INVESTIGATION OF ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF SOME GREEN LEAF PLANTS

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ABSTRACT

INVESTIGATION OF ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF SOME GREEN LEAF PLANTS

The present study aims to investigate the chemical composition, antimicrobial activity, antioxidant properties, and cytotoxic activity of extracts obtained from leaf samples that are discarded as waste products. For this purpose, two different green leaves were selected: one is the grape (*Vitis vinifera*) leaf, a by-product from the winemaking process, and the other is the cauliflower (*Brassica oleracea,* var. *botrytis*) leaf, which is a waste product of cauliflower, and they were subjected to related tests. Both leaf samples were extracted by water because of its easy accessibility and environmentally friendly properties. Leaf extracts were examined according to their total phenolic content and subjected to chemical characterization by Liquid chromatography-quadrupole time-of-flight tandem mass spectrometry system. The promising antioxidant activities of the water-extracted leaf samples were authenticated through DPPH and ABTS assays. Antimicrobial and antibiofilm activities were examined against some Gram-positive (*Bacillus cereus*, *Listeria innocua*, and *Carnobacterium divergens*) and Gram-negative (*Escherichia coli*, *Serratia liquefaciens*, and *Salmonella* Typhimurium) strains, and two fungi (*Saccharomyces cerevisiae* and *Candida albicans*) species. Both tested leaf extracts showed a dose-dependent antimicrobial activity, while the antimicrobial activity of grape leaf extract was slightly higher. However, their activities against biofilm formation were varying in different bacterial and fungi species. The cytotoxic activity of the leaf extracts was examined on the mouse fibroblast cell (L929) line. According to the presented results, neither of the leaf extract samples used in the study showed any unwholesome effects on the cell line at any time point.

ÖZET

BAZI YEŞİL YAPRAKLI BİTKİLERİN ANTİMİKROBİYAL, ANTİOKSİDAN VE SİTOTOKSİK ÖZELLİKLERİNİN İNCELENMESİ

Bu çalışma, atık ürün olarak değerlendirilen bazı yeşil yaprak örneklerinden elde edilen ekstraktların kimyasal bileşimini, antimikrobiyal aktivitesini, antioksidan özelliklerini ve sitotoksik aktivitesini araştırmayı amaçlamaktadır. Bu amaç için biri şarap yapım sürecinden çıkan bir yan ürün olan asma (*Vitis vinifera*) yaprağı, diğeri ise yenmeyen kısmı olarak nitelendirilen karnabahar (*Brassica oleracea,* var. *botrytis*) yaprağı olmak üzere iki farklı bitki örneği kullanılmıştır. Yaprak örneklerinin her ikisi de kolay erişilebilirliği ve çevre dostu olması sebebiyle su ile ekstrakte edilmiştir. Yaprak özütleri toplam fenolik içeriklerine göre incelenmiş ve sıvı kromatografi-dört kutuplu uçuş süresi tandem kütle spektrometresi sistemi ile kimyasal karakterizasyona tabi tutulmuştur. Suyla ekstrakte edilmiş yaprak örneklerinin antioksidan aktiviteleri, DPPH ve ABTS aracılığıyla doğrulanmıştır. Antimikrobiyal ve antibiyofilm aktiviteleri bazı Gram-pozitif (*Bacillus cereus*, *Listeria innocua* ve *Carnobacterium divergens*) ve Gram-negatif (*Escherichia coli*, *Serratia liquefaciens* ve *Salmonella* Typhimurium) bakteri türlerine ve iki mantar (*Saccharomyces cerevisiae* ve *Candida albicans*) türüne karşı test edilmiştir. Her iki örnek de doza bağlı bir antimikrobiyal aktivite gösterirken, üzüm yaprağı ekstraktının antimikrobiyal aktivitesinin daha yüksek olduğu gözlemlenmiştir. Ancak biyofilm oluşumuna karşı aktiviteleri farklı bakteri ve mantar türlerinde değişkenlik göstermektedir. Yaprak ekstraktlarının sitotoksik aktivitesi fare fibroblast hücre (L929) hattı üzerinde incelenmiştir. Nihai sonuçlara göre, çalışmada kullanılan yaprak özü örneklerinin hiçbiri, herhangi bir zaman noktasında hücre hattı üzerinde herhangi bir sağlıksız etki göstermemiştir.

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a*	Red–green component
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid
ACE	Angiotensin-I converting enzyme
b*	Yellow–blue component
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CFU/mL	Colony forming unit per milliliter
$CHCl_3$	Chloroform
COX-I enzyme	Cyclooxygenase 1
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
FBS	Fetal bovine serum
FRAP	Ferric ion reducing antioxidant power
GAE	Gallic acid equivalents
GLS	Glucosinolates
HAT	Hydrogen atom transfer
HGF	Human gingival fibroblast cells
HPLC	High performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with photodiode-array detection
HPLC-PDA	High-performance liquid chromatography with photodiode array detector
HPLC-RP–DAD	High-performance liquid chromatography with reversed phase photodiode-array detection
HPLC–HRESI-MS	Sensitive high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

HUVECs	Human vascular endothelial cells
IC_{50}	Half maximal inhibitory concentration
L*	Lightness
LC-Q-TOF-MS	Qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole
n-BuOH	N-Butanol
Na_2CO_3	Sodium carbonate
NMR	Spectroscopy Nuclear magnetic resonance spectroscopy
$-OCH_3$	Methoxy group
-OH	Hydroxy group
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate-buffered saline
PG	Propyl gallate
ppm	Parts per million
PPP	Pentose-phosphate pathway
R-H_2O	Residual water
Redox	Reduction-oxidation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
SET	Single electron transfer
TBHQ	Tert-butyl hydroquinone
TSB	Tryptic soy broth
UPLC-DAD-HDMS-TOF-MS	Ultraperformance liquid chromatography with diode array detector-quadrupole time-of-flight-high-definition mass spectrometry

CHAPTER 1

INTRODUCTION

Plants have been an integral part of the medicine and food industries for a long time, due to the essential nutrients and bioactive substances they contain. Traditional practices and modern pharmaceuticals use plant-derived compounds for healing. Numerous plant-derived compounds have been harnessed for their therapeutic potential, giving rise to traditional practices like Traditional Chinese and Ayurveda (Indian) Medicine. For instance, *Lamiaceae* (*Rosmarinus*) species are one of the most important aromatic and medicinal plants with many beneficial bioactive compounds in medicine, and food (Skendi et al. 2022). Even today, plants are giving inspiration to modern pharmaceuticals, with many drugs being derived from botanical sources. It has been stated that despite synthetic advances, about 25% of drugs are of plant origin (Fowler 2006).

Besides, approximately 20,000 plant taxa serving medicinal purposes worldwide, the complex plant-drug-industry relationship offers sustainable bioactive opportunities (Hoareau and Dasilva 1999). However, the use of plants for medicinal purposes is quite limited in Turkish culture. According to the reported data, about 500 plant taxa are used for medicinal purposes (Altundağ and Aslım 2005). On the other hand, most of the plants used in the field of health are in the class of medicinal plants. However, in the food industry, a lot of by-products are obtained during the processes. Although these products are mostly considered waste, many recent studies have stated that plant wastes and by-products such as seeds, leaves, stems, and pulp contain a substantial number of bioactive substances (Gyawali and Ibrahim 2014).

Recent studies include considerable reviews investigating methods of recovering phenolic compounds from vegetable wastes generated during the processing of vegetables and fruits. These studies focus on the analysis of bioactive substances in the waste product and the extraction of these substances with the highest efficiency (Skendi et al. 2022). Besides, the potential uses of the obtained high-quality bioactive substances are also considered as worth-studying subjects.

Plant secondary metabolites just as phenolic compounds, sulfur-containing compounds, alkaloids, and terpenoids are a variety of bioactive substances synthesized by plants to perform various ecological functions, often beyond their basic survival needs. (Guerriero et al. 2018). These metabolites benefit plants in many aspects such as plant flowering, intracellular communication, and insect and pathogen invasion (Teoh 2016). Besides, the plant's secondary metabolites contribute significantly to human health and well-being. For instance, many secondary metabolites exhibit antimicrobial properties by inhibiting the growth and proliferation of pathogenic microorganisms (Bakkali et al. 2008). In this way, it also contributes to the development of natural food additives and offers promising alternatives to synthetic agents.

Moreover, plant secondary metabolites exert potent antioxidant effects by controlling oxidative stress and reducing cellular damage caused by free radicals (Rice-Evans, Paganga, and Miller 1997). Thus, they lead to potential therapeutic applications in alleviating chronic diseases due to oxidative imbalance. Besides, lipid oxidation is a problem that causes off-flavor in food products and thus a decrease in sensory quality and shelf life. To overcome this problem, many synthetic antioxidant agents such as tert-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG) are used (Lourenço, Moldão-Martins, and Alves 2019). However, secondary metabolites have promising antioxidative features that can contribute to increasing food quality naturally by preventing lipid oxidation in foods (Lourenço, Moldão-Martins, and Alves 2019).

Furthermore, some plant secondary metabolites have cytotoxic properties, which prevent cancer cells from growing and surviving (Cragg and Newman 2005). The discovery of these compounds has enormous potential for developing new natural anticancer agents. Considering that medicinal plants are mostly used in the treatment phase of the disease, bioactive metabolites extracted from food waste can be added to the daily diet and may lead to studies aimed at preventing the disease rather than treating it. Overall, the versatile capabilities of plant secondary metabolites underline their important role in promoting health, advancing research, and providing sustainable solutions in various fields.

Many products that are formed during the collection and processing of foods or that have no use value are considered waste. These waste products accumulate over time and cause environmental pollution. On the other hand, with the increasing world population, access to natural resources and nutrients is decreasing day by day. For this

reason, isolating bioactive products from plant wastes is critical both in preventing waste depletion and in creating new value-added products. (Bala et al. 2023). Besides, it is also possible to develop different products without searching for new resources (Eren et al. 2021). In this way, it is possible to prevent environmental degradation and make more sustainable production.

The aim of this study is to characterize some green leafy plants evaluated as waste by chemical content analysis and to determine their antimicrobial, antioxidant, and cytotoxic properties.

The major goals of the study are as follows:

(1) Determination of the chemical composition and bioactive metabolites of plant-based waste extracts.
(2) To ensure the recognition of green leafy plants seen as waste and to encourage their use.
(3) Providing added value to plant-based waste products.
(4) Studying substances that are beneficial for human health.
(5) Developing new food supplements and additives with high-added value.

CHAPTER 2

LITERATURE REVIEW

2.1. Botanically Definition

2.1.1. Grape (*Vitis vinifera L.*) Leaves

Vitaceae is known as a medium-sized woody plant family with more than 900 species to 16 extant genera, native to the Mediterranean region (Wen et al. 2018). The family characteristically has lianas with tendrils opposite to the leaves (Simpson 2010), and the plant has a good climbing feature through the tendrils (Şendoğdu et al. 2006). *Vitis vinifera* is a species of the genus *Vitis*, which belongs to the *Vitaceae* family, and is one of the most popular crops because of the grapes.

Grapes are one of the most popular agricultural goods with cultivation land of 7.3 million hectares, and approximately 75 million tons of total production in 2021 ("Annual Assessment of the World Vine and Wine Sector in 2021 International Organisation of Vine and Wine Intergovernmental Organisation" 2021). Among the 10 thousand different grape crops, 33 of them are composed of half of the grape production all over the world (Venkitasamy et al. 2019). Although the use of grapes differs in countries' physical and politico-religious status, nearly half of the production is processed for winemaking, while 40% is reserved for fresh consumption, and the rest is for raisin production ("Annual Assessment of the World Vine and Wine Sector in 2021 International Organisation of Vine and Wine Intergovernmental Organisation" 2021; Venkitasamy et al. 2019). Italy, Spain, France, and Chile are some of the top countries in wine production, whereas a few countries including Turkey are focusing to produce table grape and raisin production("Annual Assessment of the World Vine and Wine Sector in 2021 International Organisation of Vine and Wine Intergovernmental Organisation" 2021). Turkey ranks 5th in the world in fresh grape production with an annual production

capacity of 3.7 million tons. Almost all this production (357.5 thousand tons) is processed as raisins, 1.9 million tons are consumed as table grapes, and the rest is used in grape juice and wine production ("Annual Assessment of the World Vine and Wine Sector in 2021 International Organisation of Vine and Wine Intergovernmental Organisation" 2021). Some waste products e.g., seeds, and pomace are produced during the harvesting and processing of grapes (Baroi et al. 2022). Yet, studies on the reuse or evaluation of these wastes, except for grape seeds are insufficient.

Figure 2. 1 Fresh *Vitis vinifera L.* leaves

Figure 2. 2 Front and back sight of the fresh *V. vinifera* leaves

As shown in Figure 2.1 and Figure 2.2 above, *V. vinifera* leaves are medium to large (8–1000 in width) in size, often heart-shaped with numerous lobes, and color from light to dark green with a unique taste (Cantwell et al. 2022). The leaves have an important place in folk medicine dating from Ancient Egypt to Ancient Greek times (Deliorman Orhan et al. 2009). The leaves of *V. vinifera* are used as a folk remedy in India and Europe for many health problems including bleeding, hemorrhoids, diarrhea, and vomiting (Katalinic et al. 2013). In Anatolia, the leaves of *V. vinifera* are used by diabetics to reduce blood glucose levels (Şendoğdu et al. 2006). In several traditional medicines, the juice of the *V. vinifera* leaves is also recommended as an antiseptic eyewash. (Fernandes et al. 2013). Furthermore, *V. vinifera* leaves have also been used in traditional cuisines. "Sarma"-rolled leaves with rice, herbs, and sometimes meat- is one of the most popular dishes from Turkish cuisine. However, the leaves used in cuisine are selected from young, good-quality fresh leaves (Cantwell et al. 2022). Despite the fact *V. vinifera* leaves have long been used by various civilizations for direct consumption and human health, they constitute the vast majority of waste generated during production and winemaking. The generation and accumulation of the leaves as waste have led to cause some environmental and economic problems.

2.1.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

Brassicaceae family includes about 350 genera and more than 3,000 species of annual or perennial woody plants, semi-shrubs, or shrubs (Mandrich and Caputo 2020; Jahangir et al. 2009). This plant family is also known as *Cruciferae* and has simple leaves and bisexual flowers (Simpson 2010). Many commercially important genera such as broccoli, mustard, cauliflower, cabbage, and radish belong to the *Brassicaceae* family (Mandrich and Caputo 2020; Baky et al. 2022). Although it varies around the world, the Mediterranean and its neighboring lands have an important place in terms of species diversity and distribution (Mandrich and Caputo 2020).

Cauliflower (*Brassica oleceae var. botrytis*), one of the favored members of this family, is one of the widely produced and consumed cultivars in worldwide with its glucosides, phenolic substances, and vitamins (Huynh et al. 2016). This species, which is a biennial plant, consists of a white or yellowish edible flower in the middle and dark green pointed leaves ("Tarım ve Orman Bakanlığı, Karnabahar Yetiştiriciliği," n.d.). Although it is grown in winter, very low temperatures negatively affect the development of the plant, usually, 15-20°C is the optimum growing temperature ("Cauliflower" 2021; "Tarım ve Orman Bakanlığı, Karnabahar Yetiştiriciliği," n.d.). Cauliflower and broccoli production has a production share of 2% in world vegetable production (Balkaya, Gör Şeyma Sarıbaş, and Müh Tolga Özgen Ondokuz Mayıs Üniversitesi Ziraat Fakültesi Bahçe Bitkileri Bölümü -Samsun, n.d.). With the increase in production farms in America in recent years, cauliflower has been produced in an area of about 20 thousand hectares in 2020 ("Cauliflower" 2021). In Turkey, cauliflower production, which was 180 thousand tons in an area of 0.076 hectares in 2015, reached 215 tons in 2020 ("Bitkisel Üretim Verileri" 2020). Antalya, İzmir, and Bursa are the provinces where the most cauliflower is grown in our country (Balkaya, Gör Şeyma Sarıbaş, and Müh Tolga Özgen Ondokuz Mayıs Üniversitesi Ziraat Fakültesi Bahçe Bitkileri Bölümü -Samsun, n.d.).

Figure 2. 3 Fresh *Brassica oleceae var. botrytis* leaves

The whitish middle flowers of the cauliflower are considered the sole edible part. Thus, the way of consumption creates a high amount of non-edible parts like stems, pods, and other leaves (Huynh et al. 2014). From harvest to consumption tons of cauliflower by-products are generated, which causes environmental pollution because of the present organic matter and moisture contents (Xu et al. 2017). The leaves compose almost half of the by-product generation (Zenezini Chiozzi et al. 2016). Although there are many new approaches and research studies to overcome waste generation, cauliflower leaves have been restricted to only flour and fiber production in a small portion (Zenezini Chiozzi et al. 2016). The fresh cauliflower leaves are shown in Figure 2.3 above.

2.2. Chemical Composition and Bioactive Metabolites

2.2.1. Chemical Composition and Bioactive Metabolites of Grape (*Vitis vinifera L.*) Leaves

Despite it has place in the cuisine and traditional medicine of different cultures, most of the grapevine waste products are generated by the leaves. Thus, the determination of the chemical composition and bioactive metabolites of grape leaves is important for further studies. Phenolic compounds are the most prominent bioactive compounds produced via shikimic acid and pentose-phosphate (PPP) metabolizations in plants (Lin et al. 2016). Simple or complex flavonoids, stilbenes, phenolic acids, tannins, and anthocyanins are some phenolic examples (Gan et al. 2018). These compounds have at least one hydroxylated benzene ring in their structure (Lin et al. 2016; Hooper and Cassidy 2006). The -OH groups in the structure of phenolics have been correlated with their reported capacities to reduce LDL levels, and tumor growth, as well as their anti-inflammatory and antibacterial actions (Hooper and Cassidy 2006).

V. vinifera leaves from two different cities in Tunisia were harvested in June and July, and the chemical composition of the extracted leaves was demonstrated by Aouey et al (Aouey et al. 2016). The outcomes of the study indicated that the extracted leaves were rich in flavonoids including proanthocyanins and anthocyanins, phenolic acids, minerals, sugars, amino acids, and sterols (Aouey et al. 2016). It has also been stated that high levels of polyphenols looked promise as antimicrobial, anti-cancerogenic, antioxidative, and anti-inflammatory properties (Aouey et al. 2016). In a study, chlorogenic acid, gallic acid, vanillic acid, catechin, photocathodic acid, syringic acid, ferulic acid, coumarin, catechol, caffeic acid, and pyrogallol were isolated from the red grape cultivar Sultana (Hussein and Abdrabba 2015). Turkish-origin *Vitis vinifera* leaves were extracted by water and the chemical and phenolic compounds were examined. In parallel with previous studies, the results showed that the grapevine leaves contain anthocyanins, phenolic acids, tannins, lipids, carotenoids, terpenes, enzymes, organic acids such as ascorbic acid, tartaric acid, fumaric acid, citric acid, malic acid, and non-reducing or reducing sugars. The importance of phenolics was also highlighted because

of their therapeutic properties (Deliorman Orhan et al. 2009). Accordingly, Fernandes et al. (2013) examined the *V. vinifera* leaves from 20 different red and white varieties in Portugal and stated that the phenolics are the major compounds to give some therapeutic properties to the plant (Fernandes et al. 2013). According to the study performed by Pintac et al. (2019), fresh, green, and healthy *V. vinifera* leaves were found richer in phenolic compounds e.g., phenolic acids (Pintać et al. 2019). Moreover, it has been also concluded many glycosylated compounds like quercetin-3-O-glucoside, hyperoside, and rutin were more copious in the leaves than in the pomace (Pintać et al. 2019). Grapevine leaves and tendrils grown in Romania were analyzed by Moldovan et al. (Moldovan et al. 2020). The results showed that the organic acids (gallic, caftaric, and protocatechuic acids), quercitrin, epicatechin, catechin, hyperoside, isoquercitrin, rutin were found in the leaves (Moldovan et al. 2020). Besides, it has been stated the total flavonoid content of the Romanian leaves was richer than that in Croatia, Turkey, and India. They also concluded the leaves of *V. vinifera* may be used in medicinal and cosmetic industries because of their encouraging isoquercitrin content (Moldovan et al. 2020). In another study, phenolic compounds (caffeic acid, catechin, resveratrol, epicatechin, quercetin, and rutin) were obtained from a total of six grape varieties, five of which are native to West Azerbaijan (Ghara Ghandome, Agh Shani, Hosseini, Ghara Shani, and Ghara Shira), and one international (Muscat Alexandria). It has also been concluded that the amount of total phenolics differs in different cultivars (Farhadi et al. 2016). Dresch et al. (2014) analyzed the leaves of Couderc and Cabernet Sauvignon varieties (Dresch et al. 2014). Flavonoids like quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucuronide, and rutin were mostly identified compounds, however, Couderc had more phenolic content than Cabernet Sauvignon (Dresch et al. 2014). All researchers, who included different *V. vinifera* species in their studies, agreed that the total phenolic content differs from species to species (Katalinic et al. 2013; Farhadi et al. 2016; Fernandes et al. 2013; Soylemezoglu et al. 2016; Balík et al. 2008; Dresch et al. 2014; Deliorman Orhan et al. 2009; Aouey et al. 2016). Some of the most abundant phenolics in the *V. vinifera* leaves are shown in Figure 2.4 below.

