REPUBLIC OF TÜRKİYE
DICLE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

ELUCIDATING THE BIOCHEMICAL ACTIVITY AND PHYTOCHEMICAL CONTENT OF *ANTHEMIS TRICORNIS* EIG SPECIES

Alaa HASAN

MASTER'S THESIS

ANALYTICAL CHEMISTRY DEPARTMENT

ACADEMIC SUPERVISOR

Assoc. Prof. Dr. Mustafa Abdullah YILMAZ

DIYARBAKIR- 2024

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APPROVAL OF THE THESIS

The thesis titled “Elucidation of the biochemical activity and phytochemical content of Anthemis tricornis EIG species” prepared by Alaa HASAN, a Master's student at the Department of Analytical Chemistry, Institute of Health Sciences, Dicle University, has been evaluated in terms of scope and scientific quality in accordance with the relevant articles of the Dicle University Postgraduate Education-Teaching and Examination Regulation and accepted as a Master's Thesis.

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Date: 23-10-2024

This thesis has been approved by Dicle University Health Sciences Institute Board of Directors with the decision numbered.… dated …. /…/….

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Prof. Dr. Mahmut BALKAN
Dicle University
Director of Institue of Health Science

DECLARATION

I declare that this thesis is my work, that I have not acted unethically in any phase from the planning to the writing of the thesis, that I have obtained all the information in this thesis within academic and ethical rules, that I have cited all the information and comments not obtained through this thesis and included these sources in the list of sources, that I have not violated any patent or copyright during the work and writing of this thesis, and that I have prepared my thesis in accordance with the Dicle University Health Sciences Institute thesis writing guide standards.

23/10/2024

Alaa HASAN

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Alaa HASAN

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ABBREVIATIONS

ABTS	2,2´-Azino-bis(3-Ethylbenzothiazoline-Sulfonic acid)
AChE	Acetylcholinesterase Enzyme
AchEI	Acetylcholinesterase Inhibitor
BChE	Butyrylcholinesterase Enzyme
BHA	Butylated Hydroxy Anisole
BHT	Butylated Hydroxy Toluene
CA	Carbonic Anhydrase
CAT	Catalase
DMPD	N, N-Dimethyl-Phenylenediamine
DPPH	1,1-Diphenyl-2-Picryl Hydrazyl
DTNB	5,5’-Dithio-bis(2-Nitrobenzoic acid)
EDTA	Ethylene Diamine Tetra Acetic acid
ES	Enzyme-Substrate Complex
ESI	Enzyme-Substrate-Inhibitor Complex
ETS	Electron Transport System
FCR	Folin-Ciocalteu Reagent
FRAP	Iron (III) Reducing Antioxidant Power
hCA	Human Carbonic Anhydrase Isoenzyme
HDA	Hydroxy-trans-2-decenoic acid
HMF	Hydroxy Methyl Furfural
IC	Inhibitor Concentration
IC50	Inhibitor Concentration That Halves the Current Concentration
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectroscopy
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PER	Ammonium Persulfate
PG	Propyl Gallate
***p*-PNF**	*p*-Nitrophenyl Acetate

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1. ABSTRACT

1.1. Özet

Anthemis Tricornis Eig Türünün Biyokimyasal Aktivitesinin ve Fitokimyasal İçeriğinin Aydınlatılması

Öğrencinin Adı ve Soyadı: Alaa HASAN
Tez Danışmanı: Doç. Dr. Mustafa Abdullah YILMAZ
Anabilim Dalı: Analitik Kimya Anabilim Dalı

Amaç: *Anthemis* cinsi, özellikle de *A. tricornis*, çeşitli tedavi edici özellikleri nedeniyle geleneksel tıpta, özellikle Türkiye'de önem taşımaktadır. Bu yüksek lisans tezi, *A. tricornis* ekstraktının biyokimyasal aktivitesine odaklanmakta ve fitokimyasalların, özellikle flavonoidlerin ve fenolik asitlerin tıbbi etkinliğindeki rolünü vurgulamaktadır.

Gereç ve Yöntem: Çalışmada antioksidan aktiviteler çeşitli yöntemler kullanılarak değerlendirildi. Serbest radikal temizleme yöntemleri, bitkinin oksidatif stresle mücadele etme yeteneğini değerlendiren DPPH$^{•}$, ABTS$^{•+}$ ve DMPD$^{•+}$'yi içeriyordu. Ayrıca indirgeme kapasitesi CUPRAC, FRAP ve Fe^{3+} gibi yöntemlerle ölçüldü. Toplam fenolik asitlerin ve flavonoidlerin miktarının belirlenmesi, doğru kimyasal profilleme için LC-MS/MS teknolojisi kullanılarak yapıldı.

Bulgular: Bu araştırmada, A. tricornis'ten elde edilen özütün kimyasal bileşimleri ve biyolojik aktiviteleri incelenmiştir. Etanol özütündeki toplam fenolik asit ve flavonoid bileşenlerinin miktarları, sırasıyla gallik asit ve kuersetin eşdeğerleri (57.88 µg GAE/mg), (41.16 µg QE/mg) olarak ifade edilerek belirlenmiştir. A. tricornis'ten elde edilen özüt, karbonik anhidraz II (CA II), asetilkolinesteraz (AChE) ve α-amilaz (α-Alz) gibi enzimleri inhibe etme yeteneğinin yanı sıra güçlü antioksidan aktivite göstermiş ve böylece bu bitkinin terapötik potansiyelini artırmıştır.

Sonuç: *A. tricornis*'in sekonder metabolitler açısından zengin olduğunu, antioksidan kapasite ve enzim inhibisyonu ile ilgili önemli biyolojik aktiviteler sergilediğini ortaya koymaktadır. Bu araştırma, *A. tricornis*'in psikofarmakolojik özelliklerinin daha fazla araştırılması için bir temel teşkil etmekte ve potansiyel olarak hem geleneksel hem de modern tıpta yeni tedavilerin geliştirilmesine yardımcı olmaktadır.

Anahtar Kelimeler: İkincil metabolitler, *A. tricornis*, antioksidanlar, enzim inhibitörleri, LC-MS/MS.

1.2. Abstract

Elucidating The Biochemical Activity and Phytochemical Content of *Anthemis Tricornis* EIG Species

Student's Surname and Name: HASAN Alaa
Supervisor of Thesis: Assoc. Prof. Dr. Mustafa Abdullah YILMAZ
Department: Department of Analytical Chemistry

Aim: The genus *Anthemis*, particularly *A. tricornis*, is significant in traditional medicine, especially in Turkey, due to its diverse therapeutic properties. This master's thesis focuses on the biochemical activity of *A. tricornis* extract, emphasizing the role of phytochemicals, specifically flavonoids and phenolic acids, in its medicinal efficacy.

Materials and Methods: The study assessed antioxidant activities using various methods. Free radical scavenging methods included $\text{DPPH}^{\bullet}$, $\text{ABTS}^{\bullet+}$, and $\text{DMPD}^{\bullet+}$, which evaluate the plant's ability to combat oxidative stress. Additionally, reducing capacity was measured through methods like CUPRAC, FRAP, and Fe^{3+}. The quantification of total phenolic acids and flavonoids was performed using LC-MS/MS technology for accurate chemical profiling.

Results: In this research, the chemical compositions and biological activities of extract obtained from *A. tricornis* was studied. The amounts of total phenolic acid and flavonoid components in ethanol extract was determined by expression as gallic acid and quercetin equivalents, respectively (57.88 µg GAE/mg), (41.16 µg QE/mg). The extract obtained from *A. tricornis* showed strong antioxidant activity, along with the ability to inhibit enzymes such as carbonic anhydrase II (CA II), acetylcholinesterase (AChE), and α-amylase (α-Alz) , thereby enhancing the therapeutic potential of this plant.

Conclusion: In conclusion, the thesis demonstrates that *A. tricornis* is rich in secondary metabolites, exhibiting notable biological activities related to antioxidant capacity and enzyme inhibition. This research serves as a foundation for further exploration of the psychopharmacological properties of *A. tricornis*, potentially aiding in the development of new therapies in both traditional and modern medicine.

Keywords: Secondary metabolites, *A. tricornis*, antioxidants, enzyme inhibitors, LC-MS/MS.

2. AIM AND SCOPE OF THE STUDY

It is impossible to see the human species surviving in a world without flora. Plants have provided people with sustenance since the beginning of time. Medicinal plants are often utilized as a main form of therapy (1). The oldest known evidence of therapeutic plants, namely herbs, dates back to the Sumerian culture. This period's clay tablets include allusions to a variety of medicinal herbs, including opium. The Ebers Papyrus, which dates back to ancient Egypt about 1550 BC, has a thorough chronicle of over 850 therapeutic plant treatments (2).

In his De Materie medica, Dioscorides, a Greek physician serving in the Roman army, identified and documented over a thousand prescriptions for medications derived from over 600 medicinal plants. This work established the foundation for pharmacopeias throughout the following 1500 years.

The plant kingdom employs a wide spectrum of structural diversity via the use of several biochemical substances. Exploring the chemical components present in medicinal plants resulted in the discovery of numerous novel pharmacophores. Pharmacophores have been shown to be quite effective in the medication development process. Medicinal plants include a variety of bioactive phytochemicals or bionutrients, and their complex chemical makeup provides several potentials for novel medicinal discoveries. Research conducted over the past two to three decades has demonstrated that these phytochemicals have an important role in the prevention of chronic illnesses such as cancer, diabetes, and coronary heart disease (3).

The lower incidence of side effects to plant preparations compared to existing conventional pharmaceuticals, combined with their lower cost, is prompting both consumers and national healthcare organizations to investigate plant medicines as alternatives to synthetic treatments. According to the World Health Organization (WHO), 4 billion people, or 80% of the global population, utilize herbal medicines for basic healthcare needs. The World Health Organization (WHO) recognizes herbal medicine as a key component of basic healthcare, with plants accounting for around 11% of the 252 medications (4).

The phytochemicals are divided into two categories: Primary and secondary. Primary components include chlorophyll, proteins, and carbohydrates, while secondary substances include terpenoids, alkaloids, flavonoids, and phenols (5). The positive therapeutic benefits of plant materials are often linked to the synergistic interplay of secondary chemicals found in the plant. The fact that plant therapeutic action is restricted to certain plant species or groups supports this viewpoint since combinations of secondary products within a single plant often differ taxonomically. In contrast to primary products such as carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids, which are found in all plants and play essential roles in cell development and maintenance (6).

Plant substances are an excellent source of physiologically active chemicals. The active secondary metabolites present in certain plants include bioactive chemicals of relevance to the pharmaceutical industry (7). Plant secondary metabolites are divided into three categories depending on their metabolic pathways: phenolic compounds, terpenes, and nitrogen-containing chemicals (8). Secondary metabolites have outstanding antioxidant properties and might be employed as a very efficient natural source of antioxidants in nutraceuticals. Most secondary metabolites have a variety of therapeutic actions and interact directly with receptors, cell membranes, and nucleic acids (9).

Researchers are constantly pursuing powerful compounds known as antioxidants to protect living organisms from the harmful effects of reactive species, such as free radicals. Concerns about the possible detrimental effects of routinely used synthetic antioxidants on human health have led to an increased interest in natural antioxidant molecules produced by plants, particularly phenolic compounds (10).

The wide and complex chemical makeup of plant extracts may complicate the process of determining the phytochemical content of medicinal and aromatic plants. Phenolic compounds come in various sizes and polarities, ranging from basic phenolic acids to flavonoid aglycones and glycosides. This variability may complicate the process of collecting and studying these compounds. Identification of natural compounds in medicinal and aromatic plants necessitates the use of analytical techniques that meet certain criteria such as selectivity, separation efficiency, and sensitivity (11).

Due to the complicated structure of the matrices and the existence of substances in trace levels, researchers need more dependable procedures than thin-layer chromatography (TLC) or UV/Vis (Ultraviolet/Visible) spectrophotometry. Furthermore, gas chromatography (GC) can only analyze volatile chemicals, while capillary electrophoresis may cause repeatability issues. Ultrahigh-performance liquid chromatography (U-HPLC) paired with tandem mass spectrometry (MS/MS) is the most effective technology for qualitative and quantitative analysis and measurement of various phenolic chemicals in plant species.

Enzymes are largely made up of protein and work as catalysts in biological systems, accelerating chemical processes and producing the desired outcome without producing any byproducts. These reactions are required for cellular metabolism, ATP generation, cell division, DNA synthesis, and many other important biological functions. Enzymes interact with substrate molecules, allowing them to convert into other molecules more quickly. Enzymes aid in biological processes inside the cell and are generated and used intracellularly. Without enzymes, the process is virtually insignificant. Enzymes are researched in six major groups.

The enzymes included include transferases, oxidoreductases, lyases, ligases, isomerases, and hydrolases. Enzymes serve an important role in expediting chemical processes, allowing them to finish reactions that would normally take months in a laboratory environment (12). As a consequence, enzymes are purified and characterized. Keha and Küfrevioğlu (2014) report that around 2000 enzymes have been extracted, identified, and employed in various academic and industrial operations (13).

Another important field of study in enzyme research is the involvement of enzymes in the genesis and treatment of diseases. As a result, enzyme inhibition has been and continues to be an effective treatment for some illnesses.

For example, Alzheimer's disease is treated with three FDA-licensed acetylcholinesterase inhibitors (AChEIs). Accordingly, many researchers used carbonic anhydrase inhibitors (14). Extract from *A. tricornis* species was used in this research. Previous validation studies have been conducted. The extracts' phytochemical content will be determined qualitatively and quantitatively using the LC-MS/MS technology, which is both trustworthy and thorough. Furthermore, the biological activity of the extracts will be evaluated from many perspectives. Furthermore, the total phenolic and flavonoid content will be evaluated (15).

The biological activity was assessed using antioxidant and enzyme inhibition techniques. Antioxidant capabilities were assessed using a variety of methodologies, including free radical scavenging (DPPH and ABTS), reducing power (CUPRAC and FRAP), phosphomolybdate, and metal chelation assays. The effects of enzyme inhibition were examined for acetylcholinesterase, butyrylcholinesterase, tyrosinase, amylase, and glucosidase. This was the first research to look at the biological activity and chemical makeup of *A. tricornis* species.

