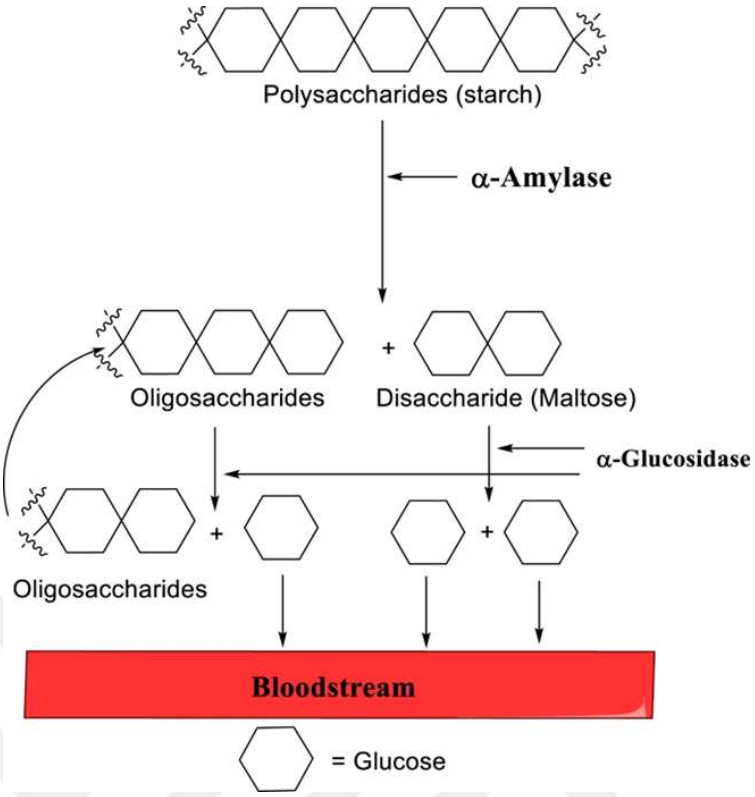


Figure 2.3 The hydrolysis of carbohydrates by α-glucosidase and α-amylase (Mohammadi Khanaposhtani et al., 2012)

2.1.6 Diabetes mellitus (DM)

Diabetes mellitus is a multifactorial chronic endocrine and/or metabolic health issue distinguished by persistent hyperglycemia. DM stems from impaired insulin production in the pancreas and/or cell response to insulin, both brought on by cellular modifications brought on by a variety of internal and external triggers, including oxidative stress, overweight, hepatic injury, and a sedentary way of life. Another thing to look at it is that excessive blood glucose causes a pathological state where more reactive oxygen species (ROS) are generated (Kandsi et al., 2024; Ge et al., 2023; Ahmed et al., 2025). In 2021, 537 million people (ranging in age from 20–79) had a diabetes diagnosis around the world. By 2045, it is predicted that their numbers is going to rise to 783 million. despite all this, about 49.7% of diabetics go undiagnosed. Diabetes is a serious global health concern since it is rising rapidly and is now ranked as the sixth most common cause of death globally (Iqbal et al., 2024; Hameed et al., 2024).

Diabetes mellitus comes in three varieties: Type 1 diabetes, often known as insulin-dependent diabetes, is characterized by little or no insulin secretion by the pancreas. Type 2 diabetes, which makes up 90–95% of cases, is caused by relatively tiny amount of secreted insulin or by cells that are resistant to it (insulin resistant) . There is also a type, which some expectant women experience throughout pregnancy and is referred to as gestational diabetes (Neagu et al., 2023). The prevalence and mortality rates associated with diabetes are rising quickly each year, making it an extremely challenging health issue. Hyperglycemia, which is brought on by ineffective diabetes management and treatment can lead to serious micro- and macrovascular consequences, comprising atherosclerosis and cardiovascular disease, and an increased risk of renal issues, heart disease, and also high blood pressure. all of that significantly burdening healthcare systems (Lam at al. 2024; Sun et al., 2022; Hsu et al., 2024).

The connection between diabetes mellitus and Alzheimer's disease is extremely complicated since it involves several matters, including insulin resistance, affected insulin signaling lipid imbalances, inflammation caused by overweight, and defective glucose metabolism. In particular, the second type of diabetes mellitus and Alzheimer's disease are strongly correlated, and those with diabetes are more likely to acquire Alzheimer's disease than people without the disease. Researchers coined the term "Type 3 Diabetes" to emphasize the link between insulin resistance and the emergence of AD pathology, as demonstrated by a number of earlier findings (Madnani, 2023; Gajendra et al., 2024).

A distinctive feature of individuals with diabetes is the occurrence of postprandial hyperglycemia. Antidiabetic medications constitute a category of pharmacological agents that function as inhibitors of enzymes implicated in hyperglycemia, thereby reducing the efficiency of various enzymes, basically α-amylase along with α-glucosidase. Since the active sites of the enzymes that break down carbohydrates are relatively similar structurally, antidiabetic medicines work by inhibiting the activity of specific enzymes. The hydrolysis of starch is intrinsically linked to hyperglycemia. Since the active sites of the enzymes that break down carbohydrates are relatively similar structurally, antidiabetic medicines work by inhibiting the

activity associated with particular enzymes. The hydrolysis of starch is intrinsically linked to hyperglycemia. Salivary α-amylase converts polysaccharides into linear and branching malto-oligosaccharides, which is the first step in the digestion of starch. After that, the pancreatic α-amylase in the small intestine continues the breakdown, producing maltotriose, maltose, as well as lower oligosaccharides. After that, the enzyme α-glucosidases, which catalyzes further breakdown, splits the α-glycosidic bonds whithen disaccharides or oligosaccharides, releasing free monosaccharides. The hydrolyzed monosaccharides are subsequently transferred to gastrointestinal epithelial tissue by specific transporters prior to their entry into the bloodstream. As a result of the previously identified mechanism , the Food and Drug Administration of the United States authorized acarbose, as foremos α-glucosidase inhibitory agent , in the year 1995. Miglitol subsequently received approval in 1996. (Lam at al,. 2024; Iqbal at al., 2024).

In spite of the fact that new medicinal compounds chiefly insulin and oral blood sugar lowering pharmaceuticals such as biguanides and also sulfonylureas have been developed, still they have many drawbacks like restricted effect, probability of causing hypoglycemia coma, and a regular consumption of these sorts of drugs causes lots of undesirable consequences such as diarrhea, anemia, bloating, gaining weight (Kandsi et al., 2024; Hsu et al., 2024; Ahmed et al., 2025), as well as hepatorenal dysfunctions (Hameed et al., 2024).

The global focus has thus switched to the seek for safer, naturally derived, and more potent hypoglycemic medications. A variety of plant and animal-based nutritional supplements are being investigated as potential substitutes for pharmaceuticals that block enzymes involved in blood glucose regulation. Fermented foods have been suggested as a safe way to manage diabetes and hyperglycemia. Over four hundred plants were initially identified as effective in treating patients with diabetes, while a relatively small amount of these have received scientific and medical recognition for additional effectiveness research. (Hameed et al., 2024).

The mechanism underlying the actions of alpha-glucosidase inhibitors involves competitive inhibition of small intestine α-glucosidase and hydrolytic enzymes and

reversible inhibition of α-amylase. The resultant inhibition causes the proximal intestine's digestion and absorption of carbohydrates to be hampered, which causes the distal intestine's L cells to release glucagon-like peptide-1 (GLP-1). This in turn helps reduce insulin levels along with postprandial blood sugar, increase while lengthening stomach emptying, suppressing hunger, and improving β-cell sensitivity to glucose. (Yang et al., 2017; Kreitman et al., 2021).

Due to flavonoids' remarkable capacity to obstruct the digestion of starch, researchers have been looking more and more towards these safe and useful phytochemicals in recent years. The amount of information about flavonoids' lab-based inhibition of α-glucosidase and α-amylase has been raised dramatically. The inhibiting activities of flavonoid subgroups concerning α-glucosidase were found to be arranged in the following decreasing order: the first flavonol > flavone > flavononol > flavanol > flavan; The second flavonoids > flavonoid glycoside; and thirdly the inhibition potency of flavonoids is greater than that of their corresponding chalcones or isoflavonoids. Still, a case-by-case comparison and consideration of the impact of substituents and sugar moieties are necessary to validate these findings. Generally, aglycone flavonoids often have more activity than their counterparts glycosides and isoflavonoids (Lam et al., 2024; Botsi et al., 2023) (Figure 4).

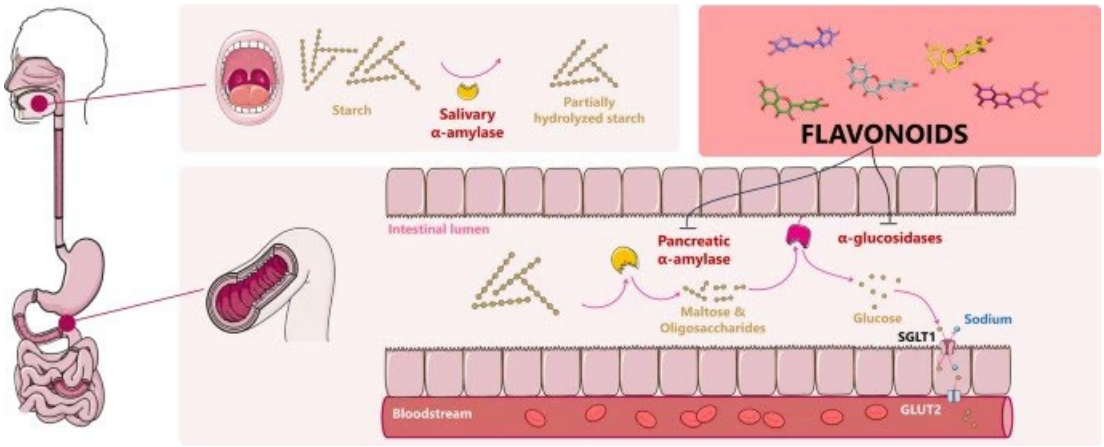


Figure 2.4 Starch hydrolysis process along with ways that flavonoids, as diabetic medications, work (Lam at al., 2024)

Plenty of investigations have been undertaken to discover novel and efficient natural remedies for diabetes for the reason that herbal remedies are preferred over synthetic

pharmaceuticals since they have less side effects, include physiologically active chemicals that work in coordinated manner, and are generally less expensive as well. Another benefit is that an increasing number of diabetes care options involve the use of antioxidantsto stop oxidative stress, the issues linked to diabetes (Ahmed et al., 2025).

Since there is no known cure for diabetes, and the drugs now on the market reduce the symptoms of diabetes rather than addressing the synthesis and operation of insulin, the majority of individuals choose to manage their disease with medicines, dietary modifications, and lifestyle changes. And we can say that there is an urgent demand for effective, exceptional antidiabetic medications with fewer harmful effects (Iqbal at al., 2024; Hsu et al., 2024).

2.1.7 Tyrosinase

The polyphenol oxidase tyrosinase is a multifunctional membrane-bound metalloenzyme that functions as a rate controlling enzyme in manufacturing the melanin. It has the EC 1.14.18.1, and also known as monophenol or o-diphenol oxygen oxidoreductase. Tyrosinase can only be produced by cells called melanocyte and is found in the melanosome membrane. It is transported to melanosomes, where the pigment melanin is produced, after being produced and then processed in the endoplasmic reticulum and also Golgi. As determined by the analysis of structure, tyrosinase is a type three copper-containing glycoprotein that has three histidine residues around two copper ions, which give it its catalytic activity. In the process of pigmentation production, the enzyme's active site exists in three states: deoxy, met, and oxy forms. Tyrosinase oxidizes arene rings by using copper, a redox catalytic cofactor, at its active site. Monophenolase function, which converts monophenols into diphenols, and diphenolase function, which converts o-diphenols into o-quinones are directly facilitated by the extremely reactive intermediate compound which is create when two copper ions and dioxygen combine at the enzyme's active site (Zolghadri et al., 2019; Pillaiyar et al., 2017; Ryu et al., 2008) (Figure 5).

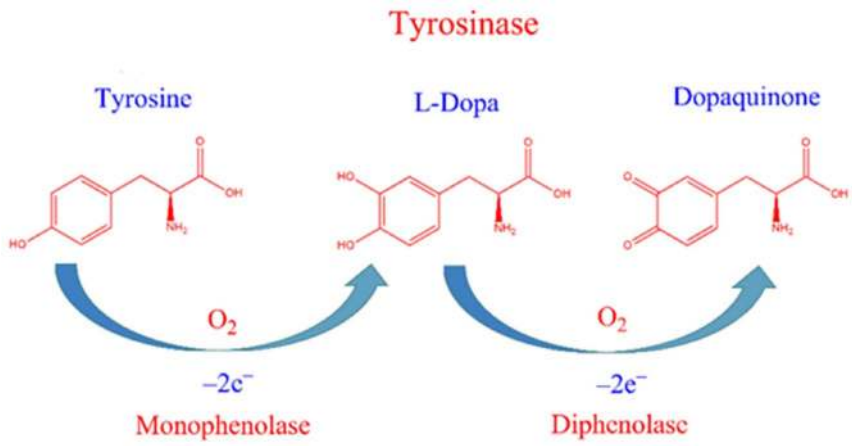


Figure 2.5 The enzymatic reactions of tyrosinase (Rocchitta et al., 2022)

Many different types of cells and tissues, including those of plants, insects, fungi, bacteria, and other mammals, include tyrosinases. Tyrosinases serve a wide range of biofunctional functions, including spore production in fungi, reproductive organ differentiation, skin melanogenesis in mammals, browning in plants, and arthropod host defense. Exposure to ultraviolet (UV) light can activate tyrosinase, which can then inductively interfere in a number of intermediary stages of the pigmentation process of human skin. Tyrosinase is a commonly used, appealing target enzyme for a variety of commercial applications. In environmental applications, it has played a significant role in the removal of pollutants of contaminated soils as well as water waste that contains phenol; the creation of biopolymers with viscoelastic functioning abilities. And the improvement of food's flavor, texture, and aroma through the polymerization process of phenol counterparts; and the cross-linked process between protein polysaccharides and also the process by which two proteins are joined together (Manickam et al., 2024).

Various disciplines have extensively studied tyrosinase during the last few years. Both prokaryotes and eukaryotes include the tyrosinase enzyme, which is widely distributed throughout nature. Other than their crucial function in the initial stage of melanin production, they also perform lots of additional functions in eukaryotes. In plants, sponges, and many invertebrates, they function as the main immune response and aid in wound healing. In arthropods, they are involved in sclerotization. Since tyrosinase is difficult to produce in significant amounts from people, the composition of its three dimension is yet unidentified (Chen et al., 2015).

2.1.8 Melanina and hyper-pigmentation

"Heterogeneous polymer derived by the oxidation of phenols and subsequent polymerization of intermediate phenols and their resulting quinones" is a common and straightforward description that would encompass all forms of melanin (Solano, 2014). Melanocytes, which are specific dendritic cells mostly found underneath or in between the epidermis' basal cells, synthesize melanin when exposed to ultraviolet B radiation through a process known as melanogenesis. Melanosomes, which are organelles present in the cells of animals and are where melanin is synthesized and stored, transport the generated melanin to nearby keratinocytes in the epidermis. Depending on the levels of oxidation, melanin can have a variety of biological forms. Melanin comes in three main types: neuromelanin, pheomelanin, and eumelanin. Phenomelanin along with eumelanin are the two basic types of melanin found in the skin of humans (Figure 6).

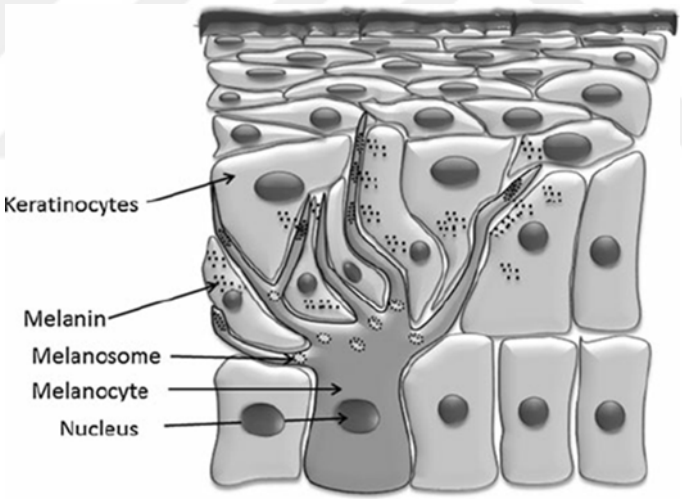


Figure 2.6 Melanin pruduction by tyrosinase within the melanocyte (Gupta et al., 2013)

Neuromelanin is present in the brain, however its exact function is unknown. In typical physiological circumstances, pigmentation serves a crucial evolutionary function in animal mimicry and camouflage and helps protect human skin from damaging UV rays. Unusual melanin loss and depigmentation can cause major dermatological and face aesthetic issues in people. Conversely, a variety of skin conditions, such as lentigines, acanthosis nigricans, periorbital hyperpigmentation, solar lentigo (age spots), post-inflammatory melanoderma cervical poikiloderma, melasma, skin cancer, freckles, and the neurodegeneration linked to Parkinson's

disease, are characterized by accumulation of melanin pigment due to its elevated production. Furthermore, prolonged exposure to ultraviolet wavelengths may result in photoaging, immune system dysfunction, damage to the DNA, genetic modification, and cancer formation (Pillaiyar et al., 2017; Zolghadri et al., 2019; Chen et al., 2015).