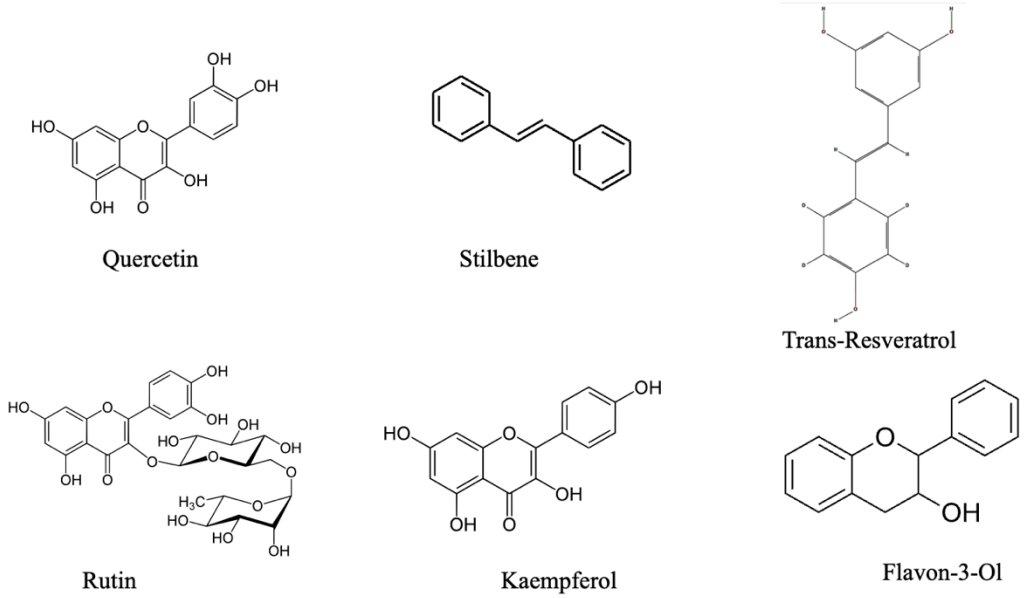


Figure 2. 4 Chemical structures of some most abundant bioactive metabolites found in grapevine leaves. (Source: https://pubchem.ncbi.nlm.nih.gov)

Beyond the variety differences, the amounts of phenolic content may be affected by different factors. *V. vinifera* leaves from six different varieties (white: Maraština, Pošip; red: Merlot, Lasin, Vranac, Syrah) were harvested during May, August, and September. September leaves were richer in total stilbenes, flavonols, phenols, and flavonoids. The presented results revealed that both varieties and picking time had an impact on the phenolic content and chemical composition (Katalinic et al. 2013). The environmental factors e.g., the soil, sun exposure, pesticides, and infections have also an impact on plant phenolics. In a study where the organic and conventionally produced grape leaves were compared, it was reported that even though there were statistical differences, the total phenolic contents were similar in both leaves. However, the amount of resveratrol was more abundant in the organic leaves, whereas the naringin was unique in the conventional leaf extract (Dani et al. 2010). A series of different enzymatic and chemical reactions e.g., oxidation of phenols by phenol oxidases and polymerization of free phenols may occur in different maturation stages, thus, the phenolic content tends to be variable (Doshi, Adsule, and Banerjee 2006). The total phenolic potential of the *V. vinifera* leaves from Hasankeyf, Turkey was examined by Selçuk et al. (Selçuk et al.

2017). According to the foundings, it has been stated that the young leaves had a higher total phenolic content than mature ones. Parallelly, young leaves had higher flavonoid content (Selçuk et al. 2017). Besides, Doshi et al. (2006) concluded that the leaves, petioles, stems, and shoots are more dependent on the maturation stage, compared to berries (Doshi, Adsule, and Banerjee 2006). In the study where the sun-exposed and grown under-the-shadow leaves were analyzed, the results showed that the leaves which are grown under the shadow had lower flavonoids and flavanols (Bodó et al. 2017).

Moreover, the processing conditions before the consumption is another factor to be considered. The leaves to be consumed are generally collected when they are young and stored in brine. In research where the fresh, brined, and unbrined vine leaves were studied, flavonoids and hydroxycinnamic acid were abundant in all the samples, where the amounts of caffeic acid were variable. Total phenolics and flavonoids were more abundant in the unbrined fermented leaves, however, the flavanol level was the lowest in the same extract (Koşar et al. 2007). Moreover, it has also been reported that the amount of quercetin tends to be increased with increased fermentation (Koşar et al. 2007). Besides, grapevine leaves become edible after proper cooking. When the leaves have been exposed to heat, their bright green color turns yellow-brownish color due to the reduction in chlorophyll. Besides, thermal processes such as boiling and blanching give a woody and herbaceous taste by providing the formation of volatiles (Lima et al. 2017).

Alongside the environmental and processing factors, the phenolic content may vary in the presence of microbial infections. Balik et al., examined the differences in phenolic compounds of healthy and grey mold-infected *V. vinifera* leaves in different varieties (Balík et al. 2008). Healthy leaves resulted in a higher polyphenol and flavanol. Besides, higher trans-resveratrol and trans-piceid were detected in both, infected and healthy leaves than in berries (Balík et al. 2008). In a different research study, Atak et al. analyzed the total phenolics of both healthy and infected by downy or powdery mildew grape leaves from 21 different cultivars (Soylemezoglu et al. 2016). Even though it differs from cultivar to cultivar, the phenolic content was increased significantly due to the infection for all varieties. Besides, *V. vinifera* was reported as less resistant to fungal infections. (Soylemezoglu et al. 2016). It has been stated that phenolics, as plant secondary metabolites, are produced under stress conditions, including UV irradiation and infections (Teixeira et al. 2014; Lin et al. 2016).

2.2.2. Chemical Composition and Bioactive Metabolites of Cauliflower (*Brassica oleceae var. botrytis*) Leaves

Vegetables in the *Brassica* family are inexpensive but very nutritious food sources as the active ingredients they contain (Jahangir et al. 2009). Cauliflower (*Brassica oleceae var. botrytis*), one of the best-known members of the *Brassica* family, has a very important place in daily nutrition and agriculture through its easy accessibility and important nutritional values such as vitamins, minerals, fiber, polyphenols, and glucosinolates (Cabello-Hurtado, Gicquel, and Esnault 2012). However, since the consumption of cauliflower is generally limited to the whitish flowering part in the middle, non-edible parts such as the outer leaves and stem cause significant waste accumulation (Elhassaneen et al. 2016; Huynh et al. 2016). About half of the waste accumulated from cauliflower production to consumption is generated by the outer leaves (Llorach et al. 2003). It is known that the accumulation of waste material causes environmental pollution and thus economic losses (Llorach et al. 2003). Hence, lately, significant research has been launched to identify the chemical composition of the waste from fruits and vegetables and to improve the economic value of these products (Jahangir et al. 2009; Cabello-Hurtado, Gicquel, and Esnault 2012; Xu et al. 2017). According to recent studies, it has been stated that the non-edible parts of cauliflower leaves contain important metabolites such as carbohydrates, amino acids, fibers, soluble sugars, and vitamins (Baky et al. 2022). Besides, in a study, it was stated that cauliflower outer leaves contain more than 20% leaf protein as a promising plant-based protein source (Xu et al. 2017).

Moreover, it has been determined that cauliflower leaves are rich in glucosinolates, organic sulfur-containing phytochemicals, and secondary metabolites, predominantly flavonol derivatives (Baky et al. 2022; Llorach et al. 2003).

Phenolic compounds, one of the secondary metabolites of plants, contain one or more hydroxylated benzene rings and are formed as a result of shikimic acid and pentose-phosphate (PPP) metabolizations (Lin et al. 2016; Hooper and Cassidy 2006). Phenolic compounds found in cauliflower leaves can be examined in two groups. The first is phenolics, which are attached to the cell wall by hydrophobic interactions, hydrogen bonds, and ester bonds, and the second is free phenolics (Huynh et al. 2014). From this

point of view, it can be concluded that the extraction method to be used has a direct effect on obtaining bioactive substances. In a study on phenolic substances obtained from the outer leaves of cauliflower by enzyme-assisted extraction, the total amount of phenolic substances was stated as 336 ± 30 mg GAE / 100 g DW, independent of enzyme type and concentration, temperature, and pH parameters (Huynh et al. 2014). In a different research, on the effect of the extraction method on the phenolic profile, cauliflower leaves were first subjected to solid-state fermentation and then extracted with methanol (Huynh et al. 2016). As a result of the study, the dominant phenolics in the unfermented sample were kaempferol-3-O-diglucoside-7-O-glucoside (34.8 mg RE/100 g FW) and kaempferol-3-O-diglucoside (24.8 mg RE/100 g FW) (Huynh et al. 2016). After 7 days of fermentation, the total phenolic content was measured as 25.3 ± 13.1 mg RE/100 g FW, which is relatively 5 times lower than the unfermented sample (Huynh et al. 2016). Besides, it was stated that cauliflower leaves are predominantly rich in kaempferol derivatives (Huynh et al. 2016; 2014). Sanz-Puig et al (2015) stated that the total phenolic content of cauliflower leaves mainly consists of organic acids such as chlorogenic acid, gallic acid, ferulic acid, and catechin (Sanz-Puig et al. 2015). Along in this study, the total polyphenol content was measured as 11359.8135 ± 747.96277 mg gallic acid/L (Sanz-Puig et al. 2015). In another study, it was stated that cauliflower leaves contain high amounts of organic acids (coumaric acid, caffeic acid, ferulic acid, and sinapic acid) and quercetin-3-O-diglucoside-7-O-glucoside alongside kaempferol and its derivatives (Gonzales et al. 2014).

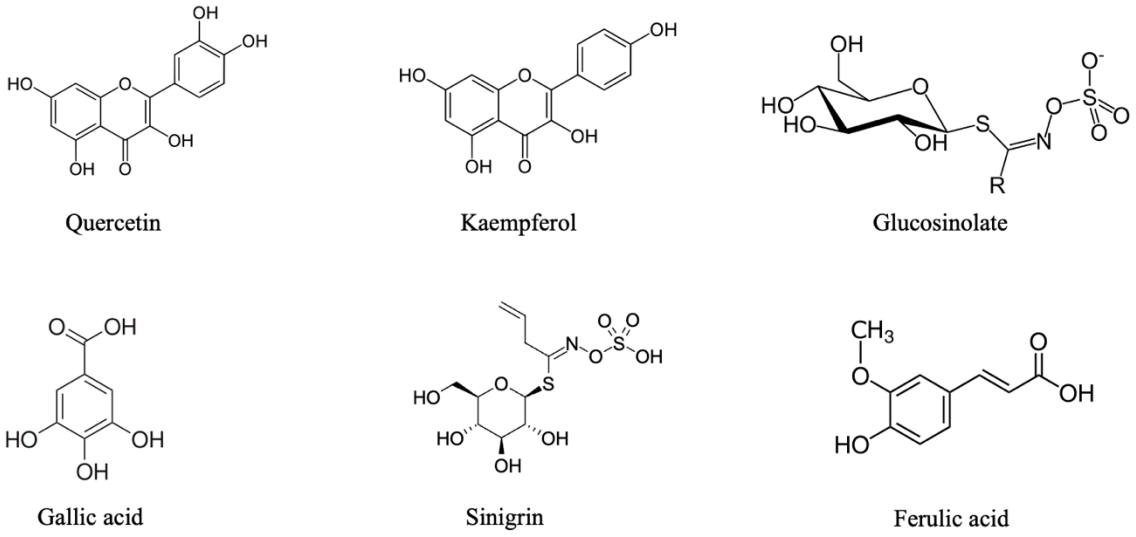


Figure 2. 5 Chemical structures of some most abundant bioactive metabolites found in cauliflower leaves. (Source: https://pubchem.ncbi.nlm.nih.gov)

On the other hand, cauliflower, like other *Brassica* vegetables, contains a significant amount of organic sulfur, which is responsible for the characteristic flavors and odors unique to these vegetables (Valette et al. 2003). Glucosinolate compounds contain a sulfur-containing fraction attached to the β-D-glucopyranose structure and a variable side chain originate some branched-chain amino acids and phenylalanine, methionine, and tryptophan (Fahey, Zalcmann, and Talalay 2001; Mithen et al. 2000). According to the amino acid precursors in the R groups, glucosinolates are examined in three main groups aliphatic, indole, and aromatic (Cartea et al. 2011; Verkerk et al. 2009). Although glucosinolates are found in almost all organs of plants, concentration differences may be observed (Verkerk et al. 2009). The stem and leaves contain lower concentrations of glucosinolate than the root and head of the plant (Cartea et al. 2011). However, Baky et al. (2022) stated that cauliflower leaves had approximately 5% more glucosinolate content than the edible parts (Baky et al. 2022). Glucosinolates create natural protection against herbivores and various microorganisms as a result of the sharp taste and odor profile they provide (Mandrich and Caputo 2020). Environmental factors such as temperature, irradiation, fertilization, and water supply, and the developmental

stages of plants are the main factors affecting the amount of glucosinolate (Mandrich and Caputo 2020). Cooking, juicing, chewing, cutting, and freezing/thawing are some physical processes that cause cellular deterioration and result in the hydrolysis of stable glucosinolates by opposing with myrosinase (Mithen et al. 2000; Verkerk et al. 2009). As a result of this reaction, isothiocyanates, nitriles, epithionitriles, and thiocyanates are formed (Shakour et al. 2022). It has been stated that these hydrolysis products have antioxidative, antimicrobial, anti-inflammatory, and anticarcinogenic properties important for health (Shakour et al. 2022). Cabello-Hurtado et al. (2012) determined that the main glucosinolates found in cauliflower leaves are gluconapin, sinigrin, glucoiberin, progoitrin, neo glucobrassicin, glucobrassicanapin, gluconasturtiin, 4-Methoxyglucobrassicin, glucobrassicin, and 4-OH-Glucobrassicin (Cabello-Hurtado, Gicquel, and Esnault 2012).

2.3. Antimicrobial Activity

Newly changing consumer preferences have led people to foods that contain natural preservatives and have much cleaner content than synthetic preservatives and foods containing them. Thanks to the secondary metabolites it contains, plant extracts have attracted a lot in this field and have been the subject of promising studies (Oulahal and Degraeve 2022; Serra et al. 2008).

2.3.1. Antimicrobial Activity of Grapevine (*Vitis vinifera L.*) Leaves

Vitis vinifera is a perennial plant belonging to the *Vitaceae* family, with a curved, woody stem and serrated, deeply lobed leaves (Venkitasamy et al. 2019). *V. vinifera* leaves contain enzymes, phenolics, organic acids, carotenoids, anthocyanins, and reducing or non-reducing sugars (Deliorman Orhan et al. 2009). Beyond the other substances, phenolic components are more attracted. Rutin, resveratrol, derivatives of quercetin-3-O-, and kaempferol are the most abundant phenolics in grape leaves (Aouey

et al. 2016; Deliorman Orhan et al. 2009; Pintać et al. 2019). In a research of the antimicrobial examination of flavonoids, stilbenes, and phenolic acids isolated from grape stems and seeds, it has been implied that these compounds may be liable for of the antimicrobial activity (Anastasiadi et al. 2009). Alike, caffeic acid, rutin, and quercetin isolated from different wines were reported as promising antilisterial agents (Vaquero, Alberto, and de Nadra 2007). In a study, the antimicrobial activity of the leaves of *V. vinifera* was tested against the bacteria (*Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 10145), fungi (*Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 10231), and viruses (*Herpes simplex- 1* (HSV-1) and *Parainfluenza* (PI)) (Deliorman Orhan et al. 2009). Although the leaves displayed antibacterial activity, the results were reportedly not significant when compared to the control agents. However, the Gram-positive bacteria were affected more than the Gram-negative bacteria. It has also been concluded the leaf extracts had no significant effect on *C. albicans* and *C. parapsilosis* (Deliorman Orhan et al. 2009). Katalinic et al (2013), compared the total phenolic contents of the six grapevine leaves and their antimicrobial activities. In their study, the most suggested health-promising flavonoids, glucoside, stilbenes, and rutin were selected against the foodborne Gram-positive (*Bacillus cereus* WSBC 10530 (clinical isolate) and *Staphylococcus aureus* ATCC 25923 (clinical isolate)) and Gram-negative (*Campylobacter jejuni* ATCC 33560 (bovine feces isolate), *Salmonella infantis* ZM9 (poultry meat isolate), and *Escherichia coli* O157:H7 ZMJ 129 (clinical isolate)) pathogens (Katalinic et al. 2013). The researchers stated that contrary to what was previously disclosed, the researchers found no discernible difference between Gram-positive and Gram-negative bacteria. Determined sensitivities of the bacteria against the leave extracts were expressed in terms of average MICs (in mg GAE/mL) as follows: *B. cereus* (0.77 ± 0.34) > *C. jejuni* (1.03 ± 0.29) > *S. aureus* (1.11 ± 0.36) > *E. coli* (1.39 ± 0.36) > *S. infantis* (1.50 ± 0.30). Besides, the leaves harvested in August and September were richer in flavonols and stilbenes. Parallelly, September leaves had the most promising effect against the bacteria (Katalinic et al. 2013). In a study, two different grape varieties, Baituni (purple) and Shami (white) from Palestine were analyzed against *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 19115, *Salmonella* Typhimurium ATCC 14028, and *Escherichia coli* (0157) ATTC 700728 (Abed et al. 2015). The results indicated that the leaf extracts were ineffective against *Listeria monocytogenes*, *Salmonella* Typhimurium, and

Escherichia coli (0157) bacteria, where a slight effect was observed against *Pseudomonas aeruginosa. Staphylococcus aureus* was the most affected strain in the study (Abed et al. 2015). Oskay and Sarı determined the antimicrobial effects of different plants in Manisa, Turkey, including *V. vinifera* (Oskay and Sari 2007). Agar well diffusion assay was employed against the selected bacteria and fungi that are resistant to some antibiotics e.g., chloramphenicol, vancomycin, nalidixic acid, novobiocin, imipenem, and penicillin G (Oskay and Sari 2007). *V. vinifera* was reported as one of the most potent plants with a wide range of antibacterial activity. The observed inhibition zones (in mm) on selected microorganisms were stated in the following order: *Proteus vulgaris* ATCC 6997 > *Salmonella* Typhimurium CCM 5445 > *Enterobacter aerogenes* ATCC 13048 > *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228 *Pseudomonas fluorescens*, *Micrococcus luteus* ATCC 9341 > methicillin-resistant *Staphylococcus aureus* ATCC 95047 (MRSA) > *Bacillus cereus* CM 99, *Enterobacter cloacae* ATCC 13067 > *Bacillus subtilis* ATCC 6683, *Serratia marcescens* CCM 583 > *Escherichia coli, Enterococcus faecalis* ATCC 29212, > *Candida albicans* (Oskay and Sari 2007). Antibacterial and antifungal activities of the Karaerik cultivar of *Vitis vinifera L*. were performed by Yiğit et al., in 2009 (Yiğit et al. 2009). In the study, both water and methanol extracts were tested against 96 clinically isolated bacterial strains of *Proteus miribalis, Escherichia coli, Enterobacter aerogenes, Staphylococcus aureus,* and *Pseudomonas aeruginosa;* and 90 *Candida* spp. of *Candida krusei, Candida albicans, Candida glabrata, Candida kefyr, Candida pseudotropicalis, Candida guillermondii, Candida tropicalis, Candida parapisilosis* and *Geotricum candidum.* It has been reported that the water-extracted leaves had the strongest effect on *Candida* spp., especially *C. glabrata, C. albicans, C tropicalis*. The same extract affected only one bacterial strain, *S. aureus* with an inhibition zone of 13 mm, and 0,625 mg/mL of MIC value (Yiğit et al. 2009).

Xia et al. stated that it would be wiser to use phenolic compounds in food applications rather than in the medical field (Xia et al. 2010). However, with a different point of view, Moldovan et al. focused on the bacterial strains associated with oral pathology instead of foodborne pathogens: *Klebsiella* sp., *Porphyromonas gingivalis* ATCC 33277, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231 (Moldovan et al. 2020). The disk/well method was applied for the antimicrobial activity. The leaves showed a successful inhibition. It was reported that the

Porphyromonas gingivalis was the most affected strain, followed by *Enterococcus faecalis, Streptococcus mutans*, and *Staphylococcus aureus*. However, any inhibition was observed for *Klebsiella* sp. and *Candida albicans* (Moldovan et al. 2020). In a study of Yordanov et al. (2008), it was stated that pure kaempferol has quite effective antifungal activity on *Candida albicans* strains (Yordanov et al. 2008). Besides, testing the antifungal activity of different variations of kaempferol, Christopoulou et al. (2008) observed activity against isolated strains of *Candida tropicalis, Candida albicans,* and *Candida glabrata*in vivo experiments (Christopoulou, Graikou, and Chinou 2008).