Anthemis, a genus comprising approximately 130 species, is mostly found in the Mediterranean area. *Anthemis* is the second biggest genus in Asteraceae family, with over 195 species that are widely employed in the medicinal, cosmetic, and food sectors. *Anthemis* is mostly found in the Mediterranean area, but its greatest variety is found in Southwest Asia, where over 150 species have been recognized (16).

Throughout history, humans have employed Asteraceae for medical and culinary uses. Family members may differ in appearance, but their chemical makeup is identical. For example, all species contain inulin, a natural polymer with strong prebiotic effects (17). Asteraceae is the biggest family of flowering plants, with around 18,000 to 20,000 species and more than 1,300 genera. Asteraceae, often known as Compositae, is a plant family that includes asters, daisies, composites, and sunflowers. Plants include phytochemicals such as polyphenols, flavonoids, and diterpenoids, which have been linked to pharmacologic effects. Numerous research has shown that Asteraceae plants contain antibacterial, antifungal, anti-inflammatory, insecticidal, and anticancer properties (18).

Anthemis species are widely employed in the medical, cosmetic, and food industries. The flowers of this genus have a long history of usage as antiseptics and medicinal herbs, with natural flavonoids and essential oils serving as the primary ingredients. In Europe, extracts, tinctures, tisanes (teas), and salves are often used to treat inflammation, infections and spasms, and promote slumber. Extracts are used to relieve pain and irritation, clean wounds and ulcers, and help prevent and cure skin damage caused by radiation, cystitis, and dental problems (19).

This thesis is intended to contribute to a better knowledge of the *A. tricornis* plant species and its possible applications in pharmacology, alternative medicine and biological sciences. Furthermore, this study may stimulate future scientific research on this subject, resulting in vital contributions to the creation of novel and successful therapies.

3. INTRODUCTION

3.1. Asteraceae Family

Asteraceae family, commonly referred to as the Aster family, is a vast group of flowering plants that belong to the angiosperms. It consists of around 1,620 genera and 23,600 species of plants that may be found worldwide. These plants include herbaceous plants, shrubs, and trees. The plants are distinguished by their composite flower heads and one-seeded achene fruits (20).

Asteraceae is widespread in many parts of the world, such as the Mediterranean Region, Southwestern America, Southwestern Asia, South Africa and Australia. Among the genera belonging to the Asteraceae family are *Centaurea* L., *Hieracium* L. and *Cirsium Mill*. They are the genera with the most species. Asteraceae has two subfamilies in Turkey: Asteroideae (Tubuliflorae) and Cichorioidae (Liguliflorae). It is known that there are 133 genera and 1156 species belonging to these subfamilies in our country. Anthemideae tribe belonging to Asteraceae family consists of approximately 111 genera and species in the world.

It has a widespread distribution with 1800 species. The genera of this tribe in Turkey are *Anthemis*, *Cota*, *Achillea*, *Otanthus*, *Arcanthemis*, *Anacyclus*, *Tanacetum*, *Tripleurospermum*, *Matricaria*, *Chamaemulum*, *Artemisia*, *Santolina*, *Leucanthemum*, *Chrysanthemum* and *Leucocylus*. These plants, which are distributed in many parts of the world, are mostly found in the Mediterranean, Central Asia and North African regions (21).

The family members have flower heads made up of florets enclosed by bracts. Every head has disk florets that resemble bells and are arranged in the center. The expanded ray florets spread from the center and may bend backward. Certain plants only produce disk or ray floret blooms. The presence of fewer sepals results in the formation of a pappus, a circular arrangement of hairs, scales, or bristles seen on ripe fruit. An achene's exocarp is long-lasting. Uncomplicated Asteraceae leaves are typically simple. The leaves on the stem might be arranged in an opposing, alternating, or whorled pattern. Occasionally, the leaves create an opposing pattern at the base of the stem before converting to a different shape as they climb.

The well-preserved fossils from the Eocene Epoch (56 million to 33.9 million years ago) indicate that the first members of this family appeared in Argentina approximately 50 million years ago (22). Because of the therapeutic values that they possess, Asteraceae species have been employed in traditional medicine for a very long time. For more than three thousand years, people have been cultivating plants from this category for the purpose of using them in culinary and therapeutic applications. Even though it may be found anywhere, the semi-arid and dry subtropical climates are where they are most often discovered. Properties that protect the liver, including as antioxidants, antibacterial agents, and anti-inflammatory agents, are possessed by the Asteraceae family (17).

3.2. Anthemis L.

Anthemis L. is classified as *Anthemis*, *Maruta*, and *Cota* in the Flora of Turkey. This genus is a member of Asteraceae family and Anthemideae tribe. Turkey is home to more than fifty species, with 54% of them being indigenous. They may reside in arid, exposed areas on wood-steppe slopes and flourish in soil that is abundant in calcium carbonate. The 210-species genus *Anthemis* is a member of Anthemideae tribe. *Anthemis* is found in the Mediterranean, eastern Africa, and western Eurasia. Including all officially described subgenera and sections, Southern Asia is home to 150–210 archaeophytic taxa. There are a limited number of species in Central Europe. North and South America are home to certain species (23).

Since Roman times, representatives from this genus have been widely employed in traditional medicine therapies. In the Mediterranean area, the most popular traditional uses of the genus species are anti-inflammatory, antioxidant, antibacterial, and antispasmodic. The chemical makeup of several *Anthemis* extracts has been intensively researched during the last two decades. Flavonoids, polyphenols, and terpenes were identified as the plant's primary ingredients. It is known that various species of *Anthemis* are valuable plants used in the pharmaceutical, cosmetic, and food industries. For this reason, research on species that have not yet been studied attracts the attention of scientists (21).

They are annual, biennial and perennial herbs, sometimes semi-shrubs or small shrubs. It is usually hairless, sparsely or densely covered with short, soft hair. The stem is simple or branched. It has a taproot or rhizome. Leaves 1-3 pinnatisect, rarely simple, primary segment usually 3 sometimes more, pinnately divided, scalloped or segmented lobed. Capitula single, discoid or radiant, peduncle bare.

The phyllaries are generally 3-series and tiled in arrangement, the inner ones are generally narrow and have membranous edges. The receptacle is bare, hemispherical, ovoid or conical in shape. Palea linear-lanceolate, subulate or oblanceolate, membranous or cartilaginous, acute at the tip, mucronate or acuminate, usually as long as the tubular flower, sometimes as long. Ligulate flowers are white, yellow, and rarely pale pink. The corolla tube sometimes remains permanently above the achene. Tubular flowers are 5-toothed, yellow, rarely pale pink, the corolla is sometimes swollen at the base. Achenes are generally obconical, cylindrical or square in cross-section, sometimes flattened in the dorso-ventral direction, with smooth surfaces, vascular or tuberculate. There is no pappus, or the edge of the elevated tip of the achene is corona-shaped, or the elongated edge behind it is circular-shaped, or the auricle is elongated like a tongue (24).

Anthemis species are used in traditional medicine due to their unique biology. They reduce pain and inflammation, clean wounds, and treat gastrointestinal diseases, hemorrhoids, coughs, stomach pains, and liver failure. Additionally, they can manage these situations. Cosmetics, pharmaceuticals, and herbal beverages use them (25). Examples of species that have been studied previously include *Anthemis nobilis* L.*,* *Anthemis Coelopoda Boiss. Var. Burgaei Boiss.* and *Anthemis Cotula.*

3.3. Anthemis tricornis

A. tricornis, sometimes known as Three-horned Chamomile, is a yearly plant with white blossoms and delicate, fern-like leaves. This species is native to Europe and often found in grasslands and meadows. A. *tricornis* is often used as an ornamental plant in gardens because of its appealing yellow blooms. It may also be used as a medicinal plant due to the anti-inflammatory properties present in the leaves. *A. tricornis* flowers have a daisy-like appearance, with white or yellow petals and a conspicuous yellow center. The seed is a small, spherical, black achene. The seedlings are little and have just one pair of leaves (26).

Figure 1. *Anthemis tricornis* Eig (was taken by Prof. Dr. Lutfi Behcet).

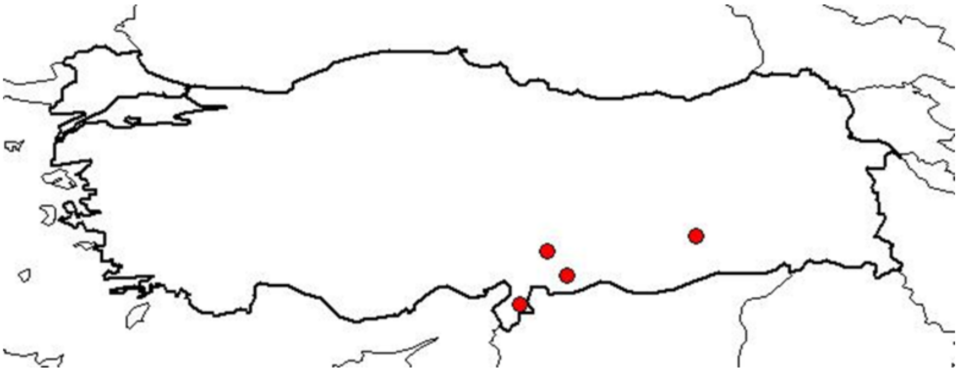


Figure 2: Based on vilayets: Gaziantep, Diyarbakir, Hatay, Kahramanmaraş. (TÜBİVES Anthemis tricornis).

3.4. Medicinal Plants

Throughout history, people have sought natural medicines to treat sickness. The usage of therapeutic herbs began spontaneously, mimicking the natural behavior of animals. Due to a lack of information about the causes of illnesses and the possible curative capabilities of plants, decision-making was primarily based on personal experience (2).

It is difficult to pinpoint the precise period and place of the early usage of plants and herbs as remedies, particularly in ancient times. An individual's bones were discovered in a Neanderthal burial site in Northern Iraq around 60,000 years ago. The finding indicated that the individual was surrounded by at least seven therapeutic plants, including Ephedra.

This shows that people initially used plants during this historical period. According to historical appraisals, more early allusions may be found in the works of Zarathustra (1000–500 BCE), who flourished inside Iran's Aryan culture about 6500 BCE. The Avesta, Zoroastrianism's sacred text, provides a complete understanding of the medicinal qualities of several plants. The complete knowledge of herbal medicines in ancient cultures is largely the result of decades of practical investigation, with the most successful cures passed down orally from generation to generation. The history of pharmacy has long been linked to the study of pharmacognosy, which examines Materia medica derived from natural sources such as plants, minerals, animals, and fungi (27).

Before the 18th century, humans were aware of numerous plants' medicinal characteristics, their effects on the human body, and treatment procedures. However, the active component that causes these effects has yet to be determined. For example, Avicenna (Ibn Sina), a Persian physician and scholar, wrote the Canon of Medicine, which was utilized until the 18th century (28).

Currently, clinical, pharmaceutical, and chemical studies on these conventional medications, which are primarily derived from plants, serve as the foundation for numerous initial drugs, including aspirin (derived from willow bark), digoxin (extracted from foxglove), morphine (derived from the opium poppy), quinine (derived from Cinchona bark), and pilocarpine. Currently, it is commonly known that medicinal plants constitute the source of more than half of all medications. Phytotherapy is becoming more popular globally. As a result, there has been a worldwide shift from synthetic medications to herbal remedies, which may be described as a return to nature in order to avoid infections and relieve pain (29).

Medicinal plants have many pharmacological activities, including antimicrobial, adaptogenic, stimulant, and sedative effects. These compounds are used to treat cholecystitis, hypotension, capillary dilatation, ulcers, anticholinesterase activity, cancer, muscular spasm relaxation, pain alleviation, and central nervous system stimulation. Medicinal plants have the benefit of supplying a combination of natural ingredients, resulting in a milder effect, greater tolerance compared to synthetic medications, and fewer adverse responses. They do not accumulate and may thus be used for a longer time. Medicinal plants and phytochemicals are used to cure and prevent a variety of human diseases, including cardiovascular, gastrointestinal, neurological, and dermatological concerns, as well as cancer (30).

Although drug development research using medicinal plants has been effective in previous decades, future initiatives face a variety of challenges. The reliability of herbal items is being questioned, and the necessity to standardize raw ingredients is becoming a major concern for the herbal business. Herbal plants may readily get polluted during cultivation, processing, and harvesting. The primary issues with herbal treatments include adulteration and heavy metal poisoning. As a result, improving the quality and number of bioactive substances is critical for developing novel herbal therapies and keeping up with other pharmacological research efforts. Researchers have undertaken detailed investigations and clinical trials on bioactive compounds found in many therapeutic plants today (31).

Many structural features of natural products (such as chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and the number and ratio of heteroatoms) are critical in medication development efforts. Furthermore, as interest in combinatorial chemistry has grown and it has become clear that these compound libraries may not always be very diverse, many synthetic and medicinal chemists are looking into the development of natural product and natural-product-like libraries that combine the structural properties of natural products with combinatorial chemistry's compound-generating potential. Medicinal plant medicines may be employed not only to develop new drugs but also as drug leads for further optimization by medicinal and synthetic chemists (32).

Botanical secondary compounds are not harmless molecules; biologically, they developed as chemical defenses capable of repelling, startling, intoxicating, or eliminating other species. It would be naive to believe that any plant extract is inherently safe for human ingestion. Poison control centers have been built on many continents expressly for this purpose. However, it would be impossible to distinguish between a useful drug and a lethal toxin. The dose is crucial in these instances, especially since certain plants with a long history of usage have been linked to possible toxicity. Comfrey (*Symphytum officinale*) has been linked to acute liver damage, whereas Yohimbe (*Corynanthe yohimbe*) is a nutritional supplement (29).

3.5. Free Radicals

Free radicals result from cell metabolism. Free radicals have electrons in their valence shell or outer orbit that are not connected with other electrons. This allows the free radical to thrive unrestricted. Well-known properties of highly reactive compounds include free radical instability, limited lifespan, and increased reactivity due to unpaired electrons. This is due to the distinct chemical composition of these two chemicals. Due to their extreme reactivity, they may grab electrons from other molecules, stabilizing them. This skill gives them stability. This technique converts the target molecule into a free radical by removing electrons. This starts a cascade of actions that harms the living cell. ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) collaborate to generate both free radicals and other non-radical reactive species (33).

