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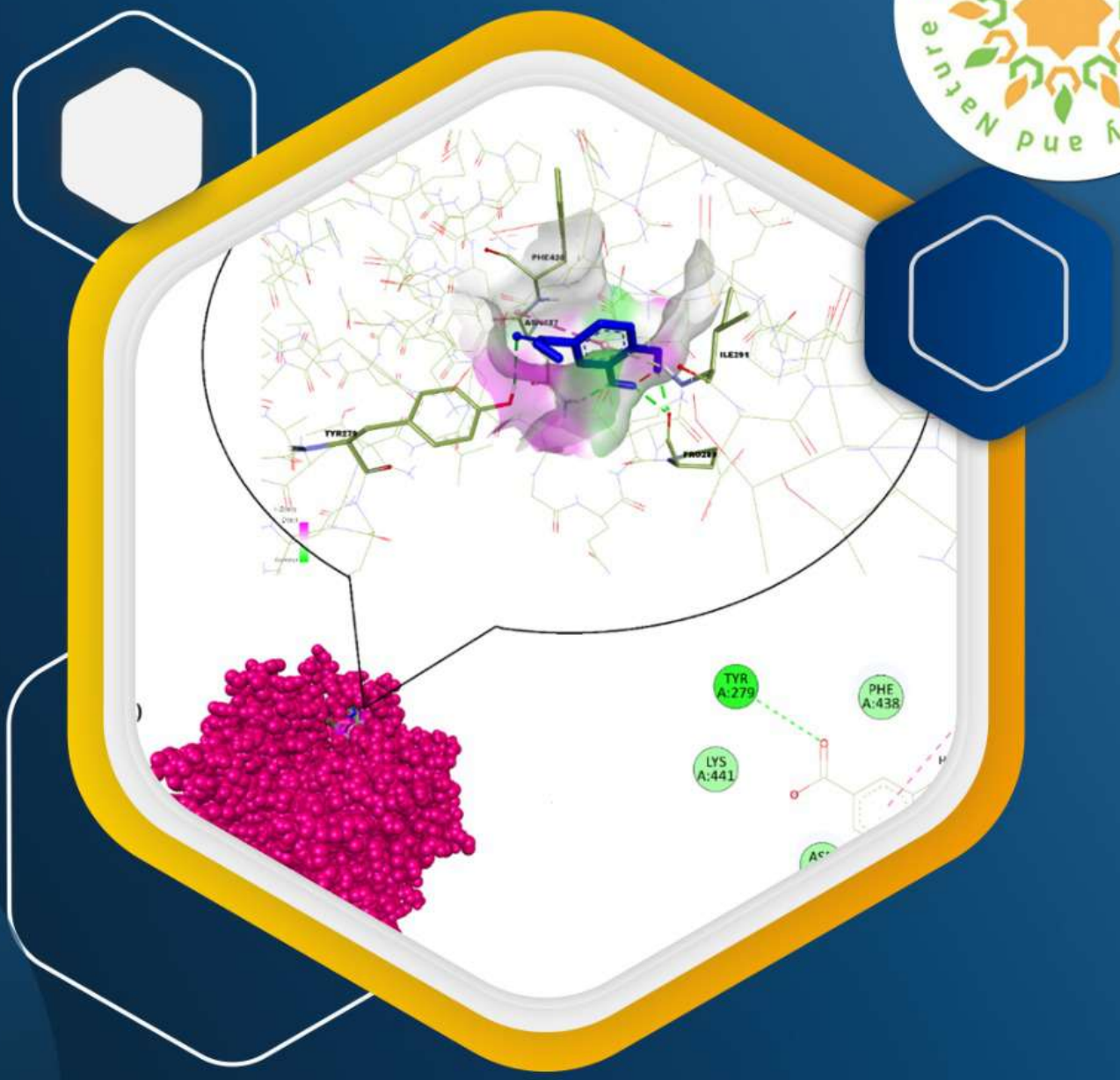


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JAN is the publication of the "Apitherapy and Nature Research Group" established within the (https://avesis.ktu.edu.tr/arastirma-grubu/apiterapivedoga) Karadeniz Technical University.

The Journal of Apitheraphy and Nature accepts English-language original articles, reviews, and letters to the editor concerning various fields of research. Main topics include:

- Apitherapy
- Bee Products (Honey, pollen, propolis, bee bread, royal jelly, bee venom)
- Food Science and Technology
- Chemistry-Biochemistry
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# *Bee Venom and Its Therapeutic Uses*

# *Arı Zehri ve Terapötik Kullanımı*

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## Abstract

The use of honey and other bee products goes back thousands of years. In fact its therapeutic benefits are mentioned in sacred books such as (Veda, the holy book of India), (the Bible, of Christians) and the Noble Quran. Apitherapy is the use of bee products for medical purposes, which including honey, royal jelly, propolis, flower pollen, and especially bee venom, is known as apitoxin. Apitherapy involves the use of various bee products for medical purposes, such as honey, royal jelly, propolis, flower pollen, and primarily bee venom, also known as apitoxin. Bee venom contains of at least 18 pharmacologically active compounds including enzymes such as phospholipases, peptide and amino acid compounds such as melittin, which has anti-inflammatory properties. Other properties such as anti-apoptotic and anti-cancer properties have also been reported for bee venom. Since, as the lethal dose ($LD_{50}$) of the venom for humans is 2.8 mg/kg per kilogram of body weight, it is a safe combination for therapeutic purposes. Bee venom has a great potential in the treatment of inflammatory diseases and the central nervous system diseases such as Parkinson's, Alzheimer's, myotrophic sclerosis and various types of cancer. Also, due to its antiviral activity, it has been effective even against the human immunodeficiency virus (HIV). Due to the prevalence of diseases in today's societies, makes it essential to find new treatment solutions. On the other hand, the drugs used in traditional medicine play an important role in the treatment of diseases. Among these natural substances is bee venom¸ which should be taken into considered in the treatment of diseases because of its many therapeutic properties.

**Keywords:** Bee Venom, Historical Records, Therapeutic Uses, Structure

## Özet

Bal ve diğer arı ürünlerinin kullanımı binlerce yıl öncesine dayanmaktadır. Aslında tedavi edici faydalarından Hindistan'ın kutsal kitabı Veda, Hıristiyanların İncil'i ve Kuran-ı Kerim gibi kutsal kitaplarda bahsedilmektedir. Apiterapi, bal, arı sütü, propolis, çiçek poleni ve özellikle arı zehirini içeren arı ürünlerinin tıbbi amaçlarla kullanılmasıdır ve apitoksin olarak bilinir. Apiterapi, bal, arı sütü, propolis, çiçek poleni ve özellikle apitoksin olarak da bilinen arı zehiri gibi çeşitli arı ürünlerinin tıbbi amaçlarla kullanılmasını içerir. Arı zehiri, fosfolipazlar gibi enzimler, peptid ve anti-enflamatuar özelliklere sahip melittin gibi amino asit bileşikleri de dahil olmak üzere farmakolojik olarak aktif en az 18 bileşik içerir. Arı zehiri için anti-apoptotik ve anti-kanser özellikleri gibi diğer özellikler de bildirilmiştir. Zehrin insanlar için öldürücü dozu ($LD_{50}$) vücut ağırlığının kilogramı başına 2,8 mg/kg olduğundan, tedavi amaçlı güvenli bir kombinasyondur. Arı zehiri, iltihaplı hastalıkların ve Parkinson, Alzheimer, miyotrofik skleroz ve çeşitli kanser türleri gibi merkezi sinir sistemi hastalıklarının tedavisinde büyük bir potansiyele sahiptir. Ayrıca, antiviral aktivitesi nedeniyle, insan immün yetmezlik virüsüne (HIV) karşı bile etkili olmuştur. Günümüz toplumlarında hastalıkların yaygınlığı, yeni tedavi çözümlerinin bulunmasını zorunlu kılmaktadır. Öte yandan, geleneksel tıpta kullanılan ilaçlar hastalıkların tedavisinde önemli bir rol oynamaktadır. Bu doğal maddeler arasında, birçok tedavi edici özelliği nedeniyle hastalıkların tedavisinde dikkate alınması gereken arı zehiri de yer almaktadır.

**Anahtar Kelimeler:** Arı Zehiri, Tarihsel Kayıtlar, Tedavi Amaçlı Kullanımları, Yapısı

## 1. INTRODUCTION

Among the many species of insects, only a-few insects have the ability to defend themselves by stinging and injecting venom when bitten. All insects that can sting belong to the order Hymenoptera, which includes ants and bees. The stinger is always at the end of the abdomen or near it. Each bee is a clear liquid that dries easily even at room temperature, odourless with a bitter taste. It forms greyish-white crystals when exposed to air. Dried venom takes on a pale yellow color, and some commercial products are brown, which is thought to be due to oxidation of some of proteins in the venom. Most venoms are sold as dry crystals (Ali et al., 2012). Bee venom is produced by female worker bees (Trumbeckaite et al., 2015). Bee venom is a natural poison produced by bees and plays an important defensive role for the bee colony. This material has an efficient and complex combination of ingredients designed to protect bees from predators (Lee et al., 2015). Bee venom contains at least 18 medically active compounds. Bee venom is safe for humans treatment, the median lethal dose ($LD_{50}$) for an adult human is 2.8 mg of venom per kilogram of body weight. Assuming that each bee injects all of its venom and that each sting contains 0.3 mg of venom, therefore 560 stings could be fatal for such a person. For a child weighing 10 kg, 93.33 stings can be fatal (Ali et al., 2012).

The idea of using BV in the field of medicine came from the belief that beekeepers hardly suffer from rheumatism or joints (Wehbe et al., 2019). This venom contains active peptides such as melittin, apamin, mast cell degranulation peptide, adolapin and enzymes such as phospholipase A2 and hyaluronidase (Trumbeckaite et al., 2015). As well as non-peptides such as histamine, dopamine and norepinephrine (Lee et al., 2015). Bee venom has been widely used in research to treat some diseases such as rheumatoid arthritis, and multiple sclerosis in traditional Eastern medicine. It is known as a natural anti-inflammatory agent (Ali et al., 2012).

One of the components of bee venom is melittin peptide.The cationic and amphipathic peptide melittin has 58 amino acids, the first 57 amino acids of this peptide are mainly hydrophobic, while the amino acids at the carboxyl end (amino acids 20 to 26) are hydrophilic with a positive electric charge. Treatment with bee products has been widely used in the past. In most countries, bee products are considered traditional medicines. Among complementary and alternative medicine methods, they have been shown to be effective in preventing some common diseases as relatively strong food supplements. Lithuania has very old beekeeping traditions and bee products have been used in folk medicine for centuries. They are used for cough, wound, tuberculosis and other diseases (Trumbeckaite et al., 2015). Interestingly, bee venom, similar to the venom from other animals, has shown a useful anti-viral and anti-cancer potential and has been effective against ovarian and prostate cancer as well as HIV (Wehbe et al., 2019). Studies have shown the ability of BV and its main component, melittin, to induce elevated levels of glucocorticoids, which may be responsible for its anti-inflammatory effects. High levels of GCs have been found after administration of BV (Racheda et al., 2010).

## 2. HISTORY

The roots of Apitherapy date: back to ancient Egypt 6000 years ago. In ancient Greece, bee products were used therapeutically. There is also evidence that honey was a part of traditional Chinese medicinal treatment. A famous ancient manuscript book with fifty-two copies from the 3rd century BC. Found in Changsha, Hunan Province, it contains two manuscripts about bees, one of which uses honey to treat diseases (Trumbeckaite et al., 2015). In the United States, the history of beekeeping (Figure 1) goes back about 100 years, which was described by several prominent physicians from So said Dr. Bodag Beck, who began treating people in his New York City office in the late 1920s. Dr. Beck's book "Bee Venom Treatment" has been used for 60 years. Dr. Beck's last surviving student is Middlebury, Vermont beekeeper Charles Marz, known by many as the "King of Bee Venom Therapy." He has been practicing apitherapy for

over 60 years with remarkable results, and most of his experience has been in the treatment of arthritis, but his success has been with multiple sclerosis (MS) (Ali et al., 2012).

Figure 1. *Apis mellifera*

## 3. MELITTIN

The main component of bee venom is bee venom. It is a fully cationic peptide of 26 amino acids (Figure 2). It is an amphoteric peptide whose terminal carboxyl region is hydrophilic and the terminal amino region is hydrophobic due to the presence of a group of positively charged amino acids. Melittin exhibits amphiphilic properties (hydrophilic and hydrophobic) when interacting with biological membranes or enzymes.

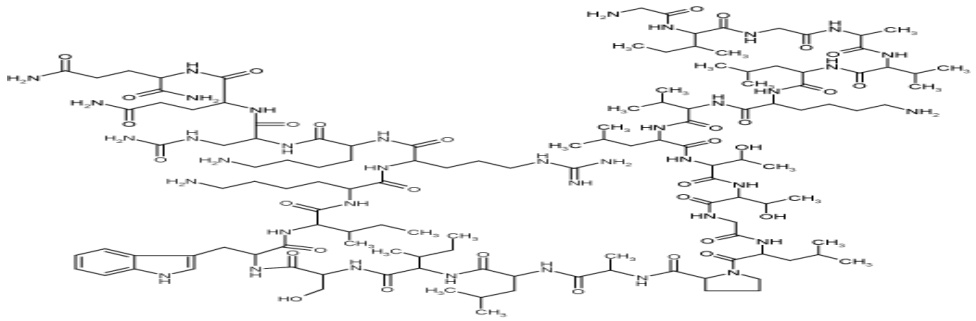

Figure 2. Structure of melittin

Melittin is the major component of bee venom, which makes accounting for approximately 40-50% of the dry powder weight of the venom. It is a small linear peptide with the chemical formula $C_{131}H_{228}N_{38}O_{32}$. Melittin forms a peptide that can penetrate the phospholipid bilayer as four polymers and is therefore able to study the interaction between the

bioactive membrane and the peptide through biological activity. Previous studies have shown that the mechanism of action of melittin to disrupt of membranes by creating pores that act non-specifically on both prokaryotic and eukaryotic cells. Melittin works with an activator called phospholipase A2, which has an increasing effect on PLA2 activity. Melittin can also act as a PLA2 activator. Interest in the medicinal properties of melittin has increased greatly in recent decades. Depending on its concentration, this biopeptide can induce both transient and persistent pores. When a transient pore is formed, only ions from the membrane can diffuse through it.When, if stable pores are formed, the membrane becomes permeable to relatively large molecules such as glucose. The formation of pores caused by melittin is responsible for its haemolytic, antimicrobial, antifungal and antitumour activities. Recently, melittin has been shown to cause smooth changes along pain signalling pathways by activating and sensitising nociceptive cells. It is also a major biologically active ingredient constituent of BV that producing analgesic, anti-inflammatory, and anti-arthritic effects after consumption (Wehbe et al., 2019).

Melittin is a compound that has been studied for a series of biological properties. The anti-inflammatory activity of melittin is mediated by several mechanisms. Basically, this mechanism involves blocking toll-like receptors (TLRs) receptors, CD14, 42) and platelet growth factor beta receptors. In addition, melittin has an inhibitory effect on a nuclear factor (kappa-B) (NF-kB). All these pathways lead to the release of molecules such as inflammatory cytokines, tumour necrosis factor (TNF), nitric oxide (NO) or prostaglandin (E2). (PGE) into the extracellular environment or blood vessels prior to inflammation. All of these molecules have inflammatory effects on tissues. Therefore, mellitin’s ability to prevent the production of these molecules, proves its anti-inflammatory properties (Klocek et al., 2009).

Melittin inhibits the pathways of TLR2, TLR4, CD4, NEMO and PDGFRβ thereby inhibiting the function of pro-inflammatory genes (Figure 3). This process leads to a reduction in the levels of pro-inflammatory molecules and a reduction in inflammation. Recently, a comprehensive review on the subject has been published, which summarising in vitro and in vivo studies and suggesting that one of the main mechanisms of melittin's antiviral activity is its interaction with enveloped viruses (or capsid proteins). Another mechanism by which shows this activity is the interaction demonstrated is that melittin, not only with the surface of the virus but also with the virus itself, which causing the host cells to avoid infection (Memariani et al., 2020). Melittin can inhibiting virus replication by stimulating type I interferon (I -IFN). Therefore, it can be an excellent method for pretreatment (Huang et al., 2012).

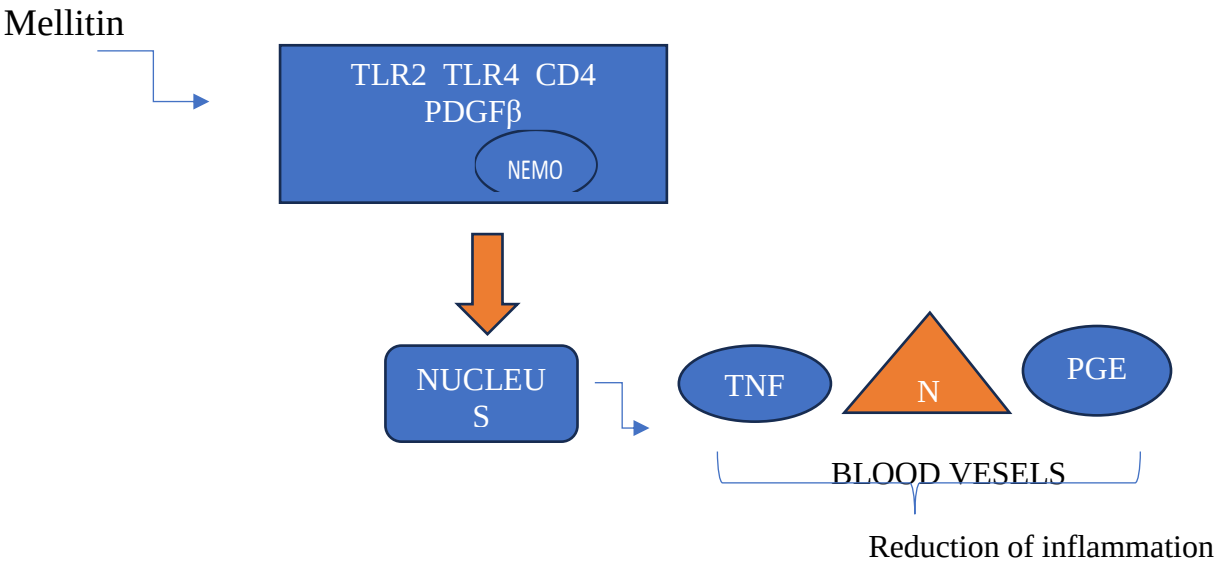


Figure 3. The mechanism of action of the anti-inflammatory effects of melittin

### 3.1. Physiological Properties of Melittin

Melittin is a polypeptide that is at a physiological pH of +6. One of the four positive charges is in the C-terminal region and the rest are two charges in the Lys-7 and N-terminal groups. Although non-polar amino acids cover a large part of the structure of the melittin, this peptide is partially soluble in methanol but very soluble in water. The three-dimensional structure of melittin tetramer by NMR method, with temperature change, shows that melittin has a structural transition between monomer and tetramer in aqueous solution, and this change has much to do with the remaining proline isomers in the melittin structure. Based on these studies, melittin is considered an important candidate for antibiotic-resistant bacteria, cancer and tumour treatment, and pathogenic viruses (Huang et al., 2016). For example, melittin can increase the cell growth of human ovarian cancer cells by increasing the expression of death receptors (DR3, DR4 and DR6) and inactivation of transcriptional signal transducers and activation of the pathway (STAT3) that ends in cell apoptosis (Carpena et al., 2020).

Recent studies have shown that melittin can induce cell cycle arrest, cell growth inhibition and apoptosis in various tumour cells. When multiple melittin peptides accumulate in the cell membrane, phospholipid packing is severely disrupted, leading to cell lysis. Melittin not only lyses a wide range of plasma membranes but also stimulates intracellular membranes such as those found in mitochondria. PLA2 and melittin act synergistically and break the membranes of sensitive cells and increase their cytotoxic effect. However, on article reported

that melittin does not disrupt the cell membrane of leukocytes at concentrations bellow 2 μM (Lee et al., 2005).

## 4. APAMIN

Apamin is an integral part of honey bee venom, accounting for approximately 2-3% of the dry weight (2111.4 daltons). It is a peptide neurotoxin consisting of 18 amino acids, which are tightly linked by the presence of two disulfide bonds (PubChem Apamin 2020). Although there are different models for the structure, studies show that the combination of an alpha helix has high stability at different pH. One of the interesting features of apamin is its permeability across the blood-brain barrier, indicating that apamin can access the central nervous system (CNS) (Palma 2013).

In addition, apamin can inhibit M2 muscarinic receptors in motor nerve endings and reduce muscle nerve transmission (Silva et al., 2010). In addition to its effects on the CNS, apamin is considered an anti-inflammatory agent that can inhibit cyclooxygenase-2 and reduce levels of TNF-, IL-1 (interleukin-1), IL-6 and NO (Shin et al., 2018; Lee et al., 2020). Apamin is known for its pharmacological properties in irreversibly blocking Ca2+-activated K+ channels (Lamy et al., 2010). These channels alter intracellular calcium by increasing K+ flux following an increase in intracellular calcium during an action potential associated membrane potential (Bond et al., 2004). Recent studies have investigated its biological and medicinal activities. However, little is known about the molecular mechanisms and levels of gene regulation involved in the anti-inflammatory process (Lee et al., 2015).

## 5. MAST CELL DEGRANULATING (MCD)

A polypeptide (401 peptide) containing 22 amino acids with a molecular weight of 2587.2 Da. and structurally similar to apamin, as both contain two disulfide bonds. It makes up 2-3% of the dry weight of BV. It also has two disulphide bridges linling aa 3, 15, 5, and 19. At physiological pH, it has a net charge of +8. (https://pubchem.ncbi.nlm.nih.gov 2020) (Ziai et al., 1990) At low concentrations, less than 0.1 mg/ml, MCD causes mast cell degranulation (Carpena et al., 2020). The name MCD reflects the biological action of histamine release from mast cells. an important inhibitor of K+ channels, and can cause a decrease in blood pressure in mice. (Hanson et al., 1974) Studies present MCD as a potent anti-inflammatory agent and may be a potential candidate for studying the mechanisms of inflammatory cells. act like mast

cells, basophils and leukocytes, which will lead to the design of compounds with therapeutic applications (https://pubchem.ncbi.nlm. nih.go, 2020)

## 6. ADOLAPIN

Adolapin is a polypeptide of 103 amino acids. This represent to 1% of the dry weight of BV. Researchers have shown that adolapin has anti-inflammatory, analgesic, and antipyretic effects by blocking prostaglandin synthesis and inhibiting cyclooxygenase activity (Park et al., 2011).

## 7. PHOSPHOLIPASE A2

PLA2, the most lethal enzyme and usually the major allergen in BV, consists of a single polypeptide chain of 128 amino acids containing four disulfide bridges. It is shown that 12-15% of the dry weight (Fenard et al., 2001) is BV (15-18 kDa). And to maintain structural stability, this enzyme has five disulfide bonds between amino acids 30-70, 31-9, 37-63, 61-95 and 113-105. There is a wide variety of PLA2 in nature, and these enzymes are classified into 16 groups. In particular, bee-derived PLA2 (bPLA2) belongs to group III (Jung et al., 2018).

This substance is very alkaline. It is interesting to note that its activity can be improved with melittin. A synergistic effect between bvPLA2 and melittin which occurs during the erythrocyte lysis process has been demonstrated and proves its existence. New experimental data have also shown that bvPLA2 elicits protective immune responses against a wide range of diseases such including asthma, Alzheimer's disease, and Parkinson's disease (Wehbe et al., 2019). It has also shown high cytotoxic activity against cancer cells with membrane disruption. Membrane disruption also confers antimicrobial activity to bPLA2 (Carpena et al., 2020) In addition, bPLA2 can act as a ligand for specific receptors. Thus, bPLA2 can bind to specific membrane receptors and generate cellular signals independent of their enzymatic activity. Two types of receptors have been identified for bPLA2: Type M and type N. (24) M Type receptors are found in skeletal muscle cells. N-type receptors are associated with the neurotoxic activity of bPLA2 (Hong et al., 2019)

## 8. HYLURONIDASE

Hyaluronidase makes up 1.5 to 2% of the dry weight of BV (Wehbe et al., 2019). It has 350 amino acids and one disulphide bridge (Carpena et al., 2020) It is known to break down

hyaluronic acid in tissues. Hyaluronidase allows the active components of BV to work in the victim's tissues by creating structural integrity and increasing blood flow to the effective area (Wehbe et al., 2019).

## 9. THERAPUTIC USES OF BEE VENOM

### 9.1. Anti-inflammatory

Inflammation is the body's protective response to harmful stimuli. Chronic inflammation can lead to the development of several diseases such as rheumatoid arthritis (RA), diabetes, cardiovascular diseases, obesity, asthma, skin disease and CNS-related diseases such as Parkinson's and Alzheimer's (Rim Wehbe et al., 2019). There are at least four major BV compounds that have anti-inflammatory properties (Lee et al., 2016).

Melittin, when administered in high doses, causes local pain, itching and inflammation. However, low doses of this BV compound can have broad anti-inflammatory effects. Many reports have investigated the anti-inflammatory mechanisms of melittin in various diseases such as rheumatoid arthritis (RA) and amyotrophic lateral sclerosis (ALS). In fact, it works by inhibiting inflammatory cytokines such as interleukin-6 (IL-6), IL-8, tumour necrosis factor- (TNF-) and interferon (IFN). The NF-κB pathway through a group of transcription factors plays a vital role in host immune and inflammatory response activities. In vitro, melittin can suppress nuclear NF-κB activation. Its anti-inflammatory effect is mediated by the reduction of IgE levels, and the release of cytokines and NF-kB (Carpena et al., 2020).

These studies showed that by blocking their primary signalling pathways, melittin inhibits inflammatory cytokines, which then leads to a reduction in inflammation in the skin, liver, joints and nervous tissue. In skin disease, a recent finding by Kim et al. showed that BV reduced atopic dermatitis, the most common chronic inflammatory allergic skin disease (Rim Wehbe et al., 2019)

### 9.2. Treatment of nervous disease

Parkinson's disease is a degenerative movement disorder that causes progressive disability in patients. The pathological hallmark of this disease is the progressive loss of dopaminergic neurons in the substantia nigra (the basal ganglia structure in the human brain) (Goldman et al., 2014; Aarsland et al., 2017). Abnormal microglial activation is also a pathological hallmark in several neurodegenerative diseases including PD (Iakovakis et al., 2018). Most clinical studies

show the effect of BV on leukocyte migration or microglial activation in animal and cellular models. Other studies have investigated the neuroprotective potential of BV acupuncture. Treatment with BV against rotenone-induced oxidative stress shows neuroinflammation and apoptosis in PD mouse models. Rotenone is a pesticide that may affect the pathophysiological mechanisms involved in PD (Aksoz et al., 2019). Interestingly, BV demonstrated its ability to prevent dopamine depletion after rotenone administration. Furthermore, locomotor activity was restored after treatment of PD with BV in a mouse model. The treatment effectively suppressed DNA damage and inhibited the expression of apoptotic genes Bax, Bcl-2 and caspase-3 in the brain of PD mice. These results show that BV normalises all markers of apoptosis and neuroinflammation after rotenone injury and restored brain neurochemistry (Khalil et al., 2015). BV has also been shown to protect against dopaminergic neuron degeneration in PD models (Wehbe et al., 2019).

Alzheimer's disease is the most common neurodegenerative disease and many pathological processes are involved in its development (Aksoz et al., 2019). Although the cause of AD remains unknown, evidence suggests that inflammatory responses may play an important role in its pathogenesis (Eldik et al., 2016; Kinney et al., 2018). Current treatments for cognitive decline in Alzheimer’s disease rely on the use of muscarinic or nicotinic receptor ligands and acetylcholinesterase (AChE) inhibitors (Terry et al., 2003). As an alternative strategy, Ye et al. (2016) showed that bvPLA2 could be used as a therapy to prevent the progression of AD in transgenic mice. The same study also shows that bvPLA2 can increase brain glucose metabolism and reduce neuroinflammatory responses in the hippocampus, thereby limiting the pathogenesis of AD (Ye et al., 2016). Amyotrophic Lateral Sclerosis (ALS) is a CNS disease that causes the death of motor neurons (Rajagopalan et al., 2019). Interestingly, BV has shown a special potential to deal with this disease (Jaarsma et al., 2000).