2.3.2. Antimicrobial Activity of Cauliflower (*Brassica oleceae var. botrytis*) Leaves

Cauliflower (*Brassica oleceae var. botrytis*) is a biennial plant consisting of edible white flower part and green leaves which belongs to the *Brassicaceae* family ("Tarım ve Orman Bakanlığı, Karnabahar Yetiştiriciliği," n.d.). Cauliflower leaves are considered the non-edible part; thus, the leaves cause a high amount of waste accumulation. Performed studies show that cauliflower leaves are rich in flavonoids like kaempferol derivatives, gallic acid, chlorogenic acid, ferulic acid, and catechin and glucosinolates like sinigrin, glucoiberin, glucobrassicin, and progoitrin (Sanz-Puig et al. 2015; Shakour et al. 2022; Volden, Bengtsson, and Wicklund 2009). It is also known that these compounds provide many beneficial health effects as antioxidative, antimicrobial, anti-inflammatory, and anticarcinogenic properties (Shakour et al. 2022).

In a study, the antimicrobial activity of cauliflower leaves against *Listeria monocytogenes* was examined (Sanz-Puig et al. 2015). In the study, the antimicrobial potential was tested in different concentrations and temperatures. It has been concluded the bactericidal effect was influenced by both concentration and temperature (Sanz-Puig et al. 2015). Besides, the cauliflower leaves showed a successful bactericidal effect at concentrations of above 5 % (w/v) in all tested temperatures (Sanz-Puig et al. 2015). In another study, cauliflower leaf juice was tested against *Salmonella enterica* subsp. enterica serovars, *Enteritidis* (*Salmonella enteritidis*), three strains of *Escherichia coli* O157:H7 (F146, 1952, and ATCC 35150) producing verotoxin (VT^+), *Escherichia coli*

HB producing thermolabile toxin (LT^+), *Enterococcus faecalis*, *Escherichia coli* nonproducing toxin (VT^-), *Listeria monocytogenes* (ATCC 9525) (Brandi et al. 2006). The leaf juice showed a dose- and time-dependent inhibitory effect. The rate of inhibition achieved 95% in the concentration of 20% leaf juice at the end of 5 hours of treatment (Brandi et al. 2006). However, the initial bacterial population has a huge effect on the antimicrobial activity. In one sample group which contains 10^6 cells/ml, the bacterial growth was inhibited within the first 5 hours, however at the end of 24 h, the bacterial population was increased (Brandi et al. 2006). Oppositely, in another sample group that contains 10^4 cells/ml initially, a successful bactericidal activity was achieved (Brandi et al. 2006). The antifungal activity of the juice of cauliflower leaves was tested against *Candida albicans* and other pathogenic fungi by Sisti et al.(Sisti, Amagliani, and Brandi 2003). Leaf juice showed a dose-dependent inhibition against *C. albicans*. On the other hand, the leaf juice was significantly effective against *Alternaria* spp., *Cladosporium* spp., *Microsporum canis,* and *Trichophyton verrucosum* (Sisti, Amagliani, and Brandi 2003). In the first 24 hours of treatment, *Trichoderma* spp. was inhibited, however, in the following days, mycelium formation was detected (Sisti, Amagliani, and Brandi 2003).

2.4. Mechanism of Antimicrobial Activity

Microorganisms may infect humans through air, water, soil, other animals, or food. It has been stated that many microorganisms develop resistance to antibiotics used in the treatment process and adversely affect the course of treatment (Kitsiou et al. 2023). For this reason, the antimicrobial activity and mechanism of action of phenolics obtained from plants have been in demand recently. Flavonoids are secondary metabolites with low molecular mass and have effective roles in the growth, development, and defense mechanisms of plants (Periferakis et al. 2022). All flavonoids have a 15-carbon skeleton chemically composed of two phenyl rings joined by a three-carbon bridge (Stan et al. 2021). Although the antimicrobial effect of flavonoids is mostly based on cell lysis by disrupting the permeability of the bacterial cell membrane, there is no definite conclusion about the mechanism of action (Stan et al. 2021). Additionally, inconsistencies can be seen in the results of the studies. For instance, Deliorman Orhan et al. (2009) had no

significant effect of water-extracted *V. vinifera* leaves on *S. aureus*, while Yiğit et al. (2009) concluded their water-extracted grape leaves were quite effective against *S. aureus* (Yiğit et al. 2009; Deliorman Orhan et al. 2009).

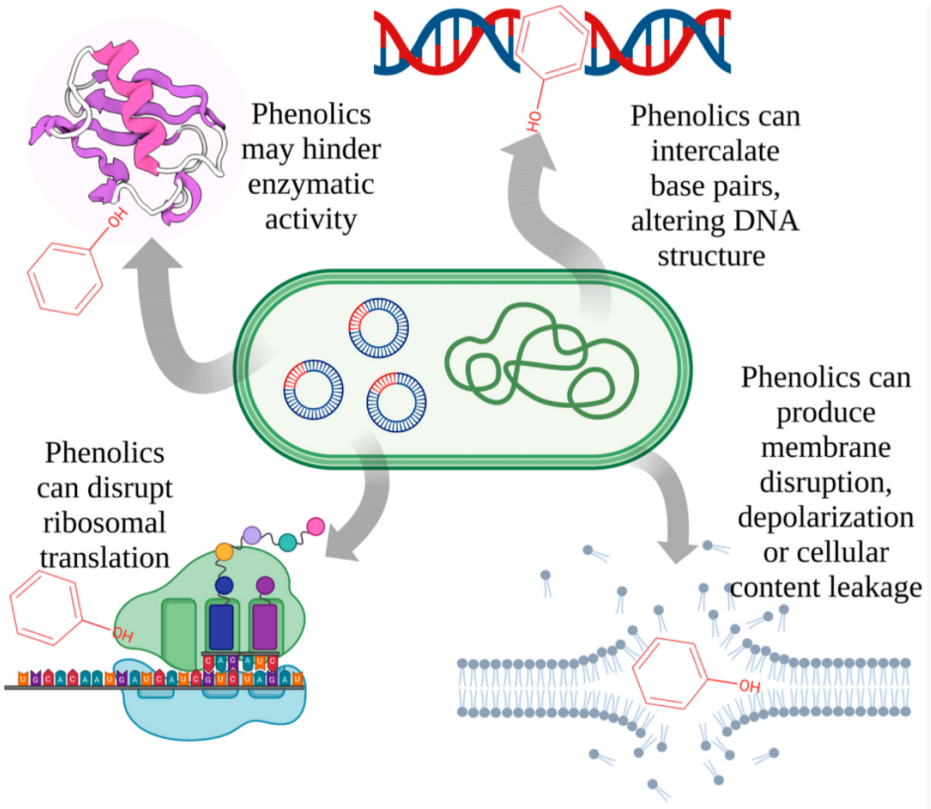


Figure 2. 6 Potential antimicrobial mechanisms of action of the phenolic compounds (Source: (Lobiuc et al. 2023)).

Kaempferol ($C_{15}H_{10}O_6$) is one of the flavanols abundant in *V. vinifera* leaves (Aouey et al. 2016; Pintać et al. 2019; Deliorman Orhan et al. 2009). The scientific studies about kaempferol are increasing to explain its role in antimicrobial activity. Although it is not easy to explain its mechanism of action because of the presence of many kaempferol derivatives, as well as the greatness and diversity in morphology and functions of the bacteria species, studies show its antimicrobial mechanism mostly depends on the destruction of the bacterial cell membrane (Periferakis et al. 2022).

Based on the study that examining the impact of a mixture containing kaempferol, quercetin, and glucopyranoside on *Micrococcus luteus* cells, it was concluded that the

antimicrobial mechanism was provided by apoptosis and DNA fragmentation due to cell membrane disruption (X. M. Li et al. 2015). According to the study by Huang et al. (2015), it was stated that kaempferol has an inhibitory effect not only on DNA gyrase but also on DNA helicase enzyme (Huang et al. 2015). In a study with *Escherichia coli*, the damage of kaempferol to the cell membrane was confirmed by the observation of protein leakage into the extracellular environment (He et al. 2014). In another study, it has been reported that kaempferol directly and effectively inhibited the DNA gyrase enzyme of *E. coli* (Wu et al. 2013).

Quercetin ($C_{15}H_{10}O_7$) is a penta-hydroxy flavone that has five hydroxyl groups and is one of the flavanols found in *V. vinifera* ("Quercetin | C15H10O7 - PubChem," n.d.). The hydroxyl groups play a crucial role in its antimicrobial activity, by providing a strong water interaction (Nguyen and Bhattacharya 2022). The hydroxyl groups in the quercetin structure interact with the bacterial cell wall and restrict or block the growth of bacteria (Nguyen and Bhattacharya 2022). In a study with treated *E. coli* cells, it was observed that quercetin caused cell death by causing many abnormalities such as cytoplasmic membrane separation, cytoplasmic substance leakage, and cell disruption in the cell wall (Wang et al. 2018). Parallelly, the same study has shown a similar effect in *S. aureus* cells causing deterioration in the cell wall and extracellular pili structure (Wang et al. 2018). As a result of a study performed by Cushnie et al. (2003), it has been reported that quercetin negatively affects DNA synthesis by inactivating the DNA gyrase enzyme in *E. coli* (Cushnie, Hamilton, and Lamb 2003). Beyond these, studies have shown that quercetin inhibits adhesion by interacting with the quorum signaling mechanism and thus exhibits antibiofilm activity (Yang et al. 2020).

Glucosinolates are plant secondary metabolites that are predominantly found in *Brassica* vegetables and contain sulfur in their structure (Romeo et al. 2018). When plant cells are deteriorated by any physical effect such as chewing or chopping, glucosinolates encounter the myrosinase enzyme and are hydrolyzed (Wilson et al. 2013). As a result of this hydrolysis, metabolites such as isothiocyanates, nitriles, thiocyanates, and epithionitriles are formed (Wilson et al. 2013). It is known that taking isothiocyanates with diet has positive effects on health (Romeo et al. 2018). Besides, isothiocyanates have been reported to provide antimicrobial activity (Borges et al. 2015; Saavedra et al. 2010). It is thought that the antimicrobial effect of isothiocyanates is due to the sulfhydryl groups they have (Borges et al. 2015; Saavedra et al. 2010). Sulphhydryl groups are thought to render bacteria inactive by oxidizing intracellular enzymes (Brandi et al. 2006b).

However, there is no clear mechanism for the mechanism of action. In a study, it was observed that isothiocyanates provide antimicrobial activity by inhibiting thioredoxin reductase and acetate kinase enzymes of *E. coli* (Wilson et al. 2013). It has been reported that the sulfhydryl groups were found to disrupt cell enzymes in a study where the antibacterial impact of cauliflower leaf juice was tested against *Salmonella* species. However, this effect was dose-dependent (Brandi et al. 2006). Another approach regarding the antimicrobial effect of isothiocyanates is that they cause cell disruption by targeting the cell membrane, changing the electrostatic potential and hydrophobicity (Borges et al. 2015). In a study with *E. coli*, it was observed that isothiocyanates cause ATP leakage by damaging the cell membrane (Brandi et al. 2006).

On the other hand, Gram-positive bacteria tend to be more resistant to antimicrobial agents due to the peptidoglycan found on their outer surface (Saavedra et al. 2010). The peptidoglycan membrane provides Gram-positive bacteria with a more stable cell surface hydrophobicity/hydrophilicity, thereby showing resistance to physicochemical changes in the environment (Bayoudh et al. 2009). The outer surface membrane of Gram-negative bacteria is higher in lipids, making them more susceptible to environmental changes (Bayoudh et al. 2009).

2.5. Antioxidant Activity

Antioxidants are the molecules that reduce or completely prevent the oxidation processes by metal chelation, hydrogen atom transfer, or single electron transfer (Kotha et al. 2022; Granato et al. 2018). These molecules may be specified in many ways including (Granato et al. 2018):

i. their chemical structure as natural or synthetic antioxidants
ii. their electric charge as polar or non-polar antioxidants
iii. their way of to involve in processes such as enzymatic or non-enzymatic antioxidants
iv. their source by our body as endogenous and exogenous antioxidants
v. their activation mechanisms

oxygen/nitrogen species (ROS/RNS), and thus an imbalance in the cell. This intracellular imbalance is defined as oxidative stress and is associated with many serious health problems, including cancer and cardiovascular diseases (Zehiroglu and Ozturk Sarikaya 2019).

Antioxidant activity is quite popular because of its positive effects in numerous fields, including food science and health. Lipid oxidation may badly affect the quality standards of food products. Undesirable taste and odor formations may be minimized by using antioxidants (Zehiroglu and Ozturk Sarikaya 2019). Besides, antioxidants may prevent the risk of many chronic diseases or cancer by decreasing oxidative stress (Granato et al. 2018).

Phenolic compounds protect plants from oxidation in even small concentrations (Granato et al. 2018). The hydroxyl groups and benzene ring in their structural composition make the phenolics able to donate hydrogen, chelate metal ions, and remove the unpaired electron among the benzene ring (Kotha et al. 2022). The mechanism of action of the antioxidant activity may be explained in three main assays as follows (Granato et al. 2018):

i. Hydrogen atom transfer (HAT): This assay includes the transformation of the hydrogen atom from the antioxidant to the substrate (Kotha et al. 2022).
ii. Single electron transfer (SET): This assay involves the redox (reduction-oxidation) reactions by transferring the electron between the antioxidant and the substrate (Kotha et al. 2022).
i. Ability to chelate transition metals: This assay is based on the chelation of Cu^{2+}, $Zn^{2+,}$ and Fe^{2+} transition metals, which are stated as the reason for many diseases and bacterial pathogenesis (Sadeer et al. 2020).

Numerous assays including ORAC, ABTS, DPPH, and FRAP may be employed to determine the antioxidant activities of the plant species. The principles, advantages, and limitations of these assays are tabulated in Table 2.1.

Table 2. 1 The principles, advantages, and limitations of the antioxidant assays

Method	Assay	Principle	Advantages	Limitations	Reference
Hydrogen Atom Transfer (HAT)	ORAC (Oxygen radical absorbance capacity) assay	Antioxidant activity is determined by measuring the fluorescent signal of the reaction between peroxyl radicals formed by APPH (2,2-azobis-2-aminopropane dihydrochloride) and antioxidants	Suitable for different matrices. Suitable for nonprotein assays	Not sufficient to determine both hydrophilic and lipophilic antioxidants	(Prior 2015; Moharram and Youssef 2014; Kotha et al. 2022)
Hydrogen Atom Transfer (HAT)	TRAP (Total radical-trapping antioxidant parameter) assay	Antioxidant activity is determined by the scavenging of luminol-derived radicals due to the AAPH decomposition.	Suitable for both both in vivo and in vitro assays	Detection of the unstable oxygen	(Kotha et al. 2022; Moharram and Youssef 2014)
Single Electron Transfer (SET)	Folin-Ciocalteu assay	Antioxidant potential is determined by measuring the decolorization at a specific wavelength	Easy and repeatable	Ignores the reaction kinetics	(Kotha et al. 2022)
Single Electron Transfer (SET)	FRAP (Ferric reducing-antioxidant power) assay	Antioxidant capacity is dmeasured at 593 nm through the reaction of Fe^{3+} complex at low pH	Easy to apply and repeatable	Ignores the reaction kinetics. Non-specific to antioxidants	(Benzie and Strain 1996; Kotha et al. 2022)

(cont. on next page)

Table 2.1. (cont.)

Single Electron Transfer (SET)	CUPRAC (Cupric ion reducing antioxidant capacity) assay	Antioxidant activity is measured colorimetrically due to the reaction of Cu^{2+} and antioxidants	Reaction kinetics are faster than that of ferric ions. More specific to antioxidants	Ignores the reaction kinetics	(Kotha et al. 2022; Moharram and Youssef 2014)
Both (HAT and SET)	TEAC (Trolox equivalent antioxidant capacity) assay (ABTS radical cation decolorizatio n assay)	Antioxidant capacity is determined by deolorization due to the reaction between Trolox (antioxidant) and ABTS (2,2-azo-bis(3- ethylbenz-thiozoline-6-sulfonic acid)).	Suitable for both hydrophilic and hydrophobic antioxidants	Not suitable to determine the antioxidant activity of proteins. Ignores the reaction kinetics	(Schaich, Tian, and Xie 2015; Kotha et al. 2022)
Both (HAT and SET)	DPPH (2,2-diphenyl-1-picrylhydraz yl) assay	Antioxidant activity is determined by measuring the decolorization due to the reaction between DPPH and antioxidants	Easy and reproducible	DPPH is a non-physiologica l reactant. It is difficult to get the reaction kinetics.	(Kotha et al. 2022; Schaich, Tian, and Xie 2015)

2.5.1. Antioxidant Activity of Grapevine (*Vitis. vinifera L.*) Leaves

The antioxidant potential of the *Vitis* plants has been highlighted in many studies (Salehi et al. 2019). The presented outcomes of the conducted studies stated that the phenolic content of the *V. vinifera* leaves directly affects the antioxidant capacity. More specifically, it has been stated that the detected active compounds like flavonols and proanthocyanins played an important role in decreasing oxidative stress (Pari and Suresh 2008). It has been stated that the September leaves assessed the highest antioxidant activity in both DPPH and FRAP assays in the study that examined the antioxidant

potentials of leaf extracts from six *V. vinifera* cultivars (Katalinic et al. 2013). Besides, the September leaves had the highest flavanol, more specifically quercetin content (Katalinić et al. 2009; Katalinic et al. 2013). Thus, picking time influences the antioxidant activity, along with the total phenolic content. The authors also reported that the quercetin derivatives are the main flavanols responsible for antioxidant activity (Katalinic et al. 2013). In another study, Auoey et al. (2016) investigate the antioxidant capacities of the grapevine leaf extracts from South Tunisia, by reducing power and DPPH assays (Aouey et al. 2016). The IC_{50} value was reported as 11.18 ± 0,12 μg/mL in the DPPH assay. Additionally, resveratrol and quercetin were the most abundant flavonols detected. Alike to the study of Katalinic et al. (2013), it has been documented that the antioxidant activity of the extracts is related to their polyphenol content (Aouey et al. 2016).

Lipid and protein damage tends to be increased by growing oxidative stress and results in many diseases including Parkinson's and Alzheimer's diseases (Ferreira et al. 2014; Dani et al. 2010). In a study, where the antioxidant activity of the *V. vinifera* leaf extracts were tested on rats, the results showed that the polyphenol compounds in grapevine leaves showed a noticable decrease in oxidative stress in the brain tissues (Dani et al. 2010). The antioxidant activity observed in the brain tissue is also reported as the neuroprotective potential of the leaf extracts (Dani et al. 2010). Similarly, the antioxidant activity of the grape leaves was tested on normal human gingival fibroblast (HGF) cells, and it has been reported that the leaf extracts inhibit lipid peroxidation and reduced oxidative stress (Moldovan et al. 2020). Besides, a positive correlation between the polyphenolic content and reactive oxygen scavenger activity has been reported (Dani et al. 2010).

The mode of action of the phenolic compounds associated with the hydroxyl groups in their structure (Xia et al. 2010). Adding the -OH group onto the flavonoid nucleus increases the antioxidant activity, however, the addition of $-OCH_3$ groups results in a decrease in the antioxidant activity (Xia et al. 2010; Arora, Nair, and Strasburg 1998).

2.5.2. Antioxidant Activity of Cauliflower (*Brassica oleceae var. botrytis*.) Leaves

The antioxidant content of cauliflower leaves is mostly attributed to the phenolics and glucosinolates it contains (Cartea et al. 2011; Soengas et al. 2012). In accordance with Cartea et al. (2011), the antioxidant activity of *Brassica* vegetables should be associated with the flavonoid content, as it has a higher antioxidant effect compared to vitamins and carotenoids (Cartea et al. 2011). It has been proved by much research that the samples with higher flavonoid content possess higher antioxidant activity (Cartea et al. 2011).

In a study conducted by Cabello-Hurtado et al (2012), the relationship of glucosinolates obtained from non-edible cauliflower parts with antioxidant effect was investigated (Cabello-Hurtado, Gicquel, and Esnault 2012). The researchers used ABTS+, DPPH, ORAC, and SRSA assays in the study, stating that the sensitivities of different antioxidant assays were also different. As a result, it was reported that the antioxidant effects of glucosinolates measured by DPPH and ABTS+ assays were weak. The highest antioxidant activity values determined by ABTS+ assay belonged to glucoiberine and gluconapine (0.13 and 0.08 Trolox Equivalent, respectively). On the other hand, it was stated that the determined antioxidant activity by ORAC and SRSA assays was much higher (Cabello-Hurtado, Gicquel, and Esnault 2012). In these assays, the glucosinolates with the highest antioxidant activity were glucobrassicin, glucoiberin and gluconapin, respectively (Cabello-Hurtado, Gicquel, and Esnault 2012).