ROS/RNS have a dual role, acting as both beneficial and toxic compounds within living systems. ROS/RNS play a crucial role in maintaining immune function, cellular signaling pathways, mitogenic response, and redox regulation, contributing to various physiological functions (34).

Both oxidative and nitrosative stress can be brought on by an excessive amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS), both of which are capable of causing damage to biomolecules. It is possible for those two types of stress to be brought on by the same thing. The presence of antioxidants and the production of reactive oxygen and nitrogen species (ROS/RNS) are both factors that contribute to oxidative and nitrosative stress. An imbalance causes both of these types of stress. Reactive oxygen species (ROS) have the ability to cause structural disruptions in a wide variety of biomolecules, including lipids, and their presence can be extremely detrimental. This is because ROS have the ability to cause these disruptions (35).

There are many types of human problems that have been linked to oxidative stress, including diabetes mellitus, neurological disorders, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases, and the natural process of aging. Both proteins and DNA have a significant influence on the occurrence of oxidative stress, which has been linked to a number of different disorders (36).

A finding that the $^{\cdot}OH$ radical enhances the production of cyclic guanosine monophosphate (cGMP) (37). This altered our viewpoint of ROS in biological systems. Subsequently, it became evident that living organisms not only adjusted to free radicals but also acquired the ability to use them in essential physiological processes. The discovery was remarkable. This notion is supported by the discovery that reactive oxygen species (ROS) have a role in regulating several cellular activities such as proliferation, death, and signaling. ROS also governs blood pressure, cognitive function, immunological function, and other essential activities at an elevated level (34).

Table 1. Main reactive oxygen (ROS) and nitrogen (RNS) species (38).

Reactive Oxygen Species	**Superoxide**	O^{-2}
	Hydrogen peroxide	H_2O_2
	Hydroxyl radical	**OH**

Reactive Nitrogen Species

Nitric oxide	**NO**
Peroxynitrite	**$ONOO^{-}$**
Dinitrogen trioxide	**N_2O_3**
Dinitrogen tetraoxide	**N_2O_4**
Nitrogen dioxide	**NO_2**
S-nitrosothiols	**RSNO**

In addition, research has shown that the buildup of oxidative damage inside an organism eventually leads to the death of the organism (39). Interventions that enhance longevity stimulate the production of reactive oxygen species (ROS), which activate beneficial stress responses and extend the lifespan of the organism (40).

Certain compounds can have both positive and negative effects on biological systems. Understanding the importance of nitric oxide as a signaling molecule for blood vessel remodeling is crucial (41). Referred regarded as a controller of essential physiological functions process it may reduce cellular toxicity by impairing metabolic enzymes and generating peroxynitrite via interaction with superoxide (42).

The term "oxidative stress" refers to a condition in which there is an imbalance between the manufacturing of reactive oxygen species (free radicals) and the ability of the organism to neutralize these radicals via the use of antioxidants. This imbalance has the potential to cause damage or injury to the tissues of the body (43).

Several studies have shown a connection between oxidative stress and metabolic abnormalities, chronic diseases, and cancer. Over the last twenty years, there has been significant progress in comprehending the impact of oxygen's free radicals on the course of diseases, building upon a notion that is fifty years old. Biologists have conducted thorough studies on the various advantages of antioxidants (44).

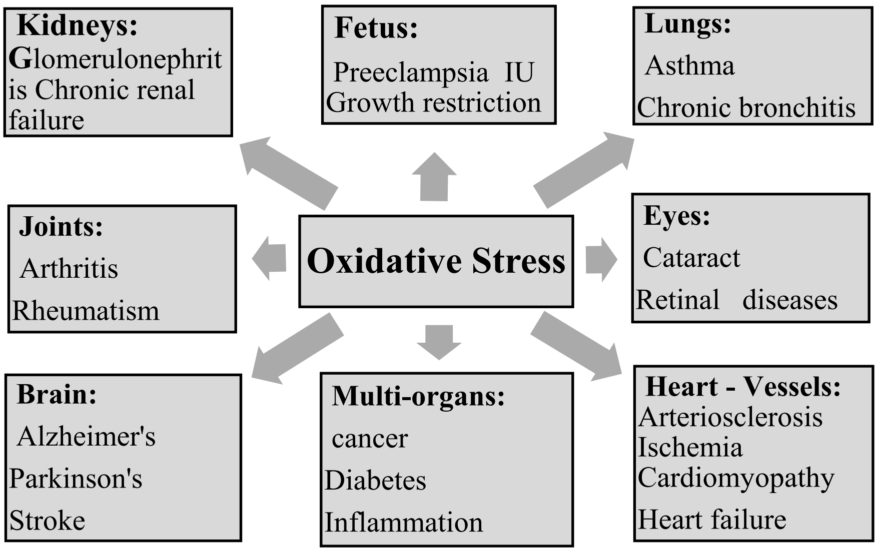


Figure 3. Oxidative stress-induced diseases in humans (45).

3.6. Antioxidants

In the past, an antioxidant was thought to be any compound that prevented the interaction of dioxygen with other molecules. This definition has been preserved. Because radicals are present in a wide variety of mechanical processes, the word "antioxidant" is now used to refer to any molecule that prevents a free radical reaction from becoming active. Oxygen is used significantly in biological processes. Reducing oxidative (radical) activities that are undesirable or excessive is necessary for organisms to continue living (46).

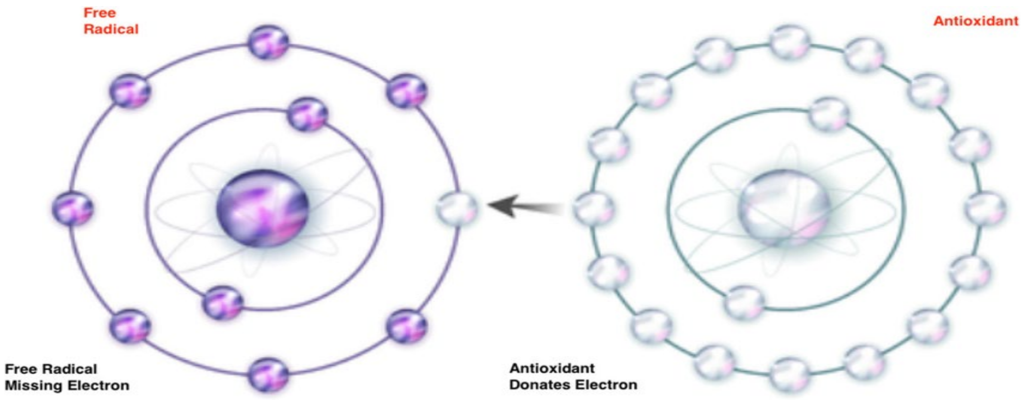


Figure 4. How antioxidants react with free radicals by donating or receiving electrons to neutralize them (47).

Antioxidants are chemical compounds that possess the remarkable capacity to restore the electron distribution of free radicals by either receiving or donating electrons, thereby neutralizing their harmful effects. Antioxidants has the ability to either absorb or donate electrons. Antioxidant molecules possess a remarkable ability to directly interact with reactive radicals, effectively eliminating them. Similarly, reactive radicals may transform into new free radicals that exhibit decreased toxicity, reactivity, and a longer lifespan in comparison to the radicals they neutralize.

The effectiveness of antioxidants may decrease when there is an abundance of antioxidants or when their radical state is terminated by other means. The antioxidant properties of glutathione, uric acid, vitamin C, and vitamin E are particularly powerful at the cellular level. Tert-butylhydroxyl-toluene, tert-butylhydroxyanisole, and tert-butylhydroquinone are three examples of artificial antioxidants that are used extensively in the food industry with the purpose of retarding the oxidation of lipids. The prevalence of toxicity issues, on the other hand, renders these synthetic antioxidants unsuitable for use in therapeutic applications. As a consequence of this, there is a growing interest in the discovery of plant extracts that have the potential to be used as dietary antioxidant supplements (48).

A large number of dietary items have significant concentrations of these naturally occurring antioxidants. Ginseng, curcuma, ginkgo, rosemary, green tea, grapes, ginger, and garlic are just some of the fruits, vegetables, spices, cereals, and herbs that are included on the list. The list also contains a broad range of other foods. There is a wide variety of antioxidant components used in their cosmetic goods. Phenolics, which comprise both phenol and polyphenols, are included in the formulation. Additionally, flavonoids, carotenoids, steroids, and thiols are components of the formulation. The presence of these molecules is a crucial component of their structure (49). These antioxidants may mitigate cellular damage caused by oxidative stress and reduce the likelihood of developing chronic illnesses (50).

Antioxidants are also essential in controlling enzymes that are involved with reactive oxygen species (ROS), which is another important function that antioxidants perform. Antioxidants can decrease the amount of free radicals that are present in cells. They can accomplish this by either inhibiting the actions or production of enzymes that generate free radicals, such as NAD(P)H oxidase and xanthine oxidase (XO), or by increasing the actions and production of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (51).

All antioxidants follow one of the mechanisms of action that vary depending on the type of oxidant. These mechanisms are:

1) Separation of free radicals from the environment,

2) Chelation of metallic ions,

3) Inhibition of enzymes that produce free radicals,

4) Activation of endogenous antioxidant enzymes,

5) Prevention of lipid peroxidation,

6) Prevention of DNA damage,

7) Prevention of protein modification and sugar destruction (40).

3.6.1. Classification of antioxidants

Molecular antioxidants might have a natural or synthetic origin, depending on the circumstances. In general, classifications are based on two criteria that are often utilized: the mechanism of antioxidant action (primary and secondary antioxidants) and catalytic considerations (enzymatic and non-enzymatic). Innate antioxidants are produced by a wide variety of species, including cells, fungi, plants, and mammals. Antioxidants that are synthetic are chemicals that are chemically varied and were made by specialists from a variety of fields in order to assist mankind.

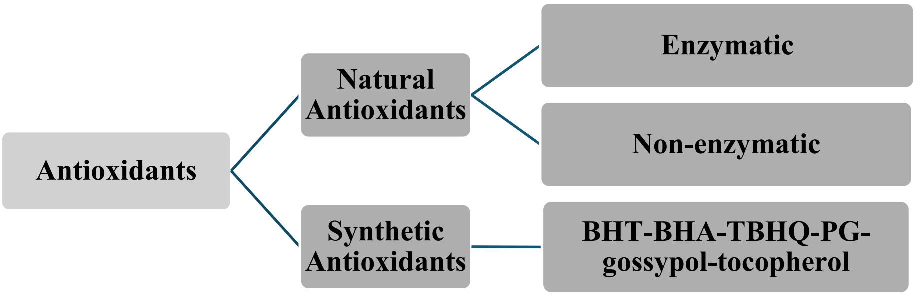


Figure 5. Types of antioxidants (52).

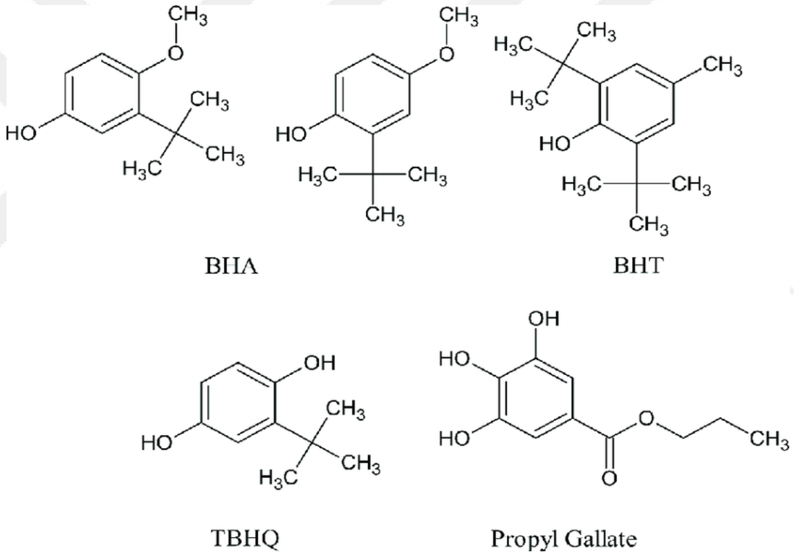
3.6.2. Natural antioxidants

Naturally occurring antioxidants are enzymatic or non-enzymatic. For antioxidant defense against reactive oxygen species (ROS), enzymes are essential. Endogenous antioxidants include SOD, KAT, GR, and GPx. Natural antioxidants glutathione and lipoic acid are synthesized by many metabolic pathways. Hydrogen peroxide and superoxide need enzymes to become water and oxygen. Non-enzymatic antioxidants quickly neutralize radicals and oxidants, protecting against reactive oxygen species. Detoxification and clearance activate enzymes that produce antioxidants.

3.6.3. Synthetic antioxidants

The majority of medicines and supplements include synthetic antioxidants. Certain chemicals prolong food freshness and edibility. Their major strategy is preventing fatty acid unsaturated double bond oxidation. Oxidized pharmaceutical substances are stabilized by antioxidants. BHA and butylated hydroxyltoluene are generally considered the three primary synthetic antioxidants. Like pharmacologists, synthetic antioxidants like propyl gallate and TBHQ are used in food production. Unfortunately, recent research has shown that synthetic antioxidants used in manufacturing may cause human cancer. Due to this, effective and safe antioxidants are sought.

Food additives called synthetic antioxidants are commonly used in food preparation to inhibit or delay the onset of lipid oxidation processes. Synthetic antioxidants commonly used include BHT, BHA, TBHQ, PG, gossypol, and tocopherol. This compound demonstrates phenolic activity with different substitutions on the ring, encompassing both singular and multiple hydroxyl groups. These compounds have impressive antioxidant properties that protect food items from the oxidative degradation of their constituents. These substances delay or hinder the process of lipid oxidation in food systems. These compounds demonstrate their antioxidant properties by effectively inhibiting the formation of free alkyl radicals and interrupting the chain reaction of lipid oxidation (53).


BHA

BHT

TBHQ

Propyl Gallate

Figure 6. Chemical structures of synthetic antioxidants (54).