Since mammalian melanins are found in tissues other than skin and hair, such as the stria vascularis of the inner ear, retinal epithelial pigment (REP), eyespots, and the tissue beneath the iris of the eye, melanin is essential for the proper operation of sight and hearing. It has long been recognized that, transparent eyes, deafness, and albinism are related in a surprising and fascinating way. Thus melanin has biological, social, and cosmetic effects. Additionally, certain mammals' adrenal glands and specific brain areas contain melanin. The substantia nigra contains the majority of neuromelanin, while the locus coeruleus has a smaller amount. It could be essential for neurodegeneration, apoptosis, and the associated Parkinson's disease. It has been demonstrated that human neuromelanin effectively binds metals like iron and also other potentially harmful substances. For mammals, adipose tissue is one of the tissues where melanin is formed ectopically. The melanin in adipose tissue appears to be functional and may act like a anti-inflammatory and oxidative damage absorbent (Najafi et al., 2024).

Despite having a protective function in common, melanins are more than just pigments that provide protection from ultraviolet light. Melanisation becomes the virulence factor in harmful microorganisms because melanin shields microbial cells from the host's defensive systems. Since certain melanins are produced in tissues that are not vulnerable to sunlight, their metal-chelating, redox, or free radical-scavenging qualities are more meaningful than their ability to absorb light. These pigments can have conflicting effects, as in certain cells, melanogenesis restriction is preferable. Research on melanin biochemistry remains ongoing from an array of disciplines as fruit technology, dermatology, biomedicine, cosmetics, and microbiology (Solano, 2014).

The browning process of vegetables, fruits, and drinks occurs when tyrosinase and its polyphenolic substrates react with oxygen following brushing, peeling, and crushing processes that break down cell structures. Plant-based meals and drinks are broken down by this enzymatic process, which also inhibits proteolytic and also glycolytic enzymes, degrades essential amino acids, and diminishes nutrition and digestibility while releasing toxic compounds, and It causes these items' quality and market worth to decline (Najafi et al., 2024).

A number of variables, including temperature, pH, oxygen availability, tyrosinase and phenolic substrate concentrations, and others, influence how rapidly an item becomes brown by enzymatic processes (Ahmad et al., 2024). While the hyperpigmentation in the mammals is known also to be induced by several inflammatory, endocrine, and hereditary disorders (Landge et al., 2024).

Tyrosinase is primarily in charge of the enzyme-mediated oxidization of phenols, which results in fungal and fruit darkening effects and hypopigmentary disorders in mammals.. As of right now, several efficient inhibitors have been found and created for use in the food and beverage bioprocessing, agricultural, medicinal, and cosmetic goods sectors as well as the environmental sector. However, only a small number of chemicals are known to function as safe and efficient tyrosinase inhibitors, despite the fact that they represent a class of significant clinical antimelanoma medications in medicine.

Sources from nature, such as bacteria, fungus, and plants, have lately drawn more attention due to their ability to produce bioactive chemicals that have antityrosinase action. Because of their superior bioavailability and lower toxicity, many researchers favor finding inhibitors coming from resources that are natural, particularly for use in food, skincare, and medicine. Simple phenols have been identified as potential inhibitors that target the tyrosinase enzyme, including the vanillin compound alongside all of its derivatives, deoxyarbutin and also the derivatives thereof, 4-n-butylresorcinol, resorcin (or resorcinol), 4-(6-Hydroxy-2-naphthyl)-1,3-bezendiol, as well as hydroquinone plus derivatives of it (Figure 7) (Zolghadri et al., 2019).

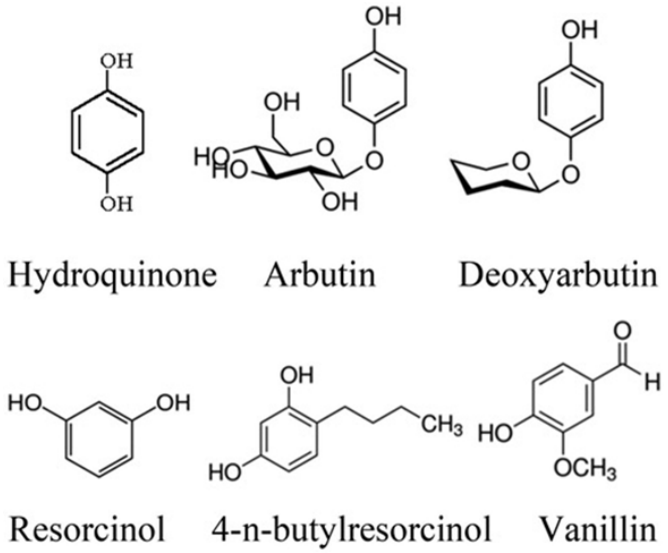


Figure 2.7 Some possible phenolic inhibitors from nature that specifically target the enzyme tyrosinase (Zolghadri et al., 2019)

Inhibitors of tyrosinase typically either combine via the ionized copper in the active site of the tyrosinase, blocking the interaction between the enzyme and substrate, or stop oxidation by an electrochemical mechanism. The ability of flavonoids to perform both functions at the same time makes them one of the most promising options for tyrosinase inhibitors (Ryu et al., 2008). In recent years, Ascorbic acid, azelaic acid, 4-n-butylresorcinol, kojic acid, deoxyarbutin, arbutin, gentisic acid, hydroquinone, and aloesin are powerful plant-based tyrosinase inhibitors that have applications in the cosmetics industry (Chen et al., 2015). Serious adverse effects include contact dermatitis, erythema, as well as paradoxical hyperpigmentation are linked to kojic acid and even arbutin, the widely recognized tyrosinase inhibitors. As a result, finding naturalli originated novel tyrosinase inhibitors may be a useful tactic for creating innovative drugs that lessen a variety of adverse effects (Jeong and Kim, 2024).

2.2 Antioxidants

Antioxidants are chemical compounds that, by neutralizing the free radicals in our biological systems, considerably slow down or even prevent the oxidation of the oxidizable substrates. Free radicals comprise highly reactive chemicals that are naturally generated as byproducts of several everyday biological operations, such as the creation of energy and the immune system's reaction. Free radicals are necessary

for a number of biological functions, besides that, the body's natural defence system continuously combats the excess free radicals. Under typical conditions, the rate at which oxidants are removed balances the rate at which they are formed. External factors that induce free radicals include radiation, air and environmental contaminants, cigarette smoking, consuming unhealthy nourishment, some medicines, and alcohol can make them pile up more quickly. Oxidative stress occurs when the rate of the body's production of antioxidants is not equivalent to the rate of formation of free radicals. Because of their high reactivity and inability to exist independently, free radical molecules exert detrimental metabolic effects on cells. Excessive ROS levels in biological cells can lead to detrimental alterations in the structure of nucleic acids, lipids, in addition to proteins, which can harm cells, hasten aging, and causing illnesses, such as cancer, diabetes, heart disease, osteoporosis, along with neurological diseases (Parkinson's and Alzheimer's) (Berk et al., 2011; Inci et al., 2023; Kwiatkowska et al., 2024).

A summary of the assumed reactive species (free-radicals and non free-radicals) are found in the Table 1 below (Munteanu and Apetrei, 2021):

Table 2.1 Reactive species (free-radicals and non free-radicals)

Non Free-Radicals		**Free-Radicals**	
Nitroxyl anion	NO^-	Hydroperoxyl radical	HOO•
Dinitrogen trioxide	N_2O_3	Superoxide radical	O_2•
Lipid hydroperoxide	LOOH	Lipid radical	L•
Ozone	O_3	Lipid alkoxyl radical	LO•
Peroxynitrite	$ONOO^-$	Peroxyl radical	ROO•
Hypochlorous acid	HOCl	Protein radical	P•
Nitryl chloride	NO_2Cl	Nitric oxide radical	NO•
Hydrogen peroxide	H_2O_2	Hydroxyl radical	HO•
Nitrous acid	HNO_2	Nitrogen dioxide radical	NO_2•
Singlet oxygen	$^1O_2$	Thiyl radical	RS•
Nitrosyl cation	NO^+	Lipid peroxyl radical	LOO•

Antioxidants' application in significant industrial processes, including the rubber vulcanization, polymerization processes of fuels in internal combustion engine fouling, and to avoid the occurrence of corrosion of metallic materials, were the subject of lots of study in the final years of the nineteenth and the beginning of 20th centuries. The use of antioxidants to stop the oxidizing process of the unsaturated fats, which results in rancidity, was the main focus of early studies on their function in biology. However, the discovery that the vitamins E, A, and C are antioxidants improved the science and made the significance of antioxidants regarding the biochemistry of organisms clear (Lobo et al., 2010). The features and capabilities of antioxidants include metal-chelating, peroxide enzymatic decomposition, singlet oxygen quenching, donation of hydrogen, donation of electrons, inhibition of enzymes, and radical scavenging. The extracellular and intracellular environments include enzymatic as well as nonenzymatic antioxidants to detoxify ROS (Lobo et al., 2010).

HAT pathway (hydrogen atom transfer), SET pathway (single electron transfer), in ddition to a mixture involving the both pathways (SET & HAT) are the main methods by which that antioxidants interact with free radicals. The HAT reaction is the coordinated movement of an electron and a proton in one kinetic step. During Hydrogen atom transfer mechanisms, the antioxidant molecule turns into a radical when the free radical takes away one of its hydrogen atoms. The bond dissociation enthalpy is a crucial metric in this process for assessing the antioxidant activity. The process of free radical inactivation will proceed more easily if the breaking down enthalpy of the H-donating molecule of the possible antioxidant is smaller.

The mechanism of the single electron transfer is simply a transmission of an electron from the nucleophile molecule of the antioxidant to the substrate, which is the rfee radical, creates a radical intermediate, whose can subsequently be engaged in a variety of processes. The most crucial energy factor to evaluating the effect of an antioxidant in this pathway is the antioxidant's ionization potential. The electron abstraction process is made easier as a result of a lower ionization potential. Differentiating between SET and HAT pathways is quite challenging. The pathway of the reaction gets determined by the solvent polarity, the partition coefficient, the

antioxidant's structure and solubility, and these two reactions typically occur concurrently (Liang and Kitts, 2014) (Figure 8).

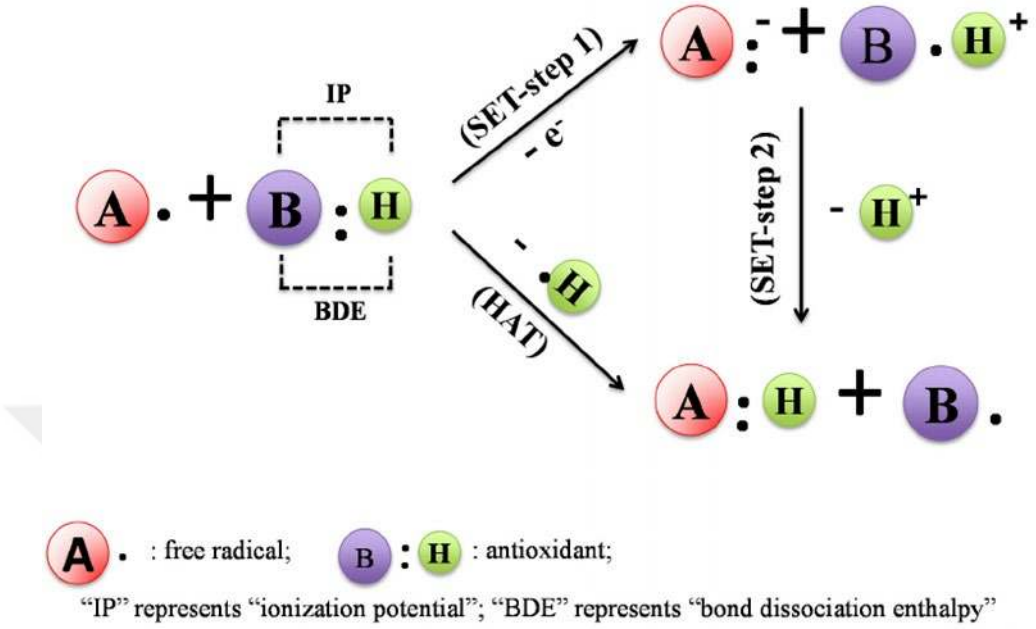


Figure 2.8 The ways in which antioxidants interact with free radicals (Liang and Kitts, 2014)

Among the most prevalent antioxidants, polyphenols typically interact with free radicals via three types of mechanisms: transition metal chelation (TMC), SET, and HAT. By breaking the O-H link and transferring hydrogen atoms, polyphenols make free radicals inert for the HAT process. Polyphenols serve as the free radicals' electron donors in the SET process. Polyphenols may bind with metal ions to generate a stable compound for the TMC process (Lang et al., 2024).

Molecules possessing antioxidant qualities can be made endogenously or obtained exogenously through dietary supplements or food being consumed. SOD, glutathion peroxydase (GSH-Px), and catalase (CAT) are the most important endogenous antioxidant enzymes. Vitamins E and C are examples of exogenous antioxidants that may be present in the organism in the fluids that are both intracellular and extracellular as well as in the cell membrane. The membranes' hydrophobic lipid interior need a specific kind of antioxidants. In an environment like this, fatty-soluble vitamin E is quite effective antioxidant, which prevents the degradation of membranes. Fatty-soluble antioxidants are essential for protecting the cellular

membranes' polyunsaturated fatty acid peroxidation. Vitamin C and other water-soluble antioxidants are crucial for scavenging ROS within the hydrophilic phases (Munteanu and Apetrei, 2021)

Also we have another primary classification which is enzymatic and non-enzymatic antioxidants as we can see in the Figure 9 below:

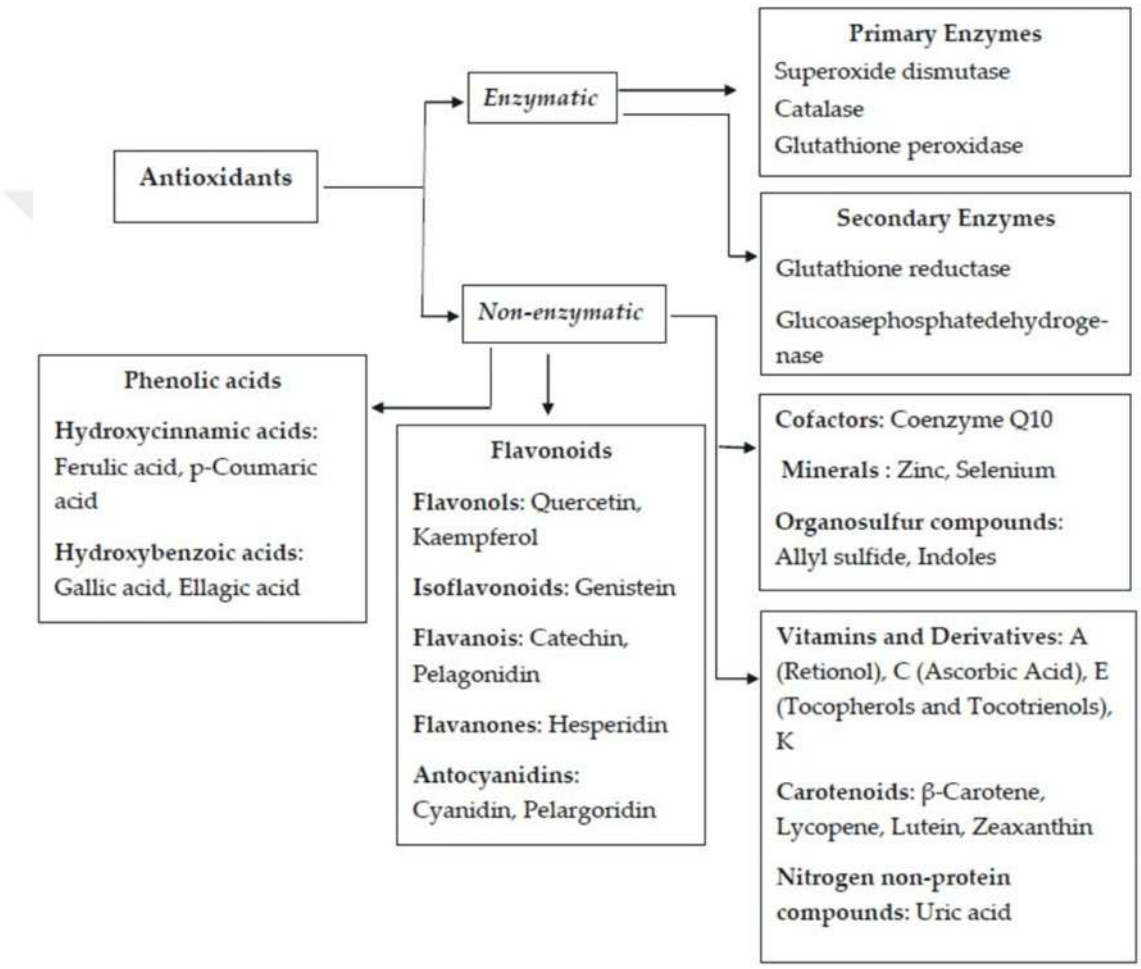


Figure 2.9 Illustration for the classification of the antioxidants (Munteanu and Apetrei, 2021)

Antioxidant compounds can be put into two categories according to where they were acquired: synthetic antioxidants, also known as artificial antioxidants, and antioxidants derived from natural sources, also known as natural antioxidants. The toxicological response of the synthesized antioxidants has been extensively examined. There are certain restrictions on the use of toxicological data, though, as it is recognized that some of these substances can be harmful if taken for an extended

period of time (Salamah and Widyasari, 2015). Thus, research on natural antioxidants has become more and more significant. In these last few decades, a number of medicinal plants have been thoroughly studdied for their antioxidant properties in an effort to find natural antioxidant sources. Because flavonoids and other phenolic compounds derived from plants have been shown to scavenge ROS, they are considered to be prospective therapeutic medicines for diseases caused by free radicals (Sarikurkcu, 2011).