The antioxidant activity of *Brassica* vegetables is highly correlated with the maturation states of the plants. Soengas et al. (2012) reported that *Brassica* vegetables reached their maximum antioxidant activity during the sprouting process, approximately 3 months after planting, then the antioxidant capacity decreased (Soengas et al. 2012). This also means that the young leaves of the plant have more antioxidant effects (Soengas et al. 2012). Besides, it was stated that the white flower part of cauliflower had less flavonoid concentration and antioxidant activity than the leaves and stems due to a lack of pigment (Soengas et al. 2012). In another study, hydrolysis and purification of the protein found in cauliflower leaves were achieved by using different enzymes (Zenezini Chiozzi et al. 2016). The antioxidant activity of the obtained protein fractions was

measured by the DPPH assay. The results obtained indicate that cauliflower leaves have only a limited antioxidant activity (4.5-13.7%) (Zenezini Chiozzi et al. 2016).

2.6. Cytotoxic Activity

The toxic effect of a therapeutic agent on a particular cell population and killing cells is called cytotoxicity (Luo et al. 2011). Cytotoxicity tests are basically based on cell viability measurements. Cell viability tests provide *in vitro* vision over the ratio of live and dead cells (Mukherjee 2019). Determination of cell viability is observed by special dyes such as Trypan blue, Coomassie blue, Alamar blue, etc. (Mukherjee 2019). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay is considered one of the most sensitive and applicable methods for cytotoxicity (Mukherjee 2019). Basically, the MTT assay is based on the principle that nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular enzymes reduce MTT (Kuete, Karaosmanoğlu, and Sivas 2017). The reduced MTT appears purple due to the dyestuff used, and the metabolic activity of the cells is measured spectrophotometrically (Kuete, Karaosmanoğlu, and Sivas 2017).

Plant secondary metabolites are grouped as alkaloids, terpenoids, polyphenols, and flavonoids according to their chemical structures, and they play a role in many activities, especially antioxidant and anti-inflammatory properties (Ramakrishna et al. 2021). Thus, the plant's secondary metabolites may have the potential to provide cytotoxic activity.

2.6.1. Cytotoxic Activity of Grapevine (*Vitis vinifera L.*) Leaves

The leaves of *V. vinifera* are used as a sedative, diuretic, and astringent due to the bioactive substances it contains such as minerals, vitamins, flavonoids, phenolic acids, and anthocyanins (Karaman and Kocabaş 2001). Besides, the wound-healing, anti-

inflammatory, and anti-inflammatory properties of *V. vinifera* leaves are known and used by different societies as traditional medicine (Karaman and Kocabaş 2001).

In a study, the relationship between bioactive substances and the cytotoxic activity of *V. vinifera* leaves was investigated (Handoussa et al. 2013). In the study, organic acids (caftaric acid, quinic acid), quercetin derivatives (quercetin-glucuronide, rutin, isoquercitrin), luteolin-7-O-glucoside, cyanidin-3-O-glucoside, and kaempferol-coumaroyl-glucoside substances were isolated (Handoussa et al. 2013). The cytotoxic activity was tested with different assays using adult male Wister albino rats. The results of the study revealed that the *V. vinifera* leaves had cytotoxic activity (Handoussa et al. 2013). Besides, it has been stated that quercetin derivatives have a similar effect to Ibuprofen, which is known to inactivate the COX-I enzyme and contribute highly to cytotoxic activity (Handoussa et al. 2013). In another study, where the cytotoxic activity of the different *V. vinifera* cultivars' leaves from two different regions of Palestine against lung cancer (A549 cells) cells was investigated (Abed et al. 2015). The results showed that the Baituni leaves had insufficient cytotoxic activity against lung cancer, whereas the Shami leaves had an effective cytotoxic activity (Abed et al. 2015). Besides, it was reported that the biological activity was dose-dependent in all tested extracts. The difference in the cytotoxic activity of the different cultivars was associated with the phytochemical composition of the leaves (Abed et al. 2015). The cytocompatibility and cytoprotective effect of the grapevine leaves against nicotine-induced cytotoxicity were tested on normal human gingival fibroblasts (HGF) cells (Moldovan et al. 2020). It was reported that the chemical composition and bioactive metabolites such as caffeic acid and quercetin derivatives provide protection against nicotine-induced cytotoxicity (Moldovan et al. 2020). Plant secondary metabolites provide natural protection to the plants against pathogens and other external threats (Ramakrishna et al. 2021). Thus, there is a hypothesis that claims that infectious diseases cause an increment in the phenolic content of plants.

Considering this point, Esfahanian et al. (2013) tested the cytotoxic activities of *V. vinifera* leaves from both virus-free and virus-infected cultivars against the human embryonic kidney normal cell line (HEK 293) and breast cancer cell line (MDA- MB-231) (Esfahanian et al. 2013). Consequently, both virus-free and infected leaves showed a wide board of cytotoxic activity from limited to moderate, whereas the virus-infected cultivars showed strong cytotoxic activity against breast cancer cells in different concentrations (Esfahanian et al. 2013).

2.6.2. Cytotoxic Activity of Cauliflower (*Brassica oleceae var. botrytis*.) Leaves

Cauliflower, one of the *Brassica* vegetables, is rich in sulfur compounds and glucosinates, which are responsible for its bitter and unique aroma, as well as bioactive compounds such as flavonoids, lutein, organic acids, vitamins A, B6, C, E, and K (Cuellar-Nuñez et al. 2022; Cabello-Hurtado, Gicquel, and Esnault 2012). It has been stated that cauliflower leaves have antioxidant, anti-inflammatory, and anticarcinogenic properties, which are very beneficial to human health, thanks to the rich bioactive substances they contain (Shakour et al. 2022).

Cauliflower leaves have been suggested as a promising source of antioxidant and angiotensin-I converting enzyme (ACE) inhibitor peptides. These peptides are in an inactive form in their native states, however, an external threat like bacterial fermentation, or enzymatic hydrolysis helps to convert their active form (Caliceti et al. 2019). From this point of view, Caliceti et al. (2019) investigated the biological and cytotoxic activity of the cauliflower leaves by recovery of the leaf peptides (Caliceti et al. 2019). For the determination of the cytotoxic activity, lactate dehydrogenase (LDH) release from human vascular endothelial cells (HUVECs) was monitored spectrophotometrically (Caliceti et al. 2019). It has been reported that the recovery peptides from cauliflower leaves showed promising protection activity against endothelial dysfunction and atherogenesis development (Caliceti et al. 2019). Similarly, the cytotoxic activity of proteins recovered from cauliflower leaves was tested on human HepG2 cells by the MTT method (Xu et al. 2017). Leaf proteins isolated in the experiments were reported to cause ACE inhibition in a promising way (Xu et al. 2017). Isothiocyanates, which are created when glucosinates are hydrolyzed by the myrosinase enzyme, activate antioxidant systems and result in apoptosis, which kills cells, alike peptides (Cuellar-Nuñez et al. 2022). In a study, it was reported that isothiocyanates isolated from cauliflower leaves significantly inhibited metabolic activity in human colorectal adenocarcinoma HT-29 (ATCC HTB-38) and colorectal carcinoma HCT116 (ATCC CCL-247) cell systems and increased reactive oxygen species in the cell (Cuellar-Nuñez et al. 2022).

CHAPTER 3

MATERIAL AND METHOD

3.1. Materials

3.1.1. Microbial Strains

In this study, spoilage, pathogenic, and non-pathogenic bacteria and fungi species (Table 3.1) were handled to determine the antimicrobial activity. The microorganisms used in the experiments were selected among the most important strains in the food safety field. Gram-positive strains include *Bacillus cereus* (ATCC 11778), *Carnobacterium divergens* (NRRL B-14830), and *Listeria innocua* (NRRLB-33314), whereas the Gram-negative strains include *Serratia liquefaciens* (NRRL B-41553), *Salmonella* Typhimurium (CCM 5445), and *Escherichia coli* (ATCC 25253). *Candida albicans* (DSM 5817) and *Saccharomyces cerevisiae* were the fungi strains.

Here, *Bacillus cereus* (ATCC 11778), *Candida albicans* (DSM 5817), and *Salmonella* Typhimurium (CCM 5445) were the pathogenic microorganisms. However, *Carnobacterium divergens* (NRRL B-14830), *Listeria innocua*, *Saccharomyces cerevisiae*, and *Escherichia coli* (ATCC 25253) are considered non-pathogenic bacterial strains. Besides, *Serratia liquefaciens* (NRRL B-41553) was the opportunistic pathogenic bacteria and *Carnobacterium divergens* (NRRL B-14830) was the spoilage bacteria, which is associated with meat spoilage.

Bacterial and fungal strains were preserved in nutrient broth containing glycerol (20%) at -80 °C. All the bacterial strains were grown in Tryptic Soy broth, aerobically, at 37 °C for 24 hours, whereas the fungal strains handled in this study were grown in the Yeast Extract broth, at 37 °C for 24 hours.

Table 3. 1 Bacterial and fungal species handled in the study.

Gram-Positive Bacteria	**Origin**
Bacillus cereus (ATCC 11778)	Unknown
Listeria innocua	Unknown
Carnobacterium divergens (NRRL B-14830)	Minced meat
Gram-Negative Bacteria	**Origin**
Serratia liquefaciens (NRRL B41553)	Ground beef
Esherichia coli (ATCC 25253)	*Unknown*
Salmonella Typhimurium (CCM 5445)	Unknown
Fungi	**Origin**
Candida albicans (DSM 5817)	Unknown
Saccharomyces cerevisiae	Commercial

3.1.2. Plant Materials

Fresh and healthy grapevine (*V. vinifera L.*) leaves were kindly obtained from Urlice Vineyards, Urla, İzmir in August 2022. Fresh cauliflower (*Brassica oleracea var. botrytis*) leaves were provided by Macrocenter, Çeşme, İzmir in January and February 2023.

The fresh leaves obtained were brought to the Food Microbiology and Mycology Laboratory at the Department of Food Engineering, İzmir Institute of Technology Urla, İzmir. The leaves were gently washed with tap water and allowed to air dry. Completely dried leaves were manually ground into fine particles of about 0.5 mm. Grinded leaves were used for the extraction as performed in the study of Deliorman Orhan et al. (2009), with small modifications (Deliorman Orhan et al. 2009).

Completely dried and grounded plant leaves were weighed 20 g and extracted with 400 ml of distilled water. The extraction was performed by stirring at 45°C for 8 hours. Then the mixture was filtered through the filter paper (0.40 µm) under atmospheric pressure for 12 hours (Deliorman Orhan et al. 2009). The extracts were kept at -20°C until further analyses.

3.2. Chemical Characterization

3.2.1. Total Phenolic Content

Total soluble phenolic content was determined by Folin-Ciocalteu assay as performed in the study by Akbulut et al. (2021) previously. For this assay, a serial dilution was prepared from 50 ppm to 250 ppm for the gallic acid calibration curve. Then diluted leaf extracts (distilled water/leaf extract, v/v, 2/8) were mixed with 2.5 ml of (0.2 N) Folin-Ciocalteu's reagent then the mixture was kept in the dark for 5 minutes at room temperature. After the incubation period, 2.5 ml of sodium carbonate (Na_2CO_3) solution (7.5% w/v) was added to the mixture, then the tubes were completed with distilled water up to 25 ml. The tubes were kept in the dark for 2 hours, then the absorbance values against to blank sample were measured at 760 nm. The same procedure was applied to the gallic acid for obtaining the calibration curve. The results were expressed as mg gallic acid equivalents (GAE) per liter (AKBULUT et al. 2021; Vasco, Ruales, and Kamal-Eldin 2008).

3.2.2. High-Performance Liquid Chromatography (HPLC) Analyses

After the total soluble phenolic content determination, an HPLC column was employed for the identification of the phenolic compounds. For the phenolic compound determination, thawed leaf extracts were homogenized by vortexing, and filtered through a 0.2 μm filter, and 20 μL of samples were injected into an analytical HPLC system (Agilent 1200) equipped with a Nucleosil 100-5C8 (25 cm x 4.6 mm) column with a 5μm particle size. A mixture of water/acetic acid (98/2, v/v) (solvent A) and methanol (solvent B) were applied to the chromatographic system with a flow rate of 10 ml/min (Fernandes et al. 2013). The gradient elution procedure was applied as follows: initial concentrations of mobile phase B was 5–15%, then increased to 15–25% from 3 to 13 min, then it was increased to 25-30% from 13 to 25 min, then increased to 30-40% from 25 to 35 min,

then increased to 40-43% from 35 to 40 min, then increased to 43-47% from 40 to 50 min, and it was increased to 47-100% from 50 to 54 min (Fernandes et al. 2013). For the detection, a diode Array and Multiple Wavelength detector SL was employed. All chromatograms were recorded at 280, 320, 350, and 500 nm.

Agilent ChemStation software (Hewlett-Packard ChemStation System) was used to process all the data obtained. All the measurements were done in triplicate.

3.2.3. LC-Q-TOF-MS Analyses

The chromatographic separation for determination and identification of the phenolic content was conducted using an Agilent 1260 Binary LC system.

For gradient elution, mobile phases A and B were employed, comprising a mixture of water and 0.1% formic acid, and acetonitrile, respectively. The column temperature was maintained at 30 °C, with a sample injection volume of 2 µL and a flow rate of 0.5 mL/min. The applied gradient elution was as follows. 0-0.5 min, 5% B; 0.5-2 min, 25% B; 2-4 min, 50% B; 4-6 min, 75% B; 6-10 min, 95% B, followed by a column conditioning step from 10 to 16 min with 5% B.

The MS analysis utilized the Agilent 6550 high-resolution Accurate Mass QTOF-MS instrument, boasting femtogram-level sensitivity, a resolution of 40,000, and a scanning rate of 50 spectra per minute.

Mass spectra were recorded over a mass range of 20-100,000 m/z. Integration and data processing were executed using the "MassHunter Workstation" software.

3.3. Antimicrobial Activity

3.3.1. Bacterial and Fungal Suspension

The selected species of bacteria and fungi used in the study were grown in appropriate media and incubation periods. Then the bacterial suspensions were adjusted approximately to 10^6- 10^7 CFU/mL by a densitometer (HVD DEN-1) (equivalent to 0.5 McFarland standard) in a TSB medium. All bacterial and fungal cultures were ten-fold diluted for further steps.

3.3.2. Broth Microdilution

For broth microdilution assay, 20 µL of bacterial suspension was added into the wells of a sterile 96-well flat bottom plate containing 180 or 150 µL of the leaf extracts. The final volume of the vessels was completed up to 200 µL by adding TS broth. Control vessels were prepared by adding 20 µL of bacterial suspension into the vessels with 180 µl of nutrient broth. The control wells do not contain leaf extracts. The inoculated plate was allowed to be incubated for 24 h at 37°C. Turbidity was determined spectrophotometrically (Thermo, VarioSkan Flash) at 600 nm wavelength with 120 min kinetic interval. After the incubation period, 100 µL samples were taken from each well and spread onto agar plates to check the bacterial growth. The same procedure was repeated for the fungal species. The results were obtained by using the software (Skan It Software 2.4.3 RE for VarioSkan Flash).

3.3.3. Antibiofilm Activity

Antibiofilm activities of the leaf extracts were determined in vitro by the spectrophotometric measurements of the 96-well plates which contain crystal violet dye. Bacterial and fungal suspensions in the TSB medium were inoculated into a sterile 96-well microtiter plate in a volume of 20 µL. Then 180 and 150 µL of grapevine and cauliflower leaf extracts were added to the vessels. The final volume of the wells was completed with TSB up to 200 µL. Then the plate was kept at 37°C for 24 h incubation. The absorbance of the plates was determined by a spectrophotometer (Thermo Scientific Varioskan® Flash) at 630 nm. Then each of the vessels was emptied by a micropipette. The emptied wells were washed with Phosphate-buffered saline (PBS) solution and the plate was allowed to dry completely. Then 125 µL of 0.1% crystal violet solution was transferred to each vessel and incubated for 20 minutes. At the end of the incubation period, the wells were emptied, then the washing with PBS solution was repeated to remove excess dye. Finally, 200 µL of 95% ethanol was added, and the absorbance of each well was recorded at 492 nm (A. Zhang et al. 2013). Measured initial and final absorbance values were substituted into the following equation (1) below:

$$B = \frac{A_{492}}{A_{630}} \qquad (1)$$

where the A_{492} and A_{630} are the recorded absorbance values at 429 nm and 630 nm respectively.

Biofilm formation was determined by the criteria determined by Zhang et al (A. Zhang et al. 2013):

$B < 0.1$ (No biofilm formation)

$0.1 < B < 0.5$ (Weak biofilm formation)

$0.5 < B < 1$ (Moderate biofilm formation)

$B \geq 1$ (Strong biofilm formation)

3.4. Antioxidant Activity

3.4.1. DPPH Assay

For the determination of the antioxidant activity by DPPH assay, 1 mL of leaf extract samples were mixed with the same amount of 0.8mmol/L DPPH solution. The well-shaked mixture was incubated at room temperature in a completely dark environment. Then the absorbance was measured at 517 nm (Pavithra and Vadivukkarasi 2015). Here, gallic acid was used as the standard. The measured absorbances were substituted in the equation (2) (Pavithra and Vadivukkarasi 2015) below:

$$\%\ Decolorization: \left[1 - \frac{A_{sample}}{A_{control}}\right] x100 \quad (2)$$

where the A_{sample} and $A_{control}$ are the absorbance values of the sample (mixtures of leaf extracts and DPPH solution) and control (DPPH) respectively.

3.4.2. ABTS Assay

For antioxidant determination by ABTS assay, 7 mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) were dissolved in pure water. The resulting reagent was kept at room temperature and in a completely dark environment for 12-16 hours for stabilization. Then, the stabilized reactive mixture was diluted 10 times in pure water to an absorbance value of approximately 0.7 at 734 nm. 100 µL of reagent and the same amount of sample were mixed and kept in the dark at room temperature for 5 minutes, and the absorbance value was measured at a wavelength of 734 nm (Rajurkar and Hande 2011). Pure water, the extraction medium, was used as a control. The antioxidant values of the samples were calculated using the following equation (3) below (Rajurkar and Hande 2011):

$$\text{ABTS}^{+} \text{ Radical-Scavenging (\%): } \frac{A_{control} - A_{sample}}{A_{control}} x100 \quad (3)$$

where the $A_{control}$ and A_{sample} are the absorbance values of control (pure water) and sample (mixture of reagent and leaf extract samples) at 734 nm respectively.

3.5. Cytotoxicity Analysis

3.5.1. Cell Culture

Mouse fibroblasts L929 cell line (P4-P15) was kept within Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and %0.5 penicillin-streptomycin (P/S). Cells were cultured in a T75 flask at 37 °C in an incubator with 5% CO_2 supplementation. The medium was changed every 2–3 days. The cells were used for experiments or once they reached 80–90% confluence.

3.5.2. Cytotoxicity Assessment

First, stock solutions were prepared for each vine and cauliflower leaf extract from freeze-dried powder samples. For this, freeze-dried samples were prepared in non-supplemented DMEM at a concentration of 50 mg/mL. Stock solutions were sterilized using a 0.22 µm cellulose acetate syringe filter. Then, 5, 50, and 500 µg/mL working solutions of the extracts were prepared in DMEM supplemented with +10% FBS + 0.5% P/S from the stock solution. Cells were trypsinized and seeded into 24-well plates at $2x10^4$ cells/cm^2 density and cultured until subconfluency. Freshly prepared culture media supplemented with the extracts at different concentrations were added to cells for 72 hours.

ISO 10993-5 (Annex-C) has been adapted in a modified version of it and was used in the direct contact cytotoxicity tests where viability greater than 70% compared to

controls indicated no cytotoxicity (ISO 2009). AlamarBlue® cell viability assay was performed at 24, 48, and 72 hours. Basically, a 1 mM AlamarBlue® stock solution was diluted 10-fold with growth medium to get a working solution of 0.1 mM AlamarBlue®. The growth media were then taken out, and PBS was used to wash the wells. Each well received 1 mL of the AlamarBlue® working solution before being incubated at 37 °C for 4 hours. Following the incubation period, 200 µL of the solution was placed into a 96-well plate and the fluorescence readings were done at an excitation wavelength of 540 nm and an emission wavelength of 635 nm using a plate reader (Varioskan LUX Plate Reader, Thermo Scientific™).

The reduction in the metabolic activity in the sample is associated with the number of living cells and the blue-violet formazan formed as monitored optical density at 570 nm. Therefore, the optical density results were substituted in equation (4), and the reduction of viability was calculated (ISO 2009):

$$Viability\ \% = \frac{100\ x\ OD_{570e}}{OD_{570b}} \qquad (4)$$

Herein, OD_{570e} refers to the mean value of the measured optical density of the extracts and OD_{570b} is the mean value of the measured optical density of the blanks. According to the handled standard, the samples whose viability reduction is lower than 70% have a cytotoxic potential (ISO 2009).