3.7. Secondary Metabolites

Enzymes produce metabolites in biological pathways. They originate from the Greek word "μεταβολιτησ" (meaning "changed"). Metabolites are a broad group of compounds with different chemical configurations. Not all chemicals engaged in enzymatic processes are metabolites, and there is no uniform definition. Small chemicals are used with biopolymers such as proteins, polynucleotides, and polysaccharides, but not lipids. It is also used to exclude pathway intermediates without distinct functionality and limit it to chemicals having a biological purpose (55).

Secondary metabolites are a vast variety of organic compounds that may be produced by plants. Plants have the remarkable capacity to manufacture these molecules. There are unique carbon skeleton architectures that are present in plant secondary metabolites. Secondary metabolites are not necessary for the life of a cell or organism; nonetheless, they do play a role in the way in which the cell or organism interacts with its surroundings. Through this contact, the creature is able to assist in ensuring that it will continue to exist within its habitat. SMs are normally generated in a way that is unique to the organ, tissue, and cell that is involved in the process. This group of chemicals has a molecular weight that is not very high (9).

The bioactive substances that are present in plants and contribute to the medicinal benefits of those plants are known as secondary metabolites. These compounds are also known as phytochemicals or natural products. There is a unique mix of chemical compounds that is produced by each plant family, genus, and species. These chemical compounds may be used as taxonomic features for placing plants into different categories (56).

Plants create several secondary metabolites to fight predators and microorganisms. These chemicals kill microorganisms and repel herbivores. Secondary metabolites help plants communicate and protect them from abiotic stresses like UV-B radiation. These secondary metabolites are crucial for plant growth and development (57).

Recent findings suggest that secondary metabolites serve as antioxidants and antiradicals, assisting plants in dealing with oxidative stress in difficult settings. Substances such as ascorbic acid, lipoic acid, quercetin, aliphatic and aryl amines, unsaturated fatty acids, and carotenoids, which include hydroxyl and thiol groups or O dihydroxy groups, are among the compounds included in the expanding list. Flavonoids, like quercetin, inhibit the formation of reactive oxygen species (ROS) via the Fenton process by binding to transition metals. This leads to a potent antioxidant and antiradical activity (58). Enhancing the immune system, protecting the body from dangerous free radicals, removing hazardous microbes, and improving general physical fitness are just a few of the defensive functions that secondary metabolites contribute to the human body (59).

Secondary metabolites produced by plants were primarily employed for nourishment, medicinal reasons, and as unpleasant compounds during the course of four thousand years, beginning with their discovery. Their origins may date back to 2600 BC. Morphine was extracted from the opium poppy (Papaver somniferum) in 1806, ushering in a new era in the research of secondary metabolites (60).

There are two primary pathways for metabolite synthesis: the shikimate pathway and the Krebs cycle. In order to produce secondary metabolites, it is essential to have primary metabolites. Primary metabolites are distinguished from secondary metabolites by their chemical, biological, and plant-based properties. Metabolites play a crucial role in all plant tissues, as most plant species have the ability to synthesize these important compounds (61).

The retention of these metabolic pathways led to a very rudimentary metabolic structure. Various metabolic processes, including glycosylation, methylation, hydroxylation, acylation, oxidation, phosphorylation, and prenylation, have caused significant variations in fundamental structures. Additionally, enzyme-mediated chemical alterations have also contributed to these changes (55).

Secondary metabolites have been classified based on many factors, including its chemical structure (whether or not it contains rings or sugars), its composition (whether or not it contains nitrogen), its solubility in organic solvents or water, and its biochemistry pathway. When it comes to identifying secondary metabolites in plants, the biosynthetic pathway is the indicator that is applied the most often (62).

Secondary metabolites in plants may be classified into three chemical groups: phenolic compounds, terpenes and steroids, and nitrogenous substances. These categories are derived based on the pathways by which they are synthesized throughout the biosynthesis process (63).

3.7.1. Phenolic compounds

Phenolic compounds are a wide range of bioactive secondary metabolites that hold significant importance and possess considerable financial value. These molecules could potentially be characterized by the presence of a phenol moiety. Phenol is composed of a benzene ring with a hydroxyl group attached to it. Hydroxybenzene is the name used by scientists to refer to it (64). These chemicals are synthesized through the shikimic acid route in plants and the pentose phosphate pathway via phenylpropanoid metabolism. These compounds consist of benzene rings and hydroxyl substituents and can vary from basic phenolic molecules to complex polymerized compounds.

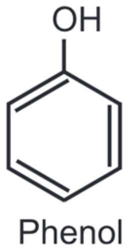


Figure 7. Phenol structure (65).

To begin the production of phenolic compounds, the initial stage involves directing glucose into the pentose phosphate pathway (PPP) and permanently transforming glucose-6-phosphate into ribulose-5-phosphate. The initial stage of the conversion to ribulose-5-phosphate is performed by glucose-6-phosphate dehydrogenase (G6PDH). Converting to ribulose-5-phosphate has a notable benefit - it enables the synthesis of nicotinamide adenine dinucleotide phosphate (NADPH), a crucial component for various cellular anabolic processes. In addition, PPP generates erythrose-4-phosphate and phosphoenolpyruvate through the process of glycolysis. These chemicals are then utilized in the phenylpropanoid pathway to create phenolic compounds. The shikimic acid pathway plays a crucial role in the production of phenylalanine (66).

They are found throughout the plant kingdom and are the most prevalent secondary compounds in plants. Nature exhibits a broad spectrum of phenolic compounds owing to their diverse structures. Presently, there is knowledge of around 8000 structures of phenolic compounds (67).

The well-structured interactions between plants and their living and non-living surroundings have played a significant role in the development of distinct natural substances. Phenolic buildup in plant tissues is a widespread adaptive response to unfavorable environmental circumstances, which enhances evolutionary success (68).

Phenolics are widely present in a wide variety of plant-based foods and beverages, including but not limited to fruits, vegetables, cereals, olives, legumes, and chocolate. Phenolics may also be found in beverages such as tea, coffee, beer, and wine. They play a crucial part in establishing the degree to which these plant-based meals are characterized by their sensory properties. Phenolics, namely procyanidin, interact with glycoprotein in saliva, which is the cause of the bitterness and astringency that are found in fruit and fruit juices. In addition to being a subset of flavonoids, anthocyanins are plant polyphenol components that are responsible for the orange, red, blue, and purple colors that are seen in fruits and vegetables. Apples, berries, beets, and onions are some examples of such substances (67).

There are several environmental challenges that plants are exposed to, including bright light, low temperatures, pathogen infection, herbivores, and nutrient deprivation. Plant phenolics are vital for protecting plants from these environmental pressures. When plants are subjected to these conditions, it is possible for them to produce harmful free radicals and other oxidative species (69).

Phenolic chemicals that are derived from natural sources have lately been linked to a number of various advantageous effects. These benefits include antioxidant, anti-inflammatory, anti-allergic, anti-carcinogenic, antihypertensive, cardioprotective, anti-arthritic, and antibacterial capabilities. As a result, the field of natural product development has made the identification of novel phenolic compounds originating from natural sources a main subject of research in recent years (70).

In recent studies, scientists have discovered a range of positive effects linked to natural phenolic compounds. These effects include antioxidant properties, anti-inflammatory benefits, allergy relief, protection against cancer, lowering blood pressure, safeguarding the heart, reducing arthritis symptoms, and fighting against bacteria. As a result, scientists have been dedicated to studying and discovering new phenolic compounds found in nature for the purpose of natural product development (71).

To use these molecules successfully, several difficulties must be overcome. The low concentration of these chemicals in plant tissues may make satisfying industry demands difficult. Their susceptibility to light and heat may also degrade and remove their benefits. It is also important to study and improve its bioavailability to maximize its medicinal and functional effects (72).

To determine dietary phenolics' health benefits, one must understand their absorption and metabolism. Phenolic structure, including conjugation, glycosylation/acylation, molecular size, and solubility, affects these advantages. The contents pass from the small intestine into the circulation and subsequently to the liver via the portal vein. Since polyphenol metabolites are rapidly removed from plasma, plant products must be consumed daily to maintain blood metabolite concentrations (73).

3.7.2. Simple phenolic compounds

Single-phenol or derivative phenolic compounds are called "simple" compounds. A substitute for phenol compounds. Simply depicting C6 skeleton structures helps identify phenolic compounds. View Figure 9 for the overall structure. Organic groups may be ortho (o), meta (m), or para (p) on the aromatic ring. This group may be alkyl, alkenyl, aryl, hydroxy, alkoxy, amino, and more. These explanations explain the hydroxyl group at position one and phenol's carbon linkages. Their discussion covers carbons at positions 1,2, 1,3, and 1,4 (74)

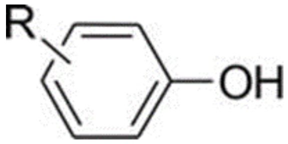


Figure 8. The general structure of simple substituted phenols (75).

3.7.3. Phenolic acids

There are many different kinds of plants that contain phenolic acids, which are secondary metabolites that are widely dispersed. When taken in fruits and vegetables, phenolic acids have the capacity to defend against illnesses that are caused by oxidative damage. These diseases include coronary heart disease, stroke, and cancer. This is the reason why phenolic acids are now receiving a lot of attention (76).

Phenolic acids differ from other acids in that they have a carboxylic acid group. This particular acid group is the only one that exists. Plants may contain polyphenol phytochemicals in addition to phenolic or carboxylic acids. Both of these substances are phenolic compounds. The majority of the elements that make up these compounds originate from plants. Seeds have a higher quantity of these compounds than fruit skins or vegetable leaves. Under some circumstances, coupling may result in the formation of amides, esters, or glycosides (77).

As a result of their chemical composition, phenolic acids may be divided into two separate categories: those that are produced from benzoic acid and those that are formed from cinnamic acid. Both of these categories can be distinguished from one another. Benzene is attached to a carboxylic group (in the case of benzoic acids) or to propionic acid (in the case of cinnamic acids) to form these kinds of compounds. The presence of varied degrees of hydroxylation may be found to result in a variety of structures (78).

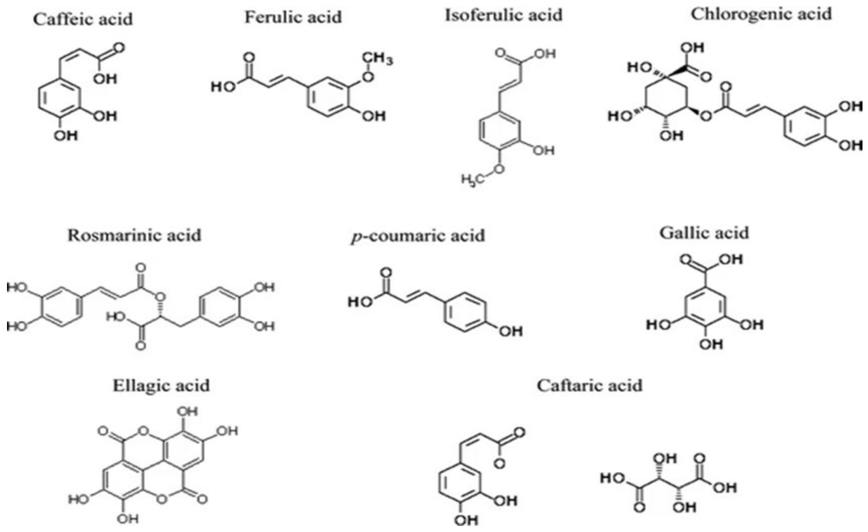


Figure 9. Main phenolic acid structures (79).

Extensive research in laboratories and with living organisms has shown that phenolic acids have the ability to neutralize harmful free radicals and lower the production of singlet oxygen. Phenolic acids possess antioxidant properties due to their ability to donate electrons and transfer hydrogen atoms to free radicals. Phenolic acids have the potential to impact cellular signaling processes and act as antioxidants (80)**.** According to the compelling evidence found in the literature, phenolic acids and their derivatives have shown great potential as drug molecules (81).

3.7.2.2. Flavonoids

According to the compelling evidence found in the literature, phenolic acids and their derivatives have shown great potential as drug molecules. Flavonoid compounds are derived from a fifteen-carbon skeleton. On a basic level, the structure is made up of two phenyl rings (A- and B-rings) linked by a three-carbon bridge (C-ring) (82). Molecular structures may range from the most fundamental phenolic molecule to the most complex high-molecular-weight polymer on the planet.

Three distinct kinds of flavonoids may be distinguished from one another based on the manner in which the aromatic ring is attached to the heterocyclic ring. Flavonoids, also known as 2-phenylbenzopyrans, isoflavonoids, also known as 3-phenylbenzopyrans, and neoflavonoids, sometimes known as 4-phenylbenzopyrans, are all included in these groups. Different classes of flavonoids may be distinguished from one another according to the degree of oxidation and saturation that occurs inside the heterocyclic C-ring (83,84).

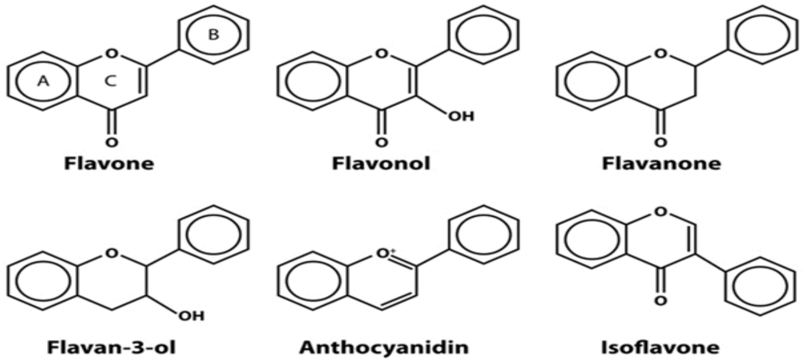


Figure 10. Basic types of flavonoid structures (85).

Flavonoids may be categorized into several subfamilies based on the level of oxidation of the oxygen heterocycle and the patterns of substitution. Some examples of these subfamilies are flavones, flavonols, isoflavones, anthocyanins, flavanols, and flavanones (86) Flavonoids in plants are formed through two different pathways: the phenylpropanoid pathway, which creates the phenylpropanoid skeleton (C6-C3), and the polyketide pathway, which generates the building blocks for polymeric C2 units. Chalcone synthase is responsible for creating the 2′-hydroxychalcone scaffold, also known as (E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one. This compound is formed by combining p-coumaroyl CoA and malonyl CoA, which are then utilized in various enzymatic processes to generate different flavonoids (87).