### 9.3. Use of bee venom in cancer

The use of apitoxin, especially its major component melittin, as a new strategy for cancer treatment has recently gained great importance (Junget al., 2018; Lim et al., 2019). Indeed, melittin is known to be a non-specific cytolytic peptide that can attack the lipid bilayer, thus resulting in significant toxicity when administered intravenously (Hong et al., 2019). However, many optimization approaches, including the use of melittin nonparticle-based delivery, have been exploited. It is noteworthy that raw BV as well as anti-tumour melittin have shown activity

against various types of cancer cells including breast, liver, leukaemia, lung, melanoma and prostate cancer cells (Liu et al., 2002; Jung et al., 2018; Hong et al., 2019). Park et al. (2011) also reported that BV and its major component, melittin, inhibited cancer cell growth both in vitro and in vivo through activation of caspase 3 and 9 pathways and inhibition of NF-κB signalling and anti-proliferative gene products. Apoptosis such as Bcl-2, cIAP-2, iNOS, COX-2 and cPLA2 (Park et al., 2011). Similarly, Zheng et al. (2019) showed that BV has an anti-proliferative effect and induces apoptosis through the activation of death receptors. Another interesting finding about melittin came from by highlighting its anti-growth and anti-metastatic properties (Figure 4). In cancer, metastasis and malignant cell attack are the main causes of disease progression (Wehbe et al., 2019). Therefore, cancer researchers have focused on understanding the molecular mechanisms that regulate malignant cell migration and possible ways to prevent it, as an important step in the fight against cancer (Rajabi et al., 2017; Zuazo-Gaztelu et al., 2018). In another study, results showed that bee venom can be used as a selective DNA(de)methylatorr in cancer. And suggest the use of bee venom or any component for epigenetic therapy in cancer cells (Uzuner et al., 2021).

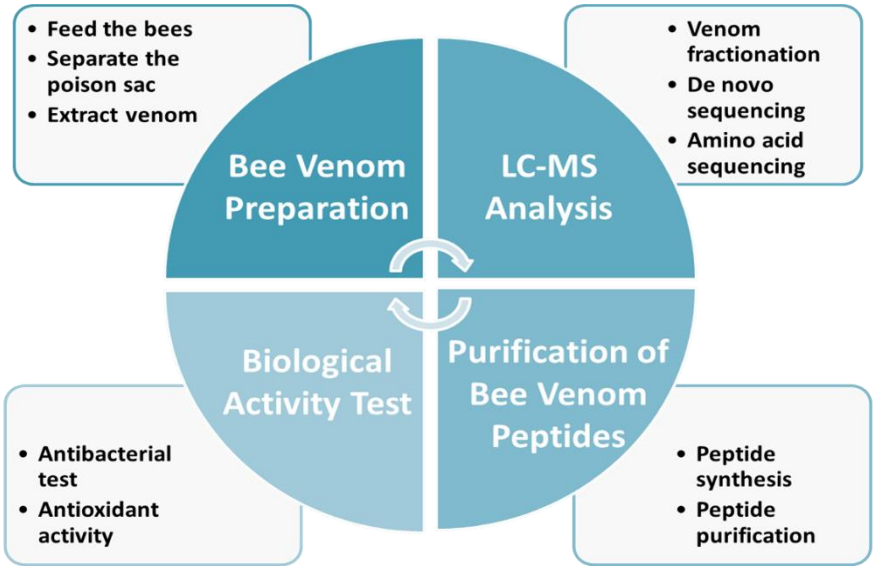


Figure 3. Schematic drawing of the main purification of action of Bee venom as an anti-bacterial agent

## 9.4. Antibacterial and antiviral properties

It is well known that BV with its two main components (melitin and PLA2) has antimicrobial activity and therefore can be used as an antibacterial supplement. These compounds act their

effects against bacteria by creating pores in their membranes, which leading to their splitting and then lysis (Park et al., 2004). BV components have antibacterial activity against gram + and gram bacteria and It is antifungal (Carpena et al., 2020). However, the antiviral effect of BV has not been reported much. A recent study investigated the antiviral potential of BV and yielded interesting results both in vivo and in vitro. This study showed that BV and melittin have significant antiviral effects against enveloped viruses (vesicular stomatitis virus, influenza A virus, herpes simplex virus, etc.) and non-enveloped viruses (enterovirus-71 and coxsackievirus) in There are many laboratory conditions (Uddin 2016).

The study also showed that melittin protected mice were exposed to lethal doses of H1N1 influenza A virus. Although the exact mechanism of action of BV and melittin as antiviral agents is unclear, it has been confirmed that BV directly interacts with the viral surface. In addition, BV and its components can stimulate type I interferon (IFN), thereby suppressing viral replication in the host cell (Bachis et al., 2010).

In addition, researchers at the Washington University School of Medicine in St. Louis reported the potential use of melittin-loaded nanoparticles to destroy the human immunodeficiency virus while leaving uninfected cells unharmed. It also suggests a preventive strategy in which these nanoparticles are used to make a vaginal gel that prevents the spread of HIV. The principle of its theory is as follows: the melittin molecules in the nanoparticles combine with the viral coating and form attack complexes and pores, thus breaking the virus (Hood et al., 2013). Another study showed that bvPLA2 can also prevent viral replication. The same team identified the peptide sequence of bvPLA2, which is responsible for inhibiting HIV replication. (Fenard et al., 2001)

### 9.5. Anti arthritis

Bee venom (BV) has been used as a traditional alternative medicine for pain relief and treatment of inflammatory diseases, such as rheumatoid arthritis (RA) in humans (Lee et al., 2015). RA is one of the most common inflammatory pathologies, the prevalence of which is between 0.2-0.9% (Carpena et al., 2020). Several studies have shown that BV treatment for RA in humans and experimental animals has an anti-inflammatory effect (Park et al., 2004). Bee venom contains several active pharmaceutical ingredients that can be effective in the treatment of arthritis. Regulation of radical production, suppression of gene induction of alpha-1 acid

glycoprotein, and inhibition of phospholipase A2 (PLA2) activity have all been suggested as effects of its possible anti-inflammatory mechanisms. Like snake venom, PLs are the main active components of BV (Lee et al., 2005).

These chemical mediators are normally released from phagocytic lysosomes during inflammation and cleave phospholipids from the cell membrane to produce arachidonic acid, which is ultimately converted to prostaglandins (PGs) (Zurier et al., 1973). Additionally, PLA2 has been shown to be an inhibitor to prevent acute and chronic inflammation (Garcia-Pastor et al., 1999) as it has been shown that PGs have a suppressive and preventive effect against arthritis induced by adjuvants in rats (Zurier and Quagliata, 1971). Therefore, the injection of bee venom in rats with arthritis may have the same therapeutic effect as PGs or anti-inflammatory drugs. In experimental animals, adjuvant-induced arthritis has been shown to be suppressed by long-term treatment of BV and/or or its compounds are also reported to be effective in the treatment of RA in humans) (Eiseman 1982; Hadjipetrou-Kourounakis 1984) Recently, it has been shown that BV produces anti-inflammatory effects in an arthritis model induced by complete Freund's adjuvant (CFA) (Kang et al., 2002). Due to the increase in the prevalence of side effects of the pharmaceutical approach to inflammatory diseases, there is an urgent need for better treatment to reduce the symptoms of these disorders. Overall, treatment using bee venom and its main components is considered a useful clinical approach for the treatments of inflammatory diseases. As bee venom contains a number of other components, advances in modern sequencing techniques offer new opportunities to combat other inflammation-related diseases (Lee et al., 2015).

### 9.6. Anti oxidant properties

BV contains components with antioxidant activity. This activity is usually related to the concentration of melittin, PLA2 and apamin. These effects may be due to by the ability of these compounds to inhibit the process of lipid peroxidation and increase the activity of superoxidase dismutase. Also, the increase of GST and GSH has been shown in treated rabbits (Carpena et al., 2020).

## 10. PRODUCTS

There are no known uses for the poison other than medical ones. Since the early 1980s, pure bee venom has been used for desensitizing (Bee Well, 1993). In Eastern Europe and in many Asian countries, bee venom has been used in the official treatment of various diseases for a considerable period of time. The methods of using venom include natural bee stings, subcutaneous injections, ointments, inhalations, and pills (Sharma and Singh 1980). Depending on the patient being treated, bee venom can be used as a cream, ointment or injection form. For injection, the venom can be mixed with injectable liquids such as distilled (sterile) water, saline solutions, and special oils at the time of injection, or it may be taken from ready-made ampoules. There are creams available that contain bee venom (such as Furapin and Apicosan in Germany, Apion in France and Eminin in Austria) for external application to arthritic joints. Entering this limited market requires a highly advanced laboratory and highly trained technicians and chemists (Krell 1996).

## 11. BEE VENOM SAFETY

Compared to other human diseases, accidents and other unusual cases, bee venom is very safe for human treatment (Rose 1994). Statistics for Deaths from Diseases, Accidents, and Other Unusual Causes in the United States in 1986 Of the 2,086,440 deaths in the United States in 1986, 977,700 died from heart disease. (46.86%) of all deaths. Total number of cancer deaths 641,400 (30.74%), smoking 150,000 (7.19%), asthma 3,880 (0.186%), penicillin allergy 300 (0.014%), insect stings (except bees) 24 (0.0012%), has been While there were 17 cases by honey bees. Although bee venom is safe for human treatment, it should only be used under the supervision of a qualified health care professional (Ali 2012).

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## DECLARATIONS

No conflict of interest or common interest has been declared by the authors.

## AUTHOR CONTRUBITIONS

The first draft of the manuscript was written by Haydeh Keyhan and all authors commented on previous versions of the manuscript, read and approved the article.

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# *Investigation of The Inhibition of SARS-CoV-2 Spike RBD and ACE-2 Interaction by Phenolics of Propolis Extracts*

# *SARS-CoV-2 Spike RBD ve ACE-2 Etkileşiminin Propolis Ekstraktındaki Fenoliklerle İnhibisyonunun Araştırılması*

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## Abstract

The molecules that consist of propolis are generally polyphenols, and they have many activities such as antiviral, antibacterial and antifungal activities. In this study, it is aimed to investigate the inhibiting capacity of the interaction between ACE-2 and Spike RBD by propolis samples belonging to three different cities (Trabzon, Kocaeli, Kırıkkkale). After determining the propolis sample exhibiting the highest inhibition effect (Kocaeli-1 propolis), the phenolics within aqueous and ethanolic extracts of propolis sample were identified by RP-HPLC-UV and radical scavenging activities, antioxidant capacities, total flavonoids (TFC), phenolic contents (TPC) were determined. Then, individual assessments of the inhibition effects of each phenolic compound were conducted with Spike S1 (SARS-CoV-2): ACE-2 Inhibitor Screening Colorimetric Assay Kit and supported by in silico docking studies. The substances with the greatest inhibitory effect are; protocathecuic acid, caffeic acid, and *p*-coumaric acid with the inhibition of 62.29%, 58.34%, and 59.20%, respectively. The lowest $IC_{50}$ value of the flavonoids was found to be 0.89 mM with caffeic acid. In silico, in vitro experiments, and MTT analyses conducted have demonstrated that caffeic acid and protocatechuic acid can be utilized as highly active compounds against COVID-19.

**Keywords:** Inhibition, Propolis, Protocatechuic acid, SARS-CoV-2

**Özet**

Propolisi oluşturan moleküller genel olarak polifenollerdir ve antiviral, antibakteriyel ve antifungal aktivite gibi birçok aktiviteye sahiptirler. Bu çalışmada, Trabzon, Kocaeli ve Kırıkkale şehirlerine ait propolis örneklerinin ACE-2 ve Spike RBD etkileşimini inhibe etme kapasitesi araştırıldı. Kocaeli-1 propolisinin en yüksek inhibisyon etkisini gösterdiği belirlendikten sonra, propolis örneğinin sulu ve etanolik ekstraktlarındaki fenolik bileşikler RP-HPLC-UV ile tanımlandı ve DPPH radikal temizleme aktiviteleri, antioksidan kapasiteleri, toplam flavonoid (TFC) ve fenolik madde içerikleri (TPC) belirlendi. Daha sonra, her bir fenolik bileşiğin Spike S1 (SARS-CoV-2): ACE-2 İnhibitör Tarama Kolorimetrik Test Kiti ile inhibisyon etkileri bireysel olarak değerlendirildi ve in siliko doklama çalışmalarıyla desteklendi. İnhibitör etkisi en fazla olan maddelerin; protokatekuik asit, kafeik asit, p-kumarik asit olduğu ve bu maddelerin sırasıyla, %62,29, %58,34, %59,20 oranında inhibisyon etkisi gösterdiği belirlendi. Test edilen flavonoidlerden en düşük $IC_{50}$ değerine, kafeik asitin (0,89 mM) sahip olduğu belirlendi. Yapılan in siliko, in vitro deneyler ve literatürdeki MTT analizleri, kafeik asit ve protokatekuik asidin, COVID-19'a karşı oldukça aktif bir bileşik olarak kullanılabileceğini göstermektedir.

**Anahtar Kelimeler:** İnhibisyon, propolis, protokatekuik asit, SARS-CoV-2

**Abbreviations:** ACE-2, Angiotensin Converting Enzyme 2; Spike RBD, Spike receptor-binding domain; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide)

## 1. INTRODUCTION

Propolis (bee gum) is the resinous substance that honey bees collect from plant buds and shoots, transform it with some enzymes they secrete, and store in the hives. Although propolis varies in structure and composition from region to region and according to flora, it consists of approximately 50-60% resin, 10% wax (wax), and 30-40% balsam. Bees use propolis to protect their hive (colony) as both a physical and chemical defense tool. Propolis is a bee product that has been known and used by humans for a very long time (Kuropatrichi et al., 2013; Lotfy, 2006). Propolis has antibacterial, anti-inflammatory, antiviral (Sforcin et al., 2017), antioxidant, antiprotozoal, anesthetic, antitumoral, anti-cancer, antifungal (Rajpara et al., 2009; Sforcin, 2016), antiseptic, anti-mutagenic properties. It is also used as an anti-hepatotoxic (Toreti et al., 2013). Propolis molecules predominantly comprise polyphenols, exhibiting diverse biological activities including antiviral, antibacterial, and antifungal properties. The antiviral effects of these different polyphenols have been determined on various viruses, such as Coronaviruses, Herpes Simplex viruses, Influenza, Rotaviruses, and Human Immunodeficiency Virus (HIV) (Yıldırım et al., 2019).

Coronaviruses are viruses that were first discovered in the 1960s and are found in birds and mammals, especially bats, cats, camels, and mice (Woo et al., 2012). Coronaviruses are a large family of enveloped, positive-sense, single-stranded RNA viruses with a 5' cap and a 3' polyadenyl tail (Lai & Cavanagh, 1997). The virus that causes COVID-19, has a single-stranded positive-sense RNA genome of approximately 30 kb, which is 74% similar to pangolin (*Manis javanica*) coronaviruses and horseshoe bat (*Rhinolophus sinicus*) coronaviruses (Bat-CoV-RaTG13*)* is 99% (Zhu et al., 2020). It is known that the coronavirus obtained from Malayan pangolins is 99% similar to SARS-CoV-2. There is a single amino acid difference between the Receptor Binding Domain (RBD) of the spike protein of Malayan pangolin coronaviruses and the RBD of SARS-CoV-2. Malayan pangolins infected with this virus also show similar effects to COVID-19 symptoms. Antibodies obtained from infected Malayan pangolins can react with the spike protein of SARS-CoV-2. Although RaTG13 coronaviruses isolated from bats are 96% similar to SARS-CoV-2, the RBDs of two spike proteins are differ from each other, and the binding affinity of the RBD of RaTG13 to the human Angiotensin Converting Enzyme 2 (ACE-2) receptor is low. Six critical amino acids in the receptor binding domain of SARS-CoV-2 and pangolin COV are identical. Considering all these situations, it is suggested that SARS-CoV-2 emerged as a result of the recombination of pangolin-COV and bat-COV-RaTG13 virus. Therefore, the intermediary host between humans and bats is thought to be the pangolin (Liu et al., 2020; Andersen, 2020). The absence of effective prophylactic or therapeutic agent options against viral infections remains a significant issue.

Coronaviruses have four different structural proteins whose functions are fully known and these are spike (S), envelope (E), membrane (M), and nucleocapsid (N). Additionally, several structural proteins are expressed in the viral genome whose exact function is unknown (Lai & Cavanagh, 1997). Among these proteins, the S protein is of great importance for adhesion, fusion, and entry of the virus into the cell, and thanks to these properties, it is seen as an important target for the development of antibodies, entry inhibitors, and vaccines. The spike protein, which protrudes from the virion's envelope, plays an important role in the host receptor selectivity and adhesion to cells. In most coronaviruses, the S protein is cleaved by host proteases into two functional subunits (S1 and S2) of approximately the same size. The N-terminal S1 domain forms the globular head of the S protein and this is where the receptor binding domain (RBD) is located. On the other hand, the S2 domain forms the stem (body) of the S protein, which contains the fusion peptide with two heptad repeat regions, the TM region and the cytosolic tail (Fung & Liu, 2018). During adhesion and entry into the cell, the cellular

protease TMPRSS2 cleaves the S1 and S2 domains to separate them. While the attachment of the virus to the host cell is ensured by the receptor binding domain of the S1 subunit of the nascent S protein, the fusion of the virus and host cell membranes is ensured by the S2 subunit. There is strong scientific evidence that SARS-CoV and SARS-CoV-2 interact with ACE-2 as a receptor. In addition, cellular receptors such as the C-type lectin CD209L and DC-S16S, which are effective in the attachment of SARS-CoV viruses to the cell, play secondary roles (Ortega et al., 2020). The interaction between the viral protein and its receptors on cellular membrane is constitutes a critical step in the replication cycle of the virus. Therefore, the efficiency of viral infection is tightly dependent on this process. Many physicochemical factors are associated with protein-protein interactions. These factors are determined by the nature of the amino acids in the proteins that will interact and the type of the chemical interactions that occur between the ligand and the receptor. RNA viruses that infect cells produce more RNA using host cells, and they use it both to protect their own RNA and to produce proteins to infect new cells. These proteins are the main targets for candidate vaccines and drugs to be developed to prevent COVID-19, and these targets include the spike protein of the virus, the main viral proteases that are specific for degrading the polyprotein of the virus (3-chymotrypsin-like protease (3CLpro), main protease papain-like protease) and the RNA-dependent RNA polymerase of the virus are the leading ones The RBDs of SARS-CoV and MERS-CoV viruses recognize different receptors on host cell surfaces. While SARS-CoV recognizes ACE-2 as a receptor, MERS-CoV recognizes dipeptidyl peptidase 4 (DPP4) as a receptor (Taia et al., 2023). Similar to SARS-CoV, the receptor of the S protein in SARS-CoV-2 is ACE-2. Therefore, the RBD of the S protein of SARS-CoV-2 has been identified as the most likely target for the development of virus binding inhibitors, neutralizing antibodies, and vaccines.

ACE-2, an integral membrane protein, is a protein containing HEXXH-E, a conserved zinc binding motif, consisting of 805 amino acids. ACE-2 is a type I transmembrane metallopeptidase with homology to ACE, an important component of the rennin-angiotensin system (RAS) and a target in the treatment of the hypertension (Riordan, 2003). It is mainly expressed in vascular endothelial cells, renal tubular endothelium, and testicular Leydig Cells (Kuba et al., 2010; Jinag et al., 2014). PCR analyses have shown that ACE-2 is also expressed in lung, kidney, and gastrointestinal tract tissues infected with SARS-CoV (Ksiazek et al., 2003; Harmer et al., 2002). The main substrate of ACE-2 is Angiotensin II (Tikellis and Thomas, 2012), and it breaks down this vasoconstrictor substrate to form Angiotensin 1-7 and thus negatively regulates the RAS system (Kuba et al., 2010; Tikellis & Thomas, 2012) and thus

lowers blood pressure through this hydrolysis. ACE-2 has also been shown to exert a protective function in the cardiovascular system and other organs (Kuba et al., 2010). In this way, it has become a promising drug target for the treatment of cardiovascular diseases.

As stated above, the importance of ACE-2 in terms of SARS-CoV-2 infections is that the Spike protein, with which the virus interacts with the cell in order to infect the cell, attaches to the cell via ACE-2. Considering this situation, this receptor has become one of the main targets of therapeutics to be developed against viral infections. Many studies have recently been carried out on which molecules can eliminate the interactions between the cellular ACE-2 receptor and the viral Spike protein in the development of effective therapeutics. For this purpose, mostly structural biology studies are carried out, and the results of the research conducted in the light of these studies are aimed to create an infrastructure for future studies and to guide scientists in the fight against the virus. Target molecules are tried to be determined through in silico experimental docking studies. For this purpose, it is important to screen natural resources that are thought to contain target molecules with inhibitory effects on the interactions between the cellular ACE-2 receptor and the viral Spike protein and to examine them for the desired activity.

Considering these situations, many researchers are investigating various ways of using propolis against this virus, taking into account the antiviral activity of propolis. Generally, considering the time when COVID-19 infections occur, the first steps in developing effective drugs are experimental molecular modeling studies (molecular docking) and many researchers are working in this field. Some of these studies target the RNA-dependent RNA polymerase of the virus, some target the main protease of the virus, and some aim to stop the interactions between the Spike protein of the virus and its cellular receptor, ACE-2.

In this study, inhibition studies were carried out with propolis extracts obtained from three different locations of Turkey (Trabzon, Kocaeli, and Kırklareli) and were examined in terms of inhibiting the interactions between ACE-2 / Spike protein RBD. Total flavonoid content (TFC), total phenolic content (TPC) and DPPH• radical scavenging activities of the best inhibitory extract (Kocaeli-1 propolis) were determined. Then, the phenolic content of this propolis sample was determined by RP-HPLC-UV. The phenolic substances found in high amounts in Kocaeli-1 propolis extracts were examined in terms of inhibiting the interactions between ACE-2 / Spike protein RBD. Following the in vitro study, a detailed docking study was carried out to demonstrate the interactions of the molecules deemed effective with both

molecules (ACE-2, Spike RBD) separately and interactively (ACE-2 / Spike RBD). With this study, the phenolic content of Kocaeli-1 propolis and the inhibition capacities of the phenolic compounds in its content were determined.

## 2. MATERIALS and METHODS

### 2.1. Chemicals and Kits

COVID-19 Spike Protein: The ACE-2 ELISA kit (Cat. No. 79954) was purchased from BPS Bioscience (San Diego, CA, USA). The chemicals used in the study were gallic acid, protocatechic acid, p-OH benzoic acid, catechin, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, routine, myricetin, resveratrol, daidzein, luteolin, t-cinnamic acid, hesperetin, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE), $FeSO_4.7H_2O$, Folin–Ciocalteu's phenol, diethyl ether, ethyl acetate, and acetonitrile were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie, Munich, Germany). Daidzein was obtained from Cayman Chemical (Michigan, USA) and ferric tripyridyltriazine (Fe-III-TPTZ), $FeCl_3$, $CH_3CO_2Na.3H_2O$, acetonitrile were obtained from Merck (Merck, Darmstadt, Germany).

### 2.2. Preparation of Propolis Extracts

Preparation of aqueous extract from propolis was carried out according to the method specified in application number TPE, 2015/04984. In summary, the method consists of four steps: dewaxing process, the extraction process, filtration, and evaporation process.

In the dewaxing process, propolis ground to 1-10 mm in size or collected from propolis traps in the hives was washed with water not exceeding 30°C. Propolis was laid on the sieve whose hole diameter was smaller than the propolis grinding diameter, with a thickness not exceeding 5 mm. The sieve was rolled up. The roll was placed in a container larger than its diameter and filled with pure water or drinking water until it passed the level of the roll. The temperature of the water was kept between 62-65°C. This process was done externally with a thermostat heater or in a temperature-controlled container. The process continued for no more than 5 hours. At the end of the period, the mixture was cooled. Wax and other resins were observed to collect on the surface of the water, and the resulting wax and other resins were removed from the environment. After the removal of wax and resin, the stages carried out in

the process, which we can generalize as the extraction process; The roll was opened, and the waxed propolis was poured into the same water. The temperature of the water was adjusted to 40-45°C, and it was rotated and extracted at this temperature. During this process, the mixture was acidified with any organic acid (citric acid, malic acid, tartaric acid, lactic acid, etc.) and the mixture was rotated in an acidic environment for at least 30 minutes. During this process, the phenolic compounds dissolved in the acidic environment passed into the solution. At the end of this period, the mixture was alkalized with bases (carbonates) and rotated in an alkaline environment for at least 30 minutes. In this process, those dissolved in the alkaline environment went into solution. By reusing the organic acid, the mixture was brought to the previous pH value. The mixture was filtered in the steps of the filtration and evaporation process that took place after the extraction process. In the preparation of ethanolic and water-based glycerol extracts (aqueous), frozen, ground propolis was added to 70-75% ethanol and glycerol, not exceeding 20%. It was shaken in the dark for 24 hours. It was kept in the refrigerator in the dark for 2 days and then filtered. Propolis samples obtained from three different cities (Trabzon, Kocaeli, and Kırklareli) were extracted by this method.

### 2.3. Determination of Total Flavonoids Content (TFC), Total Phenolic Content (TPC) and DPPH• Radical Scavenging Activity

The total flavonoid content (TFC) within aqueous and ethanolic extracts of Kocaeli-1 propolis were performed in accordance with the methodology established by Fukumoto & Mazza (2000). Quercetin was used for the standard calibration curve. The total flavonoid concentration was measured and expressed as mg of quercetin equivalents per g of the sample.

Phenolic substance quantification was conducted using the Folin-Ciocalteu method, which involves a redox reaction where phenolic compounds reduce the Folin-Ciocalteu reagent, converting into their oxidized state. A gallic acid standard was utilized, following the methodology outlined by Singleton & Rossi (1999). The total phenolic content of Kocaeli propolis extracts were calculated with the absorbance values corresponding to the concentration and expressed as mg of gallic acid equivalents per g of the sample.

Radical scavenging activity was assessed based on the reduction in maximum absorbance of the purple-violet commercial DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical at 517 nm in the presence of the antioxidant material. Concentrations corresponding to the observed absorbances were plotted to calculate $SC_{50}$ values (Cuendet et al., 1997).

### 2.4. Determination of Phenolic Compounds by RP-HPLC-UV

RP-HPLC-UV analysis was conducted using an HPLC system (Elite LaChrom Hitachi) equipped with a UV-VIS detector, operating at a wavelength range of 280-315 nm. The analysis utilized a reverse-phase C18 column (150 mm x 4.6 mm, 5 µm particle size; Fortis), employing a gradient program consisting of acetonitrile, water, and acetic acid.

### 2.5. Spike S1 (SARS-CoV-2): ACE-2 Inhibition Assay

Spike S1 (SARS-CoV-2): ACE-2 Inhibitor Screening Colorimetric Assay Kit was used as a colorimetric Elisa method (BPS Bioscience, 79954). This commercial kit is based on the measurement of binding between the Spike S1 (SARS-CoV-2) protein and the biotin-labeled human ACE-2 protein. Inhibition effects of extracts obtained from three different propolis of cities (Trabzon, Kocaeli, and Kırklareli), different fractions of these propolis extracts, and pure phenolic molecules known to be present in the relevant propolis samples on SARS CoV- 2 Spike RBD/ACE-2 interaction were determined following the procedure prescribed by the company.