3.6. Color and pH Analysis

The colors of the extracts of the grapevine and cauliflower leaves were instrumentally measured by the L*a*b* system by using Minolta CR-400 (Tokyo, Japan) colorimeter. First, the colorimeter was standardized against a white reference plate. Three measurements were taken from grapevine and cauliflower leaf extracts. The colorimeter directly calculated three color features of L* (lightness), a* (red–green component), and b* (yellow–blue component).

pH values of the leaf extracts were determined by a pH meter at room temperature.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Chemical Characterization of the Bioactive Metabolites

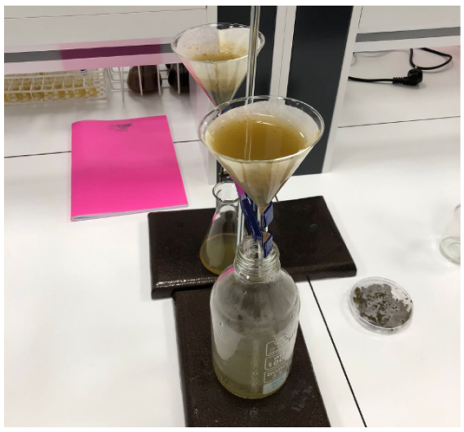
4.1.1. Extraction of the Bioactive Metabolites

Completely dried and crushed 20 gr of leaf samples were mixed with 400 mL of distilled water and extracted at 40 °C for 8 h. The mixture was filtered using filter paper for roughly 12 hours after the extraction was completed (Figure 4.1). The average extraction parameters and yields are presented in Table 4.1 below.

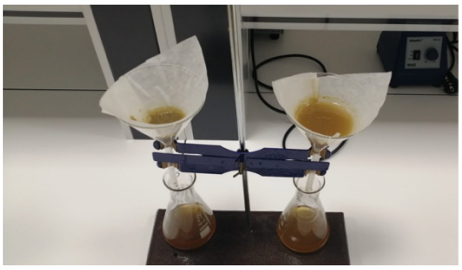
Table 4. 1 Leaf and solvent weights and extraction yields (in terms of mass)

Plant Sample	Leaf weight (g)	Water weight (g)	Extraction weight (g)	Extraction volume (ml)	Yield (%)
Grape Leaf	20.0168	389.960	315.3070	326.3333	76.9179
Cauliflower Leaf	20.0008	387.116	269.7558	276.6667	66.2669

Grape leaf extracts showed a higher extraction yield, compared to the cauliflower leaf extracts. The extraction yield of the grapevine leaves was 76.9179%, however, the yield of the cauliflower leaves was 66.2669%.



a



b

Figure 4. 1 Extracted leaf samples were left to the filter through the filter paper (a: Grape leaves, b: cauliflower leaves).

4.1.2. Total Phenolic Content

The total soluble phenolic content of the leaf extracts was determined by Folin-Ciocalteu assay. Firstly, a gallic acid calibration curve (Figure A.1) was obtained, and then the results were calculated by using the equation. Calculated results were tabulated in Table 4.2 below, the results were expressed as gallic acid equivalent (GAE). The total phenolic content of the grape leaves was calculated as 17.35183 mg GAE/L, whereas the cauliflower leaves had 13.43953 mg GAE/L. The total phenolic content of the grape leaves was found to be considerably higher than that of the cauliflower leaves, as presented in the Table 4.2.

Table 4. 2 Total phenolic contents of the leaf extracts of grape and cauliflower (mg GAE/L).

Sample	Total Phenolic Content (mg/GAE/L)
Grape Leaf	17.35183
Cauliflower Leaf	13.43953

4.1.2.1. Grape (*Vitis vinifera L.*) Leaves

The total phenolic content of the grape leaf extracts was calculated as 17.35183 mg GAE/L.

In the literature, there are some studies about the assignation of the total phenolic content of the grape leaves (Aouey et al. 2016; Soylemezoglu et al. 2016; Balík et al. 2008; Deliorman Orhan et al. 2009; Farhadi et al. 2016; Handoussa et al. 2013; Katalinic et al. 2013; Katalinić et al. 2009; Moldovan et al. 2020; Pari and Suresh 2008). In the study of Aouey et al. (2016), *V. vinifera* leaves were extracted by a mixture of water and ethanol, and the total phenolic content was expressed as 790.59 ± 7.31 mg of gallic acid/g of plant extracts (Aouey et al. 2016). Similarly, Katalinic et al. (2009 & 2013) and Moldovan et al. (2020) determined the total phenolic content of the *V. vinifera* leaf extracts extracted by a water-ethanol mixture. In the study by Moldovan et al. (2020), the total phenolic content of the leaf extracts was reported as 28.62 ± 0.24 mg GAE/g (Moldovan et al. 2020). Katalinic et al. (2009), examined the total phenolic content of the grape leaf extracts collected at different times.

The total phenolic content for the May leaves was 2910.5 ± 16.5 mg GAE/L while for the September leaves was 3338.7 ± 29.5 mg GAE/L (Katalinić et al. 2009). In another study performed by Katalinic et al. (2013), the leaves of six different *V vinifera* cultivars collected in May, August, and September were extracted by using a water-ethanol mixture, and the total phenolic contents were measured. The average total phenolic content of the May leaves was ranging between 18.8-28.0 g GAE/L, August leaves were ranging between 25.2-35.0 g GAE/L, and September leaves were ranging between 32.5-46.7 g GAE/L (Katalinic et al. 2013).

Farhadi et al. (2015), assigned the total phenolic content of the leaf extracts of six different *V. vinifera* cultivars. The phenolic substances were extracted by ultrasonication, using hydrochloric acid in methanol as the solvent (Farhadi et al. 2016). The lowest total phenolic content was recorded in the leaves of the Hosseini cultivar as 61 ± 7 mg/g dry weight (Farhadi et al. 2016). The methanolic leaf extracts from different varieties of *V. vinifera* were analyzed by Balik et al (2008). The total phenolic contents of the leaves were ranging from 15.1 mg/g to 23.8 mg/g (Balík et al. 2008).

Healthy and virus-infected leaves of different *V. vinifera* cultivars were analyzed by Söylemezoğlu et al. (2017). The leaves were extracted by methanol and the total phenolic contents of the leaf extracts were ranging from 308.98 mg GAE/100g to 509.12 mg GAE/100g, however, for the virus-infected leaves, the total phenolic content was reported in the range of 643.98- 1006.48 mg GAE/100g (Soylemezoglu et al. 2016). Differently, in a study conducted by Handoussa et al. (2013), the leaves of *V. vinifera* were extracted by using distilled water. The total phenolic content was reported as 289.33 ± 13.02 mg GAE/g dry weight (Handoussa et al. 2013).

According to the presented results, the total phenolic content differs in different studies. The differences are not surprising, because the total phenolic content is affected by many factors including the solvent, cultivar, harvest time, location, and infectious pathogens.

4.1.2.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

The total phenolic content of the cauliflower leaf extract was calculated as 13.43953 mg GAE/L.

The total phenolic content of the cauliflower leaves was determined by Sanz-Puig et al. (2015). In the study, the total phenolic content of the cauliflower leaves was reported as 11,359.8135 ± 747.96277 mg GAE/L (Sanz-Puig et al. 2015). In a different research study conducted by Huynh et al (2014), the total phenolic content of the enzyme-assisted extraction was applied to cauliflower leaves. It has been stated that the total phenolic content was 336 ± 30 mg GAE/ 100 g dry weight at the beginning of the enzyme treatment

(Huynh et al. 2014). However, it has been reported that the total phenolic content increased significantly after 24 h of enzyme-assisted extraction (Huynh et al. 2014).

The differences in the expressed results were due to the handling of different extraction procedures, the use of different solvents, and other external factors such as the location, harvest time, etc.

4.1.3. HPLC Analysis

The phenolic substances that are abundant in grape and cauliflower leaf extracts were determined by using an HPLC system equipped with a diode array detector. The obtained peaks were compared with previously presented results by Fernandes et al. (2013).

4.1.3.1. Grape (*Vitis vinifera L.*) Leaves

HPLC-DAD profiles of the phenolic compounds in grape leaves are presented in Figure 4.2 below.

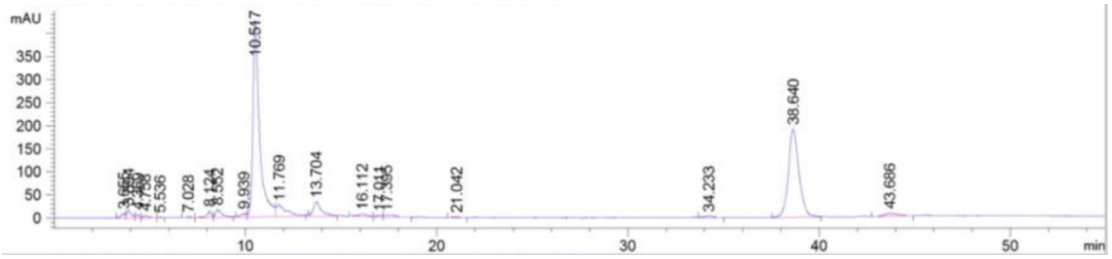


Figure 4. 2 HPLC–DAD phenolic profile of grape leaves. Detection at 320nm.

According to previous studies, grape leaves are described as good sources of phenolic substances. Fernandes et al. (2013), determined that grape leaves contain myricetin-3-O-glucoside, quercetin-3-O-galactoside, trans-coumaroyl tartaric acids, kaempferol-3-O-glucoside, quercetin-3-O-glucoside, and the derivatives of hydroxycinnamic acid (Fernandes et al. 2013). However, compared to the findings, the HPLC-DAD system handled in the study detected trans-caffeoyl tartaric acid, quercetin-3-O-galactoside and quercetin-3-O-glucoside.

4.1.3.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

HPLC profiles of the phenolic compounds in cauliflower leaves are presented in Figure 4.3 below.

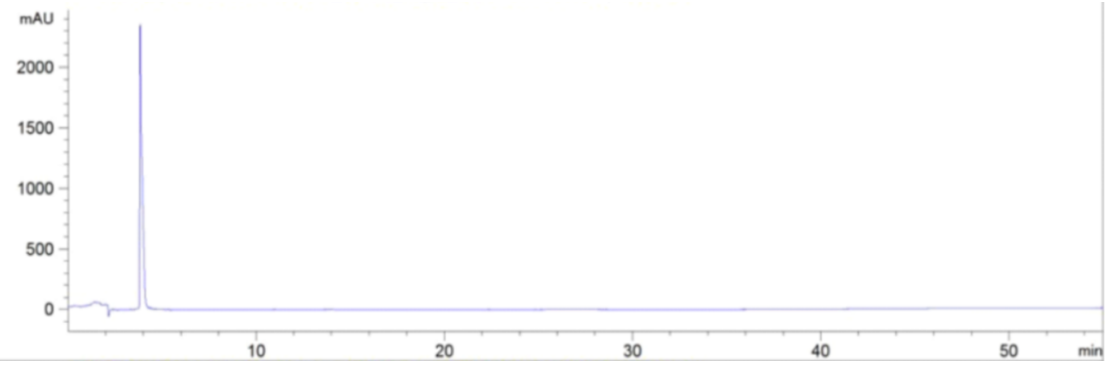


Figure 4. 3 HPLC–DAD phenolic profile of cauliflower leaves. Detection at 320nm.

Since no standard substance was used for cauliflower leaf extract, it is not possible to make a definitive comment about the result. Further studies are needed to define the phenolic content of cauliflower leaf.

4.1.4. LC-Q-TOF-MS Analysis

For the quantification and identification of phenolic substances in leaf extracts, library scanning was performed with a qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-Q-TOF-MS) system.

4.1.4.1. Grape (*Vitis vinifera L.*) Leaves

Extracted *V. vinifera* leaves were analyzed through an LC-Q-TOF-MS system. A library scanning protocol was employed for the determination of the bioactive metabolites. At the end of the protocol, 2,221 components were identified among the over 4 thousand detected components. The most abundant components detected in the grape leaf extracts were presented in Table 4.3 below.

According to the presented results, extracted *V. vinifera* leaf samples were found as rich in flavonoid compounds including isorhamnetin 3-galactoside, rutin, quercetin, kaempferol, and naringenin; organic acids including chlorogenic acid, pipecolic acid, quinic acid, and caffeic acid; some aromatic compounds like 3,4-Dihydroxybenzaldehyde, 4-Hydroxybenzaldehyde, and some essential amino acids such as valine and tyrosine.

Table 4. 3 Identified bioactive components in grape leaf extract by qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-Q-TOF-MS) system.

Name	Retention Time	Mass	Abundance
Rutin	8.774	610.1557	9724246
Kaempferol	10.065	286.0476	3054469
3,4-Dihydroxybenzaldehyde	5.294	138.0321	2459261
Chlorogenic acid	6.08	354.0941	2260462
Riboflavin	6.978	376.1395	2063852
Quinic acid	6.091	192.0639	1903905
Caffeic acid	6.607	180.043	1420377
4-Hydroxybenzaldehyde	2.498	122.0368	1314490
3-0-Methylquercetin	10.235	316.0591	1209601
4-Formyl Indole	4.687	145.0534	1121718
DL-pipecolic acid	1.385	129.0796	921073
Phlorhizin	10.694	436.1355	799999
Isorhamnetin 3-galactoside	10.257	478.1114	685813
Quercetin	12.593	302.0446	650134
(±)-Naringenin	13.838	272.0682	559971

In a study, where the extracts of grape leaves were examined according to their phenolic profile chromatographically, hyperoside, caftaric acid, gallic acid, protocatechuic acid, rutin, catechin, epicatechin, isoquercitrin, quercitrin, and quercetin were identified through liquid chromatography coupled with mass spectrometry in tandem (LC-MS/MS) system (Moldovan et al. 2020). Felicio et al. (2001) identified the compounds in *V. vinifera* leaves by using mass and NMR spectroscopy. The compounds resveratrol, ε-viniferin, balanocarpol, and β-glucopyranosyl-8'- balanocarpol have been identified from the ethanol-extracted *V. vinifera* leaves (Felicio et al. 2001). In another study, grape leaves were examined according to their phenolic substances kaempferol, catechin, naringin, rutin, resveratrol, and quercetin contents were detected by using HPLC (Dani et al. 2010). The HPLC system was used to identify the phenolic compounds in the methanol extracts of the grape leaf samples. According to the results, the phenolic substances found in the extract were expressed in descending order as follows: quercetin, rutin, catechin, epicatechin, caffeic acid, gallic acid, and resveratrol (Farhadi et al. 2016).

Besides, it has been indicated that the leaves were rich in quercetin among the other parts (skin, pulp, seeds, cane) of the plant (Farhadi et al. 2016).

The phenolic acids (gallic acid, 3-Hydroxybenzoic acid, vanillin acid, caffeic acid), flavonoids (quercetin, quercetin-4'-glucoside, (+)-Catechin, apigenin, (-)-Epicatechin, myricetin, rutin), and stilbenes (piceid /isorhapontin, astringin, *cis*-Resveratrol, *trans*-Resveratrol,) were also detected in the leaf extracts by HPLC-RP–DAD system (Katalinić et al. 2009; Katalinic et al. 2013).

The HPLC-PDA and HPLC–HRESI-MS systems were employed for the identification of the phenolic compounds of the *V. vinifera* leaf extract (Handoussa et al. 2013). The results indicated that the leaves contain quercetin derivatives (quercetin-glucuronide, quercetin-3-O-rutinoside (rutin), and quercetin-3-β -glucoside (isoquercitrin)), luteolin-7-O-glucoside, cyanidin-3-O-glucoside, kaempferol coumaroyl glucoside, organic acids (quinic acid and caftaric acid), and hesperitin (Handoussa et al. 2013).

On the other hand, rutin hydrate and gallic acid were detected when the phenolic components of the healthy and infected leaves were analyzed (Soylemezoglu et al. 2016). However, the abundance of the substances was varying among the cultivars and the infection status (Soylemezoglu et al. 2016). Similarly, Balik et al. (2008) examined the healthy and infected grape leaves according to their phenolic substances. For the healthy leaves, *trans*-resveratrol, *trans*-piceid, and caftaric acid were the most abundant compounds in ascending order (Balík et al. 2009). Besides, the positive correlation between the infection status and the phenolic content was also pointed out (Balík et al. 2009).

Pintac et al. (2019) employed a quantitative LC-MS/MS system to identify the phenolic substances. According to the results, the leaf extracts were rich in phenolic acids (2,5–dihydroxybenzoic acid, p–hydroxybenzoic acid, protocatechuic acid, gallic acid, ellagic acid, vanillic acid, syringic acid), phenolic acids (caffeic acid, *p*–coumaric acid, chlorogenic acid, ferulic acid), flavanones (esculetin, naringenin, umbelliferone, coumarins), flavonols (kaempferol, quercetin, quercitrin, quercetin-3-O-glucoside, hyperoside, isorhammnetin, rutin, kaempferol-3-O-glucoside), flavones (baicalein, luteolin, luteolin-7-O-glucoside, amentoflavone), and stilbenes (triterpenoids, resveratrol, ursolic acid) (Pintać et al. 2019). Herein, flavonols were reported as the most abundant group, and ellagic and chlorogenic acids were the dominant phenolic acids (Pintać et al.

2019). However, opposite to the other studies, resveratrol had been detected in only two varieties (Pintać et al. 2019).

When the previous studies were examined, it was seen that the most common substances in grape leaf extracts were the derivatives of resveratrol, quercetin, and kaempferol. Therefore, the identified compounds listed in Table 4.3 is supported by the literature. Herein, the extraction parameters, solvent, and the employed chromatographic instruments may create some differences among the detected components. Moreover, differences in the climatic conditions, the health status of the plants, and the cultivars may be responsible for the differences in the detected bioactive components.

4.1.4.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

Cauliflower leaf extracts were analyzed through an LC-Q-TOF-MS system. Table 4.4 below, shows the most abundant ones among the 2 thousand identified compounds in the sample.

According to the tabulated results in Table 4.4, the water-extracted cauliflower leaves were containing organic acids including malic acid, quinic acid, pantothenic acid, maleic acid; and flavanol compounds like kaempferol-7-O-glucoside.

Table 4. 4 Identified bioactive components in grape leaf extract by qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-Q-TOF-MS) system.

Name	Retention Time	Mass	Abundance
L-Phenylalanine	3.102	165.0806	6308046
DL-Phenylalanine	2.765	165.0761	4229160
Malic acid	1.305	134.0218	3962802
Quinic acid	6.043	192.0636	3820724
L-Arginine	1.137	174.1127	2451229
D-Pantothenic acid	3.439	219.1121	2145379
5-Methylcytosine	1.665	125.0595	1808669
Maleic acid	1.383	116.011	1332141
Kaempferol 7-0-glucoside	5.64	448.1013	1245233
Oxyquinoline	5.539	145.0535	1105603
4-Hydroxyindole	2.675	133.0533	686726
PAB / 4-Aminobenzoic acid	4.012	137.0486	420532
6-Methoxyquinoline	7.56	159.0694	256510
9,10-DiHOME	19.617	314.245	190022

In a study, gas chromatography equipped with a mass spectroscopy system was used for the primary metabolite identification. Here, many primary metabolites were recorded including stearic acid, oleic acid, glucose, mannose, gluconic acid, ribitol, and 4-dodecanol (Baky et al. 2022). Besides, in this study, it has been stated that the abundance of the volatile nitrogenous compounds was slightly higher in the cauliflower leaves, among the other Brassica species (Baky et al. 2022). In another study performed by Cabello-Hurtado et al. (2012), glucosinolate compounds were identified through an HPLC column. The glucosinolate compounds including sinigrin, glucobrassicin, 4-Methoxyglucobrassicin, and 4-OH-Glucobrassicin were identified (Cabello-Hurtado, Gicquel, and Esnault 2012).

The phenolic profile of the cauliflower leaf extracts was determined by using ultraperformance liquid chromatography with diode array detector-quadrupole time-of-flight-high-definition mass spectrometry (UPLC-DAD-HDMS-TOF-MS) (Huynh et al. 2016; 2014). The results were proving that the outer leaves of the cauliflower contain phenolic compounds in glycosidic form. The most abundant ones were kaempferol derivatives such as kaempferol-3-O-coumaroyldiglucoside, kaempferol-3-O-

diglucoside-7-O-glucoside, and kaempferol-3-O-diglucoside; and quercetin-7-O-diglucoside (Huynh et al. 2016; 2014).

The diversity of identified bioactive compounds in the cauliflower leaf may vary according to environmental conditions such as climate, soil, and the state of maturation, the method and type of solvent handled for extraction, as well as the method and instrument used for bioactive component identification. Thus, the difference between the components mentioned in previous studies and the experimental results tabulated in Table 4.4 is not a surprise.

4.2. Antimicrobial Activity

4.2.1. Broth Microdilution Method

The broth microdilution method was used to determine the antibacterial characteristics of grape leaf extract and cauliflower leaf extract. Gram-positive and Gram-negative bacteria, and fungi, were used to test the antibacterial activity. The microorganisms handled in the study were tabulated in Table 4.5 below.

For testing the antimicrobial activity, 180 and 150 μL of the grape leaf and cauliflower leaf extracts were applied to the inoculated vessels of the 96-well plate. The plate was inoculated at 37°C for 24 h, and the growth of the microorganisms was determined spectrophotometrically.