The extraction of flavonoids, a class of compounds that have been shown to have positive effects on health, from natural sources is now being pursued by several efforts. Flavonoids are now being used in a wide range of applications, including those in the fields of nutraceuticals, pharmaceuticals, medicine, and cosmetics. The features of these compounds are responsible for their capacity to control the activity of critical cellular enzymes. In addition, these compounds provide advantages in the areas of antioxidants, anti-inflammatory agents, anti-mutagenic agents, and prevent the development of cancer (88).

3.8. Enzymes

Initially, the designation "enzyme" was coined by Wilhelm Kuhne, a chemist from Germany. The main objective of his investigation was to study the yeast's capacity to facilitate the transformation of carbohydrates into alcohol. The term "enzyme" is derived from the Greek words "en," meaning "within," and "zume," meaning "yeast." Collectively, these individual words combine to create the English term "enzyme." Each of these individual words, when combined, forms the complete word "enzyme"(13).

Since the 1980s, ribozymes have demonstrated their enzymatic properties, challenging the traditional belief that enzymes are exclusively proteins. Every biological process involves chemical reactions, and enzymes play a crucial role in regulating most of these activities. Enzymes play a crucial role in ensuring the smooth functioning of various processes by preventing them from experiencing any slowdown. Enzymes play a crucial role in driving cellular metabolism. This involves the process of digestion, which includes breaking down large molecules such as proteins, carbohydrates, and lipids into smaller molecules. It also involves the conversion and preservation of chemical energy, along with the synthesis of cellular macromolecules from smaller building blocks. Genetic illnesses, such as albinism and phenylketonuria, occur due to a lack of enzymes (89).

Enzymes are highly effective biological catalysts that are essential for the preservation and replication of all living organisms. As a result, enzymes are crucial. Because of these attributes, they are highly suitable for specifically targeting and treating diseases that result from either genetic traits or pathological factors. One of the main objectives in the field of molecular medicine is to develop and utilize powerful compounds that can regulate the activity of numerous enzymes involved in important biological processes. Prior knowledge of the enzyme being studied is often necessary to create and characterize these small molecules, a process known as rational drug design (90).

Researchers have identified six essential kinds of enzyme operations within the field of systematic nomenclature via their observation and analysis. Every group is tasked with the job of accelerating a certain form of reaction, and it is further subdivided according to the precise parameters of the reaction that is being accelerated and the substrate that is involved. Oxidoreductases are enzymes that take part in processes that include the transfer of hydrogen, while hydrolases are enzymes that take part in the addition of water to a specific location inside a molecule. Oxidoreductases are classified as oxidoreductases. In addition, there are four other groups of reactions, which are transferases, lyases, isomerases, and ligases. Catalysis may take several forms, including substance transfer, isomerization, and ligation, and these reaction groups cover all of these types of the process. Between fifty percent and fifty percent of the enzymes that have previously been found are oxidoreductases and transferases. The table provides information on a wide variety of enzymes, including their name in common parlance, their scientific designations, and the functions they perform inside living structures (91).

Inhibition is fascinating in enzymology and needs additional study. An inhibitor, like a substrate, is a process component. The enzyme converts the inhibitor into a product. This enzyme inhibitor either inhibits the regular process or prevents the expected result. One way the inhibitor works is competitive inhibition. This occurs when the inhibitor attaches to the enzyme in the active site, where the substrate typically binds. Instead, the chemical might connect to a different enzyme site, causing noncompetitive inhibition. In this case, the inhibitor does not prevent substrate-enzyme binding. It achieves this by altering the active site to impede catalysis (92).

3.8.1. Types of inhibition

Enzyme inhibitors are capable of restricting the activity of human enzymes. This can be achieved by reducing the substrate affinity or reaction rate of the enzyme complex. Enzyme inhibitors can either be reversible or irreversible, and they work by binding to the enzyme and inhibiting its activity. Reversible medication inhibitors are advantageous due to their ability to be reversed, resulting in fewer negative effects. Drug prescriptions serve these purposes. There are different types of reversible inhibitors, including competitive, uncompetitive, and noncompetitive inhibitors. Enzymes are influenced by the way inhibitors impact them. Substrates and competing inhibitors engage in a fierce battle for dominance over the active site. Due to their highly specific affinity, inhibitors do not interact. In this field of medicinal chemistry, uncompetitive and noncompetitive inhibitors are effective (93).

3.8.2. Reversible inhibitors

Most medicines bind to enzyme targets through reversible interactions. Inhibitors can bind to the enzyme in its free form, an enzyme species formed after the enzyme-substrate complex, or both. Measuring drug affinity is best done using the equilibrium dissociation constant for the target enzyme's various forms. When comparing affinity between various inhibitors for the same enzyme or between multiple enzymes for a common inhibitor, the relative dissociation constants or the corresponding Gibbs free energy of binding are used. Most enzyme inhibitors bind to their targets through simple, reversible mechanisms. Enzyme-inhibitor binary complexes, like other protein-ligand equilibria, can be quantified using thermodynamic terms, such as the equilibrium dissociation constant (Ki) and the free energy of binding ($\Delta G_{binding}$). Enzyme catalysis offers unique opportunities for inhibitor interactions with different enzyme conformations during turnover (94).

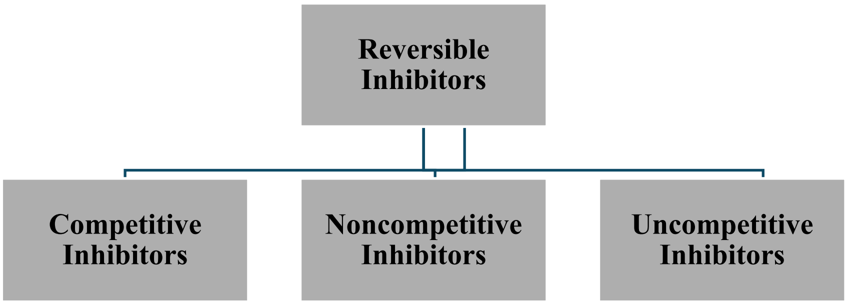


Figure 11. Reversible inhibitors (95).

Irreversible inhibitors are compounds that interact with an enzyme, causing it to lose activity permanently. They form stable covalent connections, primarily with enzyme active-site residues, and thus qualify as catalytic poisons. If such binding occurs, the inhibitors are only required in trace amounts because each inhibitor molecule eventually locates its own enzyme molecule and disables its function. Indeed, the formation of a stable covalent bond is so slow that the rate of inactivation can typically be calculated by observing the time course of residual enzyme activity after various preincubation periods with the inhibitor, even at high concentrations (90).

3.8.3. Enzyme inhibition properties

Alzheimer's disease is characterized by a decrease in the levels of presynaptic acetylcholinesterase (Ach) and the activity of choline acetyltransferase, which is the enzyme responsible for its manufacture. The discovery of this fact has led to the use of AChE inhibitors as a therapeutic method for the treatment of cognitive impairment. It has been shown that AChE inhibitors are effective in the treatment of myasthenia gravis as well as in the stimulation of the central nervous system. It was observed that the administration of tacrine (1,2,3,4-tetrahydro-9-acridinamine) resulted in a rapid arousal of the experimental animals after the administration of morphine. The subsequent research that was conducted shown that Tacrine had the capability to efficiently inhibit both AChE and BChe in a manner that is reversible, as evidenced by the references. For the treatment of Alzheimer's disease, this medicine was the first one to get approval in the United States when it was initially introduced. Despite the fact that it was somewhat successful, the application of it was eventually discontinued due to the negative consequences that it had. Along with a great number of other cholinesterase inhibitors, it has been marketed as a chemical that improves cognitive performance and is associated with the nootropic category (96).

Metallocenzymes known as carbonic anhydrases (CAs) have a significant impact on various biological processes across different organisms. This course explores the topics of gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity, disease proliferation, and pathogenicity. Carbonic anhydrase inhibitors (CAIs) are used to treat antiglaucoma, obesity, cancer, infections, and diuretics. Recent research suggests that CA stimulation could be an effective treatment for Alzheimer's disease. The primary focus of carbonic anhydrase research is on identifying and studying compounds that can inhibit or activate its activity. Diamox (acetazolamide) received approval in 1953 due to its diuretic properties. Furthermore, it provides assistance to epilepsy diets. It has the potential to decrease partial, myoclonic, absence, and primary generalized tonic–clonic seizures (97).

Research findings indicate that this particular treatment is effective for female catamenial epilepsy. Acetazolamide (ACZ), an antiepileptic medication, can induce tinnitus, kidney stones, paresthesia, anorexia, gustatory alterations, and intolerance. Therefore, ACZ is less frequently prescribed compared to other medications. The user inputted the value "40". ACZ suppresses the activity of carbonic anhydrase II, V, VII, XII, and IV. ACZ decreases the sensitivity and activity of cortical neurons in response to Maximal Electroshock (MES). This approach investigates generalized tonic-clonic seizures in animals. This substance decreases seizures by elevating brain carbon dioxide levels and suppressing GABA-A depolarization (97,98).

Managing diet-induced blood sugar increases is characteristic of diabetes management. Inhibiting digestive enzymes like alpha-amylase and alpha-glucosidase reduces intestinal carbohydrate breakdown. Alpha-amylase (EC 3.2.1.1) is a crucial enzyme in the production of synthetic medicines such as acarbose, voglibose, and miglitol. Many natural amylase inhibitors exist. Saliva and pancreas contain alpha-amylase. It breaks starch and polysaccharide glycosidic linkages. This process creates absorbable oligosaccharides and sugars. Like a pharmacologist, the pancreas delivers a lot of pancreatic amylase into the duodenum after the salivary glands produce salivary amylase. This lets the duodenum digest starch using enzymes. A single polypeptide chain of 512 amino acids makes up the HPA protein, which weighs 57 kilodaltons. Clear identification of the active location. As indicated, synthetic inhibitors that target this enzyme's active site and interrupt its catalytic activity have been discussed for diabetes therapy. They may also cause gas, diarrhea, bloating, and stomach pain. Researchers are eager to develop safer alpha-amylase inhibitors (99).

4. MATERIALS AND METHODS

4.1. Chemicals Used

AChE, BChE, CA II, α-amylase enzymes, HMF, trolox, α-tocopherol, BHT, BHA, DPPH, L-tyrosine, DMPD, ABTS, TCA, TPTZ, DTNB, PNF, CuCl2, acetone, neocuprin, FeCl3, ferrosine, K3[Fe(CN)6], serum albumin, acetylcholine iodate, butyrylcholine iodate were obtained from Sigma-Aldrich GmbH (Sterheim, Germany). Proline, fructose, Sephadex G-25, glucose, trihydroxymethyl aminomethane (Tris), naphthalene, hydrochloric acid, sucrose, sulfuric acid, naphthalene-D8 isotope, phosphoric acid, maltose, sodium bicarbonate, sodium hydroxide, coommassie brillant blue sodium sulfate, sodium acetate, sulfanilamide, acrylamide, bisacrylamide, ethanol, glycine and secondary metabolite standards G-250 were obtained from Merck. The chemicals used were supplied in analytical purity.

4.2. Collection and Extraction Procedures of *A. tricornis* Used in This Thesis Study

In this thesis study, *A. tricornis* species was collected during fieldwork carried out in southern Turkey in 2024. The classification of *A. tricornis* was determined by a member of the faculty of Pharmacy at Dicle University; Assoc. Prof. Oğuz Şakir (Herbarium No.: MARE-16706). These plant samples were shade-dried and the above-ground parts were ground into powder. Then, the powdered plant samples were subjected to extraction. For sample preparation, initially, the whole parts of the dried and powdered plant (10 g) was extracted by ethanol (3 × 50 mL) in 24 h at room temperature (extraction yield, 8.3%). The extract was subsequently filtered and evaporated under reduced pressure. Then, dry filtrate was diluted until 250 mg/L and passed through the 0.2 µm microfiber filter for LC-MS/MS.

4.3. Quantitative Analysis of Secondary Metabolites by LC-MS/MS

To determine the secondary metabolites of *A. tricornis* species by LC-MS/MS, the method developed and validated by Yılmaz through method validation was applied (100). Before applying this method, stock solutions were prepared from *A. tricornis*

EIG extract at a concentration of 10 mg/mL. Before being given to the instrument, these solutions were filtered through a 0.22 µm syringe tip filter, taken into LC-MS vials and given to the device. 53 different compounds were screened and quantitatively analyzed as secondary metabolites and three different components were used as internal standards.

Figure 12. LC-MS/MS instrument.

Table 2: LC-MS Instrument Analytical Parameters and Chromatography Conditions (101).

Analytical parameters	Chromatography conditions or model
LC-MS/MS	Shimadzu LCMS-8040 model (LC-ESI-MS/MS)
Software	Lab Solutions (Shimadzu)
Autosampler	SIL-30AC model
Degasser	DGU-20A3R model
Dual pumps	LC-30AD model
Columns	150 mm × 2.1 mm, 2.7 µm, Agilent Poroshell 120 EC-C18 model
Column temperatures	40ºC
Eluent A	Water + 0.1% Formic Acid + 5 mM Ammonium Formate
Eluent B	Methanol + 0.1% Formic Acid + 5 mM Ammonium Formate

Gradient elution profile	20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min)
Solvent flow rate	0.5 mL/min
Injection volume	5 µL
MS operating conditions	Nebulizing Gas (N_2) flow, 3 L/min; drying gas (N_2) flow, 15 L/min; DL temperature, 250°C; heat block temperature, 400°C

4.4. Determination of Total Flavonoid Content

Total Phenolic Amount Method

Solutions

- FCR was used as is.
- Na_2CO_3 solution (2%): 1 g of Na_2CO_3 was taken into a 50 mL volumetric flask and dissolved in 40 mL of distilled water. The final volume was made 50 mL by adding distilled water.
- Gallic acid solution (Standard): Prepared by dissolving 10 mg of gallic acid in 10 mL of distilled water.