### 2.6. Molecular Docking Studies

Based on the in vitro experiments described, the inhibitory effects of propolis extracts from three different provinces and the flavonoid substances detected in these extracts (analyzed using HPLC) were assessed for their impact on the interaction between SARS-CoV-2 Spike RBD and ACE-2 proteins. In this context, 3D structures of each substance showing inhibitory effects were obtained in SDF format from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database for use in docking studies. The resulting 3D ligand files were converted to pdb format using Openbabel and Pymole software to obtain 3D structures. Subsequently, these compounds were optimized using the MMFF94s force field within the Avogadro software (Hanwell et al., 2012).

The 3D crystal structures of ACE-2 (PDB ID: 1R4L: Resolution 3.00 Å) and SARS-CoV-2 Spike RBD (PDB: 6YLA: Resolution: 2.42 Å) were retrieved from the protein database ((http://www.rcsb.org/pdb), to be used as receptor proteins in the docking studies. Possible docking modes between the ligands and receptor proteins (SARS-CoV-2 Spike RBD and ACE-2) were determined. It was examined using the Autodock 4.2 (Morris et al., 2009) program and the Lamarckian genetic algorithm was used for the docking simulations. Suitable cavities

for the receptors were determined with the BIOVIA Discovery Studio 2018 program, the x, y and z coordinates were created as 126, 126, and 126, with a grid spacing of 0.375 Å. The Autodock program was set to create a total of 100 Genetic algorithms and the settings for all other parameters were saved as default. The molecular docking results, docking score, and binding affinity of each ligand on the corresponding protein target were determined. Visualization and interpretation of all obtained results were performed with BIOVIA Discovery Studio 2018 software (Dassault Systèmes BIOVIA, 2017) . The docking protocol was validated by eliminating the native inhibitor (CR3022 Fab) from complex (Spike receptor binding domain), re-docking and calculating the root mean square deviation (RMSD).

### 2.7. Statistical analysis

Experiments were performed in triplicate (n = 3) and data presented as mean ± standard deviation (SD). Data presented in figures are average of three parallel experiments and error bars are shown for SD. The statistical assessments were performed using the SPSS Version 20.0 (Statistical Package for the Social Sciences). One-way ANOVA was used to determine the statistical differences in the results. Duncan's multiple comparison test was performed to compare statistical difference between the test results. $p<0.05$ was accepted as the significance level.

## 2. RESULTS and DISCUSSION

Since the interaction between ACE-2 and Spike is a protein-protein interaction, it is known that these interactions are revealed by the methods stated below. Protein–protein interactions (PPIs) are fundamental processes for the reproduction and survival of cells and appear to be excellent targets for the development of inhibitors of host-pathogen interactions and biological processes such as cancer cell proliferation. The isolation of PPI inhibitors is quite difficult. There are several in vitro assay methods for testing PPI inhibitors, but they are generally expensive, cumbersome, and require large amounts of purified proteins. However, there are limited in vivo methods to test small molecule PPI inhibitors. While in vivo techniques such as Yeast 2 hybrid (Y2H) and Yeast 3 hybrid (Y3H) analyzes can be used to reveal protein-protein interactions, in vitro analyzes (outside of living cells) such as pull-down and coimmunoprecipitation are techniques used to reveal protein-protein interactions. However, since the yeast two-hybrid system contains artifacts and coimmunoprecipitation requires cell lysis for analysis, the exact

localization of protein-protein interactions within the cell cannot be determined. In contrast, fluorescence resonance energy transfer (FRET) allows the investigation of protein-protein interactions in situ (at their exact localization in the normally occurring cell).

The binding of ACE-2 protein to SARS-CoV-2 spike S1 protein was examined for propolis samples using the inhibitor screening colorimetric assay kit. The main point of this ELISA test is the high sensitivity of the detection of ACE-2-Biotin protein by Streptavidin-HRP. This technique is based on the binding of the active components of propolis to this SARS-CoV-2 Spike RBD/ACE-2 complex and the inhibition of binding of the second enzyme-labeled antibody to the protein. The presence of enzyme activity (horseradish peroxidase) indicates no binding. According to this method, the propolis sample with the highest inhibition effect was determined as 'Kocaeli-1 propolis'. Since ethanol also showed inhibition on HIV-RT as a negative control, the studies were continued with aqueous extracts of Kocaeli-1 propolis, not ethanol extracts.

Quantitative analyses of aqueous and ethanolic extracts of Kocaeli-1 propolis were conducted to determine the total phenolic and flavonoid contents. All results of these assays performed are summarized in Table 1.

Table 1. TPC, TFC, FRAP and radical scavenging activity of aqueous Kocaeli-1 propolis extract*

| **Propolis sample** | **Total phenolic contents (mgGAE/g)** | **Total Flavonoid contents (mgQE/ g)** | **Total Antioxidant Capacity (FRAP) (µmol$FeSO_4.7H_2O$/g)** | **DPPH• radikal Radical Scavenging Activity (SC50, mg/mL)** |
|---|---|---|---|---|
| **Kocaeli-1 (aqueous extract)** | 7.15±0,56 [a] | 2.30±0,40 [a] | 82.30±2,55 [a] | 0.56±0,10 [a] |
| **Kocaeli-1 (%70 ethanol)** | 146.20±1,20[b] | 32,.30±0,58 [b] | 380.20±3,70 [b] | 0.030±0,001 [b] |

* Lowercase letters indicate statistical difference ($p<0.05$)

The phenolic compound composition of aqueous and ethanolic extracts of Kocaeli-1 propolis were revealed by the RP-HPLC-UV method, and this content is summarized in Table 2.

Table 2. Phenolic composition of Kocaeli-1 propolis sample identified by RP-HPLC-UV

| **Standarts (µg fenolic/g sample)** | **Kocaeli-I (ethanol)** | **Kocaeli-I (aqueous)** |
|---|---|---|
| **Gallic acid** | - | 56,20 |
| **Protocatechuic acid** | - | 240 |
| **p-OH Benzoic acid** | - | 120 |
| **Catechin** | - | - |
| **Caffeic acid** | 2460 | 86 |
| **Galangin** | - | - |
| **Syringic acid** | - | 32 |
| **Epicatechin** | - | - |
| **p- Coumeric acid** | 560 | 74 |
| **Ferulic acid** | - | 28 |
| **Quarcetin** | - | - |
| **Rutin** | 4470 | - |
| **Myricetin** | 789 | - |
| **Resveratrol** | - | - |
| **Tannic acid** | - | - |
| **Daidzein** | 138 | - |
| **Luteolin** | 380 | - |
| **Chlorogenic acid** | - | - |
| **Fisetin** | - | - |
| **t-Cinnamic acid** | 530 | 133 |
| **Hesperetin** | - | - |
| **Chyrisin** | 1290 | 230 |
| **Pinocembrin** | 2560 | 142 |
| **Caffeic acid phenetyl ester (CAPE)** | 638 | - |

-: not determined

Differences in composition and TPC, TFC, FRAP and radical scavenging activity between ethanolic and aqueous forms of propolises attributed to solvent and extraction method. The literature contains numerous studies investigating the antioxidant activity of propolis extractions, with research exploring various extraction methods and solvents. (Chen et al., 2019, Turkut et al., 2019, Keskin & Kolayli, 2019, Trusheva et al., 2007). In broader scientific contexts, various types of propolis extracts such as ethanolic, glycol, supercritical fluid, oil-based, and modified aqueous extracts have been shown to exhibit distinct properties in the literature.

Then, the pure molecules known to be present in the Kocaeli-1 propolis sample (gallic acid, protocathecuic acid, p-OH benzoic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, t-cinnamic acid, chrysin) were tested against SARS-CoV-2 Spike RBD /ACE-2 and the results were shown in Figure 1.

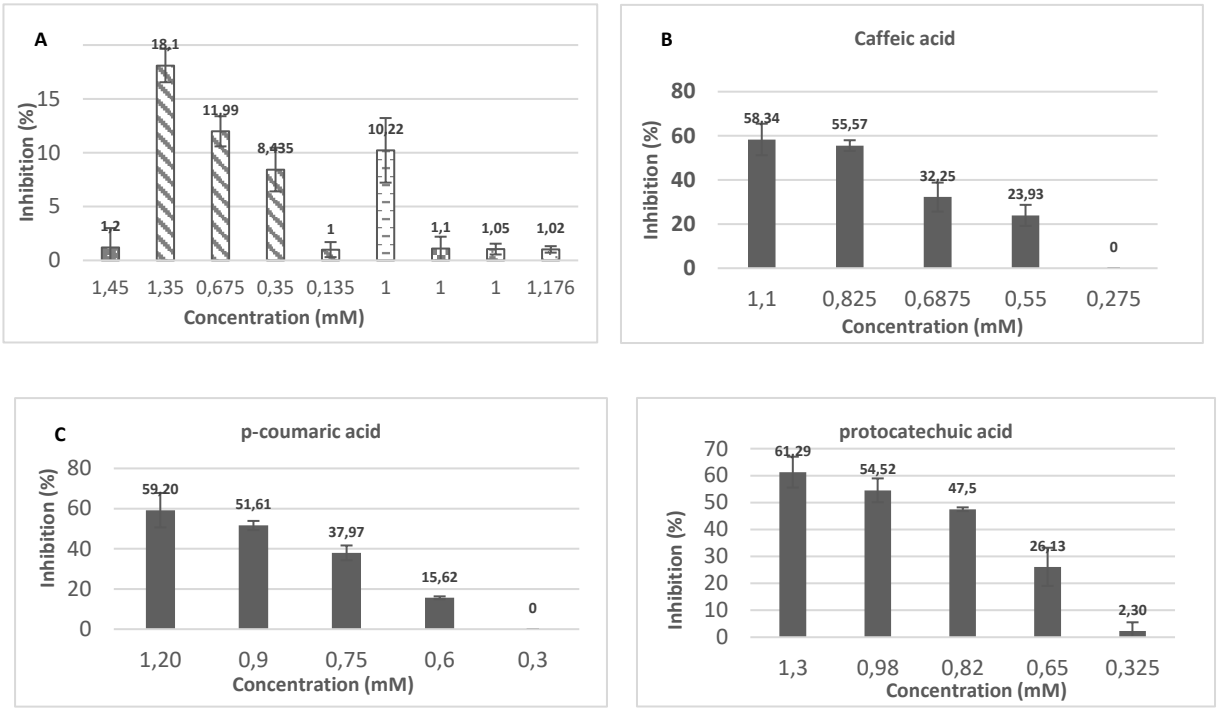


Figure 1. Effect of phenolic compounds in propolis samples on the SARS-CoV-2 Spike RBD/ACE-2 interaction A) *p*-hydroxybenzoic acid, t-cinnamic acid, chrysin, Syringic acid Ferulic acid, Gallic acid B) caffeic acid C) *p*-coumaric acid D) protocatechuic acid

According to the results of preliminary studies, it was determined that *p*-OH benzoic acid, syringic acid, ferulic acid, and gallic acid had no inhibitory effects on the SARS-CoV-2 Spike RBD/ACE-2 interaction. The inhibition values of t-cinnamic acid and chrysin at an average concentration of 1 mM were determined to be 18.1% and 10.22%, respectively (Figure 1A). Among the substances found in the propolis sample, those with the greatest inhibitory effect are; protocathecuic acid, caffeic acid, and *p*-coumaric acid (Figure 1B, 1C, 1D).

The $IC_{50}$ values (half maximal inhibitory concentration) of these substances with the strongest inhibitory effects on the SARS-CoV-2 Spike RBD/ACE-2 interaction were calculated (Table 3).

Table 3. $IC_{50}$ values for protocatechuic acid, caffeic acid and *p*-coumaric acid inhibition of SARS-CoV-2 Spike RBD/ACE-2 interaction

| **Phenolic compound** | **$IC_{50}$ (mM)** |
|---|---|
| **Protocatechuic acid** | 1±0.015 |
| **Caffeic acid** | 0.89±0.016 |
| ***p*-coumaric acid** | 0.99±0.019 |

In addition, the inhibitory effects on the SARS-CoV-2 spike RBD/ACE-2 interaction, which occurred when these substances were added together to the reaction, were also determined. Alone, protocatecuic acid at a concentration of 0.65 mM exhibited an inhibition of 26.13%, while caffeic acid at 0.55 mM demonstrated an inhibition of 23.93%. However, when co-administered, the combined inhibition increased significantly to 62.75%. Similarly, protocatecuic acid at 0.65 mM showed an inhibition of 26.13%, while coumaric acid at 0.6 mM displayed an inhibition of 15.62%. When these compounds were applied together, the resultant inhibition was 58.875%. Moreover, coumaric acid alone at 0.6 mM inhibited 15.62%, whereas caffeic acid alone at 0.55 mM inhibited 23.93%; however, when administered in combination, the inhibition rate was increased to 49.875% (Figure 2). These findings indicate that the combined application of these substances yields higher inhibition rates compared to single application.

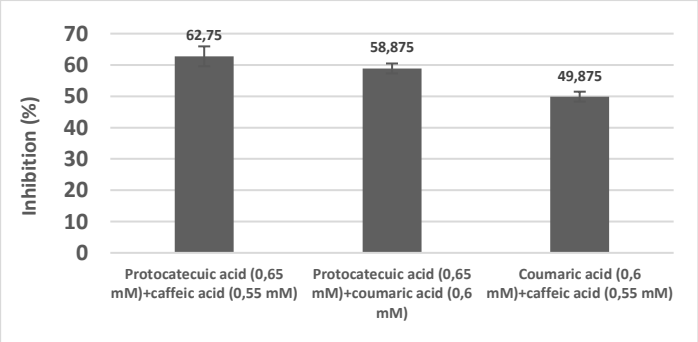


Figure 2. Effect of the combinations of phenolic compounds on the SARS-CoV-2 Spike RBD/ACE-2 interaction

The cytotoxic effects of pure molecules exhibiting the most inhibitory effects on the ACE-2/ Spike RBD interaction were investigated in the literature. In the study conducted by Rezaei-Seresht et al. in 2019; to determine whether caffeic acid is lethal to the cells, using the 3-(4,5-

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide method, MCF-7 cells (human breast cancer cell line) were treated with different concentrations of caffeic acid ( 5-200 µg/ml) for 48 and 72 hours (MTT assay) and the $IC_{50}$ values were determined. The $IC_{50}$ values of caffeic acid on MCF-7 cells were calculated as 159 µg/ml after 72 hours. The $IC_{50}$ value of caffeic acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated as 160 µg/ml (0.89 mM) (Rezaei-Seresht et al. 2019). Compared to all other experiments in this study, considering the toxic effects of caffeic acid on breast cancer cells and the morphological changes it causes, the potential of this substance to be used as an antitumor agent in the future is revealed by this study. In another study, *p*-coumaric acid was applied to neuroblastoma N2a cells at concentrations of 1 and 200 µmol/L and kept for 72 hours. The cytotoxic effects of this substance were examined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide method (MTT assay). According to this; The $IC_{50}$ value was calculated as 104 µmol/L. It was determined that 150 µmol/L *p*-coumaric acid caused 81.23% cell apoptosis after 72 hours (Shailasree et al., 2014). In another study on *p*-coumaric acid; the cell inhibition effects were examined (by MTT assay) on a colon cancer cell line (HT29 and HCT116) and a non-cancer cell line (Vero). *p*-coumaric acid was used at a concentration of 0-3 mM and cells were treated with this agent for 24, 48, and 72 hours. The results show that, the $IC_{50}$ value for HDAC enzymes in the HeLa nuclear extract is 2.6 mM. The antiproliferative effect on the HT29 colon cancer cell line after 72 hours was calculated as $1.0 \pm 0.2$ mM ($IC_{50}$). Again, its antiproliferative effect on the HCT116 colon cancer cell line at the end of 72 hours was calculated as $1.3 \pm 0.8$ mM ($IC_{50}$) (Saenglee et al. 2016). The $IC_{50}$ value of *p*-coumaric acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated to be 0.99 mM.

In a study conducted for protocatechuic acid, after protocatechuic acid treatment, cell viability was determined by the SRB assay, which is based on the measurement of the ability of SRB to adhere to cell proteins, the total protein amount or the number of cells associated with the SRB dye. After treatment of the cells with protocatechuic acid, incubation was performed for 48 hours. As a result, the $IC_{50}$ values of the cytotoxic effects of protocatechuic acid on MCF-7 and Jurkat cell lines were calculated as $5.97 \pm 0.36$ and $3.15 \pm 0.64$ (mM), respectively. The $IC_{50}$ value of protocatechuic acid used in SARS-CoV-2 Spike RBD / ACE-2 interaction experiments was calculated as 1 mM and is seen to be below this value. According to the studies of Yin et al. 2009; protocatechuic acid has antitumor properties with an effect that increases apoptosis or prevents invasion and metastasis in human breast cancer cell line (MCF-7), lung cancer cell

line (A549), HepG2 cell line, HeLa cell line, cervical cancer cells and LNCaP prostate cancer cells (Yin et al. 2009).

Molecular docking studies were carried out to investigate the inhibitory effects of ligand and reference molecule in silico, that were shown to be effective as a result of the studies carried out with the SARS-CoV-2 Spike RBD / ACE-2 interaction inhibition kit, on the RBD of ACE-2 and SARS-CoV-2 Spike protein. The conformations with high negative binding energy are shown in Figure 3.

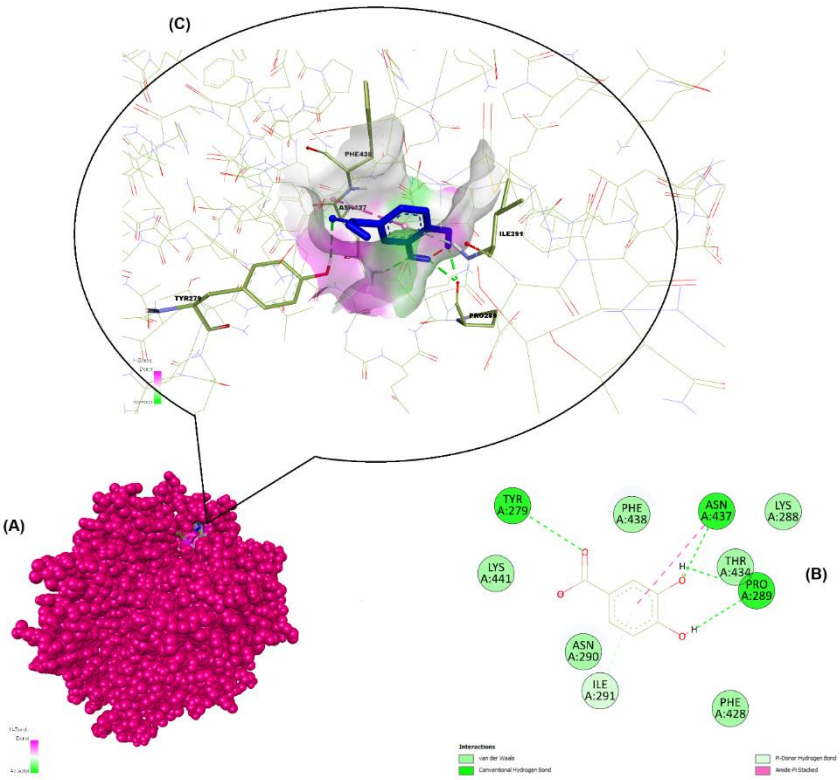


Figure 3. The binding pose profile of protocatechuic acid in the target protein ACE-2 (A) is depicted, showing the magenta-colored molecule receptor and the blue-colored molecule ligand. Two-dimensional (2D) (B) and three-dimensional (3D) (C) interaction analyses of ACE-2 protein with protocatechuic acid are presented.

Molecular docking is a crucial tool for exploring interactions between a target protein and a small molecule. Binding energy data (kcal/mol) allows us to examine and compare the binding affinity of different ligands/compounds with their respective target receptor molecules. Lower binding energy indicates a higher affinity of the ligand for the receptor. The ligand with the highest affinity can be selected as a potential drug for further studies. For this study, protocatechuic acid with a wide range of biological activities were used along with hydroxychloroquine (positive control), which demonstrated activity against SARS-CoV-2. The binding affinities of these ligands with the SARS-CoV-2 Spike Protein RBD and ACE-2 which

were used as receptors, were investigated. In a study conducted by Guler and his colleagues in 2021, docking analyses were performed with many flavonoids using the same receptors, and effective binding profiles were observed. Similarly, effective results were found in these docking analyses performed with protocatechuic acid, which was not included in that study (Guler et al., 2020).

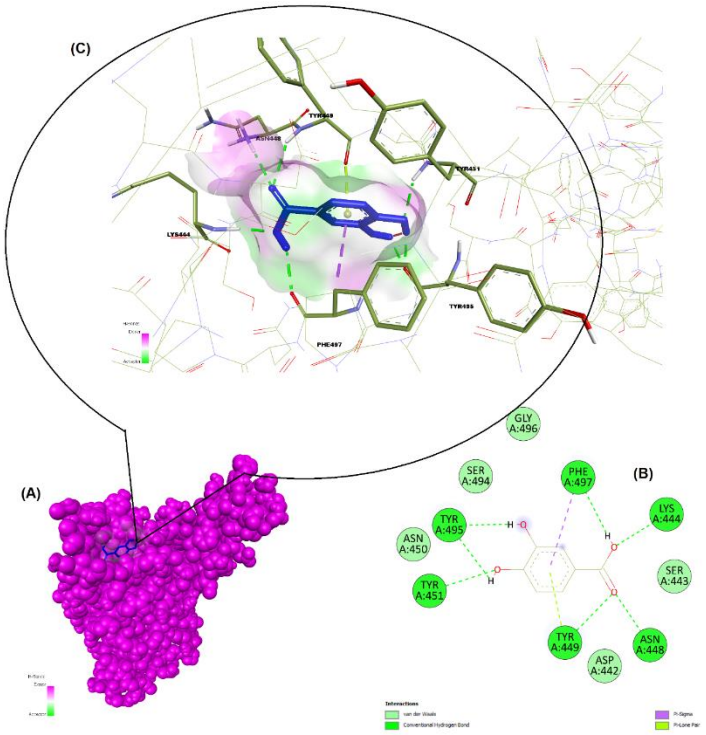


Figure 4. The binding pose profile of protocatechuic acid in the target protein SARS-CoV-2 Spike RBD (A) is depicted, showing the magenta-colored molecule receptor and the blue-colored molecule ligand. Two-dimensional (2D) (B) and three-dimensional (3D) (C) interaction analyses of SARS-CoV-2 Spike RBD with protocatechuic acid are presented.

Ligand protocatechuic acid and a reference molecule were individually docked to ACE-2 and SARS CoV-2 Spike RBD, respectively. After successful docking of all ligands used in these docking experiments, the results showed us that the protocatechuic acid performed significant interactions with the target receptors. The results indicate that, the ligand bound to the ACE-2 protein is effectively similar to the reference molecule. When the docking results with ACE-2 protein were examined, it was seen that protocatechuic acid has formed three conventional hydrogen bonds, one amide pi-stacked bond, one donor-donor bond and one pi-donor bond, three of these bonds had an atomic distance lower than 3 Å. The strongest bond of that interaction formed at position Pro289 with a length of 2.13 Å. When the docking results with Spike RBD were examined, it was seen that protocatechuic acid had better binding than the

reference molecule. Protocatechuic acid was found to be the molecule that bound strongly to the relevant receptor (-7.54 kcal/mol and 2.98 μM). It was observed that 8 conventional hydrogen bonds and 1 Pi-lone bond were formed in this docking and that conventional hydrogen bonds formed very effective bonds with a length of 1.88 Å at the Try495 position and 1.76 Å at the Lys444 position (Figure 3). The binding levels and details of the best interacting ligands are shown in detail in Table 4. For docking protocol validation, Spike receptor binding domain and its original native inhibitor (CR3022 Fab) were redocked and RMSD value was calculated. RMSD value of 1.94 Å between the docked conformation of the inhibitor and native conformation depicted the accuracy of the docking program.

Table 4. Estimated binding affinity (Kcal/mol), Ki values, and amino acids interacting at binding sites of docked ligands against ACE-2 and SARS-CoV-2 Spike receptor

| **Receptor Name / PDB ID** | **Ligand Name** | **Binding Energy (kcal/mol)** | **$K_i$ (μM)** | **H bonds** | **Interacted residues with ligand** |
|---|---|---|---|---|---|
| **Angiotensin-converting enzyme 2 (ACE-2) EC: 3.4.17.23 / 6M0J (Chain A) Res: 2.45 Å** | Protocatechuic acid | -5.25 | 141.22 | 3 | Ile291, Pro289, Asn437, Phe438 |
| | *Hydroxychloroquine | -7.90 | 1.61 | 4 | Arg393, Phe390, Leu391, Asn394, His378, His401, Asp350 |
| **SARS-CoV-2 Spike receptor binding domain / 6YLA (Chain A) Res: 2.42 Å** | Protocatechuic acid | -7.54 | 2.98 | 8 | Lys444, Asn448, Tyr449, Tyr495, Tyr451, Phe497 |
| | *Hydroxychloroquine | -6.32 | 23.35 | 7 | Leu517, Tyr396, Val382, Phe392, Thr430, Phe515 |

*reference molecule

## 4. CONCLUSION

The composition of propolis extracts depends on many factors, such as the flora of the region where the raw propolis is collected, the time of collection, and the extraction techniques. Therefore, it is not easy to standardize propolis extracts. In this study, propolis samples were collected from three different regions, and their effects on SARS-CoV-2 spike S1 protein and the ACE-2 receptor interaction were investigated. The propolis sample demonstrating the highest inhibition effect was identified as Kocaeli-1 propolis. Through this study, the phenolic content of Kocaeli-1 propolis was determined, and the effects of these phenolics on SARS-CoV-2 spike S1 protein and the ACE-2 receptor interaction were individually examined and docking studies were carried out to demonstrate the interactions of the molecules deemed effective with both molecules (ACE-2, Spike RBD) separately and interactively. It was observed that many molecules in propolis effectively bind to the ACE-2 protein. When comparing in silico results with in vitro findings, caffeic acid and protocatechuic acid were observed to have considerable binding affinities to both the SARS-CoV-2 spike S1 protein and the ACE-2 receptor.

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## DECLARATIONS

No conflict of interest or common interest has been declared by the authors.

## AUTHOR CONTRUBITIONS

The authors confirm contribution to the paper as follows: Study conception and design, data collection: Sabriye CANAKCI and Ali Osman BELDUZ**;** analysis and interpretation of results: Halil İbrahim GÜLER, Fulya AY**;** draft manuscript preparation: Fulya AY**.** All authors reviewed the results and approved the final version of the manuscript.

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# *Determination of the Effect of Different Extraction Methods on Aloe barbadensis Miller (Aloe Vera) Extract and its Usability in Ayran*

# *Farklı Ekstraksiyon Yöntemlerinin Aloe barbadensis Miller (Aloe Vera) Ekstraktı Üzerine Etkisinin ve Ayran Üretiminde Kullanılabilirliğinin Belirlenmesi*

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## Abstract

The *Aloe barbadensis Miller (Aloe vera)* plant has been gaining increasing popularity in recent years, especially in the fields of health and cosmetics. Its use in the food industry has also been on the rise late. There is particularly growing interest in its application as an edible film and coating because of its unique structure and composition. In this study, *Aloe vera* gel and leaf parts were evaluated separately. As extraction methods, maceration and ultrasonic wave-assisted extraction methods were used, and ethanol, water, and ethanol/water (1:1) mixtures were used as solvents. The effects of different extraction methods and solvents on the extracts were investigated. The total phenolic substance content, DPPH scavenging activity, and total flavonoid substance content of the obtained extracts were calculated. The usability of the obtained *Aloe vera* gel was then investigated in ayran. Sensory analyses were performed on ayran samples obtained by adding ayran at different rates. The highest amount of phenolic substance in *Aloe vera* gel was calculated as 508.80 mg GAE /g in the extract obtained using water as the solvent and the ultrasonic wave-assisted extraction method as the method. The highest DPPH scavenging activity 27.06% was detected in *Aloe vera* gel under the same extraction conditions. When we evaluated the total amount of flavonoids, the highest amount was found to be 163.79 mgQE/g when ethanol/water mixture was used as a solvent in the maceration method. In the extract obtained from *Aloe vera* leaf, where ethanol:water (1:1) solvent was used as solvent and ultrasonic wave-assisted extraction, the highest amount of phenolic substance was 597.63 mg GAE /g, the highest DPPH activity was 66.38%, and the highest total amount of flavonoid substance was 409.20 mg QE /g. When we evaluated the

results generally, the amount of total phenolic substance, DPPH scavenging activity, and total amount of flavonoid substance increased in direct proportion to each other. Compounds with phenolic and flavonoid properties increased with DPPH scavenging activity. *Aloe vera* gel was added to ayran samples at different rates (%1.25, 2.5 and 5). Samples of ayran added at a rate of 1.25% received scores similar to those of the control group without any addition. Other samples of ayran also received high scores from the panelists. In this study, the extraction conditions of *Aloe vera* gel and leaf were optimized and their usage possibilities in ayran were evaluated.