Since the primary bioactivity of polyphenols is their antioxidant capacity, effective detection techniques have been established. These techniques may be categorized into chemical, cell, along with in vivo tests. researchers could decide on an applicable test depending on their features, such as liposolubility or water solubility, ease of use, quickness, and precise results, and strong connections to biological reactions (Lang et al., 2024). PFRAP (potassium ferricyanide reducing power), DPPH, FTC (ferric thiocyanate), FRAP, CUPRAC (cupric reducing antioxidant power) assay, ABTS, the Folin–Ciocalteu assay along with ferrous oxidation-xylenol orange (FOX) assay are among the ET-based tests. The majority of ET-based assays replicate the antioxidant response by using a proper redox-potential probe, meaning that in exchange for peroxyl radicals, antioxidants interact via a colored or fluorescent indicator (oxidizing agent). The ability of an antioxidant to decrease an oxidant, which is color-changing when reduced, is measured by using spectrophotometric ET-based assays. The amount of antioxidants present in the sample is connected with the degree of color change, which can be either a rise or fall in the absorbance of the sample at a specific wavelength.

By donating a H atom, tests using HAT measure an antioxidant's ability to neutralize free radicals, typically peroxyl radicals, which are believed to have more biological significance. The reaction below describes the HAT processes of antioxidant activity:

$$ROO\cdot + AH\text{-}rOH \rightarrow ROOH + A\text{-}rO\cdot$$

The hydrogen atom of the phenol is shifted to a the free radical, and then the resonance stabilizes ArO• (aryloxy radical), which is created when antioxidant's phenol reacts with a peroxyl radical. Biomolecules that are protected and phenolic antioxidants are shown by the AH and ArOH species, respectively. In order to

provide protection for the biomolecules from the oxidation, antioxidants is required to react with free radicals much more rapidly than biomolecules (Xiao et al., 2020).

2.3 Secondary Metabolites in Plants

Plants synthesize molecules called secondary metabolites (phytochemicals), that aren't directly engaged in the main metabolic pathways of their development and growth (Samal et al., 2024). The Plant secondary metabolites, also known as phytochemicals, are produced in different parts of plant providing a natural defensive mechanism against adverse environmental conditions and microbial attacks.

These substances have several well-known therapeutic uses in addition to offering protection since they are connected to several metabolic processes both within and outside of plants. Ever since the early ages, plant's secondary metabolites have been widely benefited from in treatment of illnesses due to their remarkable biological activity, and culinary (Kumar et al., 2022).

Important functions of secondary metabolites include luring pollinators and protecting plants from diseases and animal assaults. The use of secondary metabolites in oils, colors, fibers, glues, waxes, medicinal medications, flavors, and scents is enormous. Antibiotics, pharmaceuticals, insecticides, as well as herbicides are developed using many of the secondary metabolites as active ingredients (Wani et al., 2023).

It is estimated that about 200,000 different specific secondary metabolites are produced by plants (Murthy et al., 2024). Secondary metabolites produced by plants are classified into different categories (Figure 10).

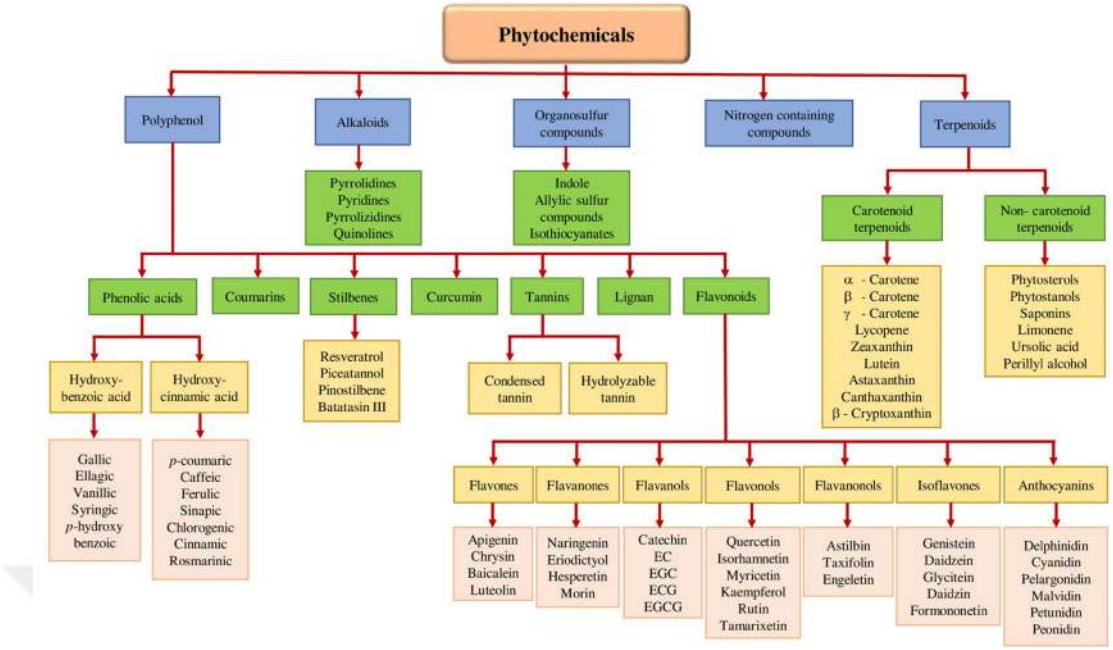


Figure 2.10 Secondary metabolites produced by plants (Santhiravel et al., 2022)

Plant-based phenolics are a diverse and complex class of chemicals. These substances are classified into several classes, with phenolic acids, flavonoids, lignans, together with stilbenes being the primary categories. They all feature a minimum of an aromatic ring that contains at least one hydroxyl (OH) group in their structure. Fruits and vegetables are frequently discovered to contain polyphenols. A number of clinical research studies have shown that some polyphenols have cardioprotective, antidiabetic, and antioxidant properties, as well as the ability to modify the composition of gut microbiota as prebiotics (Dziadowiec et al., 2024).

Alkaloids are a broad class of chemicals that include nitrogen as a common structural constituent. Alkaloids are utilized extensively in contemporary medicine and have a wide range of biological effects due to their structural variety. Chemotherapy, pain relievers, respiratory illness treatment, nutritional supplements, and several more uses are examples of alkaloids in healthcare. Alkaloids found in many plant species are categorized according to their chemical structure, biological antecedents, and occurrence (Dziadowiec et al., 2024).

Flavonoids are naturally occurring phenolic chemicals that have a (C_6-C_3-C_6) structure made up of two benzene rings (A and B rings) connected by a bridge of

three carbons. This three-carbon structure often results in a heterocyclic pyran ring. Their many health-promoting qualities, including as their anti-inflammatory, anti-oxidative, anti-infective, and anti-obesity benefits, are well documented. Significantly, a number of studies have demonstrated the ability of flavonoids as being anti-diabetic medicines because of their moderate inhibition of α-amylase and substantial inhibition of α-glucosidase, which makes them attractive candidates for the creation of anti-diabetic medications with few gastrointestinal adverse effect (Lam et al., 2024).

2.4 Main Analytical Methods for Phytochemicals

Plant extracts' abundance of metabolites may make identification procedures difficult, thus it is essential to screen for biologically active and industrially significant phytochemicals using accurate and repeatable analytical techniques. Analytical techniques that identify the natural compounds in aromatic as well as therapeutic plants should meet requirements for sensitivity, selectivity, in addition to separation efficiency. Phenolic compounds,which researchers find it to be the most fascinating subject differ in size as well as the polarization, so its extracting in addition to investigating it is exceptionally challenging.

The researchers have no choice but to search for more dependable methods than TLC or UV/Vis spectrophotometry since the matrices are mostly quite complex and the substances of interest may be present in small concentrations. Apart from that, the use of gas chromatography, or GC, is only available to compounds with volatile properties in addition to the fact that the use of capillary electrophoresis might sometimes encounter repeatability issues. Thus, the most efficient method for the qualitative as well as quantitative characterisation and identification of various types of phenolic compounds within various species of plants is U-HPLC complemented by tandem mass spectrometry (Yilmaz, 2020).

2.4.1 Liquid chromatography-mass spectrometry

The most common technique for quantifying and characterizing the structure of low as well as high molecular weight polyphenols is liquid chromatography combined with mass spectrometry, often known as LC-MS/MS. High resolution and lengthy analysis durations are necessary for the LC determination of such chemicals in complex matrices; the latter can occasionally be a significant constraint when high-throughput analysis is the goal. UHPLC, by using either columns with sub-2 mm particle packs or porous-shell columns containing superficially porous particles less than 3 mm, has created new opportunities for enhancing analytical techniques for complex sample matrices in recent years. UHPLC can achieve 5- to 10-fold faster separations than traditional LC while preserving or even improving resolution.

Typically, the chromatographic separation is accomplished by reversed-phase mode with gradient elution utilizing C18 columns and mobile phases of methanol or acetonitrile combined with acidified water. Since polyphenols are easily ionized by ESI in negative ionization mode, ESI is the most often employed ionization source. Though APPI and APCI have also been documented for polyphenol determination, the few findings reported in the literature have demonstrated that APPI is a superior ionization source for some polyphenols with the benefit of having a smaller matrix impact.

In the examination of polyphenols, a great deal of focus will need to be placed on alternative ionization sources like APPI, particularly when it comes to the categorization and authentication of naturally derived products in order to avoid fraud. Because of their highest sensitivity, triple quadrupole mass analyzers in MRM acquisition mode remain the most commonly used instruments for low resolution mass analysis. However, ion-trap based analyzers, like QTraps, are also being chosen, particularly when structural characterization and elucidation are the goals (Lucci et al., 2017).

The basic concept of LC is the idea that different compounds have different affinities, which influence how those compounds interact with the stationary and mobile phases. Following elution from the column, the chemicals will be converted

to the gaseous phase and then ionized for the mass analysis. After the molecules are converted to an ionized state, the mass spectrometers may evaluate the ions and any fragment ions created during the ionization process by calculating their mass to charge ratio. The basic idea behind an ordinary mass spectrometer is the combination of an ionization device and an ion analysis device (Figure 11).

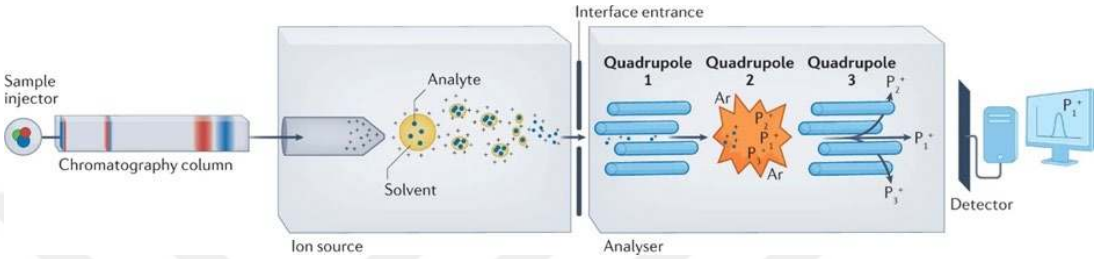


Figure 2.11 The basic idea behind the LC-MS/MS (Thomas et al., 2022)

It has been demonstrated that LC–MS/MS is an efficient method for phenolic chemical detection at low quantification values. Thus, we can say that one of the most trustworthy techniques for characterizing phenolic compounds is LC-MS/MS. However, because of the LC-MS/MS instrument's accuracy, this approach has challenges as well. A polluted instrument might make it not achievable for the operator to identify the issue, producing an inaccurate result. Additionally, it is difficult to popularize LC-MS/MS because of the instrument's expensive cost. LC-MS/MS is not appropriate for every analytical case as the choice of analytical techniques is determined by the matrix and the properties of phenolic compounds (Shi et al., 2022).

How sensitive HPLC-MS/MS is might vary due to changes in operational parameters. For instance, the mobile phase's composition, the amount of the sample, fragmentor voltage, nebulizer pressure, injection velocity, drying gas parameters (temperature and also the rate of flow), and the energy of the collision all have an impact on sensitivity. Selecting the most crucial variables is essential to maximizing HPLC-MS/MS sensitivity in order to minimize labor and expense costs and prevent tiresome trials (Bae et al., 2018)

2.5 Santalaceae Family

The Santalaceae, or sandalwood family, has about 1,000 different semiparasitic species of trees, shrubs, and plants in around 44 genera. Santalaceae are mostly root parasites, with the remainder being stem parasites. Although part of the chlorophyll in their green leaves enables the plants to produce food, all members of the Santalaceae family are parasites to some degree and attach themselves to their hosts in order to get nutrients and water. Even while some species have clusters of flowers in the leaf axils or located on short spikes, the majority have tiny, unobtrusive, bisexual or simply unisexual flowers that appear individually. There may be a nut-like structure with vibrant colors surrounding the single-seeded fruit (Encyclopaedia Britannica, 2022).

Santalaceae are found all over the world, with half of the genera found in wet tropical biomes and the other half in dryish or temperate regions. There are several exceptions to the rule that most genera are only found in the regions of the New World or in the Old World. For example, *Pyrularia* as well as *Buckleya* are found in Asia along with eastern North America, *Thesium* has species found in Brazil and the Old World, and *Comandra* is found in Europe and North America. It is challenging to differentiate Santalaceae from other families in the Santalales (especially Opiliaceae) due to their lack of distinct synapomorphies and plesiomorphic and missing characteristics. A few species are trees, most notably *Okoubaka* and *Santalum*, although the majority are perennial herbaceous species or tiny woody shrubs. They typically have simple, whole leaves, however they might also be modified to spines or even scales. In temperate species, thin leaves that fall off are typical, whereas thick leathery and permanent leaves are more prevalent in tropical and also the subtropical species. Many species feature dimorphic branches, which might be a succession of alternating squamate and leaved branches or both lengthy and short shoots (Der and Nickrent, 2008).

The herbaceous genera, which include *Thesium* with 325 species, *Phoradendron* with 235 species, *Viscum* with 65 species, and *Dendrophthora* with 65 species, represent the majority of the family's members. Approximately 25 different species of trees, ranging from Indo-Malaysia to the islands of the Pacific are classified as

sandalwood. The sole member of the family of commercial significance is the fragrant sandalwood (*S. album*), which is used to make furniture and make perfumes (Encyclopaedia Britannica, 2022).