Table 4. 5 Gram-positive, Gram-negative bacteria and fungi handled in the study.

Gram-Positive Bacteria	**Origin**
Bacillus cereus (ATCC 11778)	Unknown
Listeria innocua	Unknown
Carnobacterium divergens (NRRL B-14830)	Minced meat
Gram-Negative Bacteria	**Origin**
Serratia liquefaciens (NRRL B41553)	Ground beef
Esherichia coli (ATCC 25253)	*Unknown*
Salmonella Typhimurium (CCM 5445)	Unknown
Fungi	**Origin**
Candida albicans (DSM 5817)	Unknown
Saccharomyces cerevisiae	Commercial

4.2.1.1. Grape (*Vitis vinifera L.*) Leaves

The antimicrobial activity of the *V. vinifera* leaf extracts against the bacteria species handled in the study was shown in Figure 4.4 below. According to the tabulated results in the figure, it can be said that the grape leaf extract has promising antimicrobial activity against tested microorganisms in this study. However, the wells that applied 180 μL of the grape leaf extract showed a higher inhibition compared to the wells containing 150 μL of the extract. Thus, the antimicrobial activity is dose-dependent.

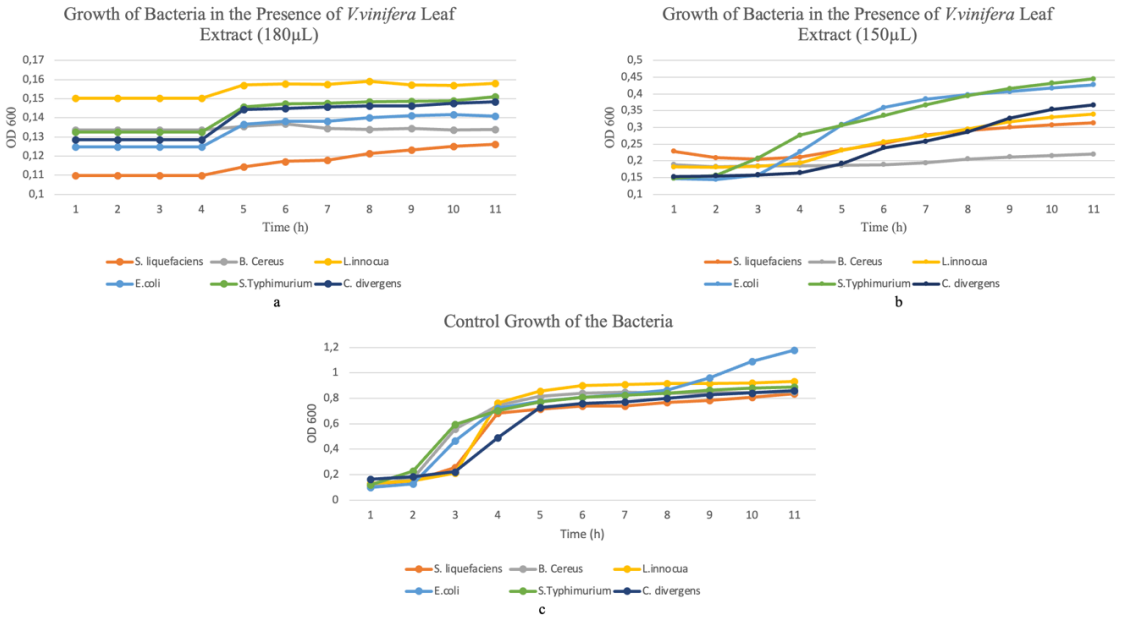


Figure 4. 4 Antimicrobial activity of the grape leaf extract against all tested bacteria in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

Among all tested Gram-positive bacteria, in 180 µL applied dose, *L. innocua* was. the most resistant (Figure 4.5-a). In that dose, the antimicrobial activity of the grape leaf extract is *B. cereus* > *C. divergens* > *L. innocua* in descending order. However, *C. divergens* was the most resistant in the dose of 150 µL. The antimicrobial activity in 150 µL in the following order is *B. cereus* > *L. innocua* > *C. divergens* (Figure 4.4-b). In both test doses, *B. cereus* was the most affected among all the Gram-positive bacteria species.

The antimicrobial activity of the grape leaf extracts against Gram-negative bacteria (*S. liquefaciens, E. coli,* and *S.* Typhimurium) is shown in Figure 4.6 below. According to the figure, *S. liquefaciens* was the least resistant specie among the others. In 180 µL, *S.* Typhimurium showed the biggest resistance, followed by *E. coli*, and *S. liquefaciens* (Figure 4.6-a). The antimicrobial activity of 150 µL grape leaf extract was recorded as *S. liquefaciens* > *E. coli* >*S.* Typhimurium. *S.* Typhimurium was found the most resistant species, followed by *E. coli* in 150 µL of applied dose (Figure 4.6-b). According to the results, all gram-positive bacteria were more affected compared to the gram-negative bacteria in the 180 µL applied dose.

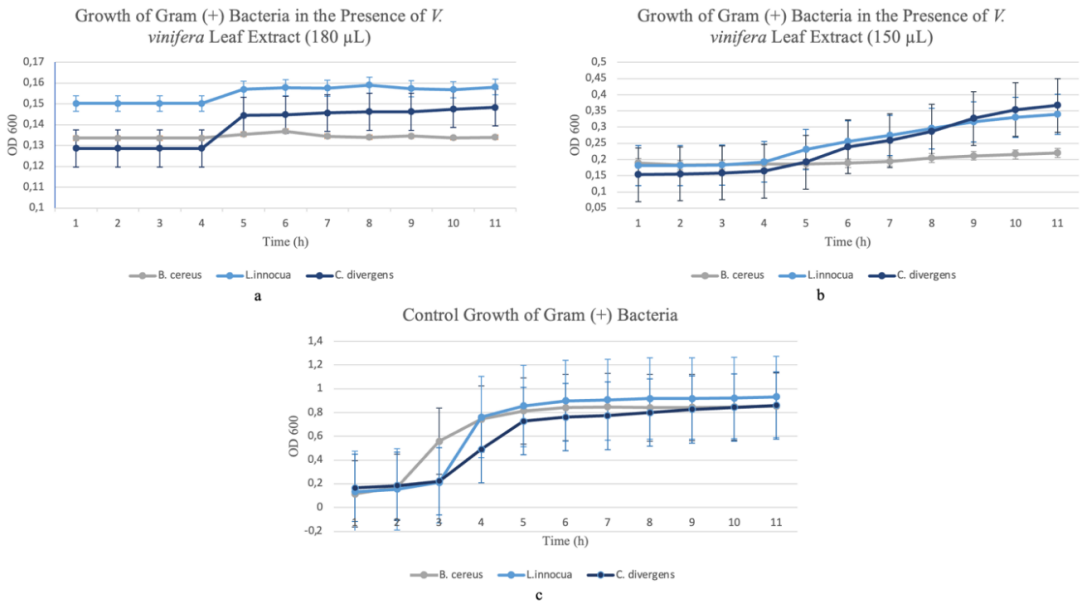


Figure 4. 5 Antibacterial activity of the grape leaf extract against Gram-positive bacteria handled in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

In a study where Turkish medicinal plants were examined according to their antimicrobial activities, *V. vinifera* leaf extracts were reported as one of the most promising plants that show antimicrobial activity against Gram-positive and Gram-negative bacteria (Oskay and Sari 2007). Therefore, the antimicrobial activity of grape leaves is not surprising when the results are compared to the literature. Besides, the antimicrobial activity of the grape leaf extracts was determined in several studies (Abed et al. 2015; Deliorman Orhan et al. 2009; Katalinic et al. 2013).

Abed et al. (2015), studied the antibacterial activity of two different cultivars of grape leaves against *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* Typhimurium*, Listeria monocytogenes,* and *Escherichia coli* (0157). According to their results, the leaf extracts showed promising antimicrobial activity against *S. aureus* followed by *P. aeruginosa,* however, the leaf extracts were ineffective against *E. coli* (0157), *S.* Typhimurium*,* and *L. monocytogenes* (Abed et al. 2015). Here, *E. coli* and *S.* Typhimurium are Gram-negative species, whereas *L. monocytogenes* is Gram-positive. It can be said that the difference in antimicrobial activity between Gram-positive and Gram-

negative species is not clear. However, in the study performed by Deliorman Orhan et al. (2009) where the antimicrobial activity was determined against *Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*, it has been concluded that the grape leaf extract did not show significant antimicrobial activity compared to the control strains (Deliorman Orhan et al. 2009). However, according to their results, the antibacterial activity was more successful against Gram-positive bacteria species (*E. faecalis* and *S. aureus*) (Deliorman Orhan et al. 2009), which is a different situation than in the study performed by Abed et al. (2016). Similarly, Katalinic et al. (2013) reported that the antimicrobial activity against gram-positive (*S. aureus,* and *B. cereus*) and gram-negative (*C. jejuni, E. coli* O157:H7, and *S. infantis*) bacteria species was not significant (Katalinic et al. 2013)

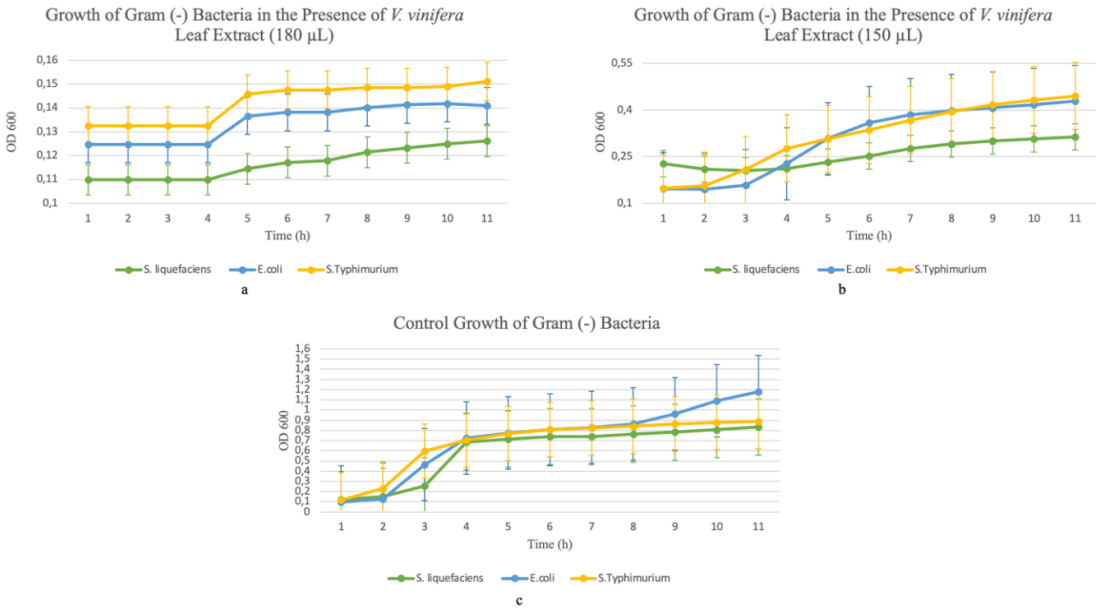


Figure 4. 6 Antibacterial activity of the grape leaf extract against Gram-negative bacteria handled in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

The findings of the present study indicated that the leaf extracts of *V. vinifera* showed successful antibacterial activity. *B. cereus* and *S. liquefaciens* were found as the least resistant species in both applied doses. Besides, the leaf extract was found ineffective against the Gram-negative strains, *E. coli* and *S.* Typhimurium. Similar results were reported in the study conducted by Abed et al. (2015). Besides, the unclear difference in the antibacterial activity against Gram-positive and Gram-negative bacteria was also supported by Katalinic et al. (2013) and Abed et al. (2015).

The antifungal activity of the grape leaf extracts was also determined against *C. albicans* and *S. cerevisiae.* According to the obtained results, *C. albicans* was more resistant than *S. cerevisiae* (Figure 4.7-a). In the study of Oskay and Sarı (2007), the leaf extract of *V. vinifera* was reported as one of the plants which have anticandidal activity. However, the inhibition zone was significantly smaller compared to the other microorganisms tested in the study (Oskay and Sari 2007).

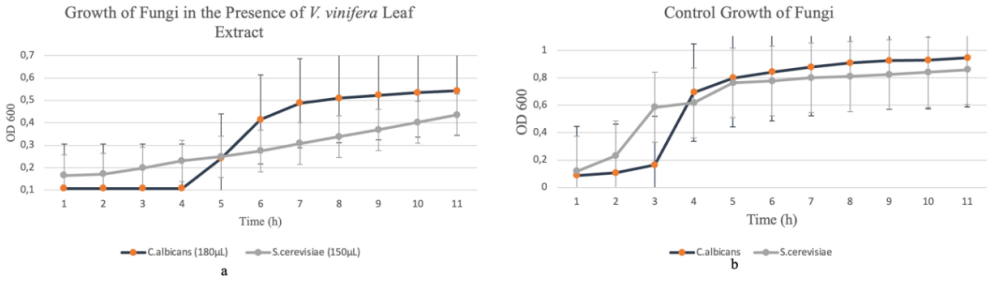


Figure 4. 7 Antimicrobial activity of the grape leaf extract against fungi (a represents the presence of *V. vinifera* leaf extract (180 µL for *C. albicans* and 150 µL for *S. cerevisiae*) and b represents the absence of *V. vinifera* leaf extract (control)).

The antimicrobial activity of the leaf extracts is associated with the phenolic content. In a study, where the grape leaf extracts were analyzed according to their phenolic content and antimicrobial activities, it has been concluded that there might be a correlation between the phenolic content and antimicrobial activity (Katalinic et al. 2013; Katalinić et al. 2009). The extracts of grape leaves harvested in May August, and

September had different antimicrobial activities. September leaves were richer in bioactive compounds such as quercetin derivatives, resveratrol, flavonols, and stilbene compounds among the May and August leaves, and it has been concluded that the antimicrobial activity of the September leaves was more effective ((Katalinić et al. 2009). The mechanism behind this activity is not clear. However, these compounds mainly interact with the bacterial cell wall and create abnormalities in the cell wall which results in cell death through -OH groups (Wang et al. 2018).

The antimicrobial activity of the *V. vinifera* leaf extracts against many microorganisms have been supported by the outcomes of many studies. The activity is mainly dose-dependent, and the phenolic substances are associated with that activity.

4.2.1.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

The water-extracted cauliflower leaves were examined according to their antimicrobial activity against *S. liquefaciens, S.* Typhimurium*, L. innocua, E. coli, C. divergens,* and *B. cereus.* The growth curves are presented in Figure 4.8 below. According to the results shown in the figure, the extract of cauliflower leaves shows antibacterial activity against different bacterial species.

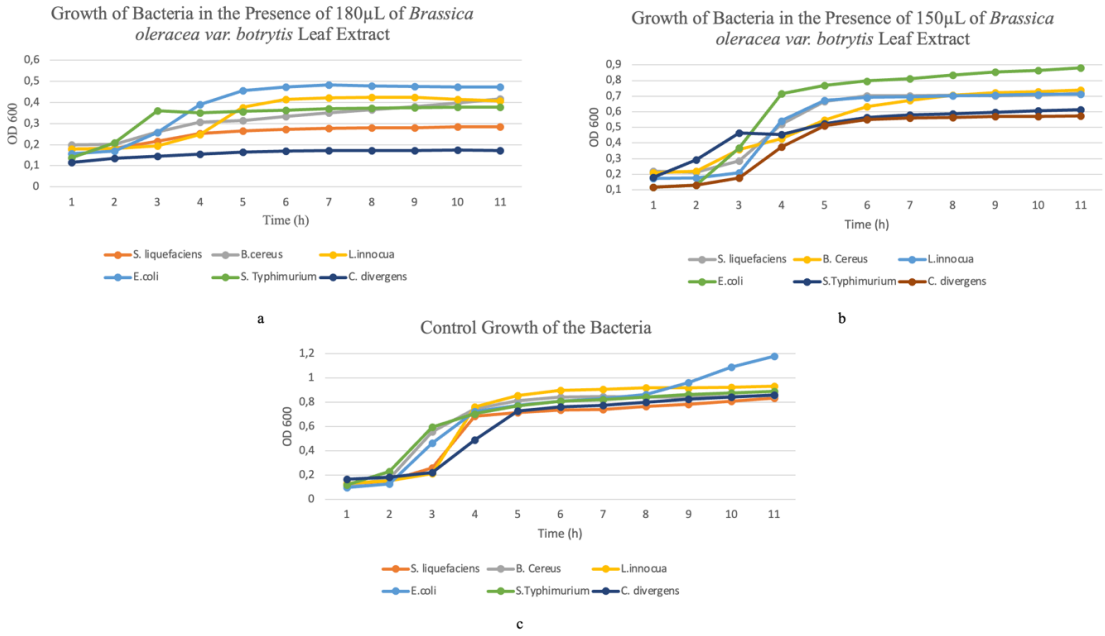


Figure 4. 8 Antimicrobial activity of the cauliflower leaf extract against all tested bacteria in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

At the dose of 180 µL, *C. divergens* was the most affected bacterial specie, among the other tested bacteria, followed by *S. liquefaciens,* and *S.* Typhimurium. At that dose, *E. coli* showed the biggest resistance against the cauliflower leaf extract. The antibacterial activity at 150 µL was very similar to the dose of 180 µL. *C. divergens* was the most affected one, followed by *S. liquefaciens,* and *S.* Typhimurium. *E. coli* was the most resistant specie. However, it should be highlighted that the antimicrobial activity is slightly lower in the lower dose (150 µL). Thus, the activity is dose-dependent.

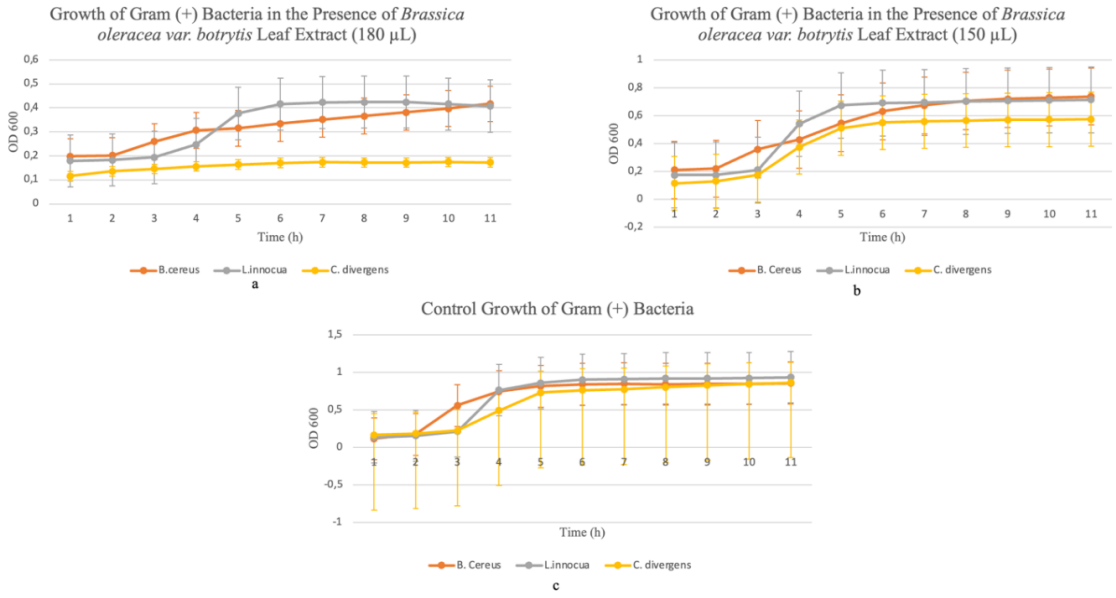


Figure 4. 9 Antibacterial activity of the cauliflower leaf extract against Gram-positive bacteria handled in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

According to the results presented in Figure 4.9, the antimicrobial activity among Gram-positive species was *C. divergens* > *L. innocua* > *B. cereus* in descending order. Although a similar order in antimicrobial activity was observed, a higher inactivation was recorded at 180 µL, compared to the 150 µL.

The antimicrobial activity in Gram-negative species handled in this study shows that *E. coli* showed the biggest resistance compared to the other species (Figure 4.10). At the dose of 180 µL, *S. liquefaciens* was the most effective bacteria. However, at 150 µL, the most affected specie was *S.* Typhimurium. Besides, Gram-negative bacteria (*E. coli, S.* Typhimurium, and *S. liquefaciens*) were found to be more affected than Gram-positive (*B. cereus, L. innocua*, and *C. divergens*) bacteria. The most possible explanation for this difference is that Gram-positive bacteria have a peptidoglycan membrane.