**Keywords:** *Aloe barbadensis Miller (Aloe vera)*, Extraction, Ayran.

## Özet

*Aloe barbadensis Miller (Aloe vera)* bitkisinin son yıllarda kullanım alanı giderek artmaktadır. Özellikle sağlık ve kozmetik sektörlerinde yaygın olarak kullanılmaktadır. Gıda endüstrisindeki kullanımı da son dönemde artış göstermektedir. Bitkinin kendine özgü yapısı ve bileşimi nedeniyle, yenilebilir film ve kaplama olarak kullanımı ile ilgili çalışmalar özellikle ilgi çekmektedir. Bu çalışmada *Aloe vera* jel ve yaprak kısımları ayrı ayrı farklı olarak değerlendirilmiştir. Ekstraksiyon yöntemleri olarak maserasyon ve ultrasonik dalga destekli ekstraksiyon yöntemleri ve çözücü olarak etanol, su ve etanol:su (1:1) çözücüsü kullanılmıştır. Farklı ekstraksiyon yöntem ve farklı çözücülerin ekstraktlara etkisi araştırılmıştır. Elde edilen ekstraktların toplam fenolik madde miktarı, DPPH süpürme aktivitesi ve toplam flavonoid madde miktarı hesaplanmıştır. Sonrasın da elde edilen *Aloe vera* jelinin ayranda kullanılabilirliği araştırılmıştır. Farklı oranlarda ayrana ilave edilerek elde edilen ayran örneklerinde duyusal analizler gerçekleştirilmiştir. *Aloe vera* jelinde en yüksek fenolik madde miktarı çözücü olarak su ve yöntem olarak ultrasonik dalga destekli ekstraksiyon yönteminin kullanılmasıyla elde edilen ekstraktta 508.80 mg GAE/g olarak hesaplanmıştır. *Aloe vera* jelinde aynı ekstraksiyon şartlarında en yüksek DPPH süpürme aktivitesi %27.06 tespit edilmiştir. Toplam flavonoid miktarını değerlendirdiğimizde maserasyon yönteminde etanol/su karışımını çözücü olarak kullandığımızda en yüksek miktar olan 163.79 mg QE/g tespit edilmiştir. *Aloe vera* yaprağında ise etanol/su karışımının çözücü olarak kullanıldığı ve ultrasonik dalga destekli ekstraksiyon ile elde edilen ekstraktta en yüksek fenolik madde miktarı 597.63 mg GAE/g, en yüksek DPPH süpürme aktivitesi %66.38 ve en yüksek toplam Flavonoid madde miktarı 409.20 mg QE/g olarak tespit edilmiştir. Genel olarak değerlendirdiğimizde toplam fenolik madde miktarı, DPPH süpürme aktivitesi ve toplam flavonoid madde miktarı birbiriyle doğru orantılı şekilde artış göstermiştir. Fenolik ve flavonoid özellik gösteren bileşikler DPPH süpürme aktivitesinin artmasını sağlamıştır. Ayran örneklerine *Aloe vera* jeli farklı oranlarda % (1.25, 2.5 ve 5) eklenmiştir. %1.25 oranında eklenen ayran örnekleri hiç eklenmeyen kontrol grubuyla benzer puanlar almıştır. Diğer ayran örnekleride panelistlerden yüksek puanlar almıştır. Bu çalışma ile *Aloe vera* jel ve yaprağının ekstraksiyon şartları optimize edilmiştir ve ayranda kullanım olanakları değerlendirilmiştir.

**Anahtar Kelimeler:** *Aloe barbadensis Miller (Aloe vera)*, Ekstraksiyon, Ayran

## 1. INTRODUCTION

*Aloe barbadensis Miller (Aloe vera);* has been revered as a medicinal plant for centuries, with its use dating back to ancient civilizations (Christachi & Florou-Paneri, 2010). Discovered by Greek scientists around 2000 years ago, *Aloe vera;* has earned the moniker "universal panacea" or "cure-all" for its purported wide range of health benefits. The Egyptians, in particular, associated *Aloe vera;* with immortality (Surjushe et al., 2008).

*Aloe vera* is remarkably rich in polysaccharides (Eshun and He, 2004). Polysaccharides are a crucial class of high-molecular-weight carbohydrates derived from microorganisms, animals, and plants (Li et al., 2018). These polysaccharides exhibit a remarkable array of bioactivities, including antimicrobial (Luiz et al., 2017), antitumor (Nazeam et al., 2017), antiviral (Xie et al., 2016), and antioxidant (Chen et al., 2016) properties. Because of their exceptional and versatile properties, polysaccharides have gained widespread use in healthcare products and medicines.

*Aloe vera* is one of the most widely used medicinal plants globally for disease prevention and treatment. It is particularly effective in addressing skin disorders, metabolic diseases, cardiovascular ailments, and cancer. Research has consistently demonstrated that *Aloe vera* leaves possess immunomodulatory, antimicrobial, antiviral, anticancer, and anti-inflammatory properties (Reynolds & Dweck, 1999; Strickland, 2001; Harlev et al., 2012).

*Aloe vera* leaves are rich sources of bioactive compounds with remarkable antioxidant properties. These compounds include mannose-rich polysaccharides (mannans), anthraquinones, C-glycosides, and lectins, which have found extensive applications in the food industry (Rodriguez et al., 2010).

*Aloe vera* leaf is composed of two primary components: latex and gel. Latex, also known as "aloe juice" or "aloe extract," accounts for approximately 20-30% of the entire leaf. This bitter, yellow liquid is exuded from the pericyclic tubules located beneath the leaf's epidermis (Boudreau & Beland, 2006). *Aloe vera* latex is particularly rich in phenolic compounds and exhibits potent antibacterial activity against Gram-positive bacteria (Boudreau & Beland, 2006; Surjushe et al., 2008; Alemdar & Agaoglu, 2009).

*Aloe vera* gel is a remarkable substance extracted from the parenchymal cells of fresh *Aloe vera* leaves. This colorless, sticky gel constitutes approximately 70-80% of the *Aloe vera* product. Its notable properties include therapeutic, antibacterial, and antifungal effects, and biodegradability. Owing to its flavoring and preservative attributes, *Aloe vera* gel finds

extensive use as a functional food ingredient in beverages, ice creams, and confectionery products (Eshun & He, 2004; Boudreau & Beland, 2006).

*Aloe vera* gel's versatility extends beyond its culinary applications. It serves as an edible and bio-safe protective film and coating material for various food items (Valverde et al., 2005). The gel's rich composition includes bioactive compounds such as salicylic acid and magnesium lactate. Additionally, it harbors mucopolysaccharides, enzymes, and sterols, including the antioxidant superoxide dismutase (Vogler & Ernst, 1999).

Biologically active components from *Aloe vera* gel can be obtained through traditional methods like maceration or novel extraction techniques like ultrasound-assisted extraction. These methods enable the efficient and rapid extraction of valuable compounds from plant material (Elferjane et al., 2023).

In the maceration method, the plant material is first shredded, mixed with the appropriate solvent, and maintained at room temperature. After the process, the mixture is filtered with filter paper (Azwanida, 2015; Majekodunmi, 2015; Ingle et al., 2017). The maceration method ensures the preservation of phenolic and aroma compounds. It minimizes the loss of aroma compounds and aids in the extraction of phenolic substances by modifying plant cell walls. The extraction process takes a long time (Olejar et al., 2015). Ultrasonic sound waves are characterized by a frequency range generally exceeding 16-18 kHz. These waves propagate as mechanical vibrations in solid or liquid media (Mason & Lorimer, 2002). During the extraction process, acoustic waves propagate in the liquid medium and cause the displacement of plant particles. Cavitation occurs when mechanical vibrations are transmitted to the plant (Büyüktuncel, 2012; Turan et al., 2021). This extraction method can be applied to both solid and liquid samples. Extraction results in a shorter time and higher yield (Büyüktuncel, 2012).

This study compares the maceration method and the ultrasonic wave-assisted extraction method. Two different extraction techniques were used in this study. Additionally, three different solvents were used. The total phenolic substance content, DPPH scavenging activity, and total flavonoid content of the obtained extracts were determined. According to the results of these analyses, the most suitable solvent and extraction method for the *Aloe vera* plant was determined. In addition, *Aloe vera* gel was used in ayran at different rates, and the optimum usage rate was determined.

## 2. MATERIALS and METHOD

### 2.1. Material

*Aloe vera* plant used in the research was grown in the Laboratory of Amasya University Suluova Vocational School. The leaves and gel of the plant were separated. Then, they were used in extraction. Ethanol (Merck.Darmstadt. Germany) and water were used as solvents. Ayran was purchased from a local grocery store. All chemicals were of analytical grade and were obtained from Merck Darmstadt. Germany.

### 2.2. Method

#### 2.2.1. Preparation of Extract

The gel part and leaves of the *Aloe vera* plant were separated. Afterwards, they were ground into small pieces. Ethanol, water and ethanol-water (1:1) mixtures were used as solvents. Extraction processes were performed using the ultrasonic wave-assisted extraction method at 40 °C and 40 min. (Çalışkan Lab. Ult 4010, Turkey). In the maceration method, the extraction process was performed at room temperature for 3 days. The mixtures were then filtered. And The filtrates were evaporated using with rotary evaporator (Buchi R100, Türkiye). The total phenolic component, DPPH scavenging activity, and total flavonoid analyses were applied to the obtained extracts. *Aloe vera* plant extraction stages are shown in Figures 1 and 2.

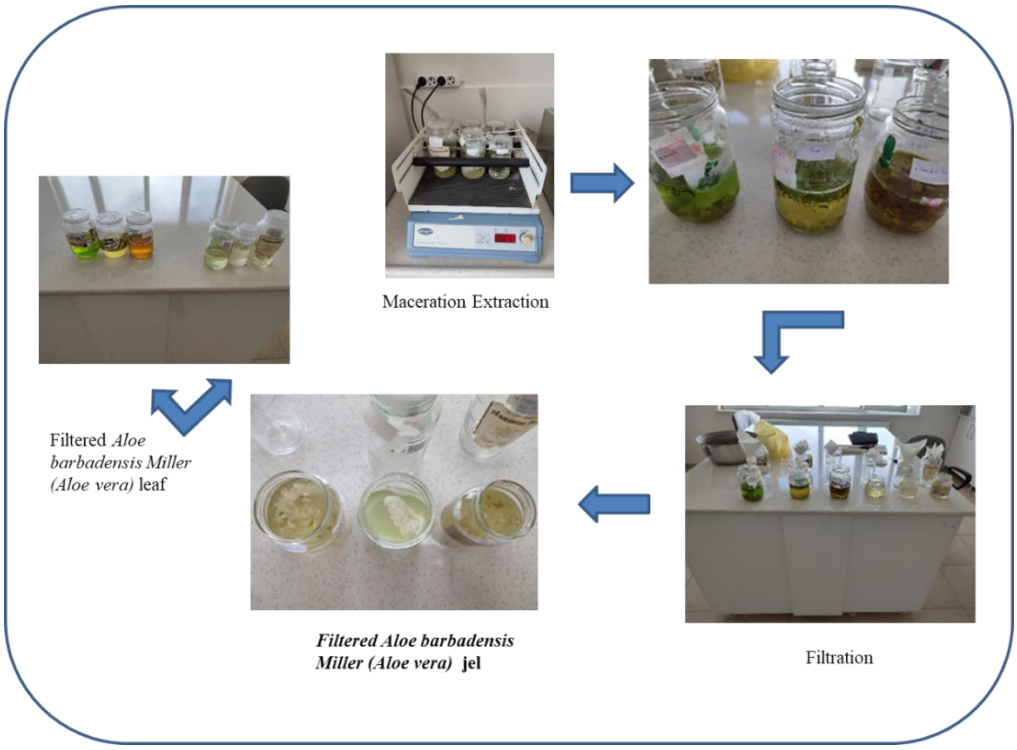


Figure 1. *Aloe vera* extraction with maceration technique

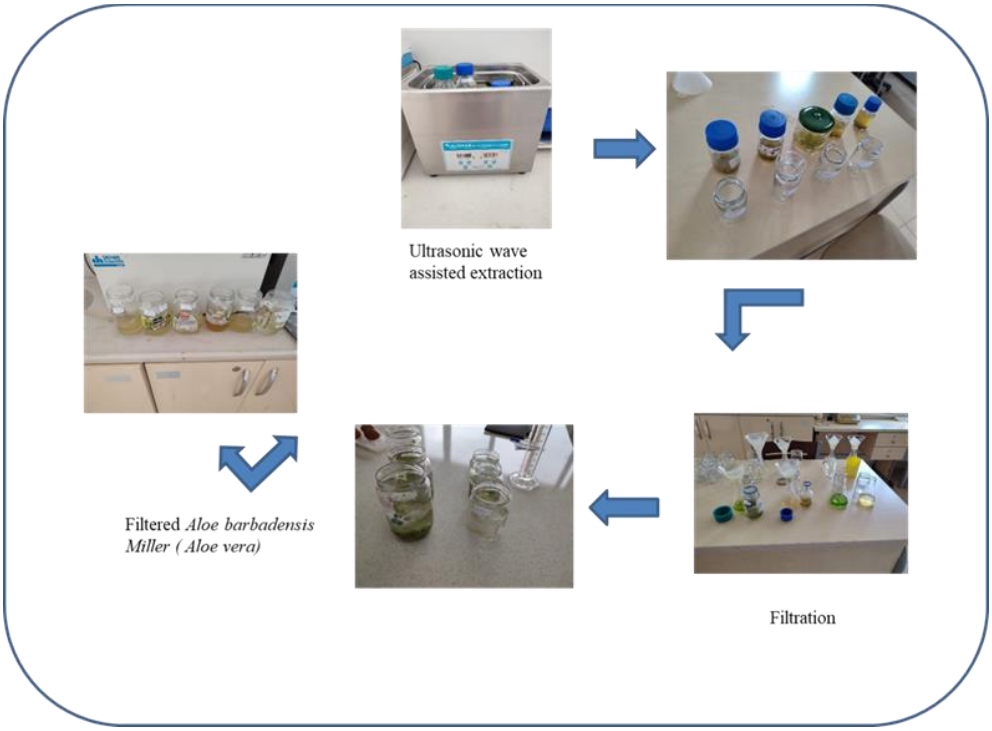


Figure 2. *Aloe vera* extraction using ultrasonic assited extraction technique stages

### 2.2.2. Total Phenolic Compounds

The total phenolic content of *Aloe vera* extracts was determined using the Folin-Ciocalteu method described by Singleton and Rossi (1965). First, 40 µL of the prepared extract (1 mg/mL) was mixed with 2.4 mL of distilled water and 200 µL of Folin-Ciocalteu reagent. After 30 seconds, 600 µL of saturated $Na_2CO_3$ and 760 µL of distilled water were added. The mixture was vortexed and incubated for 2 h at room temperature. The absorbance of the mixture was measured at 650 nm using a spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan). Results: Gallic acid was given in gallic acid equivalent (GAE) using the standard calibration curve (y = 0.001x+0.0557).

### 2.2.3. DPPH Free Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of *Aloe vera* extracts was evaluated according to Singh et al. (2002). 200 µL (1 mg/mL) of the extract was mixed with 3.8 mL of diluted DPPH solution ($1.0 \times 10^{-3}$ M). The mixture was vortexed for 15 s and kept in the dark for 60 min. At the end of the incubation period, the absorbance of the mixture was measured at 515 nm using a spectrophotometer. The DPPH scavenging activity was calculated as the percentage inhibition (Aksoylu, 2012).

### 2.2.4. Total Flavonoid Component

The total flavonoid content of the extracts was determined according to the method described by Zhishen et al. (1999). A certain volume of (1 mg/mL) was taken and mixed with 0.15 mL of 5% $NaNO_2$ solution. The mixture was incubated for 6 min, and then 0.15 mL of 10% (w/v) $AlCl_3$ solution was added and incubated for another 6 min. At the end of the incubation period, 2 mL of 1 M NaOH solution was added to the solution in the test tube, and the total volume was completed to 5 mL with pure water.The absorbance values of the extracts were read at 510 nm using a spectrophotometer at room temperature after a 15-min incubation period.Total catechin was used as the standard in the flavonoid determination studies.The total flavonoid content of the extract was calculated as mg (+) catechin/100 g sample using the calibration curve.

### 2.2.5. Production of Ayran

For the study, ayran samples obtained from the market were used. The research involved preparing control group ayran, ayran containing 1.25% *Aloe vera* extract, ayran containing 2.5% *Aloe vera* extract, and ayran containing 5% *Aloe vera* extract. Sensory analyses were performed on the prepared ayran samples.

### 2.2.6. Sensory Analysis

Sensory analysis was conducted on the prepared ayran samples by faculty members at Suluova Vocational School. The sensory analysis evaluated appearance, color, texture, odor, taste-aroma, and overall liking using a scale of 1 (very poor) to 9 (very good). Sensory analyses were performed by a panel of 10 panelists. The results were statistically evaluated (Seyrekoğlu, 2020).

### 2.2.7. Statistical Analysis

All analyses were performed in triplicate, and the mean standard deviations were calculated. The effects of the samples on the total phenolic content, DPPH scavenging activity, and total flavonoid content were determined by one-way ANNOVA analysis. All data were evaluated by variance analysis (ANOVA) using the SPSS program (SPSS 16.0). Statistical significance ($p=0.05$) was determined using Tukey's test (IBMCorp., 2011).

## 3. RESULTS and DISCUSSIONS

### 3.1. Total Phenolic Content of *Aloe vera* Extracts

In this study, two different extraction methods were used: ultrasonic-assisted extraction and maceration. Two different parts of the plant, namely the gel and leaf parts, were used in the extraction. Water, ethanol, and ethanol-water mixtures (1:1) were preferred as solvents. The total phenolic content values of *Aloe vera* extract were showed in Table 1.

Table 1. Total phenolic content of *Aloe vera* extracts

| **Samples** | **Parts of plant** | **Solvent Type** | **Extraction Method** | **Total phenolic content (mg GAE/g)** |
|---|---|---|---|---|
| **1** | *Aloe vera* gel | Ethanol | Ultrasonic wave-assisted extraction | $53.80^{g}$ ± 1.50 |
| **2** | *Aloe vera* gel | Water | Ultrasonic wave-assisted extraction | $508.80^{b}$ ± 0.50 |
| **3** | *Aloe vera* gel | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $208.30^{f}$ ± 2.00 |
| **4** | *Aloe vera* leaf | Ethanol | Ultrasonic wave-assisted extraction | $522.97^{b}$ ± 30.23 |
| **5** | *Aloe vera* leaf | Water | Ultrasonic wave-assisted extraction | $358.63^{e}$ ± 10.50 |
| **6** | *Aloe vera* leaf | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $597.63^{a}$ ± 23.96 |
| **7** | *Aloe vera* gel | Ethanol | Maceration extraction | $224.97^{f}$ ± 28.93 |
| **8** | *Aloe vera* gel | Water | Maceration extraction | $443.97^{c}$ ± 35.11 |
| **9** | *Aloe vera* gel | Ethanol: Water (1:1) | Maceration extraction | $452.80^{c}$ ± 21.36 |
| **10** | *Aloe vera* leaf | Ethanol | Maceration extraction | $329.13^{e}$ ± 44.22 |
| **11** | *Aloe vera* leaf | Water | Maceration extraction | $402.47^{d}$ ± 2.92 |
| **12** | *Aloe vera* leaf | Ethanol: Water (1:1) | Maceration extraction | $529.97^{b}$ ± 27.06 |

Different superscript letters in the same column indicate a significant difference (p>0.05).

In ultrasonic-assisted extraction of *Aloe vera* gel, the highest total phenolic content of 508.80 mg GAE/g was observed with water as the solvent, whereas the lowest total phenolic content of 53.80 mg GAE/g was observed with ethanol as the solvent. In the maceration method extraction of *Aloe vera* gel, the highest total phenolic content of 452.80 mg GAE/g was calculated when ethanol/water (1:1) was used as the solvent, whereas the lowest total phenolic content of 224.97 mg GAE/g was calculated when ethanol was used as the solvent.

In the maceration method, the total phenolic content was higher than that of the ultrasonic-assisted extraction method when water and ethanol-water mixture was used as the solvent, whereas the opposite was observed when ethanol was used as the solvent.

In ultrasonic-assisted extraction of *Aloe vera* leaves, the highest total phenolic content of 597.63 mg GAE/g was found when ethanol-water (1:1) mixture was used as the solvent, whereas the lowest total phenolic content of 358.63 mg GAE/g was found when water was used as the solvent. In the maceration method, similarly, ethanol-water (1:1) mixture was calculated as the highest phenolic compound with 529.97 mg GAE/g. When the two methods were compared, the use of ethanol and ethanol-water (1:1) mixture as the solvent provided higher total phenolic compound amounts in the ultrasonic-assisted extraction method, whereas the use of water as the solvent provided higher total phenolic compound amounts in the maceration method.

In this study, the total amount of phenolic substances in *Aloe vera* samples was calculated as 53.80-508.80 mg GAE/g in the ultrasonic wave-assisted extraction method and 224.97-452.80 mg GAE/g in the maceration method. In the *Aloe vera* leaf, it was determined as 358.63-597.63 mg GAE/g in the ultrasonic wave-assisted extraction method, whereas it was determined as 329.13-529.97 mg GAE/g in the maceration method. Elferjane et al. (2023) determined the maximum amount of phenolic compounds in, *Aloe vera* extracts as 9.95 mg GAE/g in the maceration method and 6.74 mg GAE/g in the ultrasonic wave-assisted extraction method. In another study conducted with *Aloe vera* samples, the total amount of phenolic components was determined as 56.11-93.96 mg GAE/g (Başaran, 2020).

In their study, Vega-Gálvez et al. (2011) investigated the total phenolic content of *Aloe vera* gel after subjecting it to different pressures (300, 400, and 500 MPa) for 35 days. All samples subjected to pressure (43.40±19.53–76.12±19.60 mg GAE/100), total phenol compared with the non-pressurized sample (178.45±14.76 mg GAE/100), a significant decrease

in their content was observed. Ray et al. (2013) used the freeze-drying method for *Aloe vera*. The total phenolic values in the samples were 30.11±1.89-35.77±1.07 µg GAE/mg.

Phenolic contents change during the growth periods of the plant and They also stated that there is a decrease in phenolic contents depending on the age of the plant. In their study, Miladi and Damak (2008) compared the effects of *Aloe vera* leaf on ethanol extracts. They applied distillation using hexane, ethyl acetate, chloroform-ethanol, and butanol. Chloroform-ethanol had the highest total phenolic content (40,500±0,041 µg GAE/mg) as a fraction.

In another study, *Aloe vera* peel extract exhibited the highest total phenolic content (7.99±0.26 mg GAE/g). In contrast, gel extracts displayed significantly lower phenolic content, nearly three times lower for Soxhlet extraction and four times lower for ultrasound extraction. These findings highlight the crucial role of the extraction method in determining the phenolic content. Soxhlet extraction, which involves prolonged heating, may lead to the degradation of heat-sensitive phenolic compounds (Vidic et al., 2014). Supporting this notion, previous studies (Miladi and Damak, 2008; Kammoun et al., 2011) have demonstrated that water extracts exhibit low phenolic content (2mg GAE/g), while chloroform-ethanol extracts possess a substantially higher phenolic content of approximately 40 mg GAE/g.

The total amount of phenolic compounds in both the leaf and gel parts of our *Aloe vera* samples were found to be significantly higher than that reported in the literature. Aldayel et al. (2020) showed the differences in the analysis results based on the phytochemical properties of *Aloe vera* plant composition; geography where the plant grows, climate, soil type, sun exposure, and seasonality. Changes were associated with elements such as the age of the plant. Based on the findings of this study, the most suitable extraction method for *Aloe vera* gel is ultrasound-assisted extraction using water as the solvent. For *Aloe vera* leaves, the recommended method is also ultrasound-assisted extraction, but with a solvent mixture of ethanol and water (1:1). In line with the findings of this study, numerous studies in the literature have also demonstrated that the combination of ethanol and water as solvents yields a remarkable increase in the total phenolic content of extracts. This synergy between ethanol and water can be attributed to their ability to effectively solubilize a various phenolic compounds, including both polar and non-polar compounds.

### 3.2. DPPH Scavenging Activity of *Aloe vera* Extracts

Table 2 presents the DPPH scavenging activity values of *Aloe vera* samples. When water was used as the solvent in the ultrasonic wave-assisted extraction method, the DPPH scavenging

activity of the *Aloe vera* gel reached its highest value (27.06%), which is consistent with the total phenolic content findings. The lowest value (7.25%) was obtained when ethanol was used as the solvent in the same method. In the maceration method, the highest DPPH scavenging activity (26.96%) was again achieved with water as the solvent. The lowest inhibition (4.70%) was observed when ethanol was used as the solvent in the maceration method. In both extraction methods, the use of water as the solvent increased the DPPH scavenging activity.

Table 2. DPPH scavenging activity of *Aloe vera* extracts

| **Samples** | **Parts of plant** | **Solvent Type** | **Extraction Method** | **DPPH Scavenging Activity (%)** |
|---|---|---|---|---|
| **1** | *Aloe vera* gel | Ethanol | Ultrasonic wave-assisted extraction | $7.25^{f} \pm 2.42$ |
| **2** | *Aloe vera* gel | Water | Ultrasonic wave-assisted extraction | $27.06^{b} \pm 2.86$ |
| **3** | *Aloe vera* gel | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $26.51^{b} \pm 3.86$ |
| **4** | *Aloe vera* leaf | Ethanol | Ultrasonic wave-assisted extraction | $21.22^{c} \pm 3.44$ |
| **5** | *Aloe vera* leaf | Water | Ultrasonic wave-assisted extraction | $11.67^{e} \pm 0.50$ |
| **6** | *Aloe vera* leaf | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $66.38^{a} \pm 0.23$ |
| **7** | *Aloe vera* gel | Ethanol | Maceration extraction | $4.70^{f} \pm 1.13$ |
| **8** | *Aloe vera* gel | Water | Maceration extraction | $26.96^{b} \pm 0.62$ |
| **9** | *Aloe vera* gel | Ethanol: Water (1:1) | Maceration extraction | $16.09^{d} \pm 0.43$ |
| **10** | *Aloe vera* leaf | Ethanol | Maceration extraction | $8.15^{f} \pm 1.62$ |
| **11** | *Aloe vera* leaf | Water | Maceration extraction | $0.71^{g} \pm 0.24$ |
| **12** | *Aloe vera* leaf | Ethanol: Water (1:1) | Maceration extraction | $28.51^{b} \pm 1.16$ |

Different superscript letters in the same column indicate a significant difference ($p>0.05$).

This study investigated the DPPH scavenging activity of extracts obtained from *Aloe vera* leaves using different extraction methods. The results demonstrate that the highest DPPH

scavenging activity values were achieved when an ethanol-water mixture (1:1) was used in both extraction methods. The lowest DPPH scavenging activity (11.67%) was observed when water was used as the solvent in ultrasonic-assisted extraction. In the maceration method, the lowest activity (0.71%) was also observed with water extraction.