2.5.1 The genus *Thesium*

Thesium is concidered as the biggest genus within the family Santalaceae and also the Santalales order. All continents save Antarctica are home to it. About 175 species, or fifty percent of the genus *Thesium*, are located in the Cape Floristic Region of southern Africa. There are five species in North Africa, forty-five in Central Africa, four in West Africa, fifty in East Africa, and seven in Madagascar. Australia has only one species, Europe has 35, and Asia has 45. North America is home to only one species, *T. ramosum Hayne*, which has been imported and is regarded as a noxious plant. There are three species in South America. (Lombard et al., 2020; Mucina and Nickrent, 2024) (Figure 12).

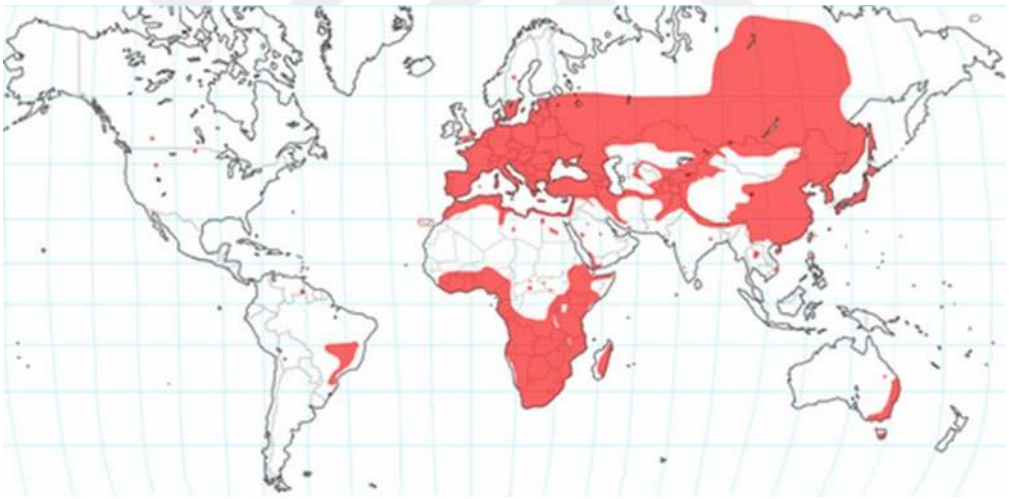


Figure 2.12 The global distribution of *Thesium* (Mucina and Nickrent, 2024)

The genus *Thesium* originated and is mostly diverse in South Africa's Cape. Although the species of *Thesium* live on a variety of substrates, deep coastal sand, limestone, quartz, sandstone, and shale are the most common types (Zhigila and Muasya, 2022). Genus *Thesium* includes perennials and also annuals whose growth types include of shrubs, scandent shrubs, herbs, and subshrubs. The majority of species are rhizomatous or stoloniferous, however those that have adapted to southern and also tropical Africa's fire-prone areas have shoots that emerge from a

caudex, indicating their ability to resprout after disturbances. The majority of species are tiny in size (less than 50 cm tall), with sparse to highly branching stems that range in color from yellow to golden to grayish to green. With a cross-section that ranges from flat to terete or triangular, the leaves are usually sessile or decurrent, scale-like or well-developed, or nearly absent. Miniature flowers that range in color from white into a greenish are either unisexual or bisexual (Mucina and Nickrent, 2024; Zhigila et al., 2020) (Figure 13).

Figure 2.13 Variability found in the vegetative characteristics of a few chosen *Thesium* species (Zhigila et al., 2020)

The Cederberg's usage of "*T. spicatum*" (lidjes tea) was first documented by Marloth (1917). When it's ready to drink, he says, the tea is black. Smith (1966) described *Thesium* strictum as tuberculosis tea, or "teringtee," but provided no information on its intended usage other than the suggestion that it was used to cure tuberculosis. *Thesium* macrostachyum, also known as lidjiestee to many residents of the Wupperthal region of the Cederberg, is used to make the most well-known and

widely consumed Thesium tea. *T. carinatum*, also known locally as jakkalstee, is another species of *Thesium* that has recently been identified as an ingredient of tea in the Wupperthal region. The chemistry and pharmacological activities of the Cape species of *Thesium* are not much investigated(Van Wyk and Gorelik, 2017).

Out of the approximately 350 species of *Thesium*, just three have been found to be toxic. *Thesium humile*, which is found throughout the north African region and the Mediterranean, has been linked to severe poisonings and fatalities in ruminant animals and livestock. Acute meteorism, convulsions, and abdominal pain are signs of T. humile poisoning. *Thesium namaquense*, a poison bush native to South Africa, has been recognized to be poisonous to bunnies and sheep, causing symptoms such as lethargy, anorexia, cyanosis, and breathing difficulties. Toxicology tests revealed that just 200 milligrams of dry plant matter was deadly when consumed by sheep, however more study is needed to determine the toxic compounds. The aboveground portions of *T. minkwizianum*, a plant native to western Asia, contain the alkaloid thesine, which is known to be extremely hazardous at high quantities. As it depresses the brain's motor centers leading to a decreased blood pressure, reduction in intestinal activity, and loss of skeletal muscular tone. (Lombard et al., 2020).

23 species of *Thesium*, 17 of which were from Africa and 6 of which were from Asia, have both traditional and modern applications. Despite the genus's almost universal occurrence, no applications for Europe, Australia, or even North America were noted. In addition to its many smaller applications, *Thesium* has been most frequently utilized as medicines, beneficial foods and drinks, charms, along with crafts. While certain modern applications, such useful foods and also Animal feeds, growth stimuli as well as fertilizers, hygiene, control of plant diseases, and medication for animals, were exclusive to Asia, charm usage were exclusive to southern and even East Africa (Figure 14).

The most widely used and adaptable species is *Thesium chinense*, which is next followed by *Thesium longifolium*. Regarding the plant's parts utilized, no particular tendencies were noted. As a pharmaceutical *Thesium* has been utilized to treat 137 diseases most of which are related to the reproductive as well as breast systems, the

respiratory system, degenerative diseases, the digestive system, and the urinary system. It has not yet been determined whether the host plants have any effect on the chemistry and pharmacology. 2 species, *T. chinense* together with *T. viride*, have been examined for their pharmacological properties, while three other species are said to be toxic. *T. viride* possesses antibacterial action, while *Thesium chinense* has anti-oxidant, cytotoxic, anti-inflammatory, chemopreventive, as well as various other generic therapeutic qualities (Lombard et al., 2020).

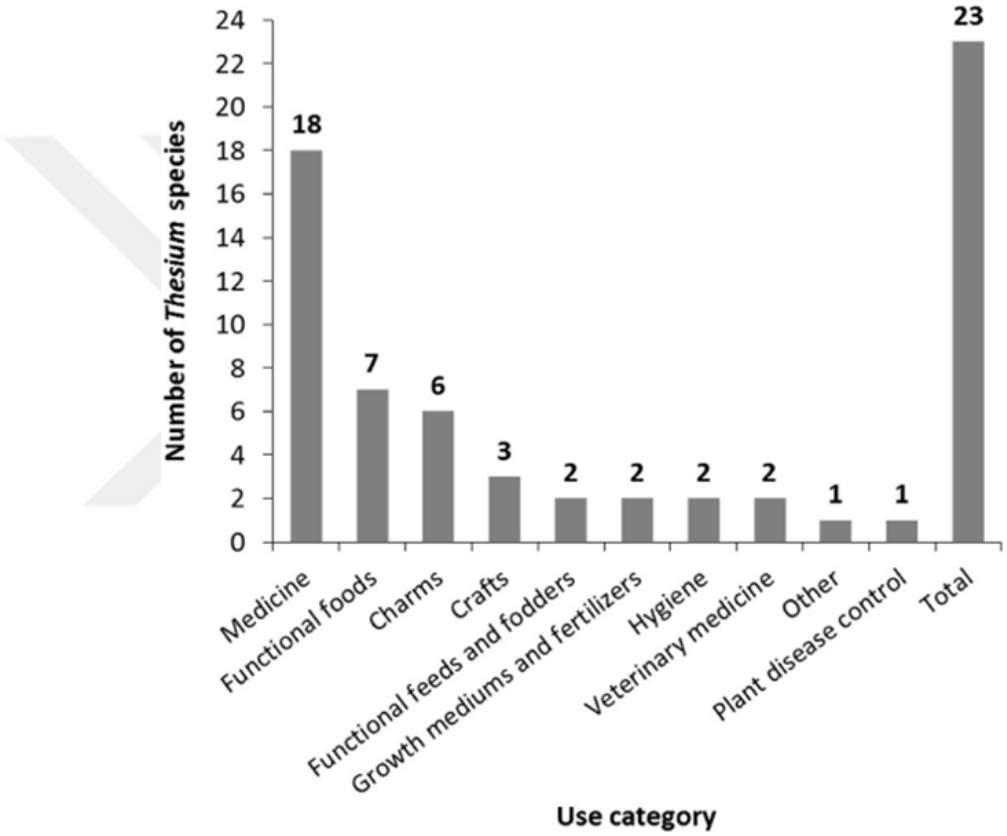


Figure 2.14 *Thesium* species' ethnobotanical and modern use worldwide, categorized into 10 distinct use groups (Lombard et al., 2020)

Eight *Thesium* species' phytochemical components have been investigated in the year 2020. A total of 70 phytochemical substances have been isolated from these species, primarily phenolics, alkaloids, in addition to fatty acids, as described in the (Table 2). Notably, just the Mediterranean region's *T. humile Vahl* and Asia's *T. chinense Turcz* had 60 of the 70 phytochemical substances extracted (Lombard et al., 2020) (Figure 15).

Table 2.2 Chemical components that have been isolated from *Thesium* species (Lombard et al., 2020)

Scientific name	**Constituent class**	**Constituent name**
Thesium himalense (Royle ex Edgew)	Others	cis-4-hydroxy-L-proline
Thesium chinense (Turcz)	Alkaloid	lupanine
		N-methylcytisine
		sophocarpine
	Fatty acid	(12E) - heptadec-12-en-8, 10-diynoic acid
		exocarpic acid
		dodec-9,11-diynoic acid
	Phenolic	kaempferol-3,7-di-O-β-D-glucopyranoside
		methyl-p-hydroxycinnamate
		kaempferol
		methyl caffeate
		kaempferol-3-O-glucoside [L
		apigenin-7-O-β-D-glucopyranuronide
		kaempferol 3-rhamnoside
		kaempferol-3-O-β-D-glucopyranoside
		luteolin-7-O-glucoside
		kaempferol-3-O-rhamnopyranosyl-(1→2)-[6-O-acetyl]-glucopyranoside
		acacetin-7-O-β-d-rutinoside
		naringenin-4-O-glucoside
		rutin
		quercetin-3-O-neohesperidoside
		apigenin-5-O-neohesperidoside
		homoplantaginin
		apigenin-8-C-α-L-arabinopyranoside
		apigenin-7-O-glucoside
		pectolinarin
		kaempferol-3-O-β-D-glucopyranoside-6"-(3-hydroxy-3-methylglutarate)
		chrysoeriol 7-O-glucuronide
		kaempferyl 5-methyl ether
		chrysoeriol
		kaempferol-3-O-rutinoside
		kaempferol-3-O-neohesperidoside
	Terpene	(−)-loliolide

		5,6-epoxy-3-hydroxy-7-megastigmen-9-ene
Thesium humile (Vahl)	Alkane	3-3dimethyl hexane
		undecane
		docosane
		eicosane
		cyclotetradecane
	Alkaloid	1-hydroxymethylpyrrolizidine
	Alkene	1-octadecene
		1-hexadecene
	Fatty acid	dodecanoic acid methyl ester
		n- tridecanoic acid methyl ester
		octadecanoic acid methyl ester
		n-tetradecanoic acid methyl ester
		hexadecanoic acid, butyl ester
		hexadecanoic acid methyl ester
		pentadecanoic acid methyl ester
		9-octadecenoic acid (Z) methyl ester
		10-octadecenoic acid methyl ester
		exocarpic acid
		ximenynic acid
	Others	tricosanol-12
		B-sitosterol
		D-mannitol
		phenol 4,6-di(1,1-dimethylethyl)-2-methyl
		1-octadecanol
	Phenolic	phenol, 2,4-bis(1,1-dimethylethyl)
		phenol 4,6-di(1,1-dimethylethyl)-2-methyl
Thesium divaricatum (Jan ex Mert. & W.D.J.Koch)	Phenolic	isorhamnetin-3-O-galactoside
		isorhamnetin-3-O-galactorhamnoglucoside
		isorhamnetin-3-O-galactorhamnoside
Thesium australe (R.Br)	Fatty acid	octadecenediynoic acid
Thesium wightianum (Wall. ex Wight)	Others	cis-4-hydroxy-L-proline
Thesium minkwitzianum (B.Fedtsch)	Alkaloid	D- isoretronecanol
		thesinicine
		thesinine
		thesine
	Others	succinic acid

		D-mannitol
Thesium hystrix (A.W.Hill)	Phenolic	quercitrin

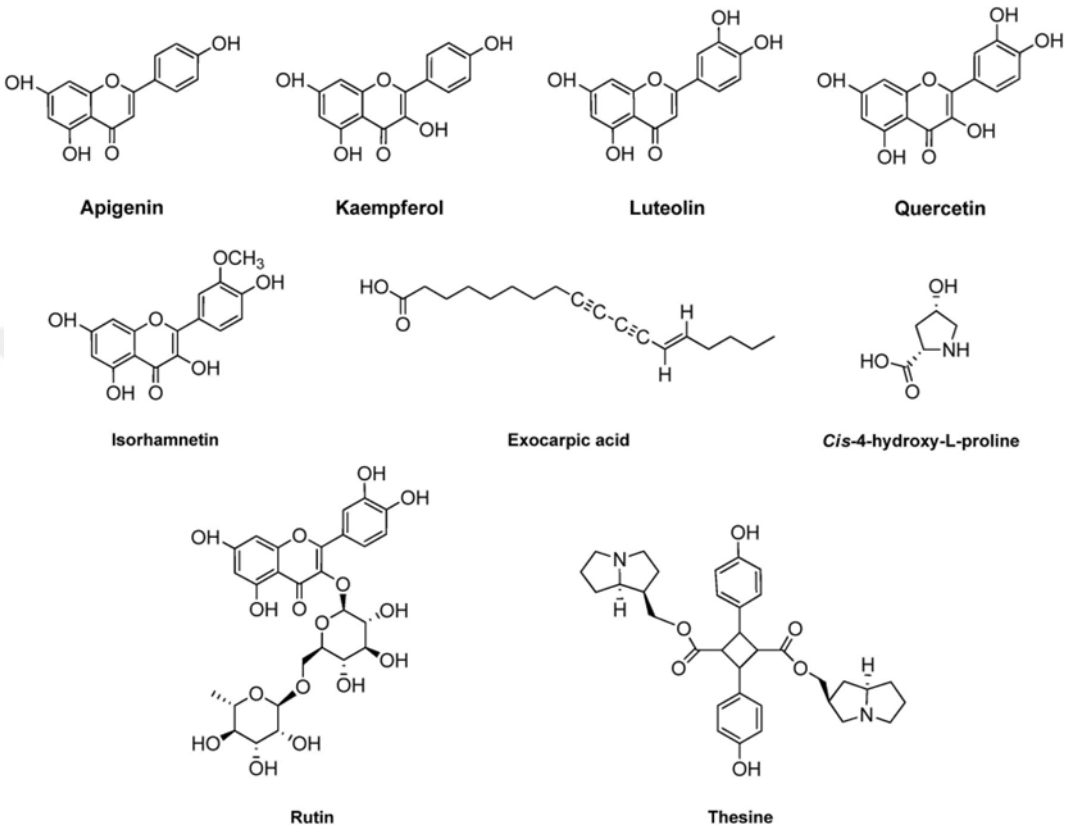


Figure 2.15 The molecular structures of compounds that have been isolated from *Thesium* species (Lombard et al., 2020)

According to studies on the medicinal plant *T. chinense*, thirty-four compounds—including Terpenoids, alkaloids, flavonoids, organic acids, and others as well—have been identified thus far. Flavonoids are the plant's main ingredients and have a significant role in *T. chinense*'s biological activity. In the form of flavonols and flavones, 19 flavonoids have been isolated from *T. chinense*. These flavonols involve rutin, kaempferol, kaempferyl 5-methyl ether, apigenin-5-O-neohesperidoside astragalin, afzelin, kaempferol-3-O-glucorhamnoside, kaem pferol-3,7-di-O-β-D-glucopyranoside, and kaempferol-3-O-rhamno pyranosyl-(1 → 2)-[6-O-acetyl]-glucopyranoside. Derivatives of the flavone type include chrysoeriol, apigenin, apigenin-8-C-α-L-arabinopyranoside, apigenin-7-O-glucoside, luteolin-7-O-

glucoside, homoplantaginin, chrysoeriol 7-O-glucuronide, linarin, pectolinarin, and kaempferol-3-O-D-glucopyranoside-6''-(3-hydroxy-3-methylglutarate).