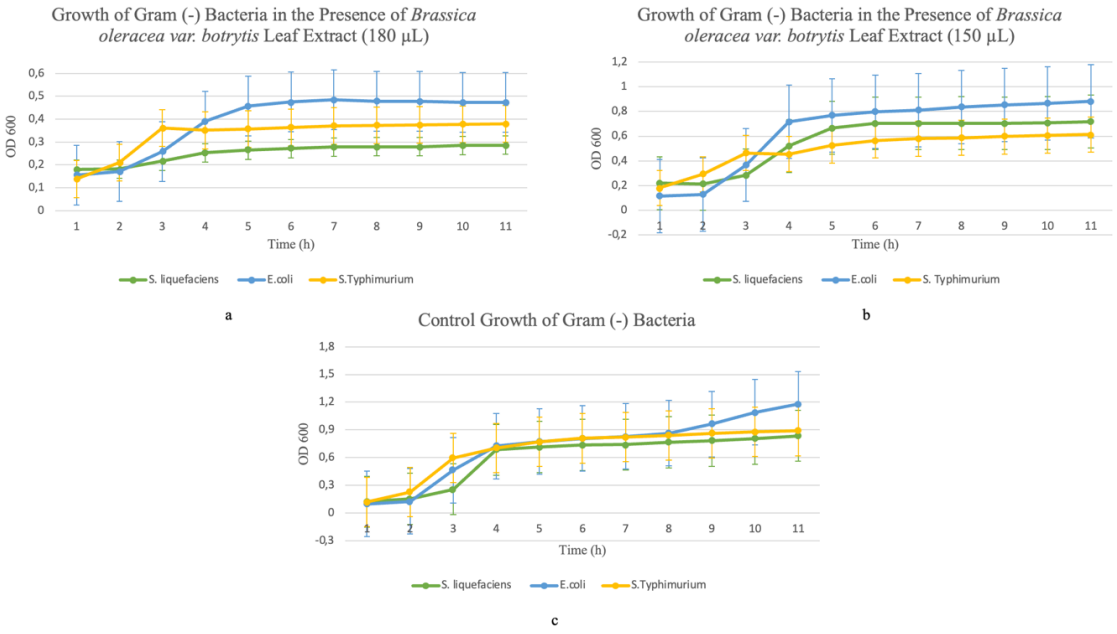


Figure 4. 10 Antibacterial activity of the cauliflower leaf extract against Gram-negative bacteria handled in the study (a, b, and c represent the applied doses, 180 µL, 150 µL, and 0 µL (control), respectively).

The antibacterial activity of cauliflower leaf extracts against *L. monocytogenes* was identified (Sanz-Puig et al. 2015). It has been stated that the cauliflower leaf extracts showed promising bactericidal activity against *L. monocytogenes* (Sanz-Puig et al. 2015). Besides, higher bactericidal activity was observed at the higher applied dose, thus the activity was dose-dependent (Sanz-Puig et al. 2015). The antibacterial activity of the cauliflower leaf extract was associated with its phenolic content. The leaves were rich in organic acids including chlorogenic acid, gallic acid, and ferulic acid (Sanz-Puig et al. 2015). In another study, conducted by Brandi et al. (2006), cauliflower leaf juice was analyzed according to its antimicrobial activity against different bacterial species (Brandi et al. 2006b). The results showed that the cauliflower leaf juice has bactericidal activity against *Salmonella Enteritidis,* various enterotoxigenic *E. coli* strains, and *L. monocytogenes* (Brandi et al. 2006). Besides, it has been stated that antibacterial activity depends on the isothiocyanate content (Brandi et al. 2006).

Isothiocyanate compounds show their antimicrobial activity in a broad spectrum of action by disrupting the outer cell membrane and redox balance (Romeo et al. 2018).

Another possible mechanism had been reposted as the blocking of sulfhydryl groups of enzymes (Tajima et al. 1998). However, it should be pointed out that the isothiocyanate activity is dose-dependent (Brandi et al. 2006). On the other hand, organic acids e.g., ferulic acid and gallic acid affect the cell surface and change its charge, hydrophobicity, and K^+ leakage (Borges et al. 2015).

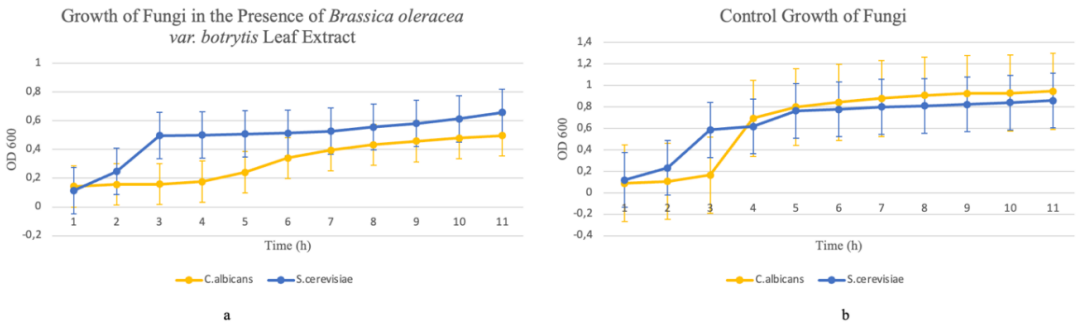


Figure 4. 11 Antimicrobial activity of the cauliflower leaf extract against fungi (a represents the presence of *Brassica oleceae var. botrytis* leaf extract (180 µL for *C. albicans* and 150 µL for *S. cerevisiae*) and b represents the absence of *Brassica oleceae* var. *botryris* leaf extract.

Cauliflower extracts were tested for antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae*. The results were shown in Figure 4.11 above. According to the figure, *C. albicans* was more affected compared to *S. cerevisiae*. However, *C. albicans* was inoculated in the presence of 180 µL of cauliflower leaf extract, whereas *S. cerevisiae* was inoculated in the presence of 150 µL of the extract. Since a dose-dependent activity was observed before, the results may be in conflict.

The antifungal activity of cauliflower leaves was analyzed by Sisti et al. (2003). The crude cauliflower juice in different concentrations was applied to the *C. albicans* suspensions. The results indicated that cauliflower leaf juice has a promising activity to inhibit *C. albicans* growth in a dose-dependent manner (Sisti, Amagliani, and Brandi 2003). The possible mechanism for the antifungal activity is associated with isothiocyanates (Sisti, Amagliani, and Brandi 2003). Isothiocyanate compounds disrupt

the sulfhydryl groups of the cellular enzymes and proteins which has a role in ATP synthesis (Sisti, Amagliani, and Brandi 2003; Tajima et al. 1998).

Cauliflower leaf extracts showed promising antimicrobial activity against different species of Gram-positive and Gram-negative bacteria, and fungi. The effect of the phenolic substances has been proposed in previous studies (Sanz-Puig et al. 2015; Brandi et al. 2006b).

4.2.2. Antibiofilm Activity

The antibiofilm activities of the leaf extracts were determined by a spectrophotometer. The initial and final absorbance of the inoculated vessels of a 96-well plate were measured at 429 nm and 630 nm respectively. Then the measured values were substituted in Equation (1) below:

$$B = \frac{A_{492}}{A_{630}} \qquad (1)$$

Then calculated B values were used to determine the biofilm formation in the following criteria (A. Zhang et al. 2013):

B <0.1 (No biofilm formation)

0.1< B < 0.5 (Weak biofilm formation)

0.5 < B < 1 (Moderate biofilm formation)

B ≥ 1 (Strong biofilm formation)

4.2.2.1. Grape (*Vitis vinifera L.*) Leaves

The biofilm formation among the tested microorganisms in the presence of *V. vinifera* leaf extract was tabulated in Table 4.6 below:

Table 4. 6 Calculated B values and biofilm formation in the presence and absence of grape leaf extracts (*C. albicans* in 150 µL and *S. cerevisiae* in 180 µL test doses have not been performed).

Dose		***S. liquefaciens***	***B. cereus***	***L. innocua***	***E. coli***	***S.* Typhimurium**	***C. divergens***	***C. albicans***	***S. cerevisiae***
180 µL	B value	0.354	0.266	0.267	0.283	0.355	0.253	0.151	-
	Biofilm Formation	Weak	Weak	Weak	Weak	Weak	Weak	Weak	-
150 µL	B value	0.293	0.319	0.269	0.227	0.233	0.246	-	0.204
	Biofilm Formation	Weak	Weak	Weak	Weak	Weak	Weak	-	Weak
Control (0 µL)	B value	0.194	0.181	0.185	1.041	1.043	1.058	1.025	0.907
	Biofilm Formation	Weak	Weak	Weak	Strong	Strong	Strong	Strong	Moderate

According to the results tabulated in the table above, *V. vinifera* leaf extract has promising antibiofilm activity. *C. divergens, E. coli*, *S.* Typhimurium, and *C. albicans* were able to form a strong biofilm structure. However, in the presence of grape leaf extract, biofilm formation was decreased (Table 4.6). Similarly*, S. cerevisiae* was forming a moderate biofilm in the absence of the leaf extract, however, a decrease in biofilm formation was observed in the presence of grape leaf extracts. Among the fungal strains handled in this study, *V. vinifera* leaf extracts were more effective against *C. albicans*,

compared to *S. cerevisiae*. On the other hand, the antibiofilm activity was expected to be dose-dependent, however, calculated B values showed that there may not be a positive correlation between the applied dose and antibiofilm activity. In 180 μL of the applied dose, the antibiofilm activity was slightly lower than in the 150 μL.

The antibiofilm activity of the grape leaf extracts was also studied by Ramadan et al (2017). In the study, ethanolic grape leaf extracts showed promising biofilm inhibitory effects against *S.* Typhimurium, *E. coli, S. aureus,* and *P. aeruginosa* (Ramadan et al. 2017). Besides, it has been stated that the antibiofilm activity is associated with phenolic content (Ramadan et al. 2017). Thus, the grape leaves were examined according to their phenolic content and bioactive composition. The leaf extracts were including quercetin derivatives, ferulic acid, chlorogenic acid, and phenolic acids (Ramadan et al. 2017). Besides, these bioactive metabolites are reported as they interact with bacterial cell wall proteins and they damage the cell membrane, and block nucleic acid synthesis, or energy metabolism (Slobodníková et al. 2016). Moreover, quercetin shows antibiofilm activity by suppressing the quorum-sensing mechanism which is essential for cell-to-cell communication and biofilm formation (Sánchez, González, and Hedlefs 2016).

4.2.2.2. Cauliflower *(Brassica oleceae var. botrytis*) Leaves

Calculated B values and biofilm formed by tested microorganisms in the presence and absence of cauliflower leaf extract are shown in Table 4.7 below:

Table 4. 7 Calculated B values and biofilm formation in the presence and absence of cauliflower leaf extracts (*C. albicans* in 150 µL and *S. cerevisiae* in 180 µL test doses have not been performed).

Dose		***S. liquefaciens***	***B. cereus***	***L. innocua***	***E. coli***	***S.* Typhimurium**	***C. divergens***	***C. albicans***	***S. cerevisiae***
180 µL	B value	0.266	0.178	0.201	0.165	0.197	0.231	0.172	-
	Biofilm Formation	Weak	Weak	Weak	Weak	Weak	Weak	Weak	-
150 µL	B value	0.168	0.136	0.174	0.119	0.166	0.182	-	0.128
	Biofilm Formation	Weak	Weak	Weak	Weak	Weak	Weak	-	Weak
Control (0 µL)	B value	0.194	0.180	0.185	1.041	1.043	1.058	1.026	0.907
	Biofilm Formation	Weak	Weak	Weak	Strong	Strong	Strong	Strong	Moderate

According to the table, water-extracted cauliflower leaves may be used as an antibiofilm agent against *C. divergens*, *S.* Typhimurium, *E. coli*, *C. albicans,* and *S. cerevisiae.* In the control samples, the microorganisms formed strong biofilm structures except for *S. cerevisiae*, which formed a moderate structure (Table 4.7). However, when the leaf extracts were applied to the vessels, the biofilm formation was affected negatively. The antibiofilm activity of the cauliflower leaf extract was most effective

against *E. coli*, followed by *S.* Typhimurium, and *C. divergens* among the tested bacterial strains. In fungal strains, *C. albicans* was affected more compared to *S. cerevisiae*.

The secondary metabolites contained in the cauliflower leaves may be the most responsible components of the antibiofilm activity. It has been reported that the derivatives of kaempferol and quercetin derivatives have antibiofilm activity against many microorganisms including *S. mutans*, *E. coli*, and *S. aureus* (Slobodníková et al. 2016; J. Zhang et al. 2014). For instance, kaempferol shows its antibiofilm activity by blocking the gene expression of surface proteins of bacteria, or by destroying the surface proteins which reduces the adhesion to fibrinogen (Ming et al. 2017).

4.3. Antioxidant Activity

The total antioxidant activities of the leaf extracts were determined through ABTS and DPPH assays (calibration curves were presented in Figure A.2 and A.3 for ABTS and DPPH assays, respectively), and the results were tabulated in Table 4.8 below:

Table 4. 8 Antioxidant activities of the leaf extracts

Sample	**Antioxidant Activity**	
	DPPH Assay (mM AAE/mL)	**ABTS Assay (mM Trolox equivalent)**
Grape Leaf	36.3 ±0.01	12.1 ±0.006
Cauliflower Leaf	1.15 ±0.005	0.43 ±0.02

It is difficult and unreliable to express the antioxidant potential of a sample by referring to a single method. Therefore, comparing different assays may help for a better outcome. In this study, two different antioxidant activity assays were used. ABTS assay, which is also known as Trolox equivalent antioxidant capacity assay, measures the relative antioxidant activity by reacting with a strong oxidizing agent and ABTS salt (Prior 2015). Trolox, a water-soluble vitamin E analogue standard, is used to compare

and the expression of the results (Prior 2015). On the other hand, the DPPH assay is frequently used for the free radical scavenging abilities of natural compounds (Pavithra and Vadivukkarasi 2015). DPPH assay measures the antioxidant activity calorimetrically, based on the color difference because of electron transfer (Prior 2015).

4.3.1. Grape (*Vitis vinifera L.*) Leaves

The antioxidant activity of the grape leaf extract was 36.3 (mM AAE/mL) in the DPPH assay, and 12.1 (mM Trolox equivalent) in the ABTS assay (Table 4.8).

The tabulated results in Table 4.8 show that the antioxidant activity of grape leaf is higher in both tested assays. However, the antioxidant activity outcomes were different. A higher activity was detected by the DPPH assay, compared to the ABTS assay. The reason that grape leaf extract had higher antioxidant activity may be attributed to its higher total phenolic content.

Previously it has been stated that grape leaves have strong antioxidant activity (Aouey et al. 2016; Balík et al. 2009; Deliorman Orhan et al. 2009; Farhadi et al. 2016; Fernandes et al. 2013; Katalinic et al. 2013; Katalinić et al. 2009; Koşar et al. 2007; Selçuk et al. 2017; Moldovan et al. 2020; Pari and Suresh 2008). However, the antioxidant activity was in a wide range, because of many factors explained in different studies. The antioxidant potential of the grape leaf extracts from different *V. vinifera* leaves ranged from 61.39% to 92.68% (Farhadi et al. 2016). The findings assessed that antioxidant activity differed between species, and it has been suggested that there may be a relationship between total phenolic content and antioxidant activity (Farhadi et al. 2016). Different fractions of water-extracted grape leaves were examined according to their antioxidant potential, in terms of their percentage of DPPH inhibition (Deliorman Orhan et al. 2009). Ethyl acetate (EtOAc) fraction showed the highest DPPH inhibition with a value of 92.8%, followed by chloroform ($CHCl_3$), n-Butanol (n-BuOH), and water (R-H_2O) fractions (6.3%) (Deliorman Orhan et al. 2009). The phenolic content of the fractions was in the following order (EtOAc > $CHCl_3$ > n-BuOH > R-H_2O), thus the correlation between the antioxidant potential and total phenolic content has been supported (Deliorman Orhan et al. 2009).

Selçuk et al. (2017) also concluded that the antioxidant potential is associated with the phenolic content. Besides, it has been reported that the mature leaves showed the lowest antioxidant activity in terms of percentage inhibition (88.48%) since they have the lowest amount of phenolic content (Selçuk et al. 2017). Besides, the difference in the antioxidant potential of fresh, brined, and unbrined grape leaves was determined by Koşar et al. (2007). The ethanolic extracts were rich in hydroxycinnamic acids and quercetin derivatives (Koşar et al. 2007). According to the results, the extracts showed similar IC_{50} values on DPPH radicals (0.3 ±0.0 mg/mL), and the antioxidant activity was associated with hydroxycinnamic acids and quercetin derivatives (Koşar et al. 2007).

Moreover, the maturation stage of the leaves also has a significant impact on their antioxidant potential. In the study, where the antioxidant properties of the grape leaves were harvested at different times, it was concluded that the maturation state affected the antioxidant potential, as well as the total phenolic content (Katalinic et al. 2013; Katalinić et al. 2009). On the contrary, the antioxidant potential may be affected by microbial infections (Balík et al. 2009). Balik et al. (2009) assessed that mold-attacked leaves were richer in antioxidant potential and total phenolic content, compared to healthy leaves (Balík et al. 2009).

Additionally, the reducing power of the grape leaf extract was analyzed according to its antioxidant potential (Aouey et al. 2016). The presented results showed that the reducing power was associated with concentration (Aouey et al. 2016). Similarly, in another study where the antioxidant activity of the leaf extracts of the red varieties of *V. vinifera* cultivars was examined, the IC_{50} values were reported as 101–191 μg/mL (Fernandes et al. 2013). Besides, it has been proposed that the antioxidant activity is dose-dependent however, above the 500 μg/mL, of concentration, all the samples showed scavenging activity at about 80% (Fernandes et al. 2013). Moreover, the grape leaf extracts showed protection against alcohol-induced toxicity through their strong antioxidant activity (Pari and Suresh 2008). Therefore, grape leaf extracts may be used as a potential antioxidant source.

The antioxidant potential of the grape leaf extracts has been associated with the cultivars, the solvent used in the extraction process, the maturation state and harvest time, etc. Besides, the antioxidant potential is strongly correlated with the factors that influence the total phenolic content. Since the grape leaf extract had higher total phenolic content

(Table 4.2), its antioxidant potential was also higher compared to the cauliflower leaf extract.

4.3.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

The antioxidant activity of the cauliflower leaf extracts was detected as 1.15 (mM AAE/mL) and 0.43 (mM Trolox Equivalent) through the ABTS and DPPH assays respectively (Table 4.8). The activity was slightly lower than the grape leaf extract. However, a higher antioxidant activity outcome was detected through the DPPH assay.

The cauliflower leaf extracts were analyzed according to their antioxidant activity by Chiozzi et al. (2016). In the study, two different extraction procedures have been followed and the impact of the extraction procedure on the antioxidant activity has been observed. The first extraction procedure was increasing the solubility of the membrane and hydrophobic proteins, which have very little solubility by using an anionic detergent (SDS); whereas the second extraction procedure was aiming to reduce the oxidation damage and chelate metal ions by using EDTA (Zenezini Chiozzi et al. 2016). The results showed that the peptide derivatives showed limited antioxidant activity, compared to the EDTA extraction. The percentage antioxidant activity of the cauliflower leaves extracted by the EDTA-trypsin mixture was reported as 13.7% (Zenezini Chiozzi et al. 2016).

On the other hand, the relationship between antioxidant activity and glucosinolate potential was examined by Cabello-Hurtado et al. (2012). Among the assays used in the study, the activities of ABTS and DPPH assays were weaker than those of ORAC and SRSA assays (Cabello-Hurtado, Gicquel, and Esnault 2012). However, it has been stated that the antioxidant activity was associated with glucosinolate (glucobrassicin, glucoiberin, and gluconapin) content found in cauliflower leaves (Cabello-Hurtado, Gicquel, and Esnault 2012).

Compared to the grape leaf extracts, the antioxidant activity of the cauliflower leaf extract was relatively lower. However, considering the limited studies in the literature, it has been observed that cauliflower leaves have substantial antioxidant activity. The difference in the results obtained depends on the phenolic content of the

plant sample, the extraction method, and the assay used for the antioxidant potential. Therefore, the results may vary.

4.4. Cytotoxic Activity

The cytotoxicity may be defined as the ability of a chemical to kill other living cells as a result of physical/environmental conditions (e.g., exposure to high temperature, pressure, or radiation), chemical stimuli, or exposure to other cells (Çelik 2018). Minimum or no toxicity levels are being crucial to a better health effect (Çelik 2018). Thus, the determination of cytotoxic substances in the human body may be an important precursor for further studies in the evaluation of cellular injury or exposure dose (W. Li, Zhou, and Xu 2015). The biological effects of a substance on cell growth or reproduction, or morphological effects can be observed through the cytotoxicity tests. Besides, it has been pointed out that different cell types have their specific handling capacities to process chemicals (W. Li, Zhou, and Xu 2015). Therefore, understanding the specific mechanisms helps to aim at the specific cell in a determined dose (Çelik 2018).