In both ultrasonic wave-assisted extraction and maceration methods, the ethanol-water mixture provided the highest DPPH scavenging activity for *Aloe vera* leaves, whereas water decreased this value.

Antioxidants are substances that protect cells from damage caused by unstable molecules called free radicals. Free radicals can be produced by the body as a byproduct of normal metabolism, or they can be introduced from external sources such as pollution or cigaret smoke. When free radicals accumulate, they can damage cells and DNA, leading to a various of health problems, including cancer, heart disease, and neurodegenerative diseases (Wang et al., 2011).

Kumar et al. (2017) investigated the antioxidant activity of *Aloe vera* from six different agroclimatic zones in India. The researchers found that the antioxidant activity of *Aloe vera* was higher in plants collected from Northern India than in those collected from Southern India. This difference in activity was attributed to the higher content of alkaloids, glycosides, phenolic compounds, flavonoids, and saponin glycosides in Northern Indian plants (Kumar et al., 2017).

The higher antioxidant activity of *Aloe vera* from Northern India may be due to several factors, including; environmental stress**,** soil conditions, and genetic variation. The findings of this study suggest that the antioxidant activity of *Aloe vera* may vary depending on the region where it is grown. This information could be used to identify *Aloe vera* plants with the highest antioxidant activity for potential use in medicinal products or dietary supplements.

*Aloe vera* has been used for centuries for its medicinal properties. Recent research has shown that aloe vera ethanol extract has potent antioxidant properties and can protect cells from oxidative stress. In vitro studies have shown that aloe vera ethanol extract can; reduce the production of ROS (Reactive Oxygen Species), scavenge free radicals, and protect cells from damage caused by hydrogen peroxide and 4-hydroxynonenal (Cesar et al., 2018).

A clinical trial involving 53 healthy volunteers investigated the effects of 14-day supplementation with *aloe vera* gel extract on the antioxidant capacity of the subjects. The results showed that *aloe vera* supplementation significantly increased the subjects' levels of glutathione, a major antioxidant in the body (Sánchez et al., 2020).

This study compared two extraction methods: ultrasonic wave-assisted and maceration. Ultrasonic waves were significantly more effective in extracting antioxidants from both *Aloe vera* gel (7.25-27.06% DPPH scavenging activity) and leaves (11.61-66.38% DPPH scavenging activity) compared to maceration (4.70-26.96% for gel, 0.71-28.51% for leaves). Sathyaprabha et al. (2010) analyzed the antioxidant capacity of dried and powdered *Aloe vera* gel. The DPPH scavenging activity was measured at 15.8%. Çandöken (2008) investigated the water extract of *Aloe vera* pulp. The study found a substance with high hydrogen donor activity (essential for antioxidant properties) at a concentration of 60 mg/ml in the leaves. The water extract of the pulp itself exhibited a DPPH scavenging activity of 70.81 ± 0.27%. Overall, the research highlights that *Aloe vera* possesses significant antioxidant activity, and the method used for extraction can significantly impact the yield of these beneficial compounds.

The antioxidant properties of aloe vera extracts are affected by various initial developments. Changes over time vary (Hu et al., 2003). In the same study, the DPPH radical scavenging fraction of the *Aloe vera* extract was reported to be equal to or greater than that of BHT and α-tocopherol. The DPPH radical scavenging activity of *aloe vera* leaf peel ethanol extract was reported to be 39.7% (Hu et al., 2005).

The reducing power of *Aloe vera* extracts on DPPH radicals has been investigated by various researchers. López et al. (2013); This study compared the DPPH radical scavenging activity of *Aloe vera* leaf skin and flower extracts. Leaf skin extract exhibited significantly higher activity than flower extract, suggesting that the location of extraction within the plant can influence antioxidant properties. In another study the DPPH radical scavenging activity of different solvent extracts of *Aloe vera* gel was observed; both ethanolic and methanolic extracts demonstrated the highest DPPH radical scavenging activity, indicating that the choice of solvent can affect the extraction of antioxidant compounds (Khaing, 2011). In this study, while the DPPH scavenging activity of *Aloe vera* gel was similar to that reported in the literature, the inhibition of *Aloe vera* leaf was observed at lower values. Overall, these findings demonstrate that *Aloe vera* extracts possess significant DPPH radical scavenging activity, which can be influenced by various factors such as extraction conditions, growth stage, and solvent selection.

### 3.3. Total Flavonoid Content of *Aloe vera* Extracts

The total flavonoid content of *Aloe vera* gel was determined to be 80.97 mg QE/g when water was used as the solvent in the ultrasonic wave-assisted extraction method, while it was 69.73 QE mg/g when an ethanol-water (1:1) mixture was used as the solvent. In contrast, the highest

total flavonoid content was calculated as 163.79 mg QE/g when an ethanol-water (1:1) mixture was used as the solvent in the maceration method, whereas the lowest total flavonoid content was calculated as 44.91 mg QE/g when water was used as the solvent.

For *Aloe vera* gel, water is a good solvent in the ultrasonic wave-assisted extraction method, whereas an ethanol-water (1:1) mixture is a good solvent in the maceration method. In the ultrasonic wave-assisted extraction method for *Aloe vera* leaf, the highest total flavonoid content was 409.20 mg QE/g when an ethanol-water mixture was used as the solvent, whereas the lowest total flavonoid content was 157.41 mg QE/g when ethanol was used as the solvent.

Similarly, in the maceration method, the use of an ethanol-water (1:1) mixture resulted in the highest total flavonoid content of 295.18 mg QE/g, whereas the use of water as a solvent resulted in the lowest total flavonoid content of 117.89 mg QE/g. An ethanol-water (1:1) mixture was the best solvent for *Aloe vera* leaves in both the ultrasonic wave-assisted extraction method and the maceration method. The total flavonoid contents of *Aloe vera* extracts are shown in Table 3.

In this study, the total flavonoid content of *Aloe vera* samples was investigated. In the ultrasound-assisted extraction method, the values ranged from 69.73-80.97 mg QE/g for *Aloe vera* gel and 157.41-409.20 mg QE/g for *Aloe vera* leaf. In the maceration method, the values were calculated as 44.91-163.79 mg QE/g for *Aloe vera* gel and 117.89-295.18 mg QE/g for *Aloe vera* leaf.

In a study where *Aloe vera* leaf waste was extracted, the total flavonoid content was calculated as 3.48 mg QE/g using the the maceration method and 2.08 mg QE/g using the ultrasound-assisted extraction method (Elferjane et al., 2023). According to this study, the values obtained are quite high. This difference is due to the different parameters of the different parts of the *Aloe vera* plant, extraction conditions, and the solvent used. Similarly, a study conducted by Abdulbasit (2014) revealed that the methanol extract of *A. vera* exhibited the highest total flavonoid content (1958.27 mg QE/100g) compared with 10 different Arabian herbs and spices. In another study, Shashank and Vidhya (2011) reported that the total flavonoid content of *A. vera* methanol's extract was determined to be 14.10 ± 1.60 mg catechin equivalents/g. According to the results of this study, our findings are quite high. The differences in the literature can be attributed to the age of the plant, the region where it is grown, and the extraction conditions.

Table 3. Total flavonoid contents of *Aloe vera* extracts

| Samples | Parts of plant | Solvent Type | Extraction Method | Total Flavonoid content (mg QE/g) |
|---|---|---|---|---|
| **1** | *Aloe vera* gel | Ethanol | Ultrasonic wave-assisted extraction | $74.23^{h} \pm 2.00$ |
| **2** | *Aloe vera*gel | Water | Ultrasonic wave-assisted extraction | $80.97^{h} \pm 1.37$ |
| **3** | *Aloe vera* gel | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $69.73^{h} \pm 6.25$ |
| **4** | *Aloe vera* leaf | Ethanol | Ultrasonic wave-assisted extraction | $157.41^{d} \pm 3.44$ |
| **5** | *Aloe vera* leaf | Water | Ultrasonic wave-assisted extraction | $190.51^{c} \pm 7.16$ |
| **6** | *Aloe vera* leaf | Ethanol: Water (1:1) | Ultrasonic wave-assisted extraction | $409.20^{a} \pm 16.95$ |
| **7** | *Aloe vera*gel | Ethanol | Maceration extraction | $97.66^{g} \pm 3.56$ |
| **8** | *Aloe vera* gel | Water | Maceration extraction | $44.91^{i} \pm 2.31$ |
| **9** | *Aloe vera* gel | Ethanol: Water (1:1) | Maceration extraction | $163.79^{d} \pm 2.19$ |
| **10** | *Aloe vera* leaf | Ethanol | Maceration extraction | $143.29^{e} \pm 0.69$ |
| **11** | *Aloe vera* leaf | Water | Maceration extraction | $117.89^{f} \pm 11.98$ |
| **12** | *Aloe vera* leaf | Ethanol: Water (1:1) | Maceration extraction | $295.18^{b} \pm 12.26$ |

Different superscript letters in the same column indicate a significant difference ($p>0.05$).

According to the results published by Hu, et al. (2003), three-year-old *Aloe vera* plants exhibited significantly higher levels of polysaccharides and flavonoids compared to two- and four-year-old *Aloe vera* plants. Moreover, no significant difference in flavonoid levels was observed between 3- and 4-year -old Aloe vera plants.

### 3.4. Sensory Properties of Ayran with *Aloe vera* Extract

In the study, samples were obtained as follows: Control group (K), ayran with 5% *Aloe vera* gel added (K1), ayran with 2.5% *Aloe vera* gel added (K2), ayran with 3% *Aloe vera* gel added

(K3). The sensory properties of the ayran samples are shown in Table 4. There was no statistically significant difference in the sensory properties of any sample. The added *Aloe vera* gel did not affect the samples negatively in terms of sensory properties. In the pre-shaking appearance scores, the control group had the highest score, followed by the K3, K1, and K2 groups. In the post-shaking appearance, the K and K3 groups scored the highest, followed by the K2 and K1 groups. The same scoring was observed for color. In the consistency feature, K, K3, followed by K2, and K1 according to the score order. In odor scores, the order was K, K3, K2, and K1, while in taste scores, the order was K3, K, K1 and K2. In general liking scores, K, K3, K1, and K2 groups came from the highest to the lowest.

Table 4. Sensory Analysis Results of Ayran Samples

| **Sensory Properties** | **Samples** | | | |
|---|---|---|---|---|
| | **K** | **$K_1$** | **$K_2$** | **$K_3$** |
| **Appearance before shaking** | $8.20^a \pm 1.09$ | $7.60^a \pm 1.51$ | $7.20^a \pm 1.48$ | $8.00^a \pm 1.00$ |
| **Appearance after shaking** | $8.20^a \pm 0.83$ | $7.40^a \pm 1.81$ | $7.60^a \pm 0.89$ | $8.20^a \pm 1.09$ |
| **Color** | $8.00^a \pm 1.22$ | $7.60^a \pm 1.67$ | $7.80^a \pm 0.83$ | $8.00^a \pm 1.00$ |
| **Consistency** | $7.80^a \pm 1.64$ | $7.00^a \pm 1.87$ | $7.00^a \pm 1.87$ | $7.20^a \pm 2.04$ |
| **Smell** | $7.80^a \pm 1.78$ | $6.60^a \pm 2.88$ | $6.80^a \pm 2.58$ | $7.20^a \pm 1.64$ |
| **Taste** | $7.00^a \pm 1.58$ | $6.00^a \pm 2.23$ | $6.00^a \pm 3.24$ | $8.20^a \pm 1.30$ |
| **General appreciation** | $8.20^a \pm 1.09$ | $6.60^a \pm 2.07$ | $6.40^a \pm 2.70$ | $7.80^a \pm 1.78$ |
| **Affordability** | $1.40^a \pm 0.54$ | $2.20^a \pm 0.83$ | $2.00^a \pm 1.00$ | $1.40^a \pm 0.89$ |

K: Control Group (Sample of ayran); $K_1$: Sample of ayran with 5% *Aloe ver)* gel added; $K_2$: Sample of ayran with 2.5 % *Aloe vera* gel added; $K_3$: Sample of ayran with 1.25 % *Aloe vera* gel added.
Different superscript letters within the same row indicate a statistically significant difference (p>0.05).

In the market research, the affordability of the yogurt samples was investigated. Groups K and K3 received the same scores, indicating that the addition of up to 3% *Aloe vera* gel did not significantly impact affordability. However, K2 received a lower score, and K1, with the highest *Aloe vera* gel content (5%), received the lowest affordability score. This suggests that as the proportion of added *Aloe vera* gel increased, the affordability of the yogurt decreased.

Overall sensory scores followed a similar trend to affordability scores. As the proportion of added *Aloe vera* gel increased, the sensory scores decreased. However, the sample

with 1.25% *Aloe vera* gel received the same sensory score as the control group, indicating that this level of addition did not negatively impact sensory properties. The study suggests that adding *Aloe vera* gel to yogurt can enhance its sensory properties, but it may also affect its affordability. The addition of up to 5% *Aloe vera* gel showed positive effects on sensory scores, whereas higher concentrations negatively impacted both sensory and affordability aspects. Further research is needed to determine the optimal level of *Aloe vera* gel addition that balances sensory improvements with cost-effectiveness.

*Aloe vera* is a valuable plant for food applications because of its phenolic and flavonoid components. This study extend the shelf life of ayran and make it functional by natural means using *Aloe vera* gel. In a study by Panesar and Shinde (2012), the use of Aloe vera gel in fortified probiotic yoghurts resulted in a decrease in syneresis values from 4.7% to 8.3% during 28 days of storage at 4°C. pH values decreased from 4.03 to 3.91, *Lactobacillus acidophilus* counts decreased from 39.7x10^9 cfu/ml to 32.1x10^9 cfu/ml, and *Bifidobacterium bifidum* counts decreased from 16.9x10^9 cfu/ml to 7.3x10^9 cfu/ml. The study concluded that yoghurts with *Aloe vera* addition can be used as a sufficient probiotic product as they contain more bacteria than the recommended level.

Mudgil et al. (2016) by; *Aloe vera* juice added to ayran at the rate of 5-20% has a positive effect on the phase in ayran. It has been reported that it reduces separation and increases viscosity. Additionally, 10% *Aloe vera* juice was added to drinking yogurt, which was given the highest score by the panelists, and *Aloe vera* juice supplement was found to be nutritious, It has been emphasized that it improves physochemical and sensory properties. Bassannavar et al. (2014) reported that the addition of Aloe vera gel powder at 0.5% and 1% rates to fermented milk increased angiotensin-converting enzyme (ACE) inhibitor activity and the degree of proteolysis.

Sensory analysis was performed in a fermented yogurt-type milk drink with the addition of aloe vera crystals and granadilla (Passiflora ligularis Juss). Samples with 15% aloe vera and 5% granadilla received the highest scores in the sensory analysis (Gutiérrez-Álzate et al., 2020). The results of this study are similar to those obtained by Wijesundara and Adikari (2017), who studied different yogurt formulations with the addition of aloe vera. In their study, the best sensory results were obtained with the formulation containing 15% aloe vera. A similar result was also found by Govindammal et al. (2017), who showed that yogurt with 15% aloe vera was the most acceptable option. Similarly, in this study, no statistical difference was

observed between the control group and the other samples. The samples with 5% aloe vera added had the highest purchase intention.

## 4. CONCLUSION

This study aimed to determine the extraction conditions of *Aloe vera* that would yield the highest total phenolic content, DPPH scavenging activity, and total flavonoid content. For the *Aloe vera* gel, ultrasonic-assisted extraction with water as the solvent was the most effective. For *Aloe vera* leaves, ultrasonic-assisted extraction with a solvent mixture of ethanol and water (1:1) was the most effective. Sensory analysis showed that *Aloe vera* gel was acceptable in ayran (a yogurt-based beverage) at a concentration of up to 5%. These findings provide valuable information for the industrial extraction of bioactive compounds from *Aloe vera*. The use of *Aloe vera* gel in ayran is a promising application that could be further explored. Future research should investigate the use of different *Aloe vera* forms in various food products to expand its use in the food industry.

## DECLARATIONS

There is no conflict of interest between the authors.

## AUTHORS' CONTRIBUTIONS

The author contributes the study on his/her own.

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# Determination of the Impact of Mating on Stress Protein in Different Honey Bee Breeds

# Farklı Bal Arısı Irklarında Çiftleşmenin Stres Proteini Üzerine Etkisinin Belirlenmesi

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## Abstract

The queen is the only individual with a colony of bees and the ability to reproduce. This study determined the level of stress proteins (Hsp70) in mated and virgin queens reared under the same conditions in colonies of honey bee breeds and ecotypes in Turkey. When the effects of mating were examined, it was found that the stress protein content of mated queens was significantly lower than that of non-mated queens ($p<0.05$). It was also found that Hsp70 stress protein levels were lower in Thrace, Yığılca and Gökçeada ecotypes, which are the sub-ecotypes of the Anatolian bee, compared to other ecotypes and races. As a result, it was found that the adaptive abilities of Trakya, Gökçeada and Yığılca ecotypes, which are the sub-ecotypes of the Anatolian bee found in our country, are better than those of other subspecies.

**Keywords:** Honey bee, Queen**, S**ubspecies, Mating effect, Stress protein (Hsp70)

**Özet**

Ana arı bir koloninin devamlılığını sağlayan ve üreme yeteneği olan tek bireydir. Bu çalışmada Türkiye'de bulunan bal arısı ırk ve ekotiplerine ait kolonilerde aynı koşullarda yetiştirilen çiftleşmiş ve çiftleşmemiş ana arılarda stres proteini (Hsp70) seviyesi belirlenmiştir. Çiftleşmenin etkisine bakıldığında çiftleşmiş ana arıların stres proteini seviyesi çiftleşmemişlere göre önemli derecede daha düşük olduğu belirlenmiştir ($p<0,05$). Ayrıca yetiştirme koşullarında Anadolu arısının alt ekotipleri olan Trakya, Yığılca ve Gökçeada ekotiplerde Hsp70 stres proteini seviyesinin diğer ekotip ve ırklara göre daha düşük olduğu tespit edilmiştir. Sonuç olarak ülkemizde bulunan Anadolu arısının alt ekotipleri olan Trakya, Gökçeada ve Yığılca ekotiplerinin adaptasyon yeteneklerinin diğer alt türlere göre daha iyi olduğu belirlenmiştir.

**Anahtar Kelimeler:** Bal arısı, Ana arı, Irk ve ekotipler, Çiftleşme etkisi, Stres proteini (Hsp70)

## 1. INTRODUCTION

The world is home to a diverse range of honey bee breeds. These breeds have adapted to the environmental conditions in which they live and display diversity in their morphological, behavioral, and yield features. They have also adapted to the environmental conditions in which they exist. Tens of thousands of years ago, honey bees were already present in the Anatolian region. Because of this, they have differentiated in order to adapt to the diverse environmental conditions. According to the findings of a number of researchers (Bodenheimer, 1942; Fıratlı, 1987; Sonmez & Settar 1987; Ruttner, 1988; Kandemir et al., 2000), the region of Anatolia is home to a wide variety of honey bee ecotypes and breeds. The Anatolian bee, also known as *Apis mellifera anatoliaca*, the *Caucasian* bee, also known as *Apis mellifera caucasica*, the Syrian bee, also known as *Api mellifera syriaca*, and the Carniole bee, also known as *Apis mellifera carnica*, are the five species of *Apis mellifera* (Kandemir et al., 2000). In addition to the five distinguishable bee breeds that have already been mentioned, regional ecotypes of bees that are specialized in terms of the morphological and genetic characteristics that they exhibit have also emerged in Turkey as a result of the country's diverse flora and climate structures across its various regions. According to Kekecoglu (2010), some of these ecotypes include the Muğla, Gökceada, native Hatay, Yığılca, Trakya, and Efe ecotypes.

A honeybee colony consists of a queen, tens of thousands of worker bees, and hundreds of drones. The number of worker bees in a colony is what determines its population. This number varies depending on the time of the year when the, a queen's ability to lay eggs, the abundance of nectar and pollen sources, the level of environmental stress, and the presence of parasites and other harmful organisms within the colony. According to Köseoğlu et al., (2017), the only individual capable of maintaining a colony as well as having the ability to breed is the queen bee. The quality of queen bees can be affected by a variety of circumstances. These

factors include the selection of the genotype, the supply of breeding material, the breeding method, the status of the starter colony, the age and number of larvae, the breeding season, the number of drones, and the nutritional status (Şahinler & Kaftanoğlu, 1997; Doğarolu, 2004; Cengiz et al., 2019; Arslan & Cengiz, 2020; Arslan et al., 2020). Doğarolu (2004) and Şahinler & Kaftanoğlu (1997), list these factors in their respective works. Honey bees are one of the most beneficial insects on Earth with both their critical roles in pollination and health-promoting products (Oskay et al., 2023). There have been few studies conducted on the subject of the link between the effects of agricultural factors on colony performance and stress reactions (Hranitz et al., 2009). Climate change is one of the most important issues in the 21st century. As a result of the increase in temperature, various changes in our climate may occur, such as changing precipitation distribution and the frequency of severe weather events. It is estimated that global warming will raise sea levels by several meters by the end of this century, and hurricanes and heat waves will be more frequent than now (Barth & Titus, 1984; Adediran et al., 2023; Oskay & Sönmez, 2023). According to many researchers, climate change is considered dangerous for many living species and biodiversity (Mahakunda & Tiwari, 2024). The first requirement for productive and profitable beekeeping is to deal with robust colonies that are led by queen bees that are still young and of excellent quality. When compared to weak colonies managed by inadequate and low-quality queens, productive output per colony is significantly higher in robust colonies managed by quality queens (Öztürk, 2014). This is something that should be taken into consideration. It is necessary for queens to mate in order to ensure the development of the colony and the continuation of production. Bees experience both short-term and long-term changes in their physiology and behavior as a result of mating. On the other hand, not a lot of research has been done to investigate the molecular pathways that are responsible for these post-mating alterations (Kocher et al., 2008). After mating, the queen's pheromone profile as well as the size of her ovaries and the maturity of her eggs undergo dramatic alterations (Plettner et al., 1997; Tanaka et al., 2004; Richard et al., 2007). Dopamine and N-acetyldopamine levels fell after mating while this was all occurring (Fahrbach et al., 1995; Harano et al., 2005).

Excessive secretion of the hormone dopamine is known to result in a stress reaction (Harris & Woodring, 1992). HSP 70 is the most widely utilized biomarker for monitoring honey bee stress. Hsp70, one of the heat shock proteins, functions as a molecular chaperone to protect cells against the deleterious consequences of protein denaturation under unfavourable circumstances. The stress protein (Hsp 70) is a system reaction that supplies the organism's stress resistance (Feder & Hofmann, 1999). Under unfavorable conditions, the expression of

Hsp70 (Ashburner, 1982), which has been documented in several animal taxa, including insects, promotes cell survival and tolerance to stress. Due to the division of work within the colony in creating the equilibrium of the hive and external influences, the complex social structure of honey bees offers a broad variety of reaction options. The worker bees' contribution to the dynamics of the hive must be balanced against the strain of colony loss periods. Stress proteins are a crucial component of the cellular-molecular response system to several environmental stressors. (Hranitz et al., 2019) The honey bee colony maintains the hive's climatic equilibrium as a collective in order to compete and thrive against specialized rivals in vastly varied environmental circumstances. As can be seen, bees employ a variety of defense systems to mitigate the damage that environmental stress might produce (Goulson et al., 2015; Li et al., 2018). Numerous biotic and abiotic stressors, including diseases (parasites, fungus, viruses, and bacteria), ecosystem changes or losses, and the use of agrochemicals, have a detrimental impact on the health and lifespan of the bee colony, either individually or in combination. All of these elements alter the bees' immune system and defensive systems (Bruutcher et al., 2015; Li et al., 2018; Larsen et al., 2019). In recent years, a number of researchers have been conducted on the origins of stress and possible preventative measures for honey bees; improper colony management, bee disease and pests, frequent colony transfers, rapid climatic changes, and flora deficit are just a few examples (Topal et al., 2019).

In this work, we assessed, for the first time, the effects of adaptation to environmental circumstances and mating on stress protein (Hsp 70) in queens generated from several honey bee subspecies and ecotypes maintained under identical conditions.

## 2. MATERIALS and METHOD

### 2.1. Queen Bees Breeding

Queen bees of different breeds and ecotypes used in the study obtained from Directorate of Aegean Agricultural Research Institute Efe bee ecotype; Macahel Beekeeping Caucasian Camili ecotype; Macahel Beekeeping Caucasian Posof ecotype; Muğla Beekeepers Association Muğla bee ecotype, Kırklareli Beekeepers Association Trakya bee ecotype; Erdoğan Çıracı Queen Bee Enterprise Yığılca ecotype, Gökçeada Beekeepers Association Gökçeada bee ecotype, Macahel Beekeeping Anatolian bee breed, Hatay Mustafa Kemal University Hatay bee breed. These queen bees were kept in the town of Ordu, Perşembe during the whole study.

Every three days, the breeding materials that were employed in the study were given sugar syrup at a ratio of 1:1 as part of their diet. A random sampling was carried out after the

queen bees that had returned from their mating flight had started to lay eggs and had sealed their chambers. Dissection was carried out on virgin queen bees that had just emerged and the bees were taken to the laboratory immediately.

### 2.2. Stress Protein (Hsp 70) Analysis

Queens of different honey bee Subspecias and ecotypes were brought under Ordu conditions and included in nucleus colonies. Queens were bred from these breeding colonies and mated with them, and the virgin queens were taken to the laboratory and kept in a freezer at -20 °C until the bee brains were removed.

Brain tissue was extracted from bee samples for study using a microscope and dry ice and then transferred to centrifuge tubes. The brain tissue in these tubes was homogenized and extensively crushed using PBS-azide-TAME buffer. Following centrifugation at 13,000 x g for 20 minutes at 4°C, total protein concentrations in the supernatant were determined using a protein assay kit (#5000112, Bio-Rad, Hercules, CA, USA). According to the standards (Hranitz et al. 2010; Güneş et al. 2017), the HSP 70 values in 2000 ng total protein were read, the appropriate dilutions were produced according to the total protein values, and the concentrations of the samples were determined. We utilized H5147-Sigma-Aldrich Monoclonal Anti-Heat Shock Protein 70 antibody as the primary antibody for coated Eliza, while our secondary antibody is Goat Anti-Mouse IgG (H + L)-HRP Conjugate #1706516 (Hranitz et al., 2010; Güneş et al., 2017; Sarıoglu –Bozkurt et al., 2022).

### 2.3. Statistical Analysis

Using the IBM SPSS Statistics 23 application, the gathered data were categorized by bee species. The data's normality was evaluated and determined to be less than P=0.05. Since the non-normal data were statistically evaluated with the non-parametric Kruskal- Wallis test and there were nine distinct groups, mean values and standard deviations were calculated.

## 3. RESULTS and DISCUSSION

In accordance with the reported Hsp-70 levels, virgin bees were observed to be more stressed than virgin bees in general. When comparing mated and virgin bees within the Yığılca Bee, Caucasian Posof Bee, and Caucasian Camili Bee populations, $p<0.05$ was shown to be statistically significant (Table 1). ($P<0.05$ was found to be significant between the different letters in the different columns.

In all breeds, the pre-breeding values were greater than the post-breeding values. Statistically significant, however, are just three bee breeds. Trakya, Yığılca, and Gokceada ecotypes, which are subecotypes of the Anatolian bee, have a lower Hsp70 stress protein concentration, as revealed by the research. During the study, it was revealed that queens from these breeds and ecotypes had shorter queen acceptance rate, mating flight, and egg-laying periods than queens from other breeds and ecotypes, as well as fewer aggressive tendencies. The Caucasian breed has a higher queen acceptance rate, longer mating flight duration, and longer egg-laying period compared to other breeds.