Method

The amount of phenolic compounds in bee products was determined using Folin-Ciocalteu reagent (FCR) (Singleton et al. 1999). Gallic acid was used as the standard substance. First, a stock solution was prepared at a concentration of 10 mg/mL for standard graphical drawing. 10 mg gallic acid was dissolved in 10 mL distilled water and 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µL gallic acid solution was taken into tubes from the prepared stock and the final volume was completed to 23 mL with distilled water. Then, 0.5 mL FCR and 1.5 mL of 2% Na_2CO_3 were added in order after three minutes and stirred under normal conditions for two hours. Finally, absorbances against blank (distilled water) were determined at 760 nm. Distilled water was used instead of sample as control. Gallic acid equivalent (GAE) corresponding to the absorbances of the samples was calculated as (µg).

4.5. Determination of Total Phenolic Acid Content

Solutions

- Quercetin solution (Standard): 10 mg quercetin was dissolved in 10 mL of distilled water.
- Na_2CO_3 solution (2%): 1 g of Na_2CO_3 was taken in a 50 mL volumetric flask and dissolved in 40 mL of distilled water. The final volume was made up to 50 mL with distilled water.
- $Al(NO_3)_3$ solution (10%): Prepared by adding 15 g of $Al(NO_3)_3$ to 135 mL of pure water and dissolving.
- CH_3COOK solution (1M): 19.8 g of CH_3COOK was taken in 160 mL of distilled water and dissolved. The final volume was made up to 200 mL with distilled water.

Method

The total amount of flavonoids in bee products was determined based on the method applied by Park and colleagues (Park et al. 1997). Quercetin was used as the standard flavonoid substance. Firstly, 10, 20, 30, 40 and 50 µg of quercetin solution from the prepared stock was added in µL in the same volumes and 0.1 mL (1.0 M) CH_3COOK (in water) and 0.1 mL $Al(NO_3)_3$ (10%) were added to the tubes. 4.3 mL of ethanol was added to the mixture and mixed and diluted. It was left to incubate for forty minutes under normal conditions and absorbances against the blank (ethanol) were measured at 415 nm. Total flavonoid concentration was determined as quercetin equivalent (QE) (µg) from the 53 equations derived from the standard quercetin graph.

4.6. Antioxidant Activity Methods

Stock solutions prepared with α-tocopherol, trolox, BHT, BHA, and ethanol at a concentration of 10 mg/mL, which are used as standards in all antioxidant activity methods, were used.

4.6.1. Fe^{3+} - Fe^{2+} reduction method

Solutions

- Phosphate buffer (0.2 M): 3.12 g Na_2HPO_4 was dissolved in 80 mL pure water and the pH was adjusted to 6.6 and the final volume was made 100 mL with distilled water.
- 1% $K_3Fe(CN)_6$ solution: 1 g of $K_3Fe(CN)_6$ was taken and dissolved in 100 mL of distilled water.
- 10% TCA solution: 5 g of TCA was taken and dissolved in 50 mL of distilled water.
- 1% $FeCl_3$ solution: 165 mg of $FeCl_3.6H_2O$ was weighed and dissolved in 100 mL of distilled water.

Method

Oyaizu's (1986) method was modified and applied (103). Pipetting into the test tubes was done in the order specified in Table 3 and vortexed. 1 mL was taken from each tube, 1 mL of distilled water was added to each, and vortexed for 10 min. After incubation in the dark, absorbances were measured at 700 nm. Distilled water was used as a blank. To prepare the control, measurements were made by adding distilled water to the contents of the test tube instead of the sample.

Table 3. Concentrations used in the Fe^{3+} - Fe^{2+} reduction method (µg/mL).

Tube content (µl)	**15**	**30**	**45**	**60**
Numune/Standart	67.5	135	202.5	270
Distilled water	432.5	365	297.5	230
Phosphate buffer	1250	1250	1250	1250
Fecl3 Solution	250	250	250	250
K3fe(cn)6 solution	1250	1250	1250	1250
TCA solution	1250	1250	1250	1250

Total	4500	4500	4500	4500

4.6.2. CUPRAC method (Cu^{2+} - Cu^{+} reduction)

Solutions

- 1 M CH_3COONH_4 buffer: 3.85 g of CH_3COONH_4 was taken and dissolved in 40 mL of distilled water. The pH was adjusted to 6.5 and the final volume was made up to 50 mL.

- 0.01 M $CuCl_2$ solution: 0.067 g of $CuCl_2$ was weighed and dissolved with 50 mL of distilled water.
- Neocuprin solution (7.5.10-3 M): 3.9 g of neocuprin was taken and dissolved in 25 mL of ethanol.

Method

- It was carried out by modifying the method of Apak et al (104). Pipetting into the test tubes was done, and after mixing with the vortex, they were incubated in the dark and at room temperature for half an hour and the absorbances were measured at 450 nm. Distilled water was used as a blank.

Table 4. Concentrations used in the CUPRAC method (μg/mL).

Tube content (µl)	15	30	45	60
Sample/Standard	15	30	45	60
Distilled water	685	670	655	640
CuCl2	125	125	125	125
Acetate buffer	125	125	125	125
Neocuprine solution	50	50	50	50
Total	1000	1000	1000	1000

4.6.3. FRAP method (Fe^{3+} - TPTZ reduction)

Solutions

- 40 mM HCl solution: 0.334 ml of 37% HCl solution was added to approximately 50 mL of distilled water and the final volume was completed to 100 mL with distilled water. 10 mM TPTZ solution: It was dissolved by adding 0.312 g of TPTZ to the HCl solution prepared above.
- 0.3 M CH_3COONa buffer: 2.46 g of CH_3COONa was dissolved in 80 mL of distilled water and after adjusting the pH to 3.6, the final volume was made 100 mL with distilled water.
- $FeCl_3$ solution (20 mM): It was prepared by dissolving 0.54 g $FeCl_{3.6}H_2O$ in 100 mL distilled water.
- FRAP reagent: 1 volume $FeCl_3$, 1 volume TPTZ solution, 10 volumes acetate buffer solution was mixed.

Method

Pipetting into the test tubes was done and vortexed.1 mL was taken from the upper parts and 1 mL of distilled water was added to each.10 min after overtaxing. It was incubated in the dark and at room temperature and absorbances were measured against the blank (sodium acetate buffer) at 593 nm. Since a 2-fold dilution was made, absorbances were calculated by multiplying by 2.

Table 5. FRAP (TPTZ) method test tube content (µg/mL).

Tube content (µl)	15	30	45	60
Sample/Standard	37.5	75	112.5	150
Acetate buffer	712.5	675	637.5	600
FeCl3 solution	1000	1000	1000	1000
Frap reagent	750	750	750	750

Total	2500	2500	2500	2500

4.6.4. Determination of DPPH radical scavenging activity

Solutions

10^{3-}M radical solution: 39.43 mg DPPH was weighed into a 100 mL volumetric flask and was completed with ethanol and the solution was prepared after stirring for 16 hours.

Method

It was carried out based on the Blois method (105). DPPH 1 mM solution was used as an oxidant. Before using the control solution consisting of radical and ethanol, its absorbance was adjusted to 1±0.025 nm. Pipetting into test tubes at the concentrations specified in Table 3. After it was made, it was vortexed and incubated at room temperature and in the dark for half an hour, and the absorbance against blank (ethanol) was determined at 517 nm. Table 3 demonstrates concentrations used in $DPPH^{\cdot}$ Removal Method (µg/mL).

Table 6. Concentration in the DPPH scavenging method (µg/mL).

Tube content	**15**	**30**	**45**	**60**
Sample/Standard	20	40	60	80
Ethanol	730	710	690	670
$DPPH^{\cdot}$ solution	500	500	500	500
Total	1250	1250	1250	1250

4.6.5. Determination of $ABTS^{\cdot +}$ radical scavenging activity

Solutions

0.1 M Phosphate buffer: Weighed 2.4 g of Na_2HPO_4 and dissolved it in 180 mL of pure water. After adjusting the pH to 7.4, the final volume was made 200 mL with distilled water. $ABTS^{\cdot+}$ solution (2 mM): 110 mg of ABTS is weighed and infused in 100 mL phosphate buffer for approximately 15 minutes. It was mixed and after dissolution, 66.23 mg K2O8S2 was added within certain limits and it was prepared by stirring for 1-1.5 hours after dissolution.

Method

The method used by Re and his colleagues (106)was taken as a basis. The absorbance of the control solution consisting of $ABTS^{\cdot+}$ solution and phosphate buffer was adjusted to 1±0.025 nm before use. Pipetting into the test tubes was done in the order given in Table 10, and after mixing with the vortex, they were incubated for half an hour in the dark and at room temperature and the absorbances were measured at 734 nm. Phosphate buffer was used as a blank.

Table 7. Concentrations used in the $ABTS^{+}$ removal method (µg/mL).

Tube content (µl)	**15**	**30**	**45**	**60**
Sample/Standard	6.25	12.5	18.75	25
Phosphate buffer	243.75	237.5	231.25	225
$ABTS^{+}$ solution	1000	1000	1000	1000
Total	1250	1250	1250	1250

4.6.6. $DMPD^{\cdot+}$ removal method

Solutions

- M DMPD solution: Weigh 105 mg of DMPD and add it to 5 mL of distilled water resolved.

- 0.05 M $FeCl_3$ solution: 67.5 mg of $FeCl_3.6H_2O$ was taken and dissolved in 5 mL of distilled water.
- M CH_3COONa buffer: 0.82 g of CH_3COONa was taken and dissolved in 80 mL of distilled water, adjusting the pH to 5.25, and then the final volume was made 100 mL with distilled water.
- M $DMPD^{\cdot+}$ reagent: 0.1 M 1 mL DMPD solution and 0.05 M 0.2 mL $FeCl_3$ solution were added to 0.1 M 100 mL sodium acetate buffer (pH: 5.25) and prepared by dissolving.

Method

It was applied based on the method of Fogliano and his colleagues. 0.1 M $DMPD^{\cdot+}$ reagent was formed by adding 0.1 M 1 mL DMPD solution and 0.05 M 0.2 mL FeCl3 solution to 0.1 M 100 mL sodium acetate buffer (pH: 5.25). The absorbance of the control solution consisting of $DMPD^{\cdot+}$ reagent and distilled water was adjusted to 1±0.025 nm before use. Pipetting into the test tubes was done and after mixing with the vortex, the mixture was mixed for 50 minutes. It was incubated in the dark and at room temperature, and finally, the absorbance was measured at 505 nm. Sodium acetate buffer was used as a blank.

Table 8. Concentrations used in the $DMPD^{+}$ removal method (µg/mL).

Tube content (µl)	**15**	**30**	**45**	**60**
Sample/Standard	22.5	45	67.5	90
Distilled water	477.5	455	422.5	410
$DMPD^{+}$ reagent	1000	1000	1000	1000
Total	1500	1500	1500	1500

4.7. Enzyme Inhibition Activity Methods

4.7.1. Acetylcholinesterase enzyme inhibition activity method

Solutions

- 10 mM acetylcholine iodate solution: 14.5 mg of acetylcholine iodate was taken and dissolved in 5 mL of distilled water.
- 1 M Tris-HCl buffer: 0.182 g EDTA, 15.14 g Tris were weighed and dissolved in 100 mL distilled water and the pH was adjusted to 8. The final volume was made 125 mL with distilled water.
- DTNB solution (10 mM): 0.1 g sodium citrate 2 mg DTNB, weighed. It was dissolved in 10 mL of pure water.

Method

AChE breaks down acetylcholine into thiolin and acetate. Thiocholine is used to determine enzyme inhibition. As a result of the reaction of thiocholine and DTNB, yellow 5-Thio-2-Nitrobenzoic acid is formed and gives absorbance at 412 nm (107). Enzyme inhibition was performed by determining the IC_{50} value. For this, pipetting was done into the test and incubation was carried out at 412 nm for 3 minutes. Absorbance measurement was made throughout.

Table 9. Cholinesterase activity determination cuvette content.

Tube content

Buffer	100
Distilled water	780
DTNB solution	50
Enzyme solution	20
Sample (inhibitor)	(Subject to change)

Substrate (acetylcholine iodate and butyrylcholine iodate)	50
Total volume	1000

4.7.2. α-Amylase enzyme inhibition activity method

Solutions

- Sodium Phosphate buffer (0.1 M): 5.99 g sodium phosphate was dissolved in 450 mL of distilled water and after adjusting the pH to 6.9, the final volume was made 500 mL with distilled water.
- Starch solution: 20 mg of starch was dissolved by heating in 20 mL of distilled water.

Method

The enzyme was purchased commercially ready. The contents of the bathtub were filled and starch, which is the enzyme-substrate, is difficult to dissolve, so it waited in the heated mixer until it dissolved. The method was applied by determining different sample concentrations and IC_{50} was determined by determining the absorbance values at 580 nm (108).

Table 10. α-Amylase activity determination cuvette content.

Tube content	
Sample (inhibitor)	- (Subject to change)
Starch	500
Buffer	250 (Subject to change)
Enzyme	250

Total volume	1000

4.7.3. Solvents used for hCA II isoenzymes

- Affinity gel buffer (200 mM): 16.8 g $NaHCO_3$ was weighed and dissolved in 950 mL distilled water, and the pH was adjusted to 8.8 with 1 N NaOH, and then the volume was completed to 1 L with distilled water.
- Gel equilibration buffer (25 mM Tris-HCl/0.1 M Na_2SO_4): 14.2 g Na_2SO_4 and 3.028 g Tris were weighed and dissolved in 950 mL distilled water. It was prepared by adjusting the pH to 8.7 with 1 M NaOH and then completing the volume to 1 L with distilled water.
- Gel washing buffer (25 mM Tris-HCl/22 mM Na_2SO_4): 3.214 g Na_2SO_4 and 3.028 g Tris were weighed and dissolved in 950 mL distilled water. After adjusting the pH to 8.7 with 1 M NaOH, the volume was completed to 1 L with distilled water.
- hCA II isoenzyme elution buffer (0.1 M CH_3COONa/0.5 M $NaClO_4$): by weighing 9.187 g $NaClO_4$ and 2.04 g $CH_3COONa.3H_2O$. It was dissolved in 120 mL of distilled water, the pH was adjusted to 5.6 with 1 N HCl, and the final volume was made 150 mL with distilled water.
- Dialysis buffer: 6 g Tris was dissolved in 900 mL distilled water, the pH was adjusted to 7.4 with H2SO4 and the final volume was completed to 1 L with distilled water.
- Tris-SO_4 buffer (0.05 M): 6.057 g Tris was weighed and dissolved in 950 mL of distilled water, and after adjusting the pH to 7.4 with 1 N H_2SO_4, the final volume was made 1 L with distilled water.
- Nitrophenyl acetate solution (PNF): 13.5 mg of PNF was weighed. It was dissolved in 1 mL of acetone. The final volume was made 25 mL with distilled water.