Three alkaloids encompassing N-methylcytisine, sophocarpine, and also lupanine were isolated from the total alkaloids extract of *T. chinense*. Two terpenoids have been identified after purification: loliolide and 5,6-epoxy-3-hydroxy-7-megastigmen-9-ene. The following components have also been isolated: (12E)-heptadec-12-en-8,10-diynoic acid, dodec-9,11-diynoic acid, methyl caffeate, succinic acid, D-mannitol, 4-hydroxybenzoic acid, 4′-hydroxyacetophenone, cinnmamic acid, methyl-p-hydroxycinnamate, and exocarpic acid. Moreover, polysaccharides, which were present in the water extraction of *T. chinense*, were shown to be significant elements of the plant with a level of around 15%.

The studies also demonstrated connections between contemporary pharmacological functions, which are mostly produced by flavonoids and alkaloids, and ethnomedical applications of *T. chinense* in folk medicine. There are anti-inflammatory, anti-oxidant, and antimicrobial properties associated with ethnomedical uses for treating enteritidis, pulmonitis, chronic bronchitis, tonsillitis, child fever, cold, and urocystitis; anti-inflammatory and anti-oxidant properties associated with mastitis, cholecystitis, and nephritis; and anti-inflammatory and analgesic properties associated with osphyalgia and abdominal pain. *T. chinense*'s strong anti-inflammatory, antiviral, and antioxidant properties also shown cardiac, neuroprotective, and hepaprotective effects (Li et al., 2021).

LC-MS analysis of 156 samples obtained from fifty different *Thesium* species from Europe, Madagascar, Africa, along with Asia revealed a diverse range of chemicals, primarily phenolic acids, flavonols, organic acids, carboxylic acids, and related derivatives. Rutin was the most prevalent chemical found in *Thesium*. Rutin was detected in all but two species (*T. cymosum* along with *T. cf. leandrianum*), and it was the primary peak in 27 species. Isorhamnetin O-glucoside O-rhamnoside, dehydrodiferulic acid, quinic acid, kaempferol O-rutinoside, quercetin robinobioside, cryptochlorogenic acid (4-caffeoylquinic acid), quercetin rhamnosyl-rhamnosyl glucoside, dehydrodiferulic acid, and citric acid. Coumaroylquinic acid,

flavonoid glycosides, and feruloylquinic acid have also been identified in good quantities. Several chemicals, including neochlorogenic acid, feruloylquinic acid, coumaroylquinic acid, cryptochlorogenic acid, eriodictyol, in addition to quercetin, were first identified in *Thesium*. Despite the fact that glycosides of apigenin, chrysoeriol, and luteolin have previously been identified in T. chinense, no flavones were discovered as significant components (Lombard et al., 2022). Numerous ethnobotanical, modern, as well as pharmaceutical applications for the genus *Thesium* demonstrate the significant functions that many of its species of plant play in societies worldwide. Nevertheless, a lot of efforts is still needed to analyze all of the information that is currently accessible, and to also assess the genus's commercial value and economic significance (Lombard et al., 2020).

2.5.2 *Thesium aureum* species

Thesium aureum Jaub and Spach. published for the first time in Ill. Pl. Orient. 2: 4 (1844). This species' natural habitat is east Türkiye. It is a subshrub or perennial that grows mostly in the moderate biome (Figure 16). There are two synonyms for it:

- *Chrysothesium aureum* (Jaub. & Spach) is a homotypic synonym .
- *Linosyris aurea* Kuntze in Revis. Gen. Pl. 2: 588 (1891) is a heterotypic synonym (Plants of the World Online, 2024).

Figure 2.16 The natural habitat of the *Thesium aureum* species (Plants of the World Online, 2024)

Thesium aureum, also known via its traditional name Anagüvelek, is a hemiparasitic plant native to the (Ir-Tr) floristic region (Sürmen et al., 2015). *Thesium aureum* is extremely similar to *T. cilicicum*, featuring a bigger fruit with reticulate surface nerves and a longer corolla tube. the phylogeny of *Thesium aureum* has not been studed yet (Mucina and Nickrent, 2024). As stated by Armağan in a 2020 study paper: In two scree-filled steppe areas, *Thesium aureum* has been identified as a Turkiye endemic and also as a novel record for Tunceli province:

1. Ovacık on June 1, 2015, at a height of 1934 m
2. Pülümür on June 18, 2014, at a height of 2400 meters

(Armağan, 2020).

Thesium species are widely used in conventional healthcare to treat a wide range of illnesses worldwide. After reviewing the literature, it was found that there is no information about the chemical composition or biological activity of *Thesium aureum* species. An investigation has been conducted to fill a gap by evaluating the biological activity of *Thesium aureum* species in term of its antioxidant capacity and its inhibitory activity with amylase, tyrosinase, cholinesterase, butyrylcholinesterase and glycosidase. Also the chemical content of *Thesium aureum* species has been analyzed via LC-MS/MS.

3. MATERIAL AND METHOD

3.1 Herbal Material

The healthy and fresh *Thesium aureum* species specimens have been gathered from its natural habitat in the Turkish districts of Elazig (Haroglu mountain,. 1500 m), and Erzincan (Sipikor mountain., 1830 m) in May 2022 (Figure 17 and 18). Then a quick rins was gave to them with distilled water to get rid of any dirt impurities or stuck insects.

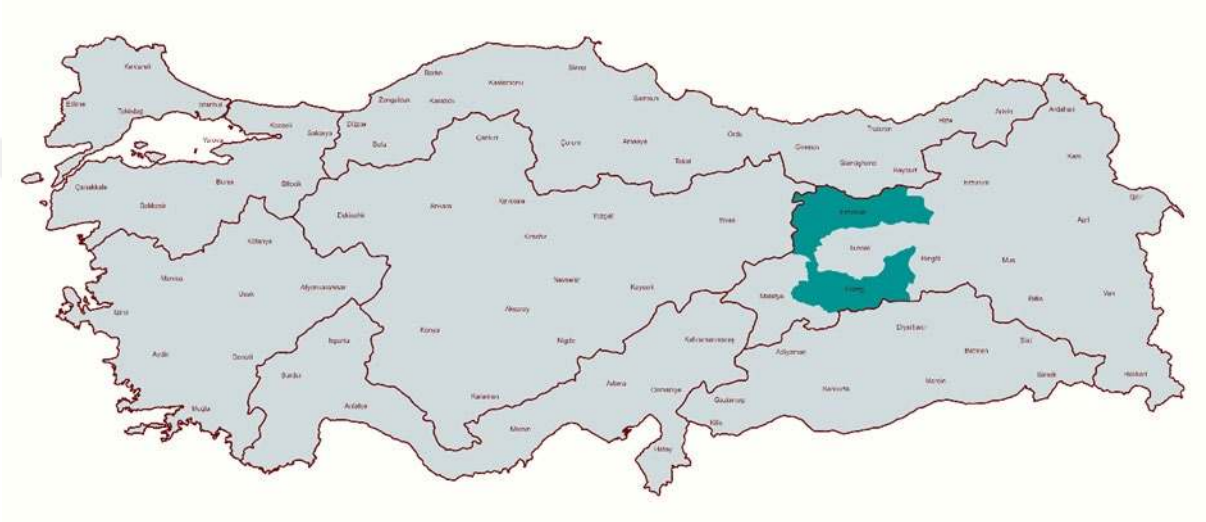


Figure 3.1 Provinces in which Samples were Collected (Elazig and Erzincan)

Figure 3.2 A picture of the gathered *Thesium aureum* species

The collected specimens were allowed to dry as quickly as possible in the open air, at the temperature of the room, and avoiding the direct sunlight as it can break down certain chemicals. The goal was to get them completely dry quickly enough so that

mold doesn't grow on them, and Before they wither up. Then they have been maintained in adequate circumstances until they were used in the research.

3.2 The Extraction Procedure

Typically, an organic compound such as ethanol is employed as a volatile extractant in solid-liquid extraction processes for capturing polyphenolic chemicals, as a conventional extraction technique (Wang et al., 2025). for the purpose of maximizing the area of the surface between the solvent and the plant material, enabling the solvent to penetrate, and extract the chemical components more efficientiy, the dried *Thesium aureum* species underwent pulverization making use of an electrically powered grinder. The procedure known as maceration was subsequently applied for obtaining the ethanol extract. To achieve the aforementioned, 10 g of the plant powder were extracted in 100 milliliters of ethanol for a whole day at a normal temperature. The extract was separated from the solid material after extraction by filtering using a filter paper, after which they were condensed using a rotating evaporator at forty degree Celsius under reduced pressure. Three duplicates of the extraction were done. After being labeled, The extract left behind was preserved under seal at 4°C in the dark for later analysis (Guha et al., 2024), as seen in the Figure 19.

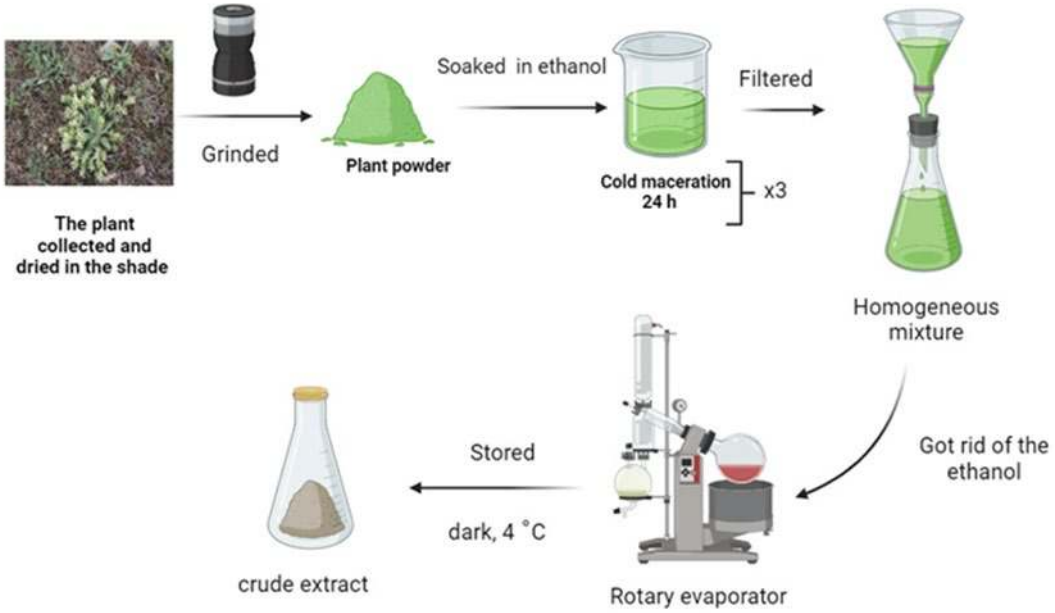


Figure 3.3 The extract preparation process

However, because of their high volatility, toxicity, and lack of renewable nature, the volatile organic solvents are not good for the environment or consumers, and makes sustainable development difficult. As a result, international laws like (REACH) tightly control the use of volatile organic solvents. Finding green solvents to replace the previously mentioned solvents is urgently needed in order to reduce environmental impact, increase downstream process efficiency, and upgrade byproducts into naturally occurring polyphenolic antioxidants that are important to industry (Wang et al., 2024).

3.3 Phytochemical Evaluation of the Extract by LC-MS/MS

Traditional techniques are ineffectual as trustworthy analytical approaches for identifying natural products in medicinal and aromatic plants because of their complicated matrices and low concentrations of chemicals. The most efficient method for both qualitative and quantitative characterization is U-HPLC combined with tandem mass spectrometry (Yilmaz, 2020) (Figure 20).

Figure 3.4 LC-MS/MS instrument

Quantitative evaluation of the botanical compounds in the ethanolic extract of *Thesium aureum* species was done via the LC-MS/MS methodology, which had been designed and tested beforehand (Yilmaz, 2020). In the utilized LC-MS/MS

method, 53 natural phytochemicals including a stilbenoid glucoside, a benzopyrone, a biflavonoid, twenty phenolic acids, three phenolic aldehydes, and fourteen flavonoid aglycones were detected in terms of quantitative as well as qualitative in the extract under study. By making up for matrix effects and analyte losses during sample preparation and analysis, three isotopically labelled internal standards—Ferulic acid D3, quercetin D3, and rutin D3 are utilized for non-flavonoid molecules, flavonoids and, flavonoid glycosides respectively.—were employed to boost the reliability of the results. For that purpose, rutin D3 (1 mg/L), quercetin D3 (5 mg/L), and (20 mg/L) of ferulic acid D3 were spiked to dry extracts. Subsequently, it was diluted with ethanol until the solution concentration reaches (1 g/L) and filtered through 0.2 μm syringe filter before undergoing LC-MS/MS analysis (Figure 21).

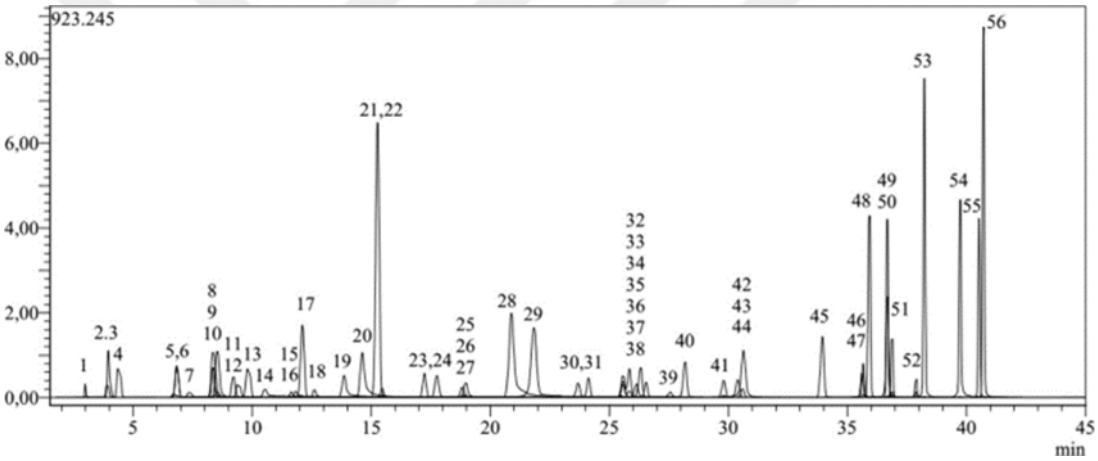


Figure 3.5 Chromatogram of the total ions for standard phenolics {[1] Quinic acid - [2] Fumaric acid - [3] Aconitic acid - [4] Gallic acid - [5] Epigallocatechin - [6] Protocatechuic acid - [7] Catechin - [8] Gentisic acid - [9] Chlorogenic acid - [10] Protocatechuic aldehyde - [11] Nicotine - [12] Epigallocatechin gallate - [13] 1,5-dicaffeoylquinic acid - [14] 4-OH Benzoic acid - [15] Epicatechin - [16] Vanilic acid - [17] Caffeic acid - [18] Syringic acid - [19] Vanillin - [20] Syringic aldehyde - [21] Daidzin - [22] Epicatechin gallate - [23] The piceid - [24] *p*-Coumaric acid - [25] Ferulic acid D3 - [26] Ferulic acid - [27] Sinapic acid - [28] Coumarin - [29] Salicylic acid - [30] Cynaroside - [31] Miquelianin - [32] Rutin - [33] Rutin D3 - [34] Isoquercitrin - [35] Hesperidin - [36] *o*-Coumaric acid - [37] Ellagic acid - [38] Rosmarinic acid - [39] Genistin - [40] Astragalin - [41] Quercitrin – [42] Cosmosiin – [43] Nicotiflorin – [44] Fisetin – [45] Daidzein – [46] Quercetin D3 - [47] Phytic acid – [48] Naringenin – [49] Hesperetin – [50] Luteolin – [51] Genistein – [52] Kaempferol – [53] Apigenin – [54] Amentoflavone – [55] Chrysin – [56] Acacetin} were examined using the newly designed LC–MS/MS technique (Yilmaz, 2020)

The above mentioned phytochemicals were screened using a Shimadzu-Nexera model of UHPLC interconnected to tandem mass spectrometer. Autosampler (SIL-30AC), column oven (CTO-10Asvp), binary pumps (LC-30CE), and degasser (DGU-20A3R) were fitted to UHPLC (reversed-phase). Regarding conducting chromatography separateness, a (150 mm x 2.1 mm x 2.7 m) column of reversed-phase - an Agilent Poroshell (120 EC-C18 model)- was adopted. The column's thermostat was 40°C, chemicals of the A eluent were 5 mM ammonium formate, water, and 0.1% formic acid. Wherase, chemicals of the B eluent were 5 mM ammonium formate, ethanol, in addition to 0.1% formic acid. Additionally, the gradient elution profile adopted was 20 – 100 % B from 0 to 25 min, 100 % B from 25 to 35 min), and then 20 % B from 35 to 45 min. Furthermore, the rate of the eluent flow has been set as 0.5 mL /min and the dose of the injection has been fixed at (5 μL).