Table 1. According to bee breeds, the HSP 70 levels of mated and virgin bees were evaluated

| **Bee breed** | **HSP-70 (ng/ul)** | |
|---|---|---|
| | Virgin | Mating |
| Muğla Bee | 19.20 ± 2.76 | 18.86 ± 2.83 |
| Anatoliaca Bee | 19.95 ± 2.72 | 18.24 ± 2.98 |
| Hatay Bee | 23.32 ± 2.21 | 18,85 ± 2,34 |
| Gökçeada Bee | 19.79 ± 2.95 | 12.48 ± 2.15 |
| Efe Bee | 21.08 ± 2.66 | 15.86 ± 2.76 |
| Trakya Bee | 14.61 ± 2.92 | 8.15 ± 2.36 |
| Yığılca Bee | 23.96 ± 1.19[a] | 10.34 ± 2.74[b] |
| Causica Posof Bee | 23.77 ± 2.05[a] | 14.92 ± 1.01[b] |
| Causica Camili Bee | 23.21±2.77[a] | 15.14 ± 2.26[b] |

Although not statistically significant, the Trakya Bee was found to have the lowest Hsp70 levels both before and after mating. This is the most ideal bee for adapting to the circumstances of the Ordu province. When our findings are analyzed, it is possible to conclude that the statistically significant bees can adjust to their new environment more readily than other varieties. It was discovered by Sarioglu et al. (2021) that the stress reaction of queenless colonies was greater than that of queenright colonies. According to the same study, the stress levels of the starting bees given sugar syrup were greater than those of the finisher bees. The low Hsp70 values in bees corroborate our findings that dopamine levels decline after mating, as demonstrated by research (Harris & Woodring, 1992) (Figure 1).

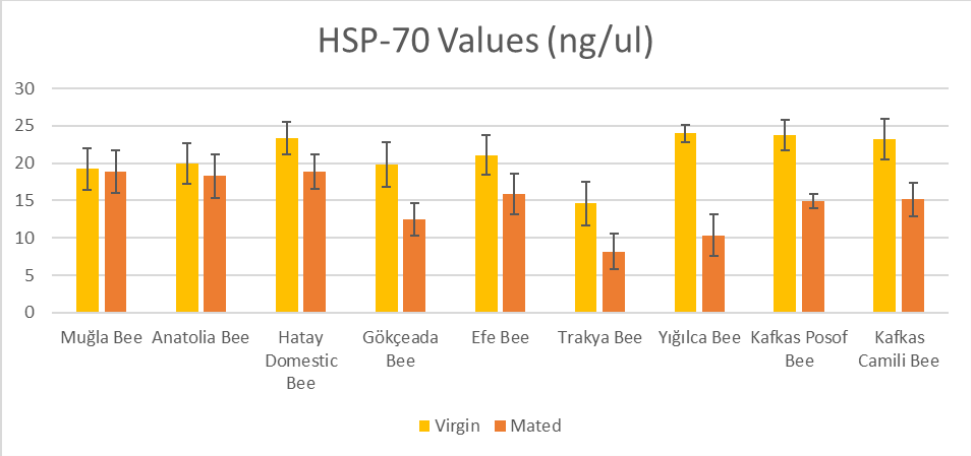


Figure 1. Differences between the HSP 70 values of mated and virgin queen according to mating queen

## 4. CONCLUSIONS

As a result of its diverse climate and flora, Turkey is a rich source of racial ecotypes for bee genes. In terms of being the mother of all members of the colony, the queen bee is the most important individual in the honeybee colony. All genetic features (chitin color, disease resistance, swarming, etc.) shown by a colony are due to the genes of the queen. Having queen bees of excellent quality in the colonies is a need for the desired efficiency/benefit in beekeeping. In this study, various breeds and ecotypes of queen bees from Turkey were maintained in Ordu. The environmental adaptability capabilities of these queens and the effect of mating on Hsp 70 stress protein were determined. According to the findings of the study, it was discovered that the ecotypes acclimated to varied geographical circumstances, and not the queen bees of different breeds, had superior adaptation capacities. In all breeds and ecotypes, Hsp70 stress protein levels were found to be lower in mated queens. As a result of the investigation, it was discovered that honey bee ecotypes in our nation had superior adaptability. The levels of stress protein (Hsp70) in Trakya, Yığılca, and Gokceada ecotypes were determined to be (8.15 ± 2.36), (10.34±2.74) and (12.48±2.14), respectively.

## DECLARATIONS

The authors have no conflicts of interest to declare.

## AUTHORS' CONTRIBUTIONS

The author contributes the study on his/her own.

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# *Work Accidents, Occupational Diseases, and Lost Workdays in Türkiye's Forestry Sector: Increasing Risks and Improvement Proposals for the 2019-2023 Period*

# *Türkiye'de Ormancılık Sektöründe İş Kazaları, Meslek Hastalıkları ve İş Günü Kayıpları: 2019-2023 Dönemi İçin Artan Riskler ve İyileştirme Önerileri*

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## Abstract

In this study, data regarding occupational accidents and diseases, as well as workday losses in the forestry sector in Turkey between 2019 and 2023, have been thoroughly analysed using official statistical annual reports from the Social Security Institution. In 2019, there were 27,025 employees in the sector, which increased by 31.42% to 35,517 by 2023. Alongside the rise in the number of employees, occupational accidents in the sector have also increased year by year, peaking in 2022 with 860 accidents, 14 of which resulted in fatalities. Over the five-year period, only one case of occupational disease was reported, indicating significant deficiencies in the identification and reporting of health risks within the sector. The increase in the number of lost workdays due to occupational accidents and diseases reached 11,125 in 2022, particularly during the COVID-19 pandemic, adversely affecting productivity in the sector. Consequently, this study suggests that the forestry sector in Turkey needs to enhance its preparedness for emergencies and develop comprehensive occupational health and safety policies along with crisis management practices.

**Keywords:** Occupational accidents, Occupational diseases, Forestry sector, Occupational health and safety, Lost workdays

**Özet**

Bu çalışmada, Türkiye'de ormancılık sektöründe 2019-2023 yılları arasındaki beş yıllık dönem içerisinde meydana gelen iş kazaları ve meslek hastalıkları ile işgünü kayıplarına ait veriler, Sosyal Güvenlik Kurumu'nun resmi istatistik yıllıklarından yararlanılarak analiz edilmeye çalışılmıştır. 2019 yılında sektörde 27.025 çalışan bulunurken, bu sayı %31,42 artışla 2023 yılında 35.517'ye yükselmiştir. Çalışan sayısının artmasına paralel olarak sektördeki iş kazalarının da yıldan yıla arttığı, 2022 yılında 860 kazayla zirveye ulaştığı ve bu kazaların 14'ünün de ölümle sonuçlandığı tespit edilmiştir. Beş yıllık dönemde yalnızca bir meslek hastalığı vakası raporlanmıştır ve bu durum sektörde sağlık risklerinin tanımlanması ve raporlanmasında ciddi eksiklikler olduğunu ortaya koymaktadır. İş kazaları ve meslek hastalıkları nedeniyle kaybedilen işgünü sayısındaki artış, özellikle COVID-19 pandemisi sırasında 2022 yılında 11.125 işgününe ulaşmış ve bu durum sektörde verimliliği olumsuz yönde etkilemiştir. Sonuç olarak bu çalışma, Türkiye'de ormancılık sektörünün acil durumlara karşı daha güçlü bir hazırlık yapması gerektiğini ve kapsamlı iş sağlığı ve güvenliği politikaları ile kriz yönetimi uygulamalarının geliştirilmesini önermektedir.

**Anahtar Kelimeler:** İş kazaları, Meslek hastalıkları, Ormancılık sektörü, İş sağlığı ve güvenliği, İşgünü kaybı

**Abbreviations:** OHS, Occupational health and safety; PPE, Personal protective equipment; SGK, Social security institution; ESAW, European statistics on accidents at work.

## 1. INTRODUCTION

The forestry sector in Türkiye plays a crucial role in the sustainable management of the country's natural resources and economic development. However, this sector carries high risks regarding work accidents and occupational diseases. Forestry activities are physically demanding and hazardous, conducted over vast geographic areas under variable conditions. Therefore, this sector's occupational health and safety (OHS) issues are essential. Workers in the forestry sector are exposed to various climates, topographies, and vegetation types, which increase the risk of accidents and occupational diseases (Akay et al., 2023).

The Occupational Health and Safety Law No. 6331 regulates OHS practices in Türkiye, which imposes significant responsibilities on employers and employees. The law mandates the identification of hazards and risks, the implementation of preventive measures, and the application of continuous improvement processes in workplaces (İnanç & Ağyürek, 2019). However, the unique working conditions and challenges of the forestry sector can limit the effectiveness of OHS practices. For instance, forestry workers are often seasonal labourers, facing low wages and inadequate working conditions (Özden et al., 2011).

Work accidents and occupational diseases in the forestry sector are commonly caused by working with cutting tools and machinery. Injuries from tools like chainsaws and axes are among the most frequent work accidents. Furthermore, the low use of personal protective equipment (PPE) makes it challenging to prevent severe and fatal accidents (Top et al., 2016; Yoshimura & Acar, 2004). Working with high-noise-producing machinery, such as chainsaws, adversely impacts health and reduces both productivity and safety on the job. Additionally, the development of occupational diseases poses a significant financial burden not only for the worker but also for the employer and the state (Albayrak et al.,2023). Job satisfaction among workers is also low, which increases the risk of work accidents and occupational diseases (Top et al., 2016).

The COVID-19 pandemic has again highlighted the importance of OHS issues in the forestry sector. A study on the perceptions of OHS among workers in the forest products industry during the pandemic revealed that workers' awareness of OHS issues increased, but this awareness needs to be sustained. Being prepared for crises like the pandemic and implementing new practices are essential for the continuous improvement of OHS in the forestry sector (Kırklıkçı & Bayram, 2024).

In Türkiye's forestry sector, occupational health and safety are critical for workers' safety and well-being and enterprises' productivity and sustainability. Effective management of OHS practices is necessary to prevent work accidents and occupational diseases and to enhance workers' job satisfaction. In this context, further research and improvement of OHS in the forestry sector are paramount for the future of workers and businesses (Küçükarslan et al., 2023).

This study aims to examine work accidents, occupational diseases, and lost workdays in Türkiye's forestry sector and its sub-activities—forest cultivation (silviculture) and other forestry activities, logging, the gathering of non-wood forest products, and support activities for forestry—using data from the Social Security Institution (SGK). Studies on the prevalence of work accidents and the risk factors causing these accidents in Türkiye's forestry sector reveal the dangerous nature of the industry. In a survey conducted within the boundaries of the Trabzon Forest Directorate, the annual accident frequency rate was 30.4%, with an accident incidence of 2052.9. Factors such as hook use, smoking, and the number of breaks taken were identified as increasing the risk of work accidents (Enez et al., 2014). Another study in the Western Black Sea region indicated that personal and organisational factors were the primary

contributors to fatal work accidents. The study found that being in a dangerous area, negligence, and irregular behaviour led to deadly accidents (Melemez, 2015).

The results of work accidents in the forestry sector are striking compared to other industries. An analysis of the 2008-2018 period revealed that the incidence rate and frequency rate of work accidents in the forestry sector accounted for 41.1% and 40.8% of all sectoral values, respectively. These rates are more favourable than the metalworking, mining, and construction sectors but more unfavourable than the textile industry (Akay et al., 2023).

Research on the causes and consequences of work accidents in the forestry sector shows that many of these accidents stem from personal and organisational factors. For instance, the leading causes of fatal work accidents in forest harvesting operations in Türkiye include being in a dangerous area, negligence, irregular behaviours, and improper worker selection (Melemez, 2015).

This situation demonstrates the need for further measures regarding occupational safety and health in the forestry sector. Work accidents and occupational diseases not only threaten the health of workers but also lead to lost workdays, which represent a significant economic burden for both workers and employers. To prevent lost workdays and occupational diseases in the forestry sector, it is necessary to increase safety training, promote the use of appropriate equipment, and conduct regular health check-ups.

Examining work accidents, occupational diseases, and lost workdays in the forestry sector and its sub-activities in Türkiye is essential for improving safety in the industry and protecting workers' health. This study will evaluate the industry's current situation based on SGK data and discuss the necessary measures for improving occupational safety. Through this analysis, it aims to contribute to the creation of safer and healthier working conditions in the forestry sector.

## 2. MATERIALS and METHOD

According to the Social Insurance and General Health Insurance Law No. 5510, work accidents and occupational diseases cover only insured individuals (SGK, 2006). Therefore, this study utilises data from the statistical yearbooks of the Turkish Social Security Institution (SGK) as its primary data source. The study examines the statistics related to “work accidents and occupational diseases,” “statistics on periods of incapacity for work,” and “statistics on insured employees and workplaces” from the five years between 2019 and 2023, aiming to analyse work accidents, occupational diseases, and days of incapacity for work in Türkiye’s Forestry

and Industrial Wood Production sector among insured employees. After 2017, due to the classification of employees under Law No. 5510 into two categories, 4a and 4b, the data for the five years from 2019 to 2023 includes the total for these two groups. The definitions of the concepts examined in this study are provided below.

Work Accident: A work accident is defined as an event that occurs while the insured person is present at the workplace and causes immediate or later physical or mental harm to the insured. It also includes events that occur outside the workplace while the insured is performing tasks assigned by the employer or working independently on behalf of themselves and cause immediate or later physical or mental harm. Additionally, a work accident can occur if an insured employee, under the orders of the employer, is sent to another location for work-related duties and, during the time not engaged in their primary task, experiences an event that causes immediate or later physical or mental harm. This also includes accidents occurring during the time provided to nursing mothers to breastfeed their children and accidents that happen while the insured is commuting to and from work using transportation provided by the employer (5510 Sayılı Kanun, 2006).

Work Accident Frequency Rate: This indicates how many insured employees out of 100 full-time workers experience a work accident. The formula is as follows:

$$Work\ Accident\ Frequency\ Rate\ =\ IAF\ /\ (Total\ Working\ Hours)\ *\ 225.000$$

In this formula, IAF refers to the number of insured employees who have experienced work accidents. In contrast, Total Working Hours refers to the product of the total number of employees and 2.250 hours, assuming a full-time employee works 45 hours per week for 50 weeks a year. The factor of 225.000 is the calculated coefficient for 100 insured full-time employees working 45 hours per week for 50 weeks in a year (Akyüz et al., 2016; Aritan & Ataman, 2017).

Fatal Work Accident: According to the definition adopted by the European Statistics on Accidents at Work (ESAW) project, “fatal work accidents are accidents that result in the death of an insured worker within one year following the accident” (Erginel & Toptancı 2017).

Occupational Disease: An occupational disease refers to any temporary or permanent illness, physical, or mental disability resulting from repeated exposure or working conditions specific to the nature of the insured's job (5510 Sayılı Kanun, 2006).

Incapacity for Work: Incapacity for work refers to when an injured employee cannot work due to a work accident. Temporary incapacity for work refers to the number of days the

insured cannot work, as indicated by medical reports from doctors or health boards authorised by SGK, due to work accidents, occupational diseases, illness, or maternity. Permanent incapacity for work refers to the number of insured individuals who, due to a work accident or occupational disease, have been found to have lost at least 10% of their earning capacity, as determined by medical boards of SGK-authorized health institutions, based on reports issued by these boards (5510 Sayılı Kanun, 2006).

## 3. RESULTS and DISCUSSION

In 2019, there were 3.367 workplaces in the forestry sector in Türkiye, both public and private. By 2023, this number decreased by approximately 0.65%, falling to 3.345. Table 1 shows the number of workplaces in the forestry and industrial wood production sector annually (SGK, 2024).

Table 1. Number of workplaces in the forestry sector by year

| Year | Permanent | Temporary | Public | Private | Total |
|---|---|---|---|---|---|
| 2023 | 1.222 | 2.123 | 454 | 2.891 | 3.345 |
| 2022 | 709 | 3.959 | 472 | 4.196 | 4.668 |
| 2021 | 1.044 | 3.369 | 449 | 3.964 | 4.413 |
| 2020 | 957 | 3.298 | 442 | 3.813 | 4.255 |
| 2019 | 886 | 2.481 | 433 | 2.934 | 3.367 |

Parallel to this decrease in the number of workplaces, the total number of insured employees in the forestry sector, which was 27.025 in 2019 (3.416 women and 23.609 men), increased to 35.517 in 2023 (3.931 women and 31.586 men), showing an increase of approximately 31.42% over the five years. Table 2 shows the number of employees in the forestry and industrial wood production sector by year (SGK, 2019; SGK, 2020; SGK, 2021; SGK, 2022; SGK, 2023).

Table 2. Number of employees in the forestry sector by year

| Year | Permanent | Temporary | Public | Private | Men | Women | Total |
|---|---|---|---|---|---|---|---|
| 2023 | 22.367 | 13.150 | 24.049 | 11.468 | 31.586 | 3.931 | 35.517 |
| 2022 | 26.705 | 19.036 | 29.896 | 15.845 | 40.173 | 5.568 | 45.741 |
| 2021 | 19.792 | 18.513 | 22.406 | 15.899 | 33.064 | 5.241 | 38.305 |
| 2020 | 17.794 | 16.785 | 20.023 | 14.556 | 30.048 | 4.531 | 34.579 |
| 2019 | 13.541 | 13.484 | 14.696 | 12.329 | 23.609 | 3.416 | 27.025 |

Between 2019 and 2023, 3.375 work accidents occurred in the forestry sector, 60 of which resulted in death. The highest number of work accidents during these five years occurred in 2022, with 860 work accidents, 14 of which resulted in death. Table 3 shows the number of work and fatal accidents per year in the forestry and industrial wood production sector (SGK, 2019; SGK, 2020; SGK, 2021; SGK, 2022; SGK, 2023).

Table 3. Number of work accidents and fatal work accidents in the forestry sector by year

| Year | Number of Work Accidents | Number of Fatal Work Accidents |
|---|---|---|
| 2023 | 792 | 12 |
| 2022 | 860 | 14 |
| 2021 | 705 | 12 |
| 2020 | 508 | 13 |
| 2019 | 510 | 9 |

The work accident frequency rate, calculated per 100 full-time employees, was lowest in 2020 at 1.47 and highest in 2023 at 2.23. Figure 1 shows the work accident frequency rates in the forestry and industrial wood production sector by year (SGK, 2019; SGK, 2020; SGK, 2021; SGK, 2022; SGK, 2023).

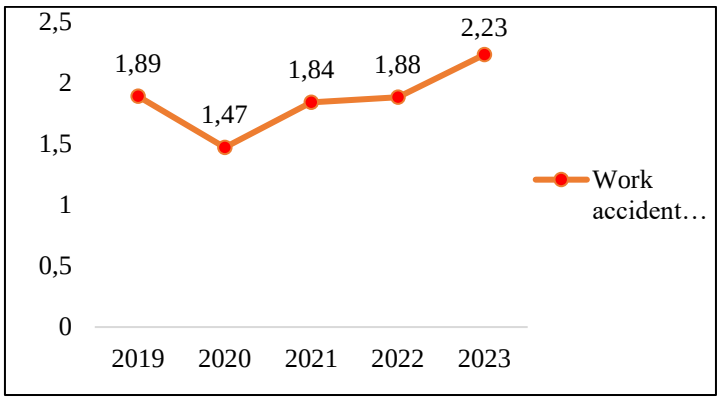


Figure 1. Work accident frequency rates in the forestry sector by year

Between 2019 and 2023, only one occupational disease was reported in the forestry sector, which did not result in death. Table 4 shows the number of occupational and fatal diseases per year in the forestry and industrial wood production sector (SGK, 2019; SGK, 2020; SGK, 2021; SGK, 2022; SGK, 2023).

Table 4. Number of occupational diseases and fatal occupational diseases in the forestry sector by year

| Year | Number of Occupational Diseases | Number of Fatal Occupational Diseases |
|---|---|---|
| 2023 | 0 | 0 |
| 2022 | 0 | 0 |
| 2021 | 0 | 0 |
| 2020 | 1 | 0 |
| 2019 | 0 | 0 |

Between 2019 and 2023, forestry sector employees took 38.218 temporary incapacity leave due to work accidents, including 35.867 days treated as outpatients and 2.351 days spent as inpatients. The highest number of days of incapacity was recorded in 2022, with 10.377 days, mainly due to the impact of the COVID-19 pandemic. Table 5 shows the number of days of temporary incapacity due to work accidents in the forestry and industrial wood production sector by year (SGK, 2019; SGK, 2020; SGK, 2021; SGK, 2022; SGK, 2023).

Table 5. Number of days of temporary ıncapacity due to work accidents in the forestry sector by year

| Year | Outpatient Treatment | Inpatient Treatment | Total |
|---|---|---|---|
| 2023 | 7.566 | 595 | 8.161 |
| 2022 | 10.377 | 748 | 11.125 |
| 2021 | 7.218 | 465 | 7.683 |
| 2020 | 5.728 | 265 | 5.993 |
| 2019 | 4.978 | 278 | 5.256 |

In Türkiye, a total of 2.590.007 work accidents occurred between 2019 and 2023, resulting in the deaths of 7.275 employees. Additionally, 5.168 employees were diagnosed with occupational diseases, and 48 employees lost their lives due to occupational diseases during this period (SGK, 2019, 2020, 2021, 2023). Considering only 2023, in which 681.655 work accidents and 1.972 fatal ones were recorded, the SGK data suggests that a work accident occurs approximately every 46 seconds in Türkiye, and five employees lose their lives each day due to work accidents.

This study examined data related to occupational health and safety (OHS) in the agricultural sector in Türkiye between 2019 and 2023, and the current situation in the Forestry and Industrial Wood Production Sector was evaluated based on the findings. The results indicate that OHS practices in this sector are inadequate and need improvement.

The data reveals an increasing work accident over the years. The 3.375 work accidents between 2019 and 2023 show that employees in this sector face significant risks. Sixty of these accidents resulted in death, with the highest number of accidents (860) and fatalities (14) recorded in 2022. On average, 1.86 out of every 100 forestry sector employees experienced a work accident during the five years. The rising trend in work accidents highlights the insufficiency of OHS measures in the forestry and industrial wood production sectors. It emphasises the need for more comprehensive policies in this area.

Common accidents in the forestry sector include operator errors or technical malfunctions when using dangerous machinery such as chainsaws, tractors, and cranes, falls from heights during tree felling, pruning, or transportation, trees falling on workers, slips, trips, and falls on uneven, slippery, or muddy terrain in forested areas, and machinery overturning or workers being crushed during the transport of cut trees or wood. Workers' education levels should be improved, safety protocols should be tightened, and modern equipment should be encouraged to reduce such accidents.

Regarding occupational diseases, only one case was reported during the five years. Based on the literature, the expected reporting rate for occupational diseases ranges from 0.4 to 1.2 per thousand annually. Still, the average reporting rate for occupational diseases in Türkiye is around 0.04 per hundred thousand (Keçeci, 2020). The low number of reported cases suggests that occupational diseases are not being adequately diagnosed or reported. Forestry sector employees are exposed to various health risks, including dust exposure, biological agents, and heavy physical workloads. Therefore, regular health screenings and training programs are crucial for the early detection and prevention of occupational diseases.

An analysis of temporary incapacity periods shows that employees took 38.218 days of temporary incapacity leave during the five years. The highest number of days (11.125) was recorded in 2022, mainly due to the impact of the COVID-19 pandemic. This indicates the sector's vulnerability to unexpected events such as pandemics and highlights the need to strengthen emergency response plans and health measures. Developing and effectively implementing emergency management plans is essential to mitigate global health crises' effects on sector employees like the pandemic.

## 4. CONCLUSION

This study thoroughly analysed occupational accidents, diseases, and lost workdays in Türkiye's forestry sector between 2019 and 2023, revealing the occupational health and safety (OHS) challenges the sector faces and the necessary measures to address these challenges. The data show a year-over-year increase in work accidents, peaking in 2022 with 860 accidents, 14 of which were fatal. This trend, combined with the inherently high-risk nature of the sector, clearly indicates that current OHS measures are insufficient and that more comprehensive policies need to be developed in this area.

The fact that only one case of occupational disease was reported over the five-year period highlights serious deficiencies in the identification and reporting of health risks. Compared to the expected reporting rate for occupational diseases, this figure is significantly low, suggesting that health risks such as dust, biological agents, and heavy physical labor faced by sector employees may be overlooked.

Moreover, the increasing number of workdays lost due to work accidents and occupational diseases has negatively impacted sector productivity. In particular, the COVID-19 pandemic resulted in 11,125 lost workdays in 2022, underscoring the sector's vulnerability to crises. The pandemic period emphasized the need for stronger preparedness for emergencies and the importance of enhancing OHS policies to include crisis management components.

One of the reasons for occupational accidents is the lack of a desired level of safety culture. It is anticipated that employing workers with higher education levels, increasing training activities, improving management’s attitudes and behaviors, eliminating the notion of fatalism, taking measures to enhance awareness and competence, ensuring active employee participation in safety, establishing effective communication, and developing a reporting culture through a strong reporting system will contribute to reducing workplace accidents. Additionally, the effective implementation of occupational health and safety (OHS) practices is expected to foster a positive safety culture within organizations and facilitate its dissemination among all employees (Albayrak & Tuna, 2021).

In light of these findings, several policy recommendations can be developed to improve occupational health and safety in the forestry sector. First, there is a need to increase OHS training for workers and promote modern and safe equipment use. Training programs should include the safe operation of dangerous machinery and emergency response procedures. In addition, steps should be taken for regular health screenings and the early diagnosis of

occupational diseases, ensuring that workers are better protected against health risks. To reduce work accidents and occupational diseases, stricter enforcement of OHS regulations and increased inspections are also crucial.

These improvements will protect workers' health and safety and contribute to the sector's sustainability. Improving the working conditions of seasonal forestry workers, such as providing adequate accommodation and hygiene facilities, is also essential from an occupational health and safety perspective.

In conclusion, occupational health and safety in the forestry sector are vital for protecting workers' health and well-being and ensuring the industry's sustainability. The findings of this study highlight the necessary measures to be taken and the areas that need improvement in OHS in Türkiye's forestry sector, providing insight into future research in this area. Future studies that use more extensive data sets and examine the sector's various subfields in more detail will contribute to developing more comprehensive and effective OHS policies.

## DECLARATIONS

There is no conflict of interest between the authors.

## AUTHORS' CONTRIBUTIONS

The author contributes the study on his/her own.

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# *Antioxidant and Antiapoptotic Effects of Primula vulgaris L. Against Methotrexate-Induced Testicular Damage in Rats*

# *Primula vulgaris L.'nin Sıçanlarda Metotreksat Kaynaklı Testis Hasarına Karşı Antioksidan ve Antiapoptotik Etkileri*

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## Abstract

The aim of this study was to investigate the antioxidant and antiapoptotic effects of *Primula vulgaris* extract against methotrexate (MTX)-induced testicular damage. In this study, 4 groups were formed with 8 rats in each group. Rats in group 1 were given 0.8 mg/kg physiological serum via gavage for 7 days. The rats in group 2 were administered a single dose (30 mg/kg) of MTX intraperitoneally on the first day of the study. The rats in group 3 were administered a single dose (30 mg/kg) of MTX on the first day of the study and then 100 mg/kg of aqueous extract via gavage for 7 days starting from the first day. The rats in group 4 were given 100

mg/kg of aqueous extract via gavage for 7 days. On the 8th day, the testicles and epididymis of the rats were removed under anesthesia and their blood was collected. The removed testicles were used for histological and biochemical analyses. When group 2 was compared with group 1, it was determined that seminiferous tubule diameter, epithelial thickness, sperm count, motility, vitality, and Johnsen scoring values decreased; tubule number that immature cells sloughed into the lumen and apoptotic index (AI) increased. In group 3, it was observed that seminiferous tubule diameter, epithelial thickness, sperm count, motility, vitality, and Johnsen scoring values increased; tubule number that immature cells sloughed into the lumen and AI decreased compared to group 2. When group 2 was compared with group 1, it was found that MDA values increased, and SOD and CAT values decreased in blood plasma and testicular tissue. According to the study results, it was determined that MTX caused damage to the testicle by creating oxidative stress, while *Primula vulgaris* reduced this damage thanks to its antioxidant effects.