4.7.4. Preparation of CA enzyme hemolysate

It was taken 10 mL of human erythrocyte and 40 mL of iced distilled water was added and incubated for 5-10 minutes. It was mixed to ensure that the cells burst within the range. 30 min at 13,000xg at +4 oC. The upper phase hemolysate was carefully collected without touching the precipitated part by centrifugation for a long time, and the pH was adjusted to 8.7 by adding tris little by little to the hemolysate. After this process, the ready hemolysate was stored at +4 oC (109).

4.7.5. Determination of esterase activity of hCA II isoenzymes

It is made depending on the esterase activity of the carbonic anhydrase enzyme. The substrate of the CA enzyme is p-Nitrophenyl acetate (PNF). The enzyme hydrolyzes PNF to form p-Nitrophenol or p-Nitrophenolate and the determination of esterase activity is based on this principle. The resulting compounds also give maximum absorption at 348 nm. While preparing the PNF solution, it is necessary to prepare it daily, wrap it in foil as it deteriorates quickly in light, and use acetone as a solvent. The reason for using acetone is that it inhibits hydrolysis less than other solvents.

Table 11. Carbonic anhydrase esterase activity cuvette content.

Quartz bathtub content	Volume (µl)
Tris-SO4 buffer	400
Distilled water	220 (May vary depending on the inhibitor)
PNF solution	360
Sample (inhibitor)	- (May vary depending on water)
Enzyme solution	20
Total volume	1000

4.7.6. Determination of hCA II isoenzymes inhibition activity

Enzyme inhibitions were determined by the esterase activity method by finding IC_{50} values. For this, 6 different concentrations of bee product extracts were pipetted from stocks prepared with DMSO and incubated at 348 nm for 3 min. absorbance measurement was made. A graph of % enzyme activity versus concentration was drawn and IC_{50} values were calculated from this graph equation.

4.8. Statistical Analysis

The experiments were carried out in triplicate for the tests. The results were shown with the standard deviation (SD) and mean values. The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with a D 0.05. This treatment was carried out using SPSS v. 14.0 program.

5. RESULTS AND DISCUSSION

5.1. Secondary Metabolites Findings by LC-MS/MS

The phytochemical analysis of an extract of *A.tricornis* revealed the presence of compounds that are recognized for their physiological and medicinal properties. Phenolic compounds, including flavonoids, phenolic acids, and tocopherols, are the primary source of natural antioxidants in plants (17,110).

By LC-MS/MS 53 different phenolic and flavonoid compounds in *A. tricornis* were quantitatively examined. The findings are given in Table (4). Quercetin-D3, routine-D3, and ferulic acid-D3 compounds were used as internal standards. The compound determined at the highest concentration in *A. tricornis* samples was determined to be quinic acid (24.006 mg analyte/g extract). The chromatogram shows the results graphically, where peaks represent the detected compounds, and the table links these peaks to the compound concentrations.

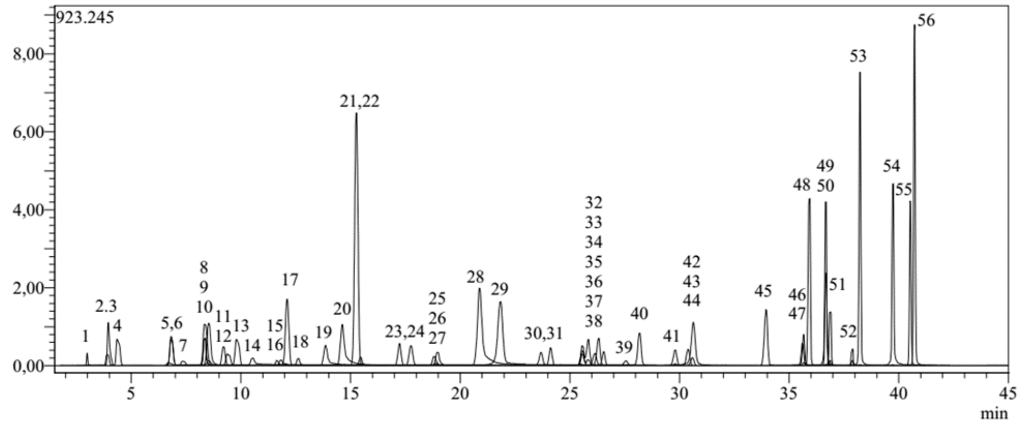


Figure 13. LC-MS/MS TIC chromatogram standard.

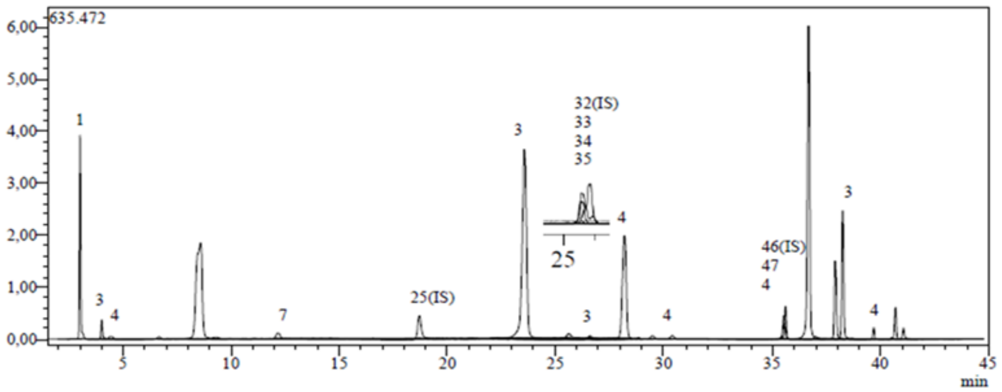


Figure 14. LC-MS/MS TIC chromatogram of *A. tricornis* ethanol extract.

Table 12. Analytical method parameters and phytochemical quantification results of A. tricornis ethanol extract (mg analyte/g extract) ^{a}R.T.: Retention time, ^{c}FI (m/z): Fragment ions, ^{b}MI (m/z): Molecular ions of the standard analytes (m/z ratio).

#	Analyte	Amount (mg/g extract)	RT$^\alpha$	F.I. (m/z)^c	M.I. (m/z)^b
1	Quinic acid	24.006	3.0	93.0	190.8
2	Fumaric acid	N.D.	3.9	40.9	115.2
3	Aconitic acid	0.228	4.0	129.0	172.8
4	Gallic acid	0.173	4.4	79.0	168.8
5	Epigallocatechin	N.D.	6.7	219.0	304.8
6	Protocatechin acid	0.153	6.8	108.0	152.8
7	Catechin	N.D.	7.4	203.1	288.8
8	Gentisic acid	N.D.	8.3	109.0	152.8
9	Chlorogenic acid	5.887	8.4	85.0	353.0
10	Protocatechuic aldehyde	N.D.	8.5	92.0	137.2
11	Tannic acid	N.D.	9.2	78.0	182.8
12	Epigallocatechin gallate	N.D.	9.4	305.1	457.0
13	1,5-dicaffeoylquinic acid	N.D.	9.8	191.0	515.0
14	4-OH Benzoic acid	N.D.	10.5	65.0	137.2
15	Epicatechin	N.D.	11.6	203.0	289.0
16	Vanillic acid	N.D.	11.8	108.0	166.8
17	Caffeic acid	0.085	12.1	134.0	179.0
18	Syringic acid	N.D.	12.6	166.9	196.8
19	Vanillin	N.D.	13.9	125.0	153.1
20	Syringic aldehyde	N.D.	14.6	151.1	181.0
21	Daidzin	N.D.	15.2	199.0	417.1
22	Epicatechin gallate	N.D.	15.5	289.0	441.1
23	Piceid	N.D.	17.2	135	391.0
24	*P*-Coumaric acid	0.037	17.8	93.0	163.0
25	Ferulic acid-D3-IS	N.A.	18.8	152.1	196.2
26	Ferulic acid	N.D.	18.8	149.0	192.8
27	Sinapic acid	N.D.	18.9	193.0	222.8

28	Coumarin	N.D.	20.9	103.1	146.9
29	Salicylic acid	0.01	21.8	65.0	137.2
30	Cyranoside	14.126	23.7	284.0	447.0
31	Miquelianin	N.D.	24.1	150.9	447.0
32	Rutin-D3-IS	N.A.	25.5	304.1	612.2
33	Rutin	0.013	25.6	301.0	608.9
34	isoquercitrin	0.272	25.6	271.0	463.0
35	Hesperidin	0.021	25.8	449.0	611.2
36	O-Coumaric acid	N.D.	26.1	93.0	162.8
37	Genistin	N.D.	26.3	239.0	431.0
38	Rosmarinic acid	0.152	26.6	197.0	359.0
39	Ellagic acid	N.D.	27.6	284.0	301.0
40	Cosmosiin	6.447	28.2	269.0	431.0
41	Quercitrin	N.D.	29.8	301.0	447.0
42	Astragalin	0.138	30.4	255.0	447.0
43	Nicotiflorin	N.D.	30.6	255.0	592.9
44	Fisetin	N.D.	30.6	163.0	285.0
45	Daidzein	N.D.	34.0	223.0	253.0
46	Quercetin	N.A.	35.6	275.9	304.0
47	Quercetinin	1.467	35.7	272.9	301.0
48	Naringenin	0.259	35.9	119.0	270.9
49	Hesperetin	N.D.	36.7	136.0	301.1
50	Luteolin	1.918	36.7	151.0	284.8
51	Genistein	N.D.	36.9	135.0	269.0
52	Kaempferol	0.039	37.9	239.0	285.0
53	Apigenin	0.481	38.2	151.0	268.0
54	Amentoflavone	0.047	39.7	417.0	537.0
55	Chrysin	0.008	40.5	145.0	252.8
56	Acacetin	0.319	40.7	239.0	283.0

- N.D.: Not detected, N.A.: Not applicable, IS: Internal standard.

The results show significant diversity in the phenolic compounds present in *A. Tricornis* extract, with substantial variation in concentrations. High-concentration compounds like quinic acid (24.006 mg analyte/g extract) and cyranoside (14.126 mg analyte/g extract) play a crucial role in the chemical characteristics of the sample. The results are consistent with previous studies that highlighted the abundance of phenolic compounds in plants and their use in chemical and medical research (111), (66) . The findings of this study enable us to have more understanding of the phytochemical compounds found in *A.tricornis* and their prospective usages.

5.2. Total Phenolic and Flavonoid Compounds Findings

Table 13. Determination of total phenolic and total flavonoid antioxidants.

Antioxidant Compounds	**Total Phenolic**		**Total Flavoind**	
A. tricornis	57.88	r^2= 0.9918	41.16	r^2= 0.9913

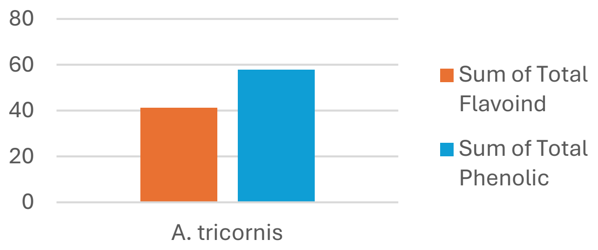


Figure 15: Total phenolic and total flavonoid antioxidants.

5.3. Antioxidant Activity Findings

5.3.1. Radical scavenging ($DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $DMPD^{\cdot+}$) methods findings

Extracts of *Asteraceae* family have free radical scavenging properties, which have been related to their phenolic component content. Nowadays, there is a growing interest in the importance of nutrition in human health, as well as natural remedy-based therapies for a variety of disorders. It has been proved that a plant-based diet, which is high in antioxidants, plays a significant role in disease prevention

In 1958, Blois proposed experimenting to scavenge the 1,1-diphenyl-2-picrylhydrazine (DPPH) radical. Several researchers have made slight adjustments to this experiment after it was completed. This test is highly regarded as one of the most prestigious methods in the field of assessing the antioxidant capabilities of plant materials. DPPH is a stable free radical that can undergo reactions in the presence of compounds that have the ability to donate hydrogen atoms. Through the addition of a reactive species or antioxidant, this method effectively removes DPPH from the solution, resulting in a change in its color. The decrease in absorbance at a wavelength of 515 nm is used to determine the amount of antioxidant activity.

$ABTS^{\bullet+}$ Method RiceEvans and Miller pioneered $ABTS^{\bullet+}$ radical scavenging in 1994 (106). improved the approach in 1999, making it more practical. Metmyoglobin may be activated by hydrogen peroxide and $ABTS^{\bullet+}$ to form a radical cation. Many individuals have adopted this improved method, which uses $ABTS^{\bullet+}$ and potassium persulfate to create a vivid blue-green $ABTS^{\bullet+}$ chromophore. This biology-based antioxidant assay for plant materials is popular. This procedure is typically employed with DPPH. Spectrophotometry at 734 nm detects hydrogen-donating antioxidants reducing the $ABTS^{\bullet+}$ radical cation.

$ABTS^{\bullet+}$ is reduced by potassium persulfate oxidation in the presence of antioxidants. This test quantifies lipophilic and hydrophilic compounds' color-elimination ability to determine their antioxidant activity. A substance's antioxidant activity depends on its antioxidant content and capacity to block radical cation absorption.

$DMPD^{\bullet+}$ radical scavenging test converts translucent N, N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) into the colorful radical $DMPD^{\bullet+}$ in the presence of Fe ions or reactive species such as hydroxyl radicals. Antioxidants that may transfer hydrogen atoms decolorize the solution and cause a drop in absorbance at 505 nm. The primary problem of utilizing this assay is that the $DMPD^{\bullet+}$ radical is only soluble in water, therefore there are restrictions in using it to determine hydrophobic antioxidants (112).