Having an electrospray ionization source -ESI - that could function in both negative as well as positive ionization modes, a LCMS-8040 model of the brand Shimadzu tandem mass spectrometer was used for the mass spectrometric detection task. Acquiring and processing the LC-ESI-MS/MS data was done via LabSolutions software (Shimadzu). Using multi-reaction monitoring (MRM) technology, the phytochemicals were measured. The MRM approach was created to identify and evaluate only phytochemical compounds. It was based on the screening of particular precursor phytochemical-to-fragment ion transitions. The MS functioning conditions had been set with the following values: DL temperature of 250 °C; 400 degrees Celsius for the heat block; interface temperature of 350 °C; flow rates of N_2 (the drying gas) was (15 L/min); and (3 L/min) for the flow of the N_2 (nebulizing gas).

3.4 The Total Phenolic Content

With a few minor adjustments, the Folin-Ciocalteu assay explained in the academic work (Slinkard and Singleton, 1977) has been employed to spectrophotometrically evaluate the total phenolic content within *Thesium aureum* species ethanolic extract. The Folin-Ciocalteu reagent reacts with phenolic chemicals exclusively in basic circumstances (10 pH, adjusted with a Na_2CO_3) when a phenolic proton split up to generate a phenolate ion, converting the yellow F-C reagent to blue phosphotungstic-

phosphomolybdenum complex, which can be measured using tools such as spectrophotometric microplate readers.

This classical reagent of F-C is only suitable for antioxidants having a solubility in water. The result is commonly calculated to be a gallic acid equivalent (GAE), as gallic acid exhibits the greatest degree of absorption when compared to other chemicals such as neochlorogenic or chlorogenic acid (Dominguez-López et al., 2024). Several scientists have demonstrated that FCR is an extremely sensitive and precise approach. Furthermore, this method has become recognized as the predominant technique for phenolic component detection and quantification in a range of matrix types.

At first, a 2 mg /mL solution has been prepared from the crude extract. Thereafter 250 µL of that solution was taken into an eppendorf tube. Then 1 milliliter of the (1:9) diluted Folin-Ciocalteu reagent (made by thinning the store-bought solution for ten times with distilled water) was added and shaked properly. After 3 minutes we added 750 µL of 1% Na_2CO_3 solution, the tube was incubated about 2 hours in the absence of light under room temp. The sign of the presence of phenols was the extracts' darkening blue colour in comparison to the blank (Figure 22).

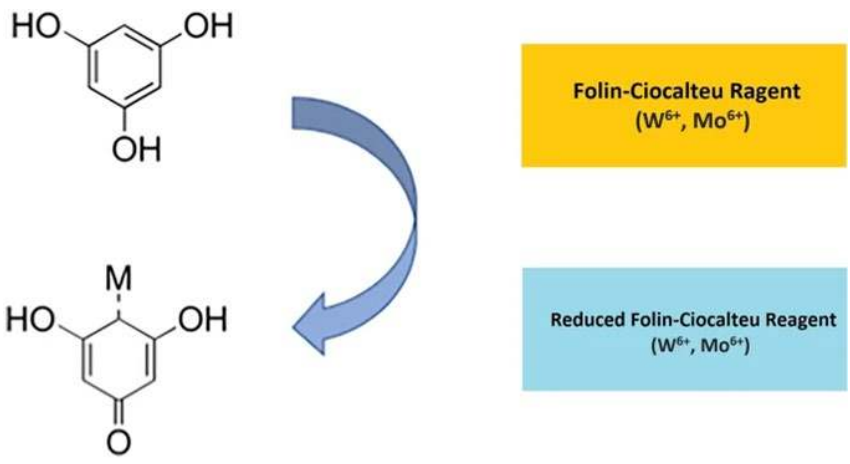


Figure 3.6 Folin-Ciocalteu method's guiding principle (Shi et al., 2022)

The same process was repeated in exactly the same way for the reference compound (gallic acid). aAfter the incubation, Absorbances have been measured against the blank sample at 765 nm. Spectrophotometric measurements were carried out using

the Shimadzu UV-1800 spectrophotometer device. Three measurements were made of the samples, and the amount of the overall output of polyphenols was determined means of gallic acid equivalent (mg GAE /g) method.

3.5 The Total Flavonoid Content

The total content of flavonoids has been determined by applying the procedure described in the published material (Berk et al., 2011). A 2 % $AlCl_3$ (aluminium trichloride) methanolic solution was prepared. 1 mL of this solution was taken and combined with exactly identical volume of the crude extract (2 mg /mL). Once ten minutes had passed, It was determined how absorbent the mixture was at a wavelength of 415 nm against the blank sample consisting of a 1 mL extract solution with 1 mL methanol without $AlCl_3$. The same procedure was repeated for rutin (standard). The total content of flavonoids has ben determined in the form of rutin equivalent (mg RE /g).

3.6 The Antioxidant Capacity

Chemical tests for determining antioxidant capacity are quick and easy, and they can provide results shortly thereafter. Nevertheless, none of these approaches are typical, and their underlying principles are very different. Most significantly, they do not accurately reflect the circumstances in vivo. As a result, many approaches should be used concurrently and must also be thoroughly and comparably examined. It is best to approach their benefits and drawbacks in a dialectic, and the realistic experiment settings and needs determine which of these procedures should be used. We employed a number of chemical assays in our investigation, including the metal-chelation assay, FRAP assay, DPPH assay, ABTS assay, phosphomolybdate assay, and CUPRAC [cupric ion reducing antioxidant capacity] assay. These assays are quick identification methods, but their reaction mechanisms differ greatly from the body's natural response.

3.6.1 Phosphomolybdate assay

The easily performed, affordable, and widely recommended phosphomolybdate assay developed by Prieto in 1999 (Prieto et al., 1999) has been utilized to find out an overall antioxidant potential of *Thesium aureum* species ethanolic extract

depending on the ammonium molybdate's reduction in acidic environments to the Keggin ion [$H_3PO_4(MoO_3)_{12}$] oxide form. The greenish phosphomolybdenum complex is formed once the resultant Keggin ion is reduced into [$(H_4PMo_8VIMo_4VO_{40})^{3-}$]in the presence of an antioxidant. Figure 23 illustrates the reaction's process and color shift. Even at the temperature of the room the phosphomolybdenum compound may come about. On the other hand, the complex yield is poor and the rate of reaction is quite sluggish. The complex's development is reliant on temperature according to (Prieto et al.,1999). Rising the temp to 95 °C has sped up the reaction rate and raised the yield of the greenish phosphomolybdenum complex. Since it is believed that all antioxidant chemicals may be involved in the test, this assessment is known as a total antioxidant capacity test.

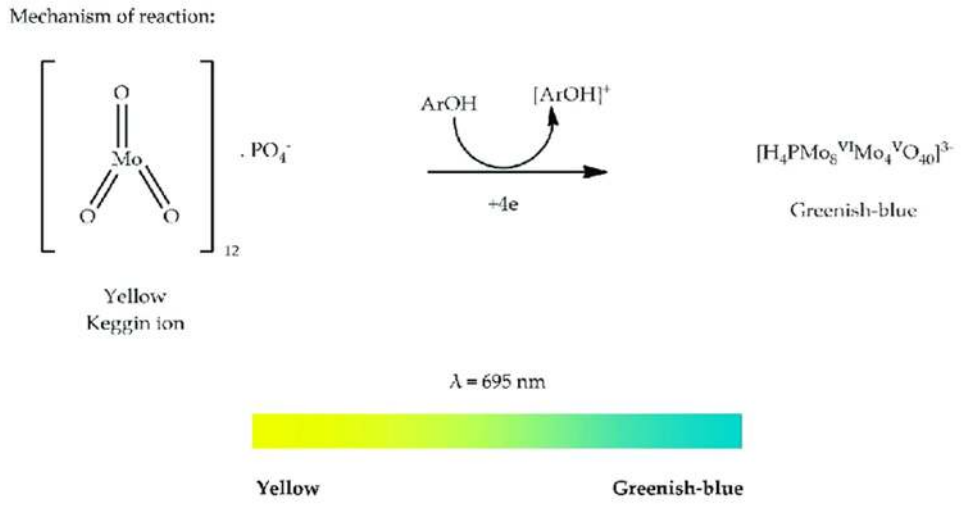


Figure 3.7 The reaction of phosphomolybdate assay (Bibi Sadeer et al., 2020)

First the following procedure was used to prepare the reagent solution for the method. 0.6 M H_2SO_4 solution: (20 mL) distilled water was taken, followed by (0.83175 mL) H_2SO_4, then completed by distilled water to the volume (25 mL). 4 mM Ammonium molybdate solution: 0.123585 g of ammonium molybdate was weighed and completed to (25 mL) with distilled water. 28 mM $Na_2HPO_4.12H_2O$ solution: 0.025 g $Na_2HPO_4.12H_2O$ was weighed and its volume was completed with distilled water to 25 mL. We obtained the reagent by mixing the three solutions. (3 mL) reagent solution was added to a tube containing 0.3 mL of the *Thesium aureum* methanolic extract solution at a concentration of 2 mg/mL. After giving it a good stir, the tube has been incubated at 95°C and for 90 minutes. The absorbance values of

the greenish colored product was measured after cooleding dowen at 695 nm. A greater absorbance value denotes a larger potential for total antioxidants in the plant extract. The same procedures were repeated for trolox. Antioxidant activity was calculated by taking Trolox equivalent (mg TE/g).

3.6.2 The metal-chelating capacity

Iron is essential for many processes within the cell, but it can additionally cause free radicals, which are responsible for oxidative stress-related illnesses. Iron is often linked to a number of disorders including diabetes, cancer, liver and heart issues, and neurological disorders. Lead, arsenic, as well as different heavy and transition metals additionally have the potential to generate free radicals. Chelation treatment is used for elimination of these disastrous metals (Bibi Sadeer et al., 2020).

The ferrous ion chelating test is carried out in compliance with the protocol designed by (Dinis et ai., 1994) to determine whether the plant extract could outcompete the iron indicator ferrozine ($C_{20}H_{12}N_4Na_2O_6S_2 \cdot xH_2O$) In somewhat acidic (pH 6) circumstances, and reduce the maximal absorption of the magenta-coloured iron(II)-ferrozine a stable, water-soluble complex. (Figure 24). Due to the fact that Fe^{2+} reacts with peptides and sulphates in the media in addition to phenolic substances, this test can't be considered as anon-specific.

First, a solution of 2 mM 0.05 mL $FeCl_2$ was added into a test tube that held 2 mL of *Thesium aureum* ethanolic extract (2 mg/mL). Then 0.2 millilitre of 5 mM ferrozine was added to start the reaction. Following ten minutes of room temperature incubation for the combined tube, the absorbance at 562 nm was measured against tha blank wich has been prepared in the absence of using ferrozine, through the combination of 0.2 mL of distilled water, 0.05 mL $FeCl_2$ (2 mM), and 2 mL of the sample's solution. The same processes were followed with EDTA, a chelating agent that is commonly utilized. The data are expressed as equivalent of EDTA (mg EDTA/g).

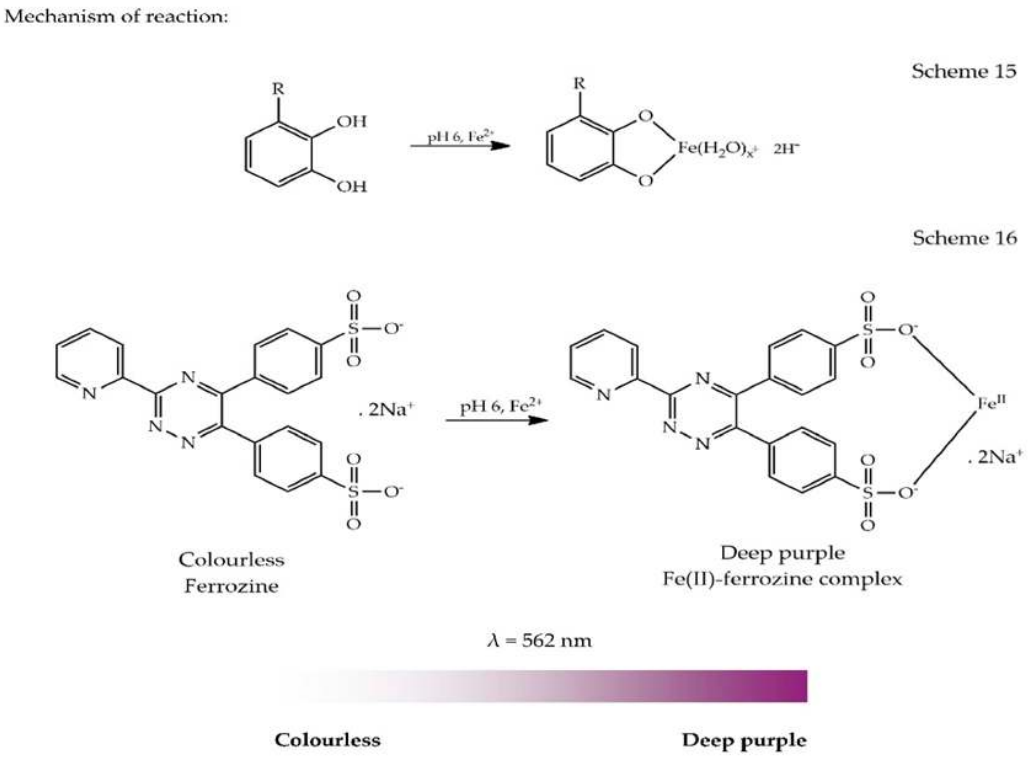


Figure 3.8 Metal chelating reactions (Bibi Sadeer et al., 2020)

3.6.3 CUPRAC assay (reduction activity)

The cupric ion reducing antioxidant capacity (CUPRAC) reagent was used to measure the overall antioxidant capacity of the ethanolic extract of our endemic *Thesium aureum*. Just seven years after the publication of the FRAP methodology, Apak, Guclu, Ozyurek, and Karademir from Istanbul University's Analytical Chemistry Department designed the CUPRAC procedure. Unlike the erroneously acidic pH 3.6 of FRAP, the experiment in this assay is run at pH 7, which is nearly physiological, the reagents are more affordable, more readily available, and somewhat better accessible compared to DPPH as well as ABTS reagents. Other advantages are the ability for assessing materials that are hydrophilic or lipophilic, rapid emergence of color, simple equipment is needed, a linear collaboration usually is noticed beside the phosphomolybdenum analyse, and it exhibits strong correlation with a variety of polyphenolics, including flavonoids and phenolic acids, as well as other antioxidant techniques like ABTS (Bibi Sadeer et al., 2020) (Figure 25).

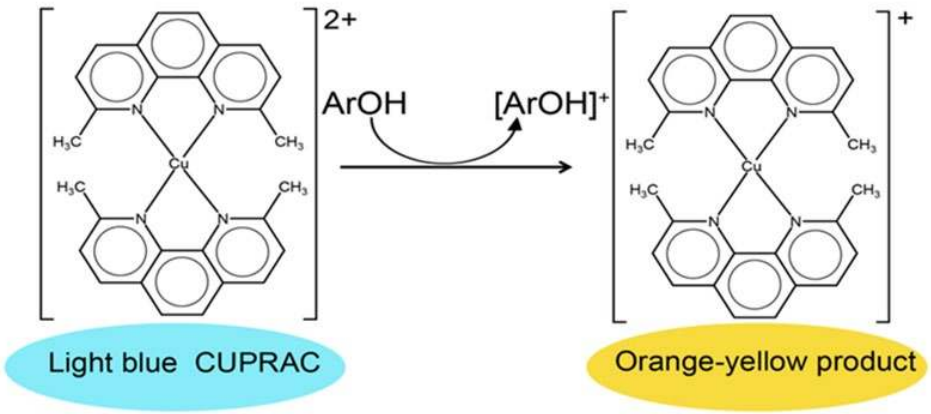


Figure 3.9 Cuprac reagent reduction reaction (Massoud rt al., 2022)

Cu^{2+} conversion to Cu^{+} is the principle of this assay. To ease absorbance measurement, a copper–ligand combination is formed using a neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline) ligand. In order to prepare the reagent: 250 mL Stock solution of copper(II) chloride (10^{-2} M) is prepared using distilled water and 0.4262 g of dihydrate salt ($CuCl_2.2H_2O$). 250 ml buffer (7 PH) from dissolving, and then diluting (19.27 g) NH_4Ac in distilled water. Solution of Neocuproine (7.5×10^{-3} M) through utilizing the same solvent to dilute 0.039 g of neocuproine (2,9 dimethyl 1-10 phenantroline) to 25 mL after dissolving it in 96 % ethanol (ought to be made immediately). One millilitre from each of the solutions ($CuCl_2$, neocuproine alcoholic, and NH_4Ac) were added to a test tube. The mixture was then well mixed. Next, (0.5) millilitre of sample solution was added and stirred together well. The absorbance was measured 30 minutes later at 450 nm. The result was put as trolox equivalent (mg TE/g) (Apak et al., 2006).