**Keywords:** Antioxidant, Apoptosis, *Primula vulgaris*, Testes

## Özet

Bu çalışmanın amacı metotreksat (MTX) kaynaklı testis hasarına karşı *Primula vulgaris* ekstraktının antioksidan ve antiapoptotik etkilerini araştırmaktır. Bu çalışmada her grupta 8 adet sıçan olacak şekilde 4 grup oluşturulmuştur. Grup 1'deki sıçanlara 7 gün boyunca 0.8 mg/kg serum fizyolojik gavaj ile verilmiştir. Grup 2'deki sıçanlara çalışmanın ilk günü tek doz (30 mg/kg) MTX intraperitoneal olarak uygulanmıştır. Grup 3'deki sıçanlara çalışmanın ilk günü tek doz (30 mg/kg) MTX uygulanmış ve daha sonra ilk günden başlayarak 7 gün boyunca 100 mg/kg sulu ekstrakt gavaj yoluyla verilmiştir. Grup 4'deki sıçanlara 7 gün boyunca 100 mg/kg sulu ekstrakt gavaj yoluyla verilmiştir. 8. gün anestezi altında sıçanların testisleri ve epididimisleri çıkarılmış, kanları alınmıştır. Çıkarılan testisler histolojik ve biyokimyasal analizler için kullanılmıştır. Grup 2, grup 1 ile kıyaslandığında seminifer tübül çapı, epitel kalınlığı, sperm sayısı, motilite, vitalite ve Johnsen skorlama değerlerinin azaldığı; lümenine immatür hücre dökülen tübül sayısı ve apoptotik indeksin arttığı belirlenmiştir. Grup 3, grup 2'ye göre seminifer tübül çapı, epitel kalınlığı, sperm sayısı, motilite, vitalite ve Johnsen skorlama değerlerinin arttığı; lümenine immatür hücre dökülen seminifer tübül sayısı ve AI'nın azaldığı gözlenmiştir. Grup 2, grup 1 ile kıyaslandığında kan plazmasında ve testis dokusunda MDA değerinin arttığı, SOD ve CAT değerlerinin ise azaldığı bulunmuştur. Çalışma sonuçlarına göre MTX'in testiste oksidatif stres oluşturarak testise zarar verdiği, *Primula vulgaris*'in ise antioksidan etkileri sayesinde bu hasarı azalttığı belirlenmiştir.

**Anahtar Kelimeler:** Antioksidan, Apoptozis, *Primula vulgaris*, Testis

**Abbreviations:** AI, Apoptotic index; CAT, Catalase; SOD, Süperoxide dismutase; MDA, Malondialdehyde; MTX, Methotrexate

## 1. INTRODUCTION

Chemotherapeutics can produce acute toxic outcomes in multiorgan systems (Kim et al., 1999). The side effects of such drugs include azoospermia and infertility in males (Schilsky et al., 1980). Methotrexate (MTX) is a folic acid antagonist agent that is widely used in the treatment of malignant tumors (including acute lymphoblastic leukemia, non-Hodgkin lymphoma, breast cancer, and malignancies of the head and neck) and non-neoplastic conditions (particularly rheumatoid arthritis) (Nouri et al., 2009). Side effects resulting from the administration of MTX observed in previous studies include injury (such as disorganization and vacuolization) to the testicular seminiferous tubules, a reduced sperm count, and impairment of sperm DNA (Padmanabhan et al., 2009; Vardi et al., 2009). Oxidative stress has been implicated in the pathogenesis of MTX-induced testicular injury (Armagan et al., 2008). It has been suggested that reactive oxygen radicals (ROS) can lead to atrophy in the testicular seminiferous tubules and apoptosis in spermatocytes (Nouri et al., 2009; Padmanabhan et al., 2009; Vardi et al., 2009). More recent research has investigated the use of antioxidants for the purpose of minimizing side effects caused by the application of MTX (Armagan et al., 2008; Vardi et al., 2009; Gulgun et al., 2010).

*Primula*, a medicinal, flowering plant, is a member of the family Primulaceae with some 400-500 known species. *Primula* herbs are widely employed in traditional medicine for their antispasmodic, vermifuge, emetic, and astringent effects. Folk doctors use various *Primula* species in the treatment of a range of conditions, including bronchitis, epilepsy, convulsions, cramps, spasms, paralysis, and rheumatic pains (Jager et al., 2006; Basbulbul et al., 2008; Orhan et al., 2012; Majid et al., 2014). The principal compounds in the genus are phenolic glycosides and saponins (Basbulbul et al., 2008). Various studies have reported that different *Primula* species exhibit cytotoxic, antibacterial, antiviral, antioxidant, antiangiogenic, anti-inflammatory and antimitotic activities. Studies have ascribed these effects to their phenolic contents (Kati et al., 2001; Tokalov et al., 2004; Buruk et al., 2006; Basbulbul et al., 2008; Orhan et al., 2012; El-Sayed et al., 2014).

This study was intended to investigate the contribution of oxidative stress to testicular injury deriving from the use of MTX, and to determine the protective potential of *Primula vulgaris* against such injury by means of histopathological and biochemical analyses.

## 2. MATERIALS and METHODS

### 2.1. Plant Material and Aqueous Extract Preparation

*Primula vulgaris* subsp. s*ibthorpii* was collected from Trabzon, Turkey, in May 2014. The plant was identified by Prof. Dr. Ufuk OZGEN. The dried powder obtained from blossom parts of *P. vulgaris* (1 g) was weighed and mixed with 20 mL methanol. This mixture was stirred on a continuous basis at room temperature for 24 hours. The suspension was then removed by centrifugation at a speed of 10,000 g for 15 mins. The supernatant was subsequently concentrated at 40 °C inside a rotary evaporator (IKA-Werke RV05 Basic, Staufen, Germany). Finally, the dry residue was resolved with distilled aqueous and filtered using a 0.45 µm filter before being stored at 4 ºC until further experiments (Kaynar et al., 2023).

### 2.2. Animals

The rats in this randomized, controlled animal study were housed at room temperature in a 12/12 h light/dark cycle with ad libitum access to standard laboratory chow and water. All animals were treated in line with the principles of the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health. The study was approved by the Karadeniz Technical University Animal Care and Ethical Committee (Ethics Board Number: 3)

### 2.3. Experimental Protocol

Thirty-two adult male Sprague Dawley rats (8 weeks old) were used. MTX was administered intraperitoneally (i.p.) and *P. vulgaris* extract (PVE) by gavage. The rats were divided at random into 4 groups of 8 animals each. Group 1 (control group) received 0.8 mL/kg saline intraperitoneally (ip) for 7 days (Berber, 2017). Group 2 (MTX group) was given 30 mg/kg methotrexate (Kocak Farma, Tekirdag, Turkey) intraperitoneally (i.p.) on the first day of the experiment (Yulug et. al., 2013; Ayan, 2016; Berber, 2017). Testicular damage was induced by giving MTX to rats in Group 2 (Yulug et. al., 2013; Ayan, 2016; Berber, 2017). Group 3 (MTX + PVE group) was given 30 mg/kg methotrexate (Kocak Farma, Tekirdag, Turkey intraperitoneally (i.p.) on the first day of the experiment, following 100 mg/kg of *P. vulgaris* extract by gavage for 7 days (Berber, 2017). Group 4 (PVE group) was administered 100 mg/kg *P. vulgaris* extract by gavage for 7 days (Berber, 2017). All rats were subjected to laparotomy on the 8$^{th}$ day of the experiment. The abdominal cavity was incised, and the bilateral testes and epididymis were removed. At the end of the procedure, all animals were sacrificed by exsanguination (Berber, 2017).

## 2.4. Biochemical Analysis

Plasma malondialdehyde (MDA) levels were calculated based on the technique previously described by Yagi (Yagi, 1994). Briefly, 2.4 mL of 0.08N $H_2SO_4$ and 0.3 mL of 10% phosphotungstic acid were added to 0.3 mL of serum. This mixture was allowed to stand at room temperature for 5 min before being centrifuged at 1600 g for a further 10 min. Discard supernatant and sediment were suspended in 4 mL of distilled water. In the next stage, 1 mL of 0.67% thiobarbituric acid was added to the mixture, which was placed in boiling water for 1 hour. The resulting color was extracted into n-butanol. The mixture was again centrifuged at 1600 g for another 10 min. The absorbance of the organic layer was read at 532 nm. Tetramethoxypropane was adopted as a standard, and MDA levels were expressed as nmol/mL.

MDA levels in testis specimens were calculated according to Uchiyama and Mihara's technique (Uchiyama & Mihara, 1978). Briefly, in the initial stage, a piece of testicular tissue was minced before being homogenized in an ice-cold 1.15% KCl solution containing 0.50 mL/L Triton X-100 with the assistance of an Ultra-Turrax T25 homogenizer. To the resulting homogenate (0.5 mL) was added 3 mL of 1% $H_3PO_4$, followed by 1 mL of 0.67% thiobarbituric acid. This mixture was placed into boiling water for 45 min. The color phase was subsequently extracted into n-butanol. Following further centrifugation, the absorbance of the resulting organic layer was read at 532 nm. Tetramethoxypropane was employed as a standard for this procedure, and MDA levels were expressed as nmol per milligram protein.

Both plasma and testis tissue specimens were used in the measurement of superoxide dismutase (SOD) and catalase (CAT) levels. Specimens were first homogenized in an ice-cold Tris-HCL buffer (50 mM, pH 7.4) containing 0.50 mL/L Triton X-100. SOD activities were determined based on the reduction of nitroblue tetrazolium by the xanthine-xanthine oxidase system (Sun et al., 1988). The formation of formazon formation was evaluated using spectrophotometric methods at 560 nm. Enzyme activity resulting in an inhibition level of 50% was adopted as one unit. Bovine erythrocyte SOD was employed as standard. The results were variously expressed as U/mg protein in testis tissue and as U/mL in plasma. CAT activity was determined using the method previously described by Aebi (Aebi, 1974). This relies on the principle that absorbance at 240 nm decreases on account of the dismutation of $H_2O_2$. The results were again expressed in the form of U/mg protein in testicular tissue and U/mL in plasma. Protein concentrations were calculated based on the method described by Lowry (Lowry et al., 1951).

## 2.5. Histopathological Staining and Analysis

The right testis and epididymis tissues were fixed and dehydrated before being embedded in paraffin. Tissues were then stained with hematoxylin and eosin (H&E). All testicular histology assessments were performed by a histologist blinded to the various experimental groups. Light microscopy (Olympus BX-51; Olympus, Tokyo, Japan) was used for evaluations. Testis sections from all experimental groups were assessed in terms of structural alterations. Johnsen's tubular biopsy score (JTBS) was adopted as a semiquantitative technique for assessing spermatogenesis in 20 seminiferous tubules from each testicular section (Kaltsonoudis et al., 2005). Testicular tubule sections were classified from 1 to 10 based on the following definitions; 10 was equivalent to full spermatogenesis and a normal structure; 9 indicated the presence of numerous spermatozoa and disorganization in tubules; 8 indicated a low number of spermatozoa; 7 indicated a complete absence of spermatozoa, but numerous spermatids; 6 indicated an absence of spermatozoa and the presence of a small number of spermatids; 5 described an absence of spermatozoa and spermatids, but the presence of numerous spermatocytes; 4 indicated the presence of a low number of spermatocytes; 3 indicated the presence of spermatogonia only; 2 indicated that no germ cells were observed, only Sertoli cells; and 1 indicated total absence of germ cells and spermatogenesis. We divided the sum of all scores by the total number of seminiferous tubules observed in order to elicit the JTBS.

## 2.6. TUNEL Analysis

Apoptosis in testicular tissue was determined using the terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate nick end labeling assay (TUNEL). This was performed with an in situ cell death detection kit, POD, (ROCHE, Mannheim, Germany) in accordance with the manufacturer's recommendations. Color was subsequently analyzed using a kit containing 3,3-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA). The presence of DNA fragmentations was investigated in seminiferous tubule germinal cells and in epithelial cells from the epididymal canal. TUNEL (+) cells staining brown were considered apoptotic. TUNEL (+) cells were counted from 20 seminiferous tubules and 20 epididymal canal sections from each testis at ×400 magnification with the assistance of Analysis 5 Research software (Olympus Soft Imaging Solutions, M¨unster, Germany). The proportion of TUNEL (+) apoptotic testicular cells to the total number of cells was calculated as the testis apoptotic index (TAI) (Patel et al., 2014), while the epididymal apoptotic index (EAI) was calculated as the proportion of TUNEL (+) apoptotic cells in the epididymis to the total number of cells.

### 2.7. Statistical Analysis

Freidman variance analysis was performed on the seminiferous tubule diameter, epithelial thickness, number of tubules with immature cells shed into the lumen, Johnsen scoring, AI, sperm count, motility, and vitality parameters of the study groups. Holm test was applied for post-hoc evaluation. $p < 0.05$ was considered significant in the Freidman test. Kruskal-Wallis analysis of variance was used to compare differences between group parameters (MDA, SOD, CAT). Dual comparisons between groups exhibiting significant values were evaluated using the Mann-Whitney *U* test. $p<0.05$ was considered statistically significant. All results were expressed as means (±) standard deviation (SD).

## 3. RESULTS and DISCUSSION

### 3.1. Biochemical Analysis

Biochemical results in testicular tissue and plasma for the experimental groups are given in Table 1 and Table 2, respectively. Tissue and plasma MDA concentrations in the group 2 were higher compared to the group 1 and group 4, while SOD and CAT activity were lower in testicular tissue. Tissue and plasma MDA concentrations in the group 3 were lower compared to the group 2, while there was a rise in SOD and CAT activities. MTX-induced sperm injury has been linked to oxidative stress. Malondialdehyde levels are frequently measured as a marker of oxidative stress. Serum and tissue MDA concentrations increased in the group 2 in this study. This finding is compatible with the results of various previous studies to the effect that MTX leads to oxidative stress in tissues by raising MDA levels (Oktem et al., 2006; Armagan et al., 2008; Cetin et al., 2008; Vardi et al., 2010).

Table 1. Biochemical results in testicular tissue

| Groups | MDA (nmol/mg protein) | SOD (U/mg protein) | CAT (U/mg protein) |
|---|---|---|---|
| **1** | 12.27 ± 1.89 | 1.86 ± 0.13 | 29.77 ± 4.60 |
| **2** | 15.381[a] ± 1.97 | 1.24[b] ± 0.12 | 19.10[b] ± 5.08 |
| **3** | 14.08 ± 2.65 | 1.41[c] ± 0.29 | 25.22 ± 9.72 |
| **4** | 12.60 ± 1.53 | 1.56[d] ± 0.54 | 26.79 ± 8.40 |

MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; MTX, methotrexate
[a]The value in the group 2 increased significantly compared to the group 1 ($p < 0.05$).
[b]The value in the group 2 was significantly lower than in the group 1 ($p < 0.05$).
[c]The value of group 3 was significantly lower than group 1 ($p < 0.05$).
[d]The value of the group 4 was significantly lower than the group 1 ($p < 0.05$).

Antioxidant defense mechanisms in the testis play a significant role in protecting sperm against ROS. Protective biomolecules including a range of antioxidants, vitamins, and glutathione enable the spermatozoa to combat ROS (Prahalathan et al., 2004). SOD is one of the most important antioxidant enzymes that protect the male reproductive organs against the deleterious effects of ROS (Fujii et al., 2003). Hydrogen peroxide ($H_2O_2$) is less effective than the superoxide group. Its effect is weakened through conversion by enzymes in tissue, including catalase and glutathione, into products with a lesser effect, such as water and oxygen (Callaghan et al., 2013). This study was also intended to determine whether the antioxidant enzymes SOD and CAT exhibit protective effects that eliminate free radicals emerging due to MTX. SOD is significantly involved in testis development and spermatogenesis. Alterations in SOD may result in a compromise of testicular functions and interrupted sperm development (Hung et al., 2002). The findings of this study show that MTX reduced SOD and CAT activity in testicular tissue. We would attribute the low levels of SOD and CAT observed to increased consumption and disequilibrium in resynthesis mechanisms.

Table 2. Biochemical results in plasma

| Groups | MDA (nmol/mL) | SOD (U/mL) | CAT (U/mL) |
|---|---|---|---|
| **1** | 0.81 ± 0.41 | 4.47 ± 3.30 | 167.77 ± 1.00 |
| **2** | 1.03[a] ± 0.01 | 2.38 ± 1.29 | 163.89 ± 2.92 |
| **3** | 0.88 ± 0.39 | 2.91 ± 2.62 | 166.24 ± 2.03 |
| **4** | 0.89 ± 0.15 | 2.46 ± 0.87 | 164.62 ± 4.59 |

MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; MTX, methotrexate
[a]The value in the group 2 increased significantly compared to the group 1 ($p < 0.05$).

The administration of *P. vulgaris* before MTX partly or entirely neutralized these effects in this study. In Group 3, tissue and plasma MDA concentrations were lower than in Group 2, while SOD and CAT activities increased. Plasma and tissue MDA concentrations were significantly lower and SOD enzyme activity was significantly higher in group 3. Previous studies have reported that antioxidant substances reduce oxidative stress by lowering MDA concentrations in tissue (Sikka, 2001; Saral et al., 2016). Antioxidant enzymes including glutathione peroxidase (GP-x), SOD, and CAT, and other antioxidants, such as vitamins C and E protect seminal plasma and sperm against cellular injury (Smith et al., 1996). Yüncü et al. founded that MDA levels increased significantly and SOD activity decreased significantly in the MTX group (Yüncü et al., 2015). Sönmez et al. determined that tissue MDA levels increased insignificantly

in the MTX group (Sönmez et al., 2016). Dagguli et al. reported that MDA, total oxidant capacity and oxidative stress index increased by inducing testicular damage with MTX in rats (Daggulli et al., 2014). *P. vulgaris* may be a free radical scavenger and enzyme regulator and therefore protect against tissue damage caused by oxidative stress.

### 3.2. Histopathological Analysis

In testicular tissue, it was determined that seminiferous tubule diameter, epithelial thickness, sperm count, motility, vitality, and Johnsen scoring values decreased in group 2 compared to group 1; tubule number that immature cells sloughed into the lumen and apoptotic index increased (Table 3).

Table 3. In the testicular tissue of the groups; seminiferous tubule diameter, epithelial thickness, and tubule number that immature cells sloughed into the lümen

| Groups | Seminiferous Tubule Diameter (µm) | Epithelial Thickness (µm) | Tubule Number that Immature Cells Sloughed into The Lumen (%) |
|---|---|---|---|
| **1** | 293.64 ± 27.12 | 59.62 ± 7.93 | 16.52 ± 11.44 |
| **2** | 229.95 [a] ± 18.69 | 42.84 ± 4.13 | 45.33 [a] ± 17.43 |
| **3** | 285.53 [b] ± 7.99 | 62.59 [b] ± 3.60 | 26.10 ± 10.91 |
| **4** | 294.50 [b] ± 12.54 | 61.34 ± 1.82 | 19.57 [b] ± 15.55 |

[a]There is a significant difference compared to the group 1 ($p < 0.05$).
[b]There is a significant difference compared to the group 2 ($p < 0.05$).

In testicular tissue, it was determined that seminiferous tubule diameter, epithelial thickness, sperm count, motility, vitality, and Johnsen scoring values increased in group 3 compared to group 2; tubule number that immature cells sloughed into the lumen and apoptotic index decreased (Table 4).

Johnsen's score was found to decrease in group 2, group 3, and group 4 compared to group 1, and this decrease was significant only in group 2. A non-significant increase was found in group 3 and group 4 compared to the group 2. A non-significant increase was observed in the group 4 compared to the group 3 (Table 5).

Table 4. Sperm count, motility, and vitality values of the groups

| Groups | Sperm count (x$10^6$) | Motility (%) | Vitality (%) |
|---|---|---|---|
| **1** | 19.12 ± 3.75 | 49.72 ± 9.60 | 61.50 ± 3.50 |
| **2** | 16.62 ± 3.92 | 30.95 ± 13.33 | 42.75 [a] ± 8.20 |
| **3** | 20.25 ± 3.91 | 34.41 ± 11.58 | 51.00 ± 7.55 |
| **4** | 21.25 [b] ± 1.90 | 49.87 ± 11.21 | 58.75 [b] ± 8.06 |

[a]There is a significant difference compared to the group 1 ($p < 0.05$).
[b]There is a significant difference compared to the group 2 ($p < 0.05$).

In the apoptotic index assessment; it was found that there was an increase in group 2, group 3, and group 4 compared to group 1, and this increase was significant in group 2. A significant decrease was found in group 3, and group 4 compared to group 2. A non-significant increase was observed in the group 4 compared to the group 3 (Table 5).

Table 5. Johnsen Scoring and apoptotic index (AI) of the groups

| Groups | Johnsen Scoring | AI (%) |
|---|---|---|
| **1** | 9.38 ± 0.11 | 12.03 ± 3.11 |
| **2** | 4.12 [a] ± 0.62 | 34.7 [a] ± 2.10 |
| **3** | 7.7 ± 0.37 | .5.16 [b] ± 3.33 |
| **4** | 8.08 ± 0.73 | .5.81 [b] ± 1.73 |

[a]There is a significant difference compared to the group 1 ($p < 0.05$).
[b]There is a significant difference compared to the group 2 ($p < 0.05$).

MTX is commonly employed in the treatment of testicular, bladder, head and neck, and breast cancer. In addition to its antineoplastic activity, it has also been used to treat psoriasis and as an immunosuppressive medication against various auto-immune diseases, including ankylosing spondylitis, Crohn's disease, dermatomyositis, Wegener's granulomatosis and rheumatoid arthritis (Kaltsonoudis et al., 2005; Patel et al., 2014; Specks, 2005; Roychowdhury et al., 2002). When MTX is employed during cancer chemotherapy normal cells begin to divide very quickly, a process that leads to various forms of toxicity. The therapeutic and toxic effects of MTX are delayed as a result of conversion to a polyglutamated form with a longer metabolic half-life. MTX also suppresses DHFR. The effect mechanism involves inhibition of DNA synthesis, which results in compromise of normal cellular processes (Kamen et al., 1981; Novakovic et al., 2003). Testicular toxicity is a particularly significant potential side-effect of

MTX, and one that may ultimately result in infertility. It is important that germinal cells be protected in the course of chemotherapeutic procedures. MTX has been reported to induce testicular toxicity through ROS generation (Yulug et al., 2013). The purpose of the present study was to investigate whether modification of oxidative stress status would occur in the rat testis following exposure to MTX and whether such effects can be attenuated through the use of *P. vulgaris*.

Sönmez et al., Yüncü et al., Yulug et al., Gökçe et al., Nouri et al., Vardi et al., Saxena et al. examined the effects of MTX on rat testes. These researchers examined the effects of MTX at different times and doses on seminiferous tubules, spermatogenic series cells and interstitial areas and determined the damages that occurred. Sönmez et al. determined significant damage in the seminiferous tubules and interstitial areas in the group to which they applied MTX. According to the study results, they detected vacuolization in the seminiferous epithelium, irregularity in germinal cells, immature germinal cell shedding into the seminiferous tubule lumen and atrophy in some seminiferous tubules (Sönmez et al., 2016).

Oxidative stress results in an imbalance between ROS and the antioxidant reserve system. ROS is the product of normal cellular metabolism. Sperm cells produce free oxygen radicals. Low levels of reactive oxygen radical production result in sperm cell capacitation, acrosome reaction, and sperm binding to the zona pellucida (de Lamirande et al., 1997). Overproduction of ROS results in sperm anomalies and infertility. The sperm membrane contains high levels of polyunsaturated fatty acids, resulting in increased oxygen-induced lipid peroxidation (Sikka, 2001). Peroxidative damage has been implicated as a significant factor in sperm function damage (Moustafa et al., 2004).

## 4. CONCLUSION

Our study shows that oxidative stress is important in MTX induced testicular damage. The administration of *P. vulgaris* reduced oxidative stress and apoptotic cell death and protected spermatogenesis in MTX-induced oxidative testicular damage. We think that this protective effect of *P. vulgaris* may be due to its antioxidant properties. Further experimental and clinical studies are now needed to confirm our findings.

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## DECLARATIONS

There is no conflict of interest between the authors.

## AUTHORS' CONTRIBUTIONS

The authors have equal contributions.

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# *Antifungal Activity Exerted by Greek Honeys and Bacteria Isolated from Them*
# *Yunan Ballarının ve Bunlardan İzole Edilen Bakterilerin Antifungal Aktivitesi*

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## Abstract

A plethora of studies provide evidence regarding honey's biological properties such as antibacterial, antioxidant, and anti-inflammatory activity. However, antifungal activity exerted by honey is rather under investigated. Due to widespread antimicrobial resistance, the emergence of novel antifungal agents, as well as the identification of alternative therapies, is crucial. This study aimed to investigate the antifungal activity exerted by heather and chestnut honeys, harvested across Greece, as well as the antifungal activity of bacteria isolated from them, against *Penicillium commune*, *Penicillium expansum*, *Aspergillus niger*, *Candida albicans M10/20* and *Candida albicans M 351/19*. Antifungal activity of tested honeys was evaluated by Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) whereas antifungal activity of bacterial isolates by antagonism assay. Fungistatic activity against all tested fungi and fungicidal activity against *C. albicans* strains was exerted by most Greek honeys. Exerted antifungal activity was comparable to Manuka honey. Furthermore, most of the identified bacterial isolates inhibited the growth of fungal strains, in antagonism assays. This study for the first time demonstrated the significant antifungal activity exerted by heather and chestnut honey produced in Greece, as well as the important role of their microbiome in observed antifungal activity. Nevertheless, our results warrant further research in order to develop novel antifungal agents and alternative therapies.

**Keywords:** Greek Heather Honey, Greek Chestnut Honey, Antifungal activity, Bacterial isolates, *Candida albicans*, food spoilage fungi.

**Özet**

Çok sayıda çalışma, balın antibakteriyel, antioksidan ve anti-inflamatuar aktivite gibi biyolojik özellikleriyle ilgili kanıtlar sunmaktadır. Ancak, balın antifungal aktivitesi oldukça az araştırılmıştır. Yaygın antimikrobiyal direnç nedeniyle, yeni antifungal ajanların ortaya çıkması ve alternatif tedavilerin tanımlanması hayati önem taşımaktadır. Bu çalışma, Yunanistan genelinde hasat edilen funda ve kestane ballarının ve bunlardan izole edilen bakterilerin *Penicillium commune, Penicillium expansum, Aspergillus niger, Candida albicans M10/20 ve Candida albicans M 351/19'a* karşı uyguladığı antifungal aktiviteyi araştırmayı amaçlamaktadır. Test edilen balların antifungal aktivitesi Minimum İnhibitör Konsantrasyon (MİK) ve Minimum Fungisidal Konsantrasyonun (MFC) Belirlenmesi ile değerlendirilirken, bakteri izolatlarının antifungal aktivitesi antagonizm testi ile değerlendirilmiştir. Test edilen tüm mantarlara karşı fungistatik aktivite ve *C. albicans* suşlarına karşı fungisidal aktivite çoğu Yunan balı tarafından uygulandı. Uygulanan antifungal aktivite Manuka balına benzerdi. Dahası, tanımlanan bakteri izolatlarının çoğu, antagonizm analizlerinde mantar suşlarının büyümesini engelledi. Bu çalışma, Yunanistan'da üretilen funda ve kestane balının uyguladığı önemli antifungal aktiviteyi ve gözlemlenen antifungal aktivitede mikrobiyomlarının önemli rolünü ilk kez gösterdi. Yine de, sonuçlarımız yeni antifungal ajanlar ve alternatif tedaviler geliştirmek için daha fazla araştırmayı gerektiriyor.