The cytotoxic activity of the leaf extracts was determined *in vitro* by MTT (methyl thiazolyl tetrazolium) assay. A purple-colored crystalline formazan that is soluble in organic solvents, such as dimethyl sulfoxide, is produced in this experiment when the tetrazole ring interacts with the mitochondrial dehydrogenase in the cytochrome b and c sites of the living cells. By means of a positive correlation between the crystals formed and the number of cells and their activities, detecting the difference in the optical density at a certain wavelength gives the number of surviving cells and metabolic activity (W. Li, Zhou, and Xu 2015). Herein, the leaf extracts were applied to the L929 cell line in 5, 50, and 500 μg/mL working volumes, and their cytotoxic activities were measured at 24, 48, and 72 hours. Figure 4.11 below shows the cytotoxicity levels of both grape and cauliflower leaf extracts, tested in 5, 50, and 500 μg/mL at different times.

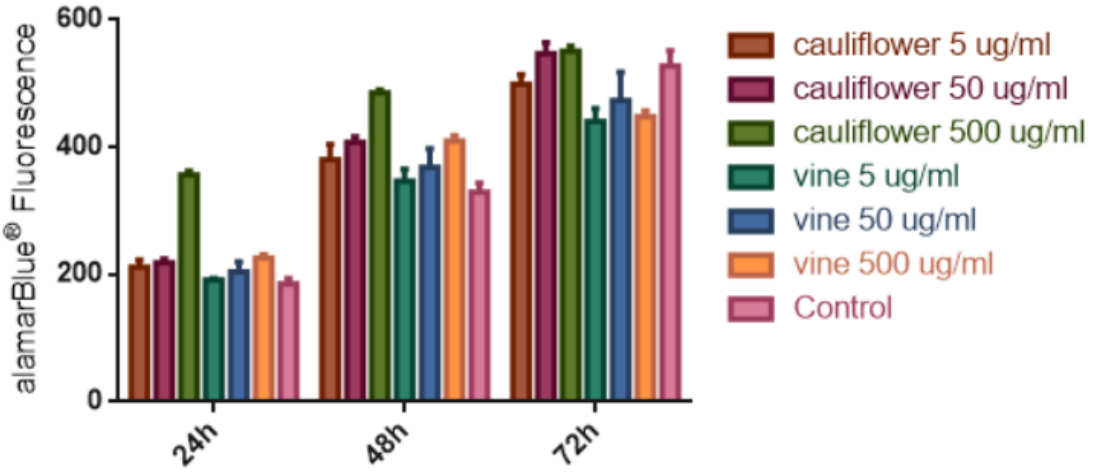


Figure 4. 12 The cytotoxicity levels of both grape and cauliflower leaf extracts, were tested in 5, 50, and 500 µg/mL at 24, 48, and 72 hours.

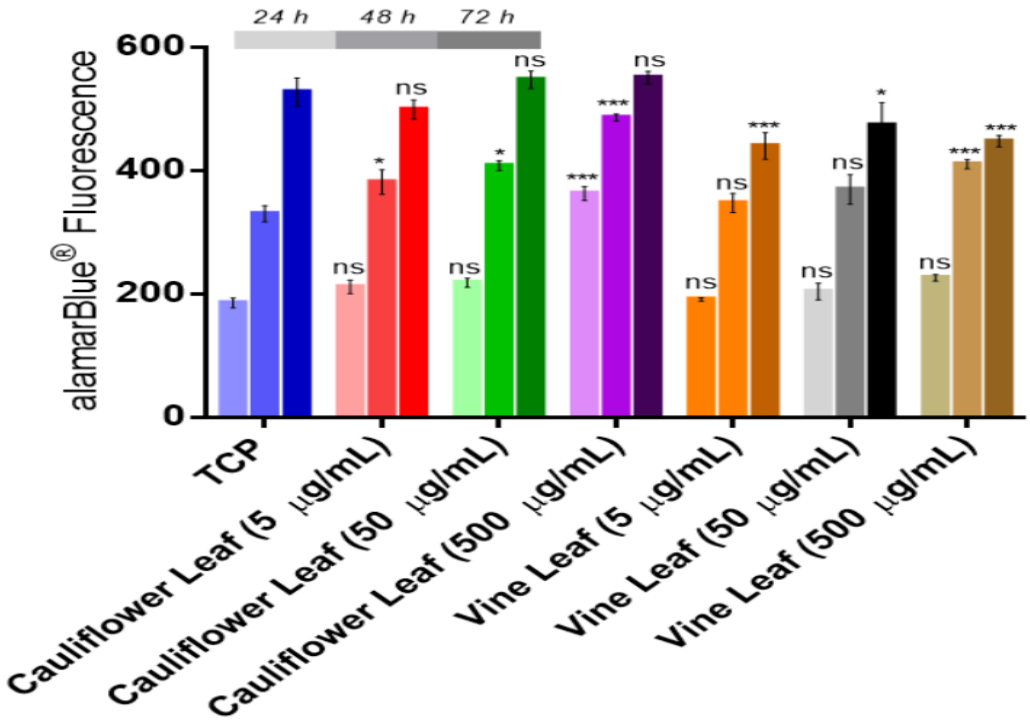


Figure 4. 13 The viability reductions of both grape and cauliflower leaf extracts (ns refers to non-significant)

4.4.1. Grape (*Vitis vinifera L.*) Leaves

The cytotoxic activity of the grape leaf extracts was tested on mouse fibroblasts L929 cell line, and the results were presented in Figures 4.11 and 4.12. According to the standard handled, if the reduction of viability compared to the blank is below 70%, there is a cytotoxic potential ((ISO 2009)). The viability reductions were higher than 70% in all working volumes at any time point (Figure 4.12). Therefore, the grape leaf extract may not be considered as a cytotoxic substance. Besides, the grape leaf extract caused an increment in metabolic activity of the cell line at 24 and 48 hours. However, the metabolic activity decreased in all working volumes at 72 hours.

The cytotoxic activity of the *V. vinifera* leaves was examined in different studies previously. Pintac et al. (2019) stated that the grape leaf extracts showed cell growth inhibition against different cancerous cells (HeLa, MCF7, and HT-29) in a dose-dependent manner (Pintać et al. 2019). The cytotoxic activity of the grape leaves was revealed by Handoussa et al. (2013). The ethanolic extracts were tested against the human osteosarcoma cell line (U2OS), human leukemia cell line (HL60), human melanoma cancerous cells, and human cell lines (LuPiCi 1936, CaCi 1962, SK-MEL28, and LiGh 1927B) and the results showed that the *V. vinifera* leaf extracts showed promising cytotoxic activity (Handoussa et al. 2013). The IC_{50} values were ranging between 8.6 and 138.6 µg/mL (Handoussa et al. 2013).

In another study examining the cytotoxic effect of different *V. vinifera* species, it was stated that the cytotoxic activity of grape leaves was limited to moderate (Esfahanian et al. 2013). Methanol-extracted grape leaves were applied in different concentrations (62.5, 125, 250, 500, 750, and 1000 µg/mL) to the human breast cancer cell line (MDA-MB-231 cells). The IC_{50} values were greater than 500 µg/mL (Esfahanian et al. 2013). Besides, since the old leaves contain more phenolic content, they showed higher cytotoxic activity compared to young leaves (Esfahanian et al. 2013). The cytotoxic activity of the two different varieties of grape leaves from arid and temperate regions in Palestine was examined (Abed et al. 2015). The cytotoxicity of the leaf extracts was ranging from 85 to 165 in terms of IC_{50} (µg/ml), and it has been stated that the leaves showed a dose-dependent inhibition against lung cancer cells (Abed et al. 2015). The leaves were containing the derivatives of myricetin, quercetin, isorhamnetin, and kaempferol, which

were quite effective inhibitors against the proliferation in murine colonocytes of HT-29 cells (Abed et al. 2015; Wenzel et al. 2000). Besides, the cytotoxic activities of quercetin and myricetin against melanoma cell lines (B16F10 cells) were indicated (Yáñez et al. 2004).

On the other hand, Moldovan et al. (2020) stated that the alcoholic and water extracts of *V. vinifera* leaves showed relatively low toxicity against cultured cells compared to the cancerous cells (Moldovan et al. 2020). The low cytotoxic activity is associated with the dose. It has been pointed pot that the *V. vinifera* tendrils extract is not toxic for humans at a dose of up to 100 mg/mL (Fraternale et al. 2016). Thus, the grape leaf extracts have promising cytotoxic activity in a dose-dependent manner. The activity is associated with the presence of hydroxy groups (Agullo et al. 1997).

Comparing the data obtained from the analysis and the literature, the grape leaf is not a cytotoxic substance. Besides, the promising cytotoxic activity of the grape leaves was supported by the data provided by previous studies. Besides, it is possible to mention a dose-dependent activity, as has been revealed by different studies before. However, the outcomes on the cytotoxicity of grape leaves may vary, as each cell has a different processing capacity.

4.4.2. Cauliflower (*Brassica oleceae var. botrytis*) Leaves

The cauliflower leaf extract was examined for cytotoxic activity on the L929 cell line. The presented results in Figures 4.11 and 4.12 shows that no cytotoxic activity was observed at any of the applied doses and durations tested. At 24 and 48 hours, it was observed that the cauliflower leaf extract increased the metabolic activity of the L929 cell line. At 72 hours, a slight decrease in the metabolic activity treated with 50 µg/mL cauliflower leaf extract was detected. However, it was no more than 16% thus, indicating a non-toxic behavior. No other reduction in metabolic activity was observed in other working volumes at that duration.

The cytotoxic activity of the soluble proteins extracted from cauliflower leaves was also determined by Xu et al. (2017). The leaf extracts were applied to human HepG2 cells in different concentrations from 1 to 500 µg/mL. The presented results showed that

cell viability did not change significantly in that concentration range (Xu et al. 2017). In another study, lignin extracts from cauliflower waste products including leaves were examined according to their cytotoxic activity against MG-63 bone cancer cell lines (Majumdar et al. 2021). The results showed that the extracts showed a significant difference in cell viability in a dose-dependent manner. It has been reported that the lignin fractions did not show a cytotoxic effect at a dose of up to 50 μg/mL (Majumdar et al. 2021). Therefore, cauliflower leaves may have the potential as an anti-cancerogenic agent.

The cauliflower leaf samples did not show cytotoxic activity in the applied doses of 5-500 μg/mL. Besides, it is possible to mention a dose-dependent activity. The cell viability reduction was associated with the applied dose. Additionally, the viability reduction of the cauliflower leaf extract was slightly higher than that of the grape leaf extracts. It may be explained by the relationship between the hydroxyl groups and cytotoxic activity (Agullo et al. 1997). Since the grape leaf samples have a higher total phenolic content (Table 4.2), this slight difference in the cytotoxic activity is not a surprise.

4.5. Color and pH Evaluation

Color and pH evaluation of the leaf extracts were presented in Table 4.9.

Table 4. 9 Color parameters and pH values of the leaf extracts (± Standard deviation)

Sample	**Color Parameters**			**pH**
	L*	**a***	**b***	
Grape Leaf	23.537 ± 0.505	-2.57 ± 0.026	4.293 ± 0.161	4.20
Cauliflower Leaf	18.43 ± 0.036	-0.933 ± 0.040	5.45 ± 0.06	5.65

The color evaluations of the leaf extracts were determined by L*, a*, b* values. The colors of the leaf extracts seemed close to each other. However, compared to the L* (lightness) values, it has been observed that the grape leaf extract was slightly lighter than the cauliflower leaf extract (Table 4.9). Besides, as illustrated in Figure 4.13, below, a* (red/green) value shows a significant difference among the leaf extracts. The color of the grape leaf extract was slightly greener compared to the cauliflower leaf extract. According to the b* (yellow/blue) values, cauliflower leaf extract has a yellowish color compared to the grape leaf extract.

On the other hand, the acidity of the grape leaf extract was slightly higher than the cauliflower leaf extract.

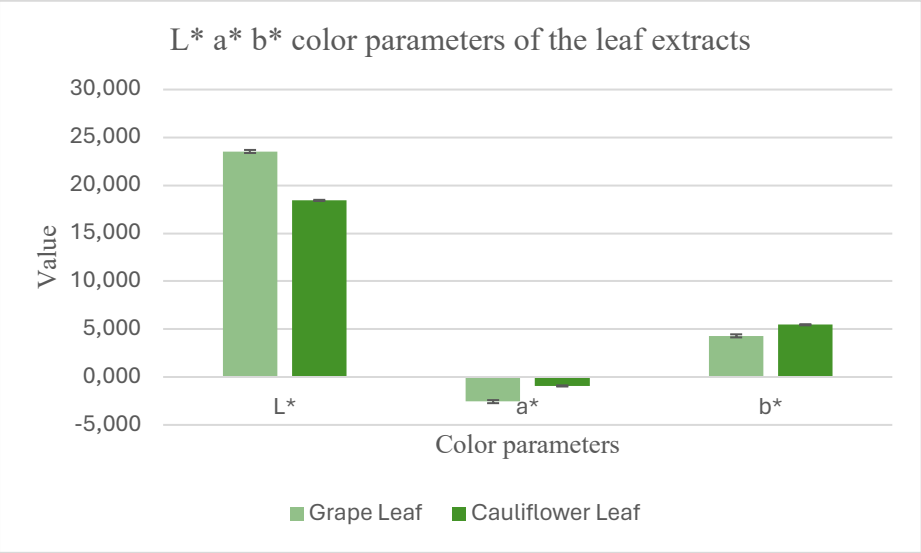


Figure 4. 14 L* a* b* color parameters of the leaf extracts.

CHAPTER 5

CONCLUSIONS

Plants contain some bioactive substances as well as nutrients. For this reason, they are used in some developing societies for the treatment of diseases as well as for nutrition, or as acute remedies. On the other hand, due to the increasing world population, agricultural lands are decreasing, and it is difficult for people to reach natural resources. Meanwhile, studies on obtaining natural substances with approaches that do not require new sources are increasing.

Secondary metabolites synthesized by plants are classified as terpenes, phenolics, alkaloids, and sulfur-containing compounds. These compounds are not directly involved in basic metabolic functions such as growth, development, and reproduction, but play critical roles in interactions with the environment, including defense against pathogens, herbivores, and environmental stressors.

On the other hand, many plant secondary metabolites provide a natural defense mechanism to the plant against pathogens because of the phenyl ring in their structure. In this way, they provide some natural antimicrobial properties to the plant. Besides, phenolic compounds are able to reduce unstable free radicals. Thus, they contribute to preventing cellular damage and oxidative stress. Moreover, they show a natural antioxidant effect by preventing the formation of off-flavors in foods. Among all these, some secondary metabolites such as phenols are important for both plant defense and medicinal applications, as they cause cell death. They have cytotoxic effects by their ability to inhibit the division and growth of cancer cells.

Based on all these, grape and cauliflower leaves, which are classified as waste, were used in this study. Leaf samples were extracted with pure water, an environmentally friendly, low-cost, and accessible solvent, in accordance with the principles of green extraction. The obtained plant leaf extracts were characterized in terms of their chemical content. Grape leaf and cauliflower leaf extracts were reported to contain 17.35183 and 13.43953 mg/GAE/L total phenolic substances, respectively. Additionally, leaf extracts were subjected to library scanning with a qualitative tandem liquid chromatography

quadrupole time of flight mass spectrometry (LC-Q-TOF-MS) system for more identified content analysis. According to the findings, phenolic substances such as rutin, kaempferol, quercetin and organic acids such as caffeic and quinic acid were detected in the vine leaves. The substances detected in the cauliflower leaf are kaempferol-7-O-glucoside and some organic acids such as malic acid and quinic acid. Then, the antioxidant effect of leaf extracts was determined by using DPPH and ABTS assays. According to the presented results, both grape and cauliflower leaf extracts had promising antioxidant activity. Interestingly, slightly higher antioxidant activity was observed in grape leaf extract, which had a higher phenolic content compared to the cauliflower leaf extract.

In the continuation of the study, the antimicrobial activities of both leaf extracts and their effects on biofilm formation were tested on various Gram-positive and Gram-negative bacteria and fungi species. According to the presented results, it was observed that both extracts contributed to the antimicrobial effect. Besides, the antimicrobial activity of Gram-positive bacteria was greater than that of Gram-negative bacteria. Moreover, just like the antioxidant activity, it was observed that the antimicrobial effect of grape leaf, which has a relatively higher phenolic content, was also higher and the indicated antimicrobial effect was dose-dependent. In parallel, it has been observed that plant extracts contribute to reducing biofilm formation. However, the dose-dependent activity observed in the antimicrobial effect has not been observed during the anti-biofilm effect. The antimicrobial activity of the leaf extracts is not a surprise. Plant secondary metabolites contribute to antimicrobial activity in several ways including enzyme inhibition, disruption of cell membranes, and quorum-sensing inhibition. However, is important to highlight that the antimicrobial mechanism of action of the phenolic compounds may vary depending on the specific type of metabolite, and the target microorganism.

In the proceeding parts of the study, the leaf extracts were examined through their cytotoxic activities. Here, it is known that plant secondary metabolites contribute to the cytotoxic effect in many ways, such as induction of apoptosis, alteration of DNA/RNA, enzyme inactivation, and alteration of cellular cycles. The findings indicate that none of the samples studied showed any deleterious effects on cells at any time point. Notably, after 24 and 48 hours, both grape and cauliflower leaf extracts exhibited an increase in the metabolic activity of L929 cells. Once the 72-hour mark was reached, a decrease in metabolic activity was observed in grape leaf extracts at all concentrations. In contrast,

only 50 µg/mL concentration of cauliflower leaf extract showed a small decrease in activity (no more than 16% indicating non-toxic behavior), while other concentrations showed no such effect.

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APPENDIX A

CALIBRATION CURVES

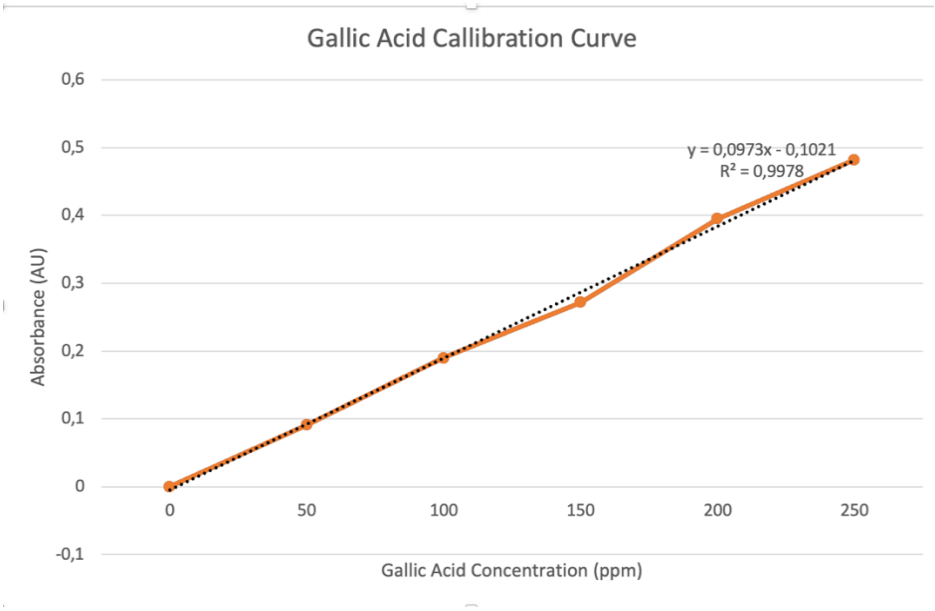

Figure A. 1 Calibration curve of gallic acid for determination of total phenolic content through Folin Ciocalteu assay.

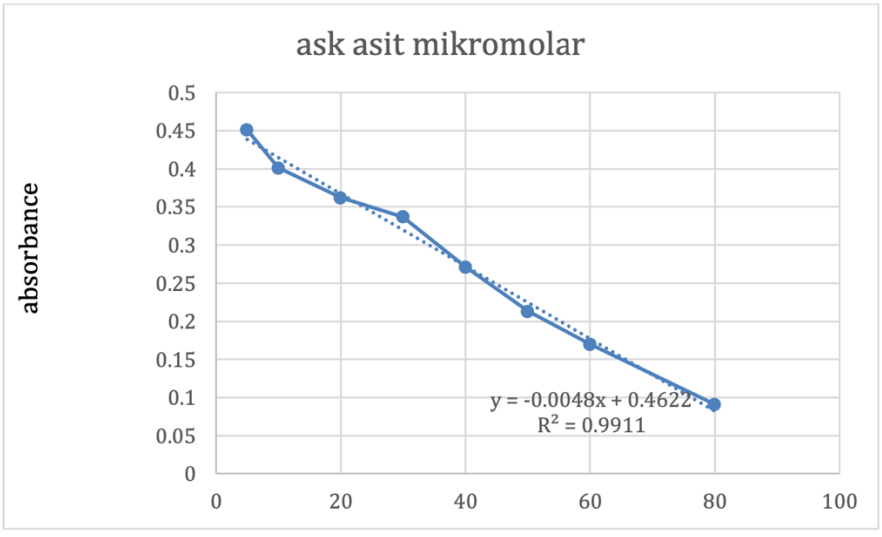

Figure A. 2 Calibration curve of ascorbic acid for the determination of the antioxidant activity through ABTS