3.6.4 FRAP assay (reduction activity)

Thesium aureum ethanolic extract's capacity to reduce ferric iron is measured by this approach (FRAP). An vivid blue colour results from FeII (ferrous), which is formed after reducing the FeIII-TPTZ complex (ferric-tripyridyltriazine) at low pH. Therefore, the sample's ability to reduce is what causes color creation. This assay is easy to use, rapid to complete, and the reaction has a linear relationship with the molecular concentration of the antioxidants involved (Figure 26).

As needed, working FRAP reagent was made by combining 2.5 mL of 10 mmol/liter TPTZ (2,4,6-tripyridyl-s-triazine) solution, 2.5 mL of 20 mmol/liter $FeCl_3r_6H_2O$ solution, and 25 mL (300 mmol /L) acetate buffer (pH 3.6). After combining 0.1 mL of (2 mg /mL) *Thesium aureum* species extract with 2 mL of the FRAP reagent. Its absorbance was measured then after half an hour at 593 nm. FRAP activity of the extract was measured in trolox equivalent (mg TE /g) (Benzie and Strain, 1996).

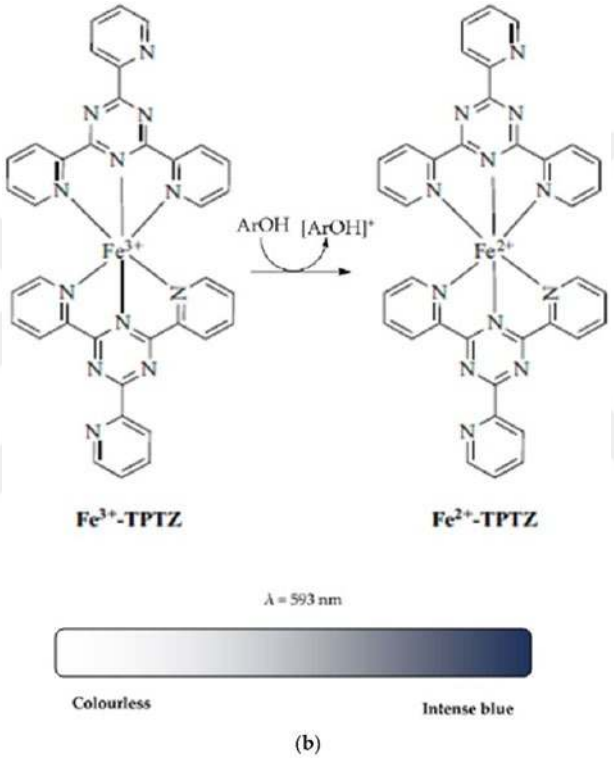


Figure 3.10 FRAP assay-based Fe $^{3+}$ reduction (Munteanu and Apetrei, 2021)

3.6.5 DPPH assay

The ethanolic extract of *Thesium aureum* species was tested for its ability to scavenge radicals. The relatively stable radical DPPH is frequently employed to evaluate the radical scavenging ability of extracts from natural sources. With this technique, the antioxidants donate hydrogen to the radical DPPH (stable in alcohol) converting it to non-radicalic DPPH-H.

First, a (0.004%) methanol solution of DPPH was made. Next, a mixture was created using 150 µL of the methanolic DPPH (0.004%) solution and 50 µL (2 mg/mL) of

the extracts. For thirty minutes, this combination was kept in complete darkness. The DPPH ruddy solution turns yellow when antioxidants are added. and after that, absorbances at 517 nm were taken. The extracts' DPPH activity was calculated as trolox equivalent (mg TEs /g) (Figure 27).

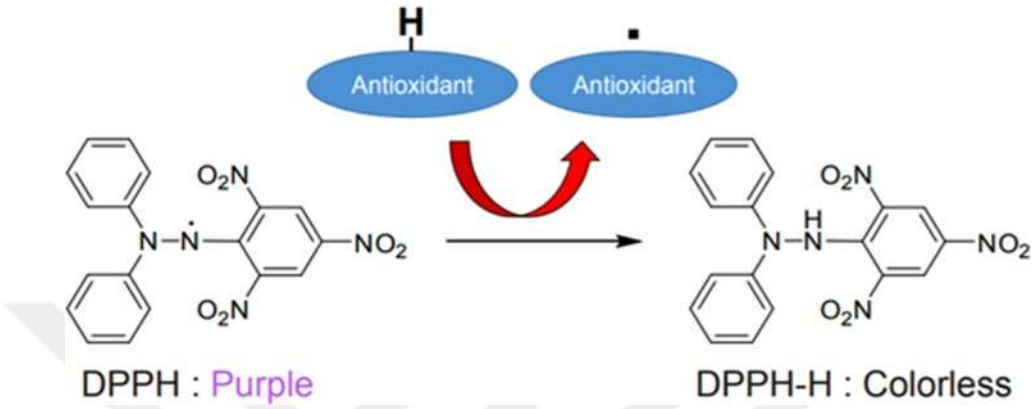


Figure 3.11 DPPH radical's interaction with antioxidant (Xiao et al., 2020)

3.6.6 ABTS assay

This experiment works with both hydrophilic along with lipophilic antioxidants. When ABTS is oxidized in the presence of potassium persulfate, the pre-formed radical blue/green monocation of ($ABTS^{\bullet 1}$) is produced (Figure 28). When placed in suitable conditions of darkness and ambient temperature, the radical remained stable in this state for over two days. This monocation is then reduced in the presence of antioxidants that donate hydrogen.

This assay has beeb started with combining (2.45 mM) $K_2S_2O_8$ (potassium persulfate) and (7.4 mM) ABTS solution. The mixture was kept in the dark for 12 to 16 hours in order to allow the formation of $ABTS^{\bullet 1}$ radicals. Ethanol was used to dilute the $ABTS^{\bullet 1}$ solution until the absorbance was 0.70 at 734 nm. One milliliter of the extract and two milliliters of ABTS solution were combined. After being allowed to sit at room temperature for thirty minutes, the sample's absorbance was taken measured at the wavelength 734 nm. The same protocols were followed for trolox. The result of the assay was expressed as trolox equivalent (mgTEs /g) (Re et al., 1999).

Figure 3.12 The interaction between ABTS and $K_2S_2O_8$ produces $ABTS^+$ (Anh-Dao et al., 2022)

3.7 The Enzyme Inhibition Capacity

Scientific experiments have found that some chemical molecules that plants produce have the ability to inhibit some enzymes. Enzymes are considered directly or indirectly responsible for causing diseases, as inhibiting the enzyme responsible for a specific disease, completely or partially, is the principle of action of most drug treatments.

3.7.1 Tyrosinase inhibition capacity

Tyrosinase inhibitory activity of *Thesium aureum* species was determined by slightly altering the modified dopachrome method, which used l-DOPA as the substrate and was previously explained (Zengin et al., 2014). 25 µL sample solution was combined with 40 µL tyrosinase solution, and 100 µL buffer (phosphate, pH 6.8). The mixture was incubated at 25 °C for 15 minutes. Thereafter, 40 µL l-DOPA was added in order to start the reaction. After deducting the blank's absorbance from the sample's, the tyrosinase inhibitory activity of *Thesium aureum* species was reported as kojic acid equivalent (mg KAEs /g).

3.7.2 Cholinesterase (AChE and BChE) inhibitory activity

The most typically employed Ellman et al. (1961)'s spectrophotometric approach was used to evaluate the tested *Thesium aureum* species' cholinesterase inhibitory activities. 25 µL of [ChE / BchE enzyme solution + Tris-HCl buffer (pH 8.0)] and 125 µL [5,5-Dithio-bis(2-nitrobenzoic) acid] were combined with 50 µL *Thesium aureum* species extract. After 15 minutes incubation, 25 µL of substrate solutions (either butyrylthiocholine iodide (BTCI) or acetylthiocholine iodide (ATCI) were added to this mixture. At 405 nm, the absorbance of the combinations incubated for 10 minutes at room temperature was measured In order to gauge 5-thio-2-

nitrobenzoic acid anion production. The standard used was galantamine, so the result of *Thesium aureum* species inhibition capacity was caculated and reported as galantamine equivalent (mg GALAE /g).

3.7.3 α-Glucosidase inhibitory activity of *Thesium aureum* species

The evaluation has beeb carried out using the technique described earlier in a published scientific paper (Lazarova et al., 2015). In a 96-well microplate, [(2 mg mL−1; 50 µL) sample solution + 50 µL α-glucosidase solution + 50 µL glutathione + 50 µL of the mixture (PNPG (4-N-trophenyl-α-D-glucopyranoside) with pH 6.8 buffer of phosphate)] has been put together. The microplate was thereafter incubated at 37 °C for a quarter of an hour. Three iterations of the test were conducted. Finally, the reaction was stoped by adding (0.2 M; 50 µL) sodium carbonate. The microplate's absorbances were measured at 400-nm wavelength. After deducting the blank's absorbance from the sample's, the activity of *Thesium aureum* species as α-glucosidase inhibitor was expressed as acarbose equivalents (mg ACAE /g).

3.7.4 α-Amylase inhibitory activity of *Thesium aureum* species

Thesium aureum sprcies extracts' ability to inhibit amylase was assessed using the Caraway-Somogyi (iodine/potassium iodide) technique (Zengin et al., 2014). Each of the microplates was filled with 50 µL of herbal extract and 50 µL of amylase enzyme solution (prepared using a pH 6.9 phosphate buffer). The microplates were incubated at 37 °C for 10 minutes. Following the incubation period, 100 µL of a 0.05 % starch solution was added and incubated for 10 minutes at 37 °C . The reaction has been stopped by adding (1M; 25 µL) HCl. After adding 100 µL of iodine-potassium iodide as a colorant, absorbances were measured at 630 nm. The findings were assessed in terms of acarbose equivalent (mmol ACAE /g), with acarbose serving as the standard.

4. RESULTS AND DISCUSSIONS

The ethanol extract made from *Thesium aureum* species, an indigenous plant obtained from locations of Elazig (Haroglu Mountain, 1500 m) and Erzincan (Sipikor Mountain, 1830 m), was examined for their capacity for antioxidants and enzyme inhibitor activity. Also LC-MS/MS investigations was carried out to identify and quantify the secondary metabolisms withen the *Thesium aureum* species' ethanol extract.

Ultrahigh performance liquid chromatograph (version Shimadzu-Nexera) in combination via tandem mass spectrometer (MS/MS) has been utilized to identify and quantify 53 secondary metabolites in the ethanol extract of the *Thesium aureum* species using the approach that Yılmaz developed for method validation. Experiments were conducted to measure the anti- diabetes (inhibition of α-amylase & α-glucosidase), anti-cholinesterase, anti-tyrosinase, in addition to anti-butyrylcholinesterase activities.

Since absolutely no one approach is capable of accurately representing the antioxidant capacity of a plant spices, many different kinds of techniques have been employed to find out the *Thesium aureum* spices' antioxidant capacity. The techniques that were used were the ones that were most often found in the literature: metal chelation, ABTS, DPPH, total antioxidant capacity, and iron and copper reducing power approaches. The extract's total flavonoid and phenolic contents were also determined as an additional to these assays.

4.1 The LC-MS/MS Analyses of *Thesium aureum* Species

By using LC-MS/MS to analyze the ethanol extract from *Thesium aureum* species in quantitative as well as qualitative terms, 53 different phenolic, flavonoid, and organic acid components were detected. As internal guidelines, quercetin-D3, routine-D3, as well as ferulic acid-D3 compounds have been used. The findings are laid out in the Table 3. Since *Thesium aureum* species is semi-parasitic, there may be an effect of the host on the nature of the chemicals present in it, as the compounds may change with the change in the host.

Table 4.1 Phytochemicals reported in *Thesium aureum* species by LC-MS/MS

Analyte	Quantitative Results (mg analyte/kg extract)	Analyte	Quantitative Results (mg analyte/kg extract)
Quinic acid	199	Salicylic acid	U
Fumaric acid	U	Cyranoside	U
Aconitic acid	U	Miquelianin	U
Gallic acid	U	Rutin-D3-IS	NA
Epigallocatechin	U	Rutin	U
Protocatechuic acid	U	Isoquercitrin	9
Catechin	U	Hesperidin	49
Gentisic acid	U	*o*-Coumaric acid	U
Chlorogenic acid	U	Genistin	U
Protocatechuic aldehyde	U	Rosmarinic acid	U
Tannic acid	U	Ellagic acid	U
Epigallocatechin gallate	U	Cosmosiin	U
1,5-dicaffeoylquinic acid	U	Quercitrin	U
4-OH Benzoic acid	U	Astragalin	U
Epicatechin	U	Nicotiflorin	U
Vanilic acid	U	Fisetin	U
Caffeic acid	U	Daidzein	U
Syringic acid	U	Quercetin-D3-IS	NA
Vanillin	U	Quercetin	U
Syringic aldehyde	U	Naringenin	3
Daidzin	U	Hesperetin	U
Epicatechin gallate	U	Luteolin	9
Piceid	U	Genistein	U
p-Coumaric acid	U	Kaempferol	U
Ferulic acid-D3-IS	NA	Apigenin	3
Ferulic acid	U	Amentoflavone	U
Sinapic acid	U	Chrysin	5
Coumarin	U	Acacetin	37

U: Undetected, NA: Not Applicable, IS: Internal Standard

The ethanolic extract of the species *Thesium aureum* revealed a total of eight distinctive secondary metabolites comprising: quinic acid (199 mg analyte/kg extract), hesperidin (49 mg analyte/kg extract), acacetin (37 mg analyte/kg extract), luteolin (9 mg analyte/kg extract), iso quercitrin (9 mg analyte/kg extract), chrysin (5 mg analyte/kg extract), naringenin (3 mg analyte/kg extract), in addition to apigenin (3 mg analyte/kg extract). The chromatogram of our ethanolic extract is presented in Figure 29.