The results of radical scavenging activity methods of *A. tricornis* were evaluated by comparing them with standard antioxidants Trolox, α-Tocopherol, BHA and BHT. Table (14) shows the half-maximum values (IC_{50}) for a group of antioxidant compounds and their ability to scavenge free radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $DMPD^{\bullet+}$). In comparison to other standards, the extract of *A. tricornis* was revealed to be less efficient in neutralizing $DPPH^{\bullet}$ radical scavenging activity (0.906±0.010). However, it was more effective in inhibiting $ABTS^{+}$ (0.713±0.024) and $DMPD^{\bullet+}$ (0.562±0.001) radical scavenging activities. To confirm these results, various studies have identified a strong relationship between total phenolic content and the ability of herbal extracts to scavenge free radicals (113).

Table 14. Determination of half-maximal concentrations (IC$_{50}$) and standards for radical scavenging DPPH$^{\cdot}$, ABTS$^{\bullet+}$.

Antioxidant Compounds	DPPH· scavenging	DPPH· r^2	ABTS$^{\bullet+}$ scavenging	ABTS$^{\bullet+}$ r^2	DMPD$^{\bullet+}$ scavenging	DMPD$^{\bullet+}$ r^2
BHA	0.077±0	0.9718	0.285±0.004	0.9830	0.546±0.009	0.9583
BHT	0.4±0.026	0.9835	0.701±0.000	0.9605	1.781±0.062	0.9755
α-TOCOPHEROL	0.083±0.002	0.9829	0.299±0	0.9749	0.129±0.019	0.9613
TROLOX	0.027±0	0.9517	0.278±0.005	0.9617	0.769±0.021	0.9843
A. tricornis	0.906±0.010	0.9752	0.713±0.024	0.9993	0.562±0.001	0.9513

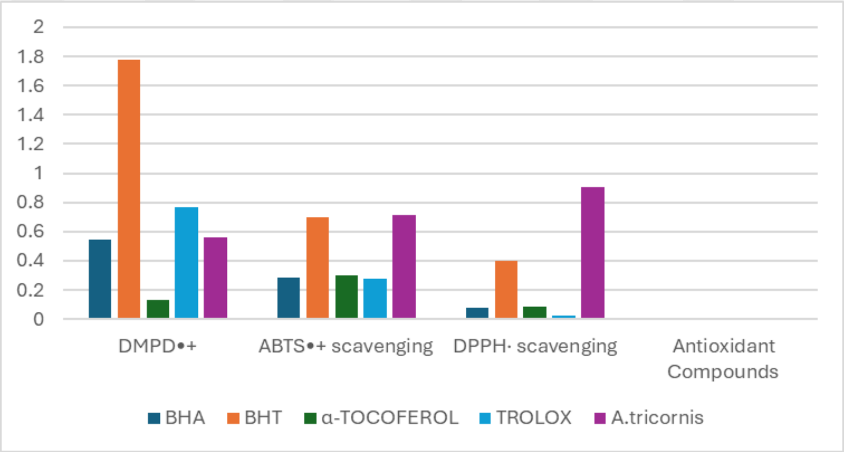


Figure 16: Determination of half-maximal concentrations (IC$_{50}$) and standards for radical scavenging DPPH$^{\cdot}$, ABTS$^{\bullet+}$.

5.3.2. Reducing power (Fe^{3+}, CUPRAC and FRAP) methods findings

Reducing power is an essential antioxidant parameter that reflects the electron-donating activities of antioxidant substances. CUPRAC and FRAP tests were used to calculate the reducing power within the scope of our investigation. The CUPRAC test has lately become one of the most widely used procedures due to its simplicity, low cost of reagents, and the fact that it happens in settings similar to physiological pH, with the reduction of Cu^{+2} to Cu^{+} monitored. The FRAP test converts the Fe^{+3}-TPTZ combination of antioxidant chemicals into Fe^{2+}-TPTZ in an acidic environment. When the reducing power data were considered as a whole, it was discovered that they were typically consistent with the overall phenolic concentration of the extracts.

This demonstrates that the phenolic and flavonoid compounds in the extract of *A. tricornis* have efficient electron-donating properties. Corroborating these results, various studies have established a robust correlation between the total phenolic content and the reducing power methods of plant extracts. In addition, some researchers have directly investigated the reducing power of some phenolic acids and flavonoids in FRAP and CUPRAC tests and reported that they have quite strong activities (114). *A. tricornis* was shown to be less effective as an antioxidant in reducing Fe^{3+} to Fe^{2+}, Cu^{2+} to Cu^{+}, and Fe^{3+}-TPTZ compared to other standards, exhibiting less effectiveness as an antioxidant in these contexts.

Table 15. Determination of reducing the power of same concentration by FRAP methods, ferric ions (Fe^{3+}) reducing and cupric ions (Cu^{2+}) reducing capacity by Cuprac method.

Antioxidants	**Fe^{3+}-Fe^{2+}reducing**		**Cu^{2+}-Cu^{+} reducing**		**Fe^{3+}-TPTZ reducing**	
	λ_{700}	**r^2**	**λ_{45}**	**r^2**	**λ_{593}**	**r^2**
BHA	2.319±0.040	0.9841	3.019±0.023	0.9572	2.142±0.012	0.9730
BHT	1.873±0.151	0.9897	2.868±0.032	0.9875	1.683±0.088	0.9960
α-TOKOFEROL	2.808±0.272	0.9905	2.184±0.109	0.9859	2.121±0.060	0.9695
TROLOX	2.333±0.167	0.9589	2.555±0.021	0.9915	2.068±0.015	0.9943
A. tricornis	0.782±0.010	0.9678	0.923±0.029	0.9751	1.066±0.008	0.9517

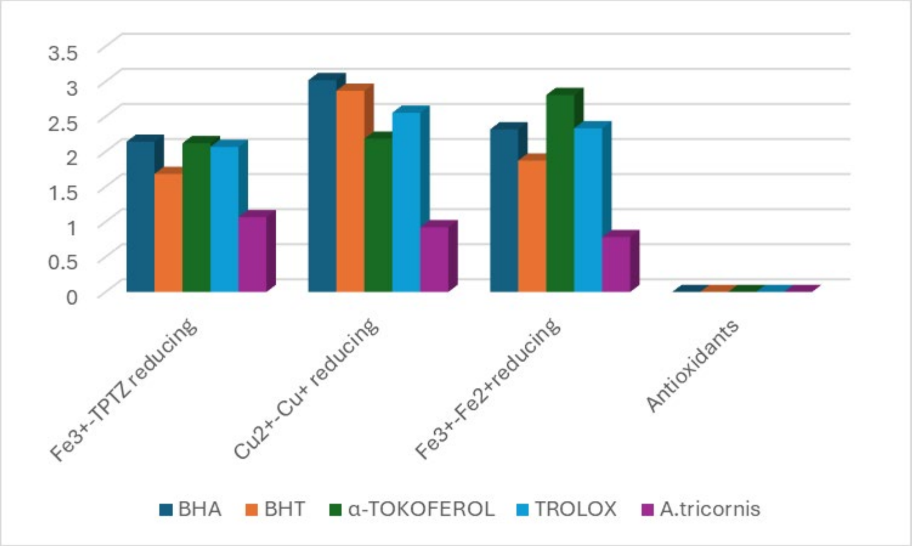


Figure 17: Reducing the power of same concentration by FRAP methods, ferric ions (Fe^{3+}) reducing and cupric ions (Cu^{2+}) reducing capacity by Cuprac method.

5.4. Enzyme Inhibition Findings

In the results of enzyme inhibition, *A. tricornis* exhibited a high ability to inhibit enzymes such as AChE, BChE, α-Amylase and CA II which indicates its effectiveness in this regard, and its consumption is expected to be beneficial for patients suffering from epilepsy, glaucoma, Alzheimer's, and diabetes. After measuring the extract's inhibition of the CA II enzyme, it was determined that it has an IC_{50} value of 11.34 (μg/mL) (r2 = 0.9808). While it indicates its effectiveness, Acetazolamide is more potent with an IC_{50} value of 3.15 μg/mL. For the AChE enzyme, the extract recorded an IC_{50} value of 23.01 μg/mL ($r^2 = 0.9955$), whereas the standard Tacrine demonstrated a significantly lower IC_{50} value of 2.25 μg/mL, indicating that Tacrine is much more effective.

Similarly, the extract's IC_{50} value for α-Alz was 32.35 μg/mL ($r^2 = 0.963$), compared to the standard Acarbose's IC_{50} value of 3.21 μg/mL, which also shows that Acarbose is considerably more effective. The findings indicate that, while *A. tricornis* extract has enzyme inhibitory action, it is less effective than standards such as Acetazolamide, Tacrine, and Acarbose. These findings indicate the extract's potential as a natural therapeutic source, emphasizing the need for more study to improve its effectiveness and applicability in medical therapies. Confirming our findings, recent research has reported on the enzyme-inhibitory characteristics of phenolic compounds, adding to the significance of our findings (84).

Table 16. Values of enzymes inhibitory.

Compounds	**IC_{50} (µg/mL)**					
	CA II	**r^2**	**AChE**	**r^2**	**α-Alz**	**r^2**
A. tricornis	11.34	0.9808	23.01	0.9955	32.35	0.9631
Standard (Acetazolamide)	3.15		-		-	
Standard (Takrin)	-		2.25		-	
Standard (Akarboz)	-		-		3.21	

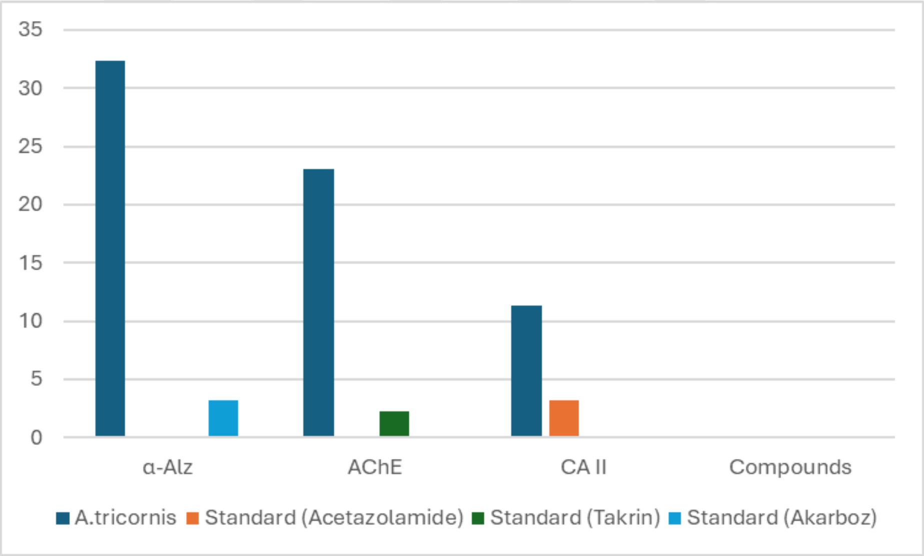


Figure 18: Values of enzymes inhibitory.

6. CONCLUSION

In this thesis study, *A. tricornis* extracts, which do not have any studies on antioxidant activity and enzyme inhibitory activity in the literature, were studied. The biochemical activity and phytochemical content of *A. tricornis* species were elucidated. After evaluation, the results showed that *A. tricornis* had antioxidant activity, and it was determined that *A.tricornis* had significant enzyme inhibitory activity. This research shows that extracts from *A. tricornis* contain significant antioxidants. Considering the key role of antioxidants, such as flavonoids and phenolic acids, in treating oxidative diseases, Current research and development efforts surrounding flavonoids and phenolic acids primarily focus on their identification, extraction techniques, exploring of novel functional properties, and applications for the enhancement of health.

Techniques such as chromatography, spectroscopy, and mass spectrometry are commonly employed to identify and quantify these compounds. In addition, understanding the principle mechanisms and advantages of the assays based on chemical reactions including Reducing Antioxidant Power tests and Radical Scavenging tests is important for the appropriate selection of method(s) for valid evaluation of antioxidant potential in various industries including medical, pharmaceutical, cosmetic, food, and nutraceutical sectors. Flavonoids and phenolic acids hold promise as natural compounds with potential health benefits, but their clinical applications require stringent investigation to overcome existing limitations.

Global opinions from varied continents give invaluable insights into dietary patterns and their associations with health findings. Future research should focus on dealing with challenges in study design and elucidating mechanisms of action to unlock the full vision of flavonoids and phenolic acids in the enhancement of human health. This research provides promising basic data on the therapeutic properties of *A. tricornis*, which warrants the need for further research that may open new prospects for developing new herbal medicines.

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8. RESUME

EDUCATION LEVEL

Name	**Alaa**	**Surname**	**HASAN**
Date of Birth		**Nationality**	
Place of Birth		**Phone**	
E-Posta			

	Name of Institution Graduated from	**Graduation Year**
Bachelor's Degree		
High School		

WORK EXPERIENCE

Position	**Institution**	**Term**
Pharmacist	Pharmacy	2016-2020

Foreign Language Exam Score								
ÜDS/YDS	YÖKDİL	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE

Verbal	**Numerical**	**Equal weight**

9. ORİGİNALİTY REPORT

Alaa Hasan YL Tez

ORİJİNALLİK RAPORU

%20	%11	%16	%5
BENZERLİK ENDEKSİ	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCİ ÖDEVLERİ

BİRİNCİL KAYNAKLAR

1	Aybeniz Yıldırım, Abdulkadir Gül, Nihal Başaran, Ali Şen, Leyla Bitiş, M.Şaban Tanyıldızı. "Anti-inflammatory activities of some Anthemis species used in the treatment of inflammation-related diseases, GC/MS and LC-MS/MS analysis with bioactivity-guided fractionation", South African Journal of Botany, 2024 Yayın	%1
2	repository-tnmgrmu.ac.in İnternet Kaynağı	%1
3	www.mdpi.com İnternet Kaynağı	%1
4	krishikosh.egranth.ac.in İnternet Kaynağı	%1
5	H. John Smith, Claire Simons. "Enzymes and Their Inhibitors - Drug Development", CRC Press, 2019 Yayın	%1
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MASTER THESIS
DİYARBAKIR-2024