**Anahtar Kelimeler:** Yunan Funda Balı, Yunan Kestane Balı, Antifungal aktivite, Bakteriyel izolatlar, *Candida albicans*, gıda bozulma mantarları

**Abbreviations:** MIC; Minimum Inhibitory Concentration, MFC; Minimum Fungicidal Concentration

## 1. INTRODUCTION

Honey is a natural product highly appreciated for its exceptional nutritional value and bioactivity. It has been used as a traditional remedy due to antimicrobial activity and wound-healing properties since ancient times. Greek honey types exert high antibacterial and antioxidant activity, verified by several studies (Anthimidou & Mossialos, 2013; Stagos et al., 2018; Tsavea et al., 2022; Tsavea & Mossialos, 2019). Physicochemical characteristics including high sugar content, low pH, hydrogen peroxide content, as well as antimicrobial peptides present in honey, modulate the antibacterial, antioxidant, and anti-inflammatory activity (Ranneh et al., 2021; Tsadila et al., 2021). However, the efficacy of honey as an antimicrobial agent has been reported to be highly variable depending on the botanical and geographic origin (Almasaudi, 2021; Ramos et al., 2018; Schiassi et al., 2021).

Chestnut honey, produced from a mixture of *Castanea sativa* nectar and honeydew by *Myzocallis castanicola,* has been shown to inhibit the growth of a diverse range of pathogens, including several *Bacillus* strains (Kačániová et al., 2022) and fungi like *Candida parapsilosis, Candida tropicalis, Cladosporium cladosporioides,* and *Rhodotorula mucilaginosa,* as

described by Kunčič et al. (2012). Likewise, heather honey, derived from *Erica manipuliflora* nectar exerts promising antimicrobial activity. Feás & Estevinho (2011) reported that monofloral heather harvested in Portugal exerted in a concentration-dependent manner antifungal efficacy against *Candida albicans*, *Candida krusei*, and *Cryptococcus neoformans* that was attributed to phytochemicals like polyphenols and flavonoids present in these honeys.

Honey from the Manuka tree (*Leptospermum scoparium*), native to New Zealand and Australia, demonstrates antimicrobial properties, which are not dependent on hydrogen peroxide but on the presence of methylglyoxal, a product of dihydroxyacetone, which affects diverse bacterial proteins and structures including fibria and flagella thus causing bacterial dysfunction (Adams et al., 2009; Rabie et al., 2016). Manuka honey has been extensively studied to date, because of its unique origin and proven ability to inhibit more than 60 bacterial species, including Gram-positive and Gram-negative, aerobic and anaerobic bacteria (Mandal & Mandal, 2011).

Although raw honey exerts antimicrobial activity is not sterile. It contains a unique microbiome consisting of microorganisms coming from plant pollen and nectar, bee digestive tract, and hive milieu (Olaitan et al., 2007). Microorganisms present in honey should adapt under conditions of high osmolarity, low moisture content, low acidity, and endogenous antimicrobial agents. Therefore, the main types of microorganisms surviving in honey are sporulating bacteria and yeasts (Xiong et al., 2023), with *Bacillus* species being the most prominent (Pomastowski et al., 2019; Tsadila et al., 2021). Within the harsh microenvironment of honey, microorganisms compete with each other to access limited resources. Strong competition among diverse microbial species, leads to synthesis and secretion of a multitude of secondary metabolites disrupting key cellular structures and functions of antagonistic microorganisms (Brudzynski, 2021).

Although the antibacterial properties of Greek honey types have been extensively reported (Anthimidou & Mossialos, 2013; Stagos et al., 2018; Tsavea et al., 2022; Tsavea & Mossialos, 2019), the antifungal activity exerted by them is under investigated. Screening of honey exerting antifungal activity and their mode of action is essential for the identification of novel antifungal agents that might combat the emerging antifungal resistance (Lee et al., 2023; Vitiello et al., 2023).

*Penicillium* and *Aspergillus* species are commonly referred to as “food spoilage fungi” (Snyder & Worobo, 2018). *Penicillium expansum* and *Penicillium commune*, are commonly

grown on fruits and dairy products respectively (Jurado & Vicente, 2020; Tannous et al., 2020). These fungi are highly adaptable in a wide range of conditions, with an optimum growth temperature at 25°C and elevated humidity levels (Li et al., 2020; Pitt & Hocking, 2009b). Both species produce toxins potentially harmful to humans (Pitt & Hocking, 2009b; Vidal et al., 2019). *Aspergillus niger*, identified often as the black mold covering rotten fruits, grows at variable temperatures, optimally at 35-37°C (Pitt & Hocking, 2009a). *A. niger* has been associated with otomycosis and might cause invasive pulmonary aspergillosis in immunocompromised patients (Person et al., 2010; Romsdahl et al., 2018).

On the other hand, *Candida albicans* is a fungal species often present in human oral and gastrointestinal microbiome (Dadar et al., 2018). However, it is an opportunistic pathogen, that under particular circumstances, might cause infections due to dysbiosis of the normal microbiota, immune dysfunction, and damage to the mucosal barrier (Talapko et al., 2021).

The aim of this study was to investigate the antifungal activity of Greek heather and Chestnut honey, along with that of their bacterial isolates, towards the fungi *Penicillium expansum, Penicillium commune, Aspergillus niger,* and *Candida albicans*.

## 2. MATERIALS and METHODS

### 2.1. Honey Samples

A total of nine (9) heather and seven (7) chestnut honey samples, harvested across Greece, as shown in Figure 1and Table 1, were provided by individual beekeepers and beekeeping associations. Each sample was recorded by a unique reference number and then was stored in a dry and cool place until testing. In the case of crystalization, samples were warmed up in a waterbath at 35-40 °C for up to 10 min and then stirred. In order to compare the antifungal activity of tested honey samples, Manuka honey UMF 24+ (MGO 1122+) (New Zealand Honey Co), was used as a reference.

Figure 1. Geographical origin of honey samples. Orange map pins indicate chestnut honey samples and green map pins indicate heather honey samples.

Table 1. Information regarding the geographical and botanical origin, as well as, the harvest period of the honey samples.

| Reference number | Botanical origin | Geographical origin | Harvest (Month/Year) |
|---|---|---|---|
| **200** | Chestnut | Veria | 07/2020 |
| **240** | Chestnut | Chania | 08/2020 |
| **271** | Chestnut | Florina | 07/2021 |
| **187** | Chestnut | Fokida | 02/2020 |
| **267** | Chestnut | Serres | 07/2021 |
| **210** | Chestnut | Pella | 08/2020 |
| **212** | Chestnut | Mount Athos | 07/2020 |
| **229** | Heather | Antiparos | 12/2020 |
| **183** | Heather | Kalamos | 11/2020 |
| **244** | Heather | Crete | 08/2020 |
| **243** | Heather | Crete | 10/2020 |
| **218** | Heather | Chania | 12/2020 |
| **269** | Heather | Kavala | 04/2021 |
| **241** | Heather | Chania | 10/2020 |
| **195** | Heather | Ios | 11/2020 |
| **233** | Heather | Andros | 01/2021 |

## 2.2. Fungal Strains

*Penicillium expansum* (DSM 62841), *Penicillium commune* (DSM 2211), and *Aspergillus niger* (DSM 2466) strains were purchased by DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. *Candida albicans M10/20* and *Candida albicans M351/19,* isolated from the upper and lower respiratory system respectively, were provided by the A' Laboratory of Microbiology, School of Medicine, Aristotle University of Thessaloniki and they were identified by standard methods.

## 2.3. Bacterial Strains

Bacterial strains were isolated from diverse honey samples and they were identified by 16S rRNA gene sequencing as described before (Tsadila et al., 2021).

Table 2. Bacterial strains used in this experiment

| Strain | Bacteria | Genbank Accession Number |
|---|---|---|
| **CTA2** | *Bacillus pumilus* | MW700013 |
| **CTA15** | *B. pumilus* | MW700019 |
| **CTA31** | *Bacillus sp.* | MW700025 |
| **CTA163** | *Bacillus licheniformis* | MW700039 |
| **CTB7** | *Bacillus safensis* | MW700041 |
| **CTB16** | *B.safensis* | MW700043 |
| **CTB21** | *B.pumilus* | MW700044 |
| **CTB89** | *B.safensis* | MW700053 |
| **CTB120** | *B.safensis* | MW700057 |
| **CTA20** | *Bacillus subtilis* | MW700021 |
| **CTB11** | *Bacillus sp.* | MW700042 |
| **CTA28** | *Bacillus paramycoides* | MW700024 |
| **CTB34** | *Bacillus cereus* | MW700048 |
| **CTA23** | *Pseudomonas fulva* | MW700022 |
| **CTA138** | *Bacillus sp.* | MW700037 |

## 2.4. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is considered the lowest concentration of honey that completely inhibits fungal growth.

Determination of the minimum inhibitory concentration (MIC) of honey samples was performed on sterile 96-well microtiter plates (Kisker Biotech GmbH & Co. KG, Steinfurt, Germany), as previously described with some modifications (Patton et al., 2006). Spore suspension of *P. commune, P. expansum, A. niger,* or *C. albicans* broth culture, of $OD_{530}$= 0.09-0.13, was further diluted, using RPMI 1640 broth, w/ L- Glutamine (BioSera, France) at a ratio of 50:1. Subsequently, 100µl of the fungal solution and 100 ml of two-fold diluted honey, were

added inside each well, resulting in final honey concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.562% (v/v). Each honey sample concentration was tested in triplicates. RPMI broth was used as a negative control and RPMI broth inoculated with fungal suspension or fungal culture was used as a positive control for fungal growth. Furthermore, Manuka honey was tested in every microtiter plate, along with the honey samples for comparison. The growth of *C. albicans* strains was calculated by measuring the optical density before (t=0) and after 24h (t=24) incubation at 37°C. Optical density (OD) was determined at 530 nm using an ELx808 Microplate reader ELx808 (BioTek Instruments, Inc., Winooski, VT, USA). In order to determine the percentage of growth inhibition of each honey dilution the following formula was implemented (Patton et al., 2006):

$$100\% \text{ Suspension} = 1 - (\text{DODsample} / \text{DODcontrol}) \times 100$$

Regarding *P. commune, P. expansum, and A. niger*, being filamentous fungi, it was not feasible to measure their growth by optical density. Therefore, the growth inhibition was recorded under an inverted microscope, after 72h incubation at room temperature.

## 2.5. Determination of Minimum Fungicidal Concentration (MFC)

Minimum fungicidal concentration (MFC) was determined by transferring a small amount of sample contained in each well of the microtiter plate on which the MIC was calculated, to Sabouraud Dextrose plates (Neogen® Culture Media, USA) using a replicator (BoekelScientific, Pennsylvania, USA). The plates were incubated at 37° C for 24 h. MFC was determined as the lowest concentration of honey at which, no fungal growth was observed. This test was performed only on *C. albicans* strains because the other tested fungi have the potential to form hyphae and spores that the replicator could not efficiently transfer to an agar plate.

## 2.6. Antagonism Assay

In order to investigate the antifungal activity of bacterial isolates against *P. commune, P. expansum,* and *A. niger*, the antagonism assay was implemented. This method tests the ability of bacteria to inhibit fungal growth as the microorganisms grow together allowing competitive exclusion (Molina-Romero et al., 2017). Potentially fungistatic/fungicidal bacterial strains were grown on Plate Count Agar (PCA) (Neogen® Culture Media, USA) for 24 hours prior to testing. Fungal spore suspension ($OD_{530}$ = 0,3-0,35) was spread on PCA plates using a sterile cotton swab. Afterward, bacterial colonies were placed on a Petri dish in triplicates and the dishes were incubated at room temperature (25°C). Depending on the growth rate of each fungal

strain, conclusive observations were made at 48h for *P. commune*, *P. expansum*, and at 72h for *A. niger*. Results were determined by subtracting the diameter of the bacterial colonies from the diameter of the inhibition zone. Mean values and standard deviation were calculated from triplicates.

## 3. RESULTS AND DISCUSSION

### 3.1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Honey Samples Against *C. albicans 10/20 and C. albicans 351/19*

The MIC assay was implemented, in order to determine the fungistatic activity of honey samples against the *C. albicans* strains. Table 3 summarizes the results regarding both strains.

Table 3. Minimum inhibitory concentrations ( %v/v) of honey samples *against C. albicans 10/20 and C. albicans 351/19.*

| **Honey samples** | ***C. albicans 10/20*** | ***C. albicans 351/19*** |
|---|---|---|
| **Manuka**<br>**195, 229, 183** | 25% | 25% |
| **233, 240** | 25% | 50% |
| **210, 244** | 50% | 25% |
| **200, 271, 187, 267, 212, 243, 218, 269, 241** | 50% | 50% |

Manuka honey as well as three heather honey samples, suppressed the growth of both strains at 25 % (v/v). Honey samples 233 and 240 were able to inhibit *C. albicans 10/20* growth at 25% (v/v), while samples 210 and 244 halted *C. albicans 351/19* growth at the same concentration. Overall, all samples exhibited antifungal activity against the *C. albicans* strains. Table 4 presents the data regarding the MFC against *C. albicans* strains.

Table 4. Minimum fungicidal concentrations (%v/v) of honey samples against *C. albicans 10/20* and *C. albicans 351/19.*

| **Honey samples** | ***C. albicans 10/20*** | ***C. albicans 351/19*** |
|---|---|---|
| **229, 244, 243, 241, 195, 233** | 50% | 50% |
| **Manuka** | ≥50% | ≥50% |
| **200, 210, 183** | >50% | 50% |
| **267, 218** | 50% | >50% |
| **240, 271, 187, 212, 269,** | >50% | >50% |

Manuka honey exhibited fungicidal activity at a concentration higher than 50% against both strains. Heather honey samples 229, 244, 243, 241, 195, and 233 surpassed Manuka honey activity since they showed fungicidal activity against both strains at 50%. A total of eight (8) out of sixteen (16) honey samples were able to kill at least one of the *C. albicans* strains at a concentration of 50% (v/v).

This is the first study presenting data on the antifungal activity of Greek honeys against *C. albicans* strains. In a recent study, Fernandes et al. (2021), tested heather and chestnut honey from Portugal against *Candida* species and determined MICs at 50% v/v, while MFCs were above 50% v/v. In the same study, Manuka honey used in comparison exerted activity at the same concentration. Concentrations of phenols, flavonoids, and hydrogen peroxide of tested honey samples and Manuka honey were similar. Furthermore, the antifungal activity of Portuguese heather honey against *C. albicans* was previously demonstrated by Feás & Estevinho (2011), who determined MIC at 60% v/v. Our data are in accordance with previous studies on Portuguese honey. However, some of the Greek honey samples, exerted fungistatic activity at 25% v/v and fungicidal activity at 50% v/v, surpassing that of Manuka (with the highest available antimicrobial activity, MGO 1122) and Portuguese honey samples.

On the other hand, Kolayli et al. (2020), implementing an agar diffusion method, observed a lack of inhibitory activity against *C. albicans* of heather and chestnut honey harvested in Türkiye, as well as Manuka honey. Kunčič et al. (2012) reached the same conclusion regarding chestnut honey of Slovenian origin. The discrepancy in the results indicates that the activity of honey could be heavily affected by the implemented method of determining the antimicrobial activity. It is generally accepted that determination of MIC is a more sensitive and quantitatively precise method to study antimicrobial activity compared to agar-well diffusion assay because diffusion rates of active substances might be slower in agar than in broth (Anthimidou & Mossialos, 2013).

### 3.2. Minimum Inhibitory Concentration (MIC) of honey samples against *P. commune, P. expansum, A. niger*

Microtiter plates of two-fold diluted tested honey and food spoilage fungi were incubated for 72 hours at room temperature and their growth was studied under an inverted microscope. The Table 5 summarizes the MICs of each honey against *P. commune, P. expansum* and *A. niger*.

Table 5. Minimum inhibitory concentrations %v/v) of honey samples against *P. commune*, *P. expansum*, *A. niger*

| Samples | *P. commune* | *P. expansum* | *A. niger* |
|---|---|---|---|
| **Manuka** | 50% | 50% | 50% |
| **200, 267, 210, 212, 229, 244, 243, 241** | | | |
| **183, 218, 269, 195, 233** | 50% | 50% | >50% |
| **178** | 50% | >50% | 50% |
| **240** | >50% | 50% | >50% |
| **271** | >50% | >50% | 50% |

Manuka honey was able to inhibit the growth of all tested spoilage fungi at 50% concentration. Eight (8) tested honeys, out of which four (4) heather and four (4) Chestnut honey samples, exerted the same activity as Manuka honey.

*Penicillium expansum* and *Aspergillus niger* were reported to be more sensitive to honey of diverse botanical origin in previous studies (Ahmad et al., 2017; Kunat-Budzyńska et al., 2023; Suhana et al., 2015; Vică et al., 2022, p. 4). Kacániová et al. (2010), implementing agar well diffusion assay, established that Chestnut honey could inhibit, though not completely, *P. expansum* growth at 10% w/v concentration. Suhana et al. (2015) determined the MIC of Manuka honey against *A. niger* at 21% v/v, surpassing the other tested honey samples. Of note, this is the first study to present data on honey antifungal activity against *P. commune*.

### 3.3. Antifungal activity against *P.commune, P. expansum,* and *A. niger* exerted by bacterial strains

Assessment of antifungal activity exerted by bacterial strains was performed by parallel growth of fungal and bacterial strains on the same growth medium. Examples of the observed inhibition zones around the bacterial colonies are depicted in Figure 2 and their values are presented in Table 6.

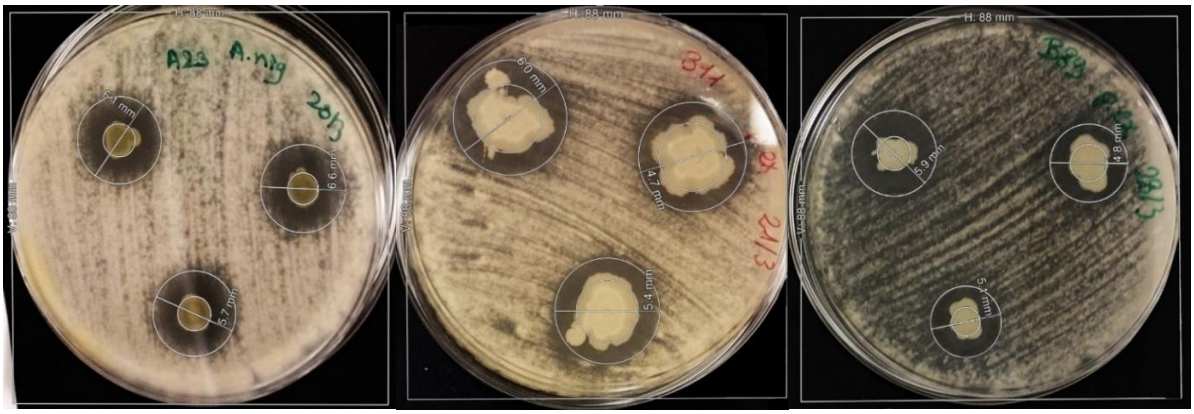

Figure 2. Examples of inhibited fungal growth around bacterial colonies (Left: inhibition of *A. niger* by A23 - *P. fulva*, right: inhibition of *P. expansum* by B11 -*Bacillus sp.*, center: inhibition of *P. expansum* by B89 - *B. safensis*

Table 6. Inhibition zone diameter of bacterial isolates against *P. commune*, *P. expansum*, *A. niger*

| **Bacterial isolates** | ***P. commune*** | ***P. expansum*** | ***A. niger*** |
|---|---|---|---|
| **A2** | 8.4 ± 0.33 mm | 17.6 ± 0.53 mm | 8.6 ± 0.32mm |
| **A15** | 8.8 ± 0.57 mm | 11.6 ± 1.81 mm | 8.5 ± 0.29 mm |
| **A31** | 8.2 ± 0.34 mm | 7.9 ± 0.30 mm | 6.8 ± 0.75 mm |
| **A163** | 4.6 ± 0.21 mm | 11.0 ± 0.60 mm | 8.0 ± 0.49 mm |
| **B16** | 4.0 ± 0.42 mm | 10.2 ± 0.78 mm | 5.2 ± 0.85 mm |
| **B21** | 8.4 ± 0.62 mm | 9.4 ± 1.92mm | 9.9 ± 0.74 mm |
| **B89** | 8.9 ± 0.23 mm | 10.5 ± 1.14 mm | 9.1 ± 0.54 mm |
| **A20** | 8.1 ± 1.60 mm | 15.1 ± 2.57 mm | 9.2 ± 0.50 mm |
| **B11** | 8.5 ± 0.41 mm | 11.1 ± 1.44 mm | 7.3 ± 0.34 mm |
| **A28** | 4.5 ± 1.16 mm | 8.2 ± 1.13 mm | 5.9 ± 1.20 mm |
| **A23** | 4.2 ± 0.74 mm | 13.1 ± 1.88 mm | 12.3 ± 0.74 mm |
| **A138** | 12.9 ± 2.00 mm | 9.4 ± 1.80 mm | 5.4 ± 0.20 mm |
| **B7** | - | 11.5 ± 1.74 mm | 6.6 ± 0.28 mm |
| **B120** | - | 13.0 ± 1.00 mm | 5.6 ± 0.34 mm |
| **B34** | - | 10.2 ± 1.00 mm | 9.0 ± 0.65 mm |

With the exception of A23-*Pseudomonas fulva*, all tested bacterial strains are *Bacilli*. In previous studies, members of this genus isolated from raw honey were reported to produce *in vitro* a variety of secondary metabolites that could inhibit the growth of other microorganisms in a competitive way. Manns et al. (2012), were able to identify an antifungal peptide produced by *B. thuringiensis* SF361 isolated from honey exerting activity against *Aspergillus*, *Penicillium*, *Byssochlamys*, and *Candida albicans*. Similarly, Xiong et al. (2022), attributed the antifungal activity exerted by two strains of *Bacillus velezensis* to iturin A, a known lipopeptide that inhibits fungal growth. *B. subtilis* and *B. licheniformis* have been the subject of extensive research by Harwood et al., 2018), intending to characterize the synthesis of antifungal non-ribosomally synthesized peptides and polyketides produced by them. Cyclic lipopeptides, such

as surfactin, iturin, pipastatin, and fengysine, applied as antifungal agents for the control of plant diseases, proved to be of outstanding importance (Xiong et al., 2022). Therefore, it is plausible that antifungal activity reported in this study could be attributed to so far unknown secondary metabolites belonging to nonribosomal peptides and /or polyketides. Nevertheless, further research regarding the biosynthetic potential of tested bacterial isolates could elucidate the mechanisms of antifungal activity described in this study.

## 4. CONCLUSION

In conclusion, this study provides evidence of the antifungal properties of Greek heather and chestnut honeys, alongside certain bacterial isolates, highlighting their potential as antifungal agents.

The results demonstrate that *Penicillium expansum, Penicillium commune, Aspergillus niger,* and *Candida albicans*, are susceptible to the majority of tested honeys, though in a variable way. To the best of our knowledge, this is the first study to investigate the inhibitory effects of honey on *P. commune* growth. Some of the tested honey samples did not only match the antifungal activity of renowned Manuka honey but in certain cases surpassed it, particularly heather honey against *Candida albicans*, exerting fungicidal efficacy at lower concentrations.

Furthermore, our research is the first to examine the *in vitro* antifungal activity of characterized bacterial strains isolated from diverse Greek honey types, against food spoilage fungi. Most isolates were able to inhibit the growth of *Penicillium expansum*, *Penicillium commune,* and *Aspergillus niger*. These findings further support the hypothesis that competitive relationships among microorganisms foster the production of secondary metabolites with antifungal properties.

Given the growing concerns regarding antifungal resistance, our data are important in the search for novel antifungal substances. Future studies should focus on specific bioactive compounds exerting the observed antifungal activity and elucidating their mode of action.

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## DECLARATIONS

The authors declare no conflicts of interest.

## AUTHORS' CONTRIBUTIONS

**Ioanna Boutrou**: Investigation, Methodology, Data curation, Writing-Original draft preparation. **Christina Tsadila**: Investigation, Methodology, Data curation. **Chiara Amoroso**: Investigation, Data curation. **Dimitris Mossialos**: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervising

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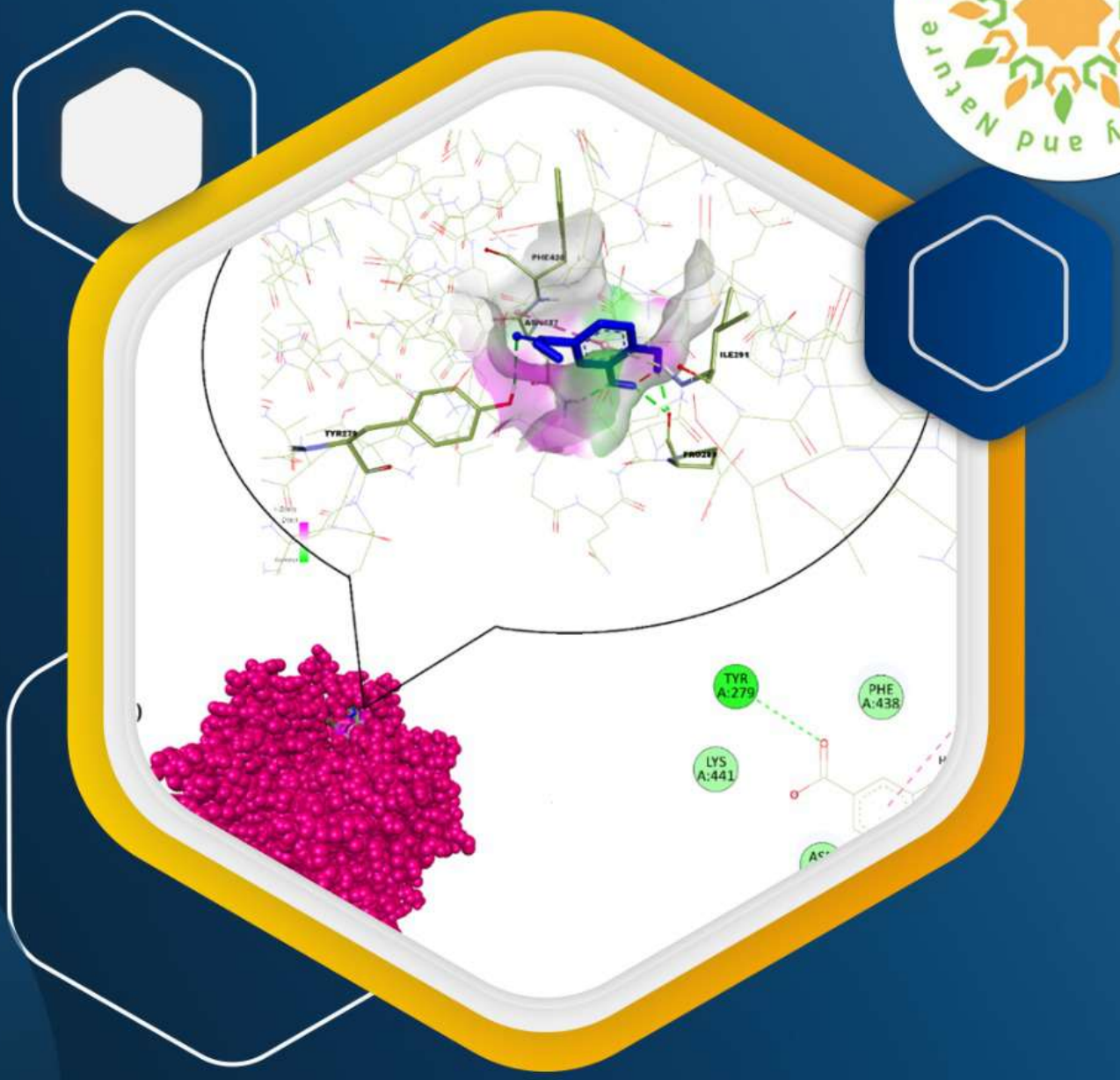


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