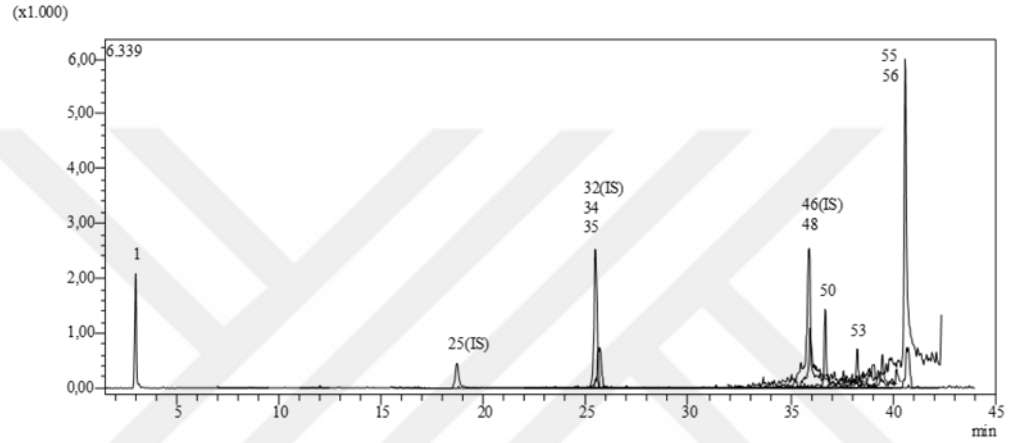
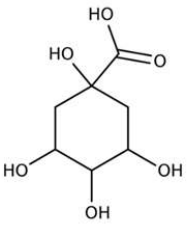


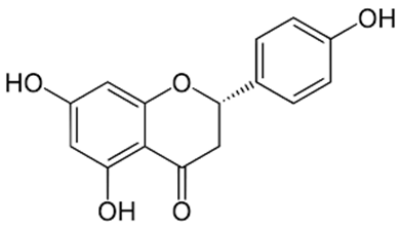
Figure 4.1 The chromatogram of the ethanolic extract of the *Thesium aureum* species

The ethanolic extract of *Thesium aureum* species did not have an abundance of phenolic chemicals. Quinic acid has been determined to represent the component exhibiting the highest concentration within the *Thesium aureum* pieces ethanolic extract (199 mg analyte/kg extract). Quinic acid was one of the seven most prevalent chemicals found in fifty *Thesium* species in accordance with Lombard (2022). When we looked at the findings of a research by Lombard (2020), we found that luteolin and apigenin are also common compounds between *Thesium aureum* species and other *Thesium* species

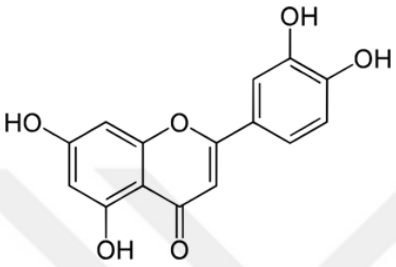
The clear chemical structures of the secondary metabolites found in the study was (Figure 30):



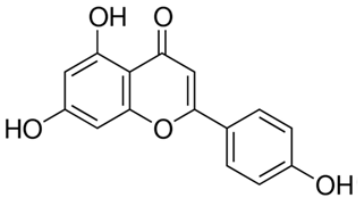
Quinic acid



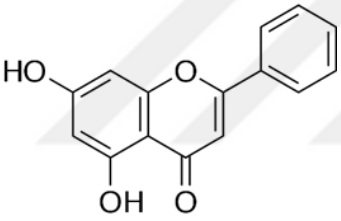
Naringenin



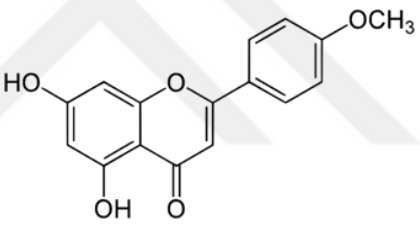
Luteolin



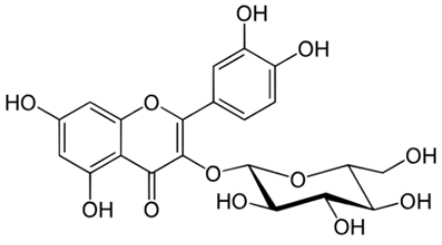
Apigenin



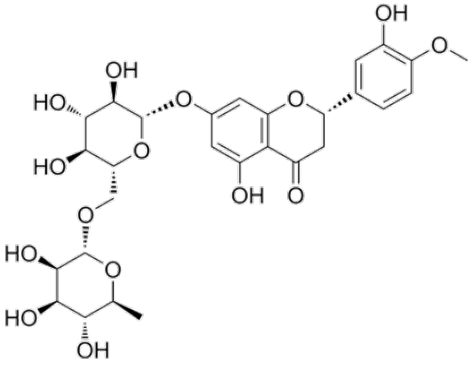
Chrysin



Acacetin



Iso quercitrin



Hespiridin

Figure 4.2 The phytochemicals of *Thesium aureum* species

4.2 The Total Phenolic and Total Flavonoid Content

Recently, scientists have started concentrating on phenolic chemicals due to the numerous health advantages they provide. A standard phenolic compound, such as gallic acid or pyrocatechol, is used for estimating the total phenolic content in an extract. Due to its simplicity and lack of particular equipment requirements, the well-known Folin-Ciocalteu assay is frequently relied on to determine the total phenolics. We applyed Folin-Ciocalteu assay, and the gallic acid standard was used. The total phenolics in the ethanolic extract of the *Thesim aureum* species was found to be (29.55±0.33 mg GAE/ g).

The total flavonoid amount withen the *Thesium aureum* species ethanolic extract was measured by spektrofotometrik. Flavonoid content was calculated as routine equivalent (RE). According to the calculations, the total flavonoids in *Thesium aureum* species ethanolic exract was (8.88±0.03 mg RE/g). RE: routine equivalent.

The results are consistent with LC-MS/MS analysis in which all of the concentrations of flavonoid compounds combined were les than half of the phynolics, while the predominant compound (the highest concentration) within phinolics was quinic acid

4.3 *Thesium aureum* Species Antioxidant Capacity

To precisely determine the antioxidant capacity of plant extracts, a variety of techniques are needed due to the diverse actions performed by antioxidant components. TEACs (trolox equivalent antioxidant capacities) of the *Thesium aureum* species' ethanolic extract were determined using a variety of procedures including DPPH, FRAP, phosphomolybdate, ABTS, CUPRAC, and metal chelation.

4.3.1 Phosphomolybdate assay

Phosphomolybdate test is typically used to assess the overall antioxidant capability. This test relies on the transport of both hydrogen and electrons in an acidic environment. It primarily depends on Mo(VI) reduction, by compounds with antioxidant properties, to Mo(V), and then spectrophotometry measuring the resulted green-colored complex (phosphate/Mo(V)) .

Because the screening process is uncomplicated and the reagents are inexpensive, it is one of the techniques for assessing antioxidant capacity that has been utilized a lot in recent years. The method's results are presented as being comparable to a chemical whose antioxidant activity is well-known. Antioxidant substances of choice often consist of ascorbic acid or trolox. A total of 1.65 ±0.07 mmol TE/g was the total antioxidant capacity of the *Thesium aureum* extract.

Studies have shown that certain flavonoids, such luteolin, reduce the activity of free radicals. Flavonoids may also lower peroxidase activity and prevent neutrophils from releasing free radicals (Jiang et al., 2023). Our LC-MS/MS investigations have demonstrated that the ethanolic extract of *Thesium aureum* species contains litholine, which might account for the species' antioxidant activity.

Exposure to heat during the incubation stage may cause the types of antioxidants in the plant extract to breakdown due to the raised temperature, which may reach 90 degrees during the incubation process that we performed while conducting the phosphomolybdate test. Following an extensive review of the literature, we found that there were many disagreements over the validity of the current in vitro antioxidant tests. In the end, there isn't yet an universally recognized and quite successful procedure for determining antioxidant strengths. There isn't a standard method that can provide us with an extensive view of a test sample's amount of antioxidant capacity. However, in order to get a true measurement of the antioxidant, a combination of a few tests (no less than three) must be conducted.

4.3.2 The metal-chelating capacity

Prooxidants, among which are iron and other transition metals, act as vital to the oxidation process. In fact, because it reduces the synthesis of hydroxyl radicals during lipid peroxidation, the ability to chelate minerals is believed to be an antioxidant mechanism. The transition metal ion Fe^{2+} can help kick off and propagate a wide range of radical processes due to its natural ability to displace single electrons (Yilmaz et al., 2023). The ferrozine technique was used in this dissertation investigation, and the EDTA equivalent was utilized to quantify the metal chelating ability. Metal chelating activity of the *Thesium aureum* species ethanolic extract was

shown to be 21.53±1.04 mg EDTAE/g. In other words, the investigated *Thesium aureum* species' extract showed a very good metal chelating activity.

4.3.3 CUPRAC and FRAP assays (the reduction power)

In order to accurately represent the capacity of antioxidant chemicals to donate electrons, reducing power is a crucial antioxidant metric. CUPRAC and FRAP tests were used for this reason in order to determine the reducing power within the scope of our study. Because of its ease of use, low cost of the reagents, ability to be performed in conditions near physiological pH, and ability to track the reduction of Cu^{+2} to Cu^{+}, the CUPRAC test has become among the most often used methods in the last few years. The copper reduction ability of the *Thesium aureum* species' ethanolic extract was found to be (71.31±3.09 mg TE/g), which indicates a very important reducing activity of the *Thesium aureum* species.

Also commonly used technique to evaluate the antioxidant capability of different substances is FRAP assay. The fundamental idea behind the FRAP assay is that in the presence of antioxidants, the ferric tripyridyltriazine complex (Fe3+-TPTZ) reduces to its ferrous form (Fe^{2+}-TPTZ). An acidic environment is required for this reaction to occur in order for the TPTZ complex to function as intended. Our extract's ability to reduce iron was shown to be (47.49±1.82 mg TE/g), which is a good reducing potential demonstrated by the ethanolic extract of *Thesium aureum* speciesc.

Since the total phenolic content of the extract was relatively low, the good result of the reduction activity of our extract could potentially be due to the reason that the diverse variety of phytochemicals in the plant extract may have a synergistic effect to enhance the overall reduction behavior. The other reason could be that, in addition to phenolic components, the plant extract may also include powerful antioxidants such vitamins and alkaloids all of which greatly enhance its reducing effectiveness. These chemicals have a capability to boost the antioxidant properties in conjunction with phenolic compounds.

4.3.4 DPPH and ABTS assays (the scavenging activity)

The most widely utilized radicals in bioactivity investigations are DPPH and ABTS radicals, which are important when assessing the manner in which herbal extracts affect radicals. The activity of the herbal extract causes the DPPH radical, a stable radical, to change colour from purple to yellow by gaining a hydrogen ion or electron to transform into a stable diamagnetic compound, and this change is measured at 517 nm. The studied extract's capacity to scavenge DPPH was measured, and the DPPH scavenging activity of the *Thesium aureum*'s ethanolic extract was discovered to be moderate (6.82±0.59 mg TE/g).

Since the ABTS radical is unstable, it should be used between twelve and sixteen hours after preparation. Our extract's ABTS radical scavenging activity was found to be low. ABTS radical scavenging activity of our extract showed the value of (20.63±0.17 mg TE/g). In both of these methods, it was discovered that the ethanol extract of *Thesium aureum* species lacked antioxidant activity.

4.4 The Enzyme Inhibition Capacity

The inhibition capacity of the ethanolic extract of *Thesium aureum* species was tested on the enzymes: tyrosinase, AChE, BChE, α-glucosidase, and α- amylase.

4.4.1 Tyrosinase inhibition capacity

Tyrosinase inhibition has several uses in agriculture and cosmetics in addition to being a target for medicinal applications. Tyrosinase is an enzyme responsible for limiting an amount at which melanin is formulated in bacteria, plants, fungus, and humans. Although the pigment melanin is what gives skin its color and protection from ultraviolet rays, Health problems including melasma, solar lentigo, pregnancy spots, acne, cancerous tumors of melanoma, freckles, and senile lentigines are initiated by an overabundance of melanin (Findik et al., 2024). The ethanol-macerated extract that was produced from *Thesium aureum* species has a relatively good value (37.54±1.84 mg KAE/ g) as a tyrosinase inhibitor .

Thesium aureum species ethanolic extract's good capacity to inhibit tyrosine enzyme is attributable to the fact that, a lot of phenolic chemicals, including hesperidin and naringenin, which we found to be present in the ethanolic extract of the *Thesium aureum* species, have been shown to have tyrosinase-inhibiting properties. It has been determined that these phenolics are competitive inhibitors that cause tyrosinase's tertiary structure to be distorted. Tyrosine activity decreases as a result of the enzyme's altered substrate affinity brought about by the hydrophobic and hydrogen bonding interactions between phenolics and the active region of the enzyme (Findik et al., 2024).

The Metal-chelating capability of our extract (21.53±1.04) is relativaly good, and that is strongly related to the inhibition of the tyrosinase, because the removing of the coenzyme cupper from the enzyme leading tho the enzyme dysfuction. Another reason for the good result of the tyrosinase inhibition assay is due to quinic acid, the compound which has the biggest value in the extract, which have relativly a similar stracture to those compunds which concider a good inhibitors for tyrosinase.

4.4.2 Cholinesterase (AChE and BChE) inhibition capacity

A vast variety of plants and herbs that could potentially be utilized to treat Alzheimer's disease have been reported in the ancient methods of traditional medical treatment. These plants have significant potential as complementary treatments, alternative medicine sources, and sources of novel pharmaceutical chemicals. In fact, the plant is the origin of galantamine, one of the most used anti-AD medications (Uddin et al., 2015). The colorimetric methodology of Ellman was followed in performing the butyrylcholinesterase (BChE) as well as acetylcholinesterase (AChE) inhibitory evaluation. The ethanolic ectract of *Thesium aureum* species was evaluated for its potential to block the BChE enzyme, and the final result has been highlighted as being comparable to galatamine (GALAE). Based on the result of the analysis, It has been discovered that the *Thesium aureum* species' ethanolic extract has a relatively small value (2.26±0.21 mg GALAE/g) regarding the BChE enzyme hindering capacity.

Furthermore, the study assessed the degree of Aceticolinesterase inhibition in *Thesium aureum*, more specifically its ethanolic extract, and determined it as 3.01±0.07 mg GALAE/g (GALAE: galantamine equivalent). The information gathered from the literature study showed that there was no study has been done on the *Thesium aureum* species regarding its AChE,and BChE enzymes inhibitory properties.

4.4.3 α-Glucosidase and α- amylase inhibition capacity

One potential approach to treating Type 2 Diabetes is the idea of reducing glucose absorption in order to keep blood glucose levels within normal ranges. Delaying the breakdown of carbohydrates can lower the amount of glucose that enters blood vessels and lower postprandial glucose levels. Acarbose was the first α-glucosidase inhibitor (AGI) licensed by the FDA in 1995, and miglitol was approved in 1996. However, gastrointestinal adverse effects such diarrhea, distention of the abdomen, and flatulence might be brought on by AGI drugs. Due to their substantial inhibition of α-glucosidase and moderate inhibition of α-amylase, flavonoids have been shown in numerous researches achievements to have the potential to be anti-diabetic agents. This makes them a good choice as anti-diabetic medications that have minimal gastrointestinal side effects (Lam et al., 2024).

The results demonstrate that the extract made from the species *Thesium aureum*, macerated in ethanol is ineffective as an anti-diabetic agent with a value of (0.56±0.00 mmol ACAE/g) for α-glucosidase hindering capacity and (0.43±0.00 mmol ACAE/g) for α-amylase hindering capacity. As far as we are aware, there has never been a study that has proven the antidiabetic benefit of *Thesium aureum* species

5. CONCLUTIONS

As a result of searches in various literatures, it was found that there is not any research on the phytochemical contents, antioxidants, or enzymes inhibitors in the *Thesium aureum* species. The data obtained at this stage filled an important gap in the literature.

This study has been showed that *Thesium aureum* species contains compounds with good reductive capabilities, in terms of antioxidants. Also, it has also proven successful in treating problems related to hyperpigmentation. Promising initial results on the therapeutic benefits of *Thesium aureum* are presented in this paper, prompting additional investigation that could lead to novel herbal medicine and cosmetic product development opportunities.

Given the fact that *Thesium aureum* is a hemiparasite, it is necessary to determine if the chemical components in trace amounts are produced by the plants itself, or are absorbed from nearby host plants. There is an urgent need for additional research to identify the antibacterial, anti-inflammatory, and toxic properties of the *Thesium aureum* species. It is also advised to carry out more scientific research to pinpoint the substances that give this species their biological activity and clarify the connection between composition, activity, and the best prescribed dosages.

More researches are needed to understand the health effects of specific phenolic compounds detected in this research. Assays for assessing antioxidant capability are constantly being created and improved. New techniques with quick, easy, and precise qualities might be the subject of future studies.

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DİCLE ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
TEZ BENZERLİK BİLDİRİMİ FORMU

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Tez Başlığı	DETERMINATION OF BIOACTIVITIES OF *Thesium aureum* JAUP.&SPACH AND ELUCIDATION OF ITS PHYTOCHEMICAL CONTENT BY LC-MS/MS		
RAPOR BİLGİLERİ			
Raporlama Aşaması	Tez Savunma Sınavı Sonrası		
Sayfa Sayısı	98		
Raporlama Tarihi	07.02.2025		
Benzerlik Oranı (%)	15		

Yukarıda bilgileri verilen tez çalışmamın toplam 98 sayfalık kısmına ilişkin, 07/02/2025 tarihinde tez danışmanım tarafından Turnitin isimli intihal tespit programından aşağıda belirtilen filtrelemeler uygulanarak alınmış olan intihal raporuna göre, tezimin benzerlik oranı % 15 olarak tespit edilmiştir.

Uygulanan filtrelemeler:

☐Başlangıç Bölümleri (Kabul ve Onay sayfası, Teşekkür sayfası, Özet/Abstract) hariç
☒Kaynaklar hariç
☐Alıntılar hariç/dâhil
☐Diğer (Açıklayınız)

Tezimin benzerlik oranı, Dicle Üniversitesi Fen Bilimleri Enstitüsü İntihal Raporu Uygulama Esaslarında belirtilen üst sınır benzerlik oranını aşmamaktadır. Tez benzerlik oranı üst sınır benzerlik oranının altında olsa dahi aksinin tespit edilmesi durumunda her türlü yasal sorumluğu kabul ettiğimi ve hukuki sonuçlarına razı olduğumu bildirir,
gereğini arz ederim.

Öğrenci: Helin SULIMAN
Tarih: 07.02.2025
İmza:

Danışman: Doç. Dr. Mustafa Abdullah YILMAZ İmza:
Tarih: 07.02.2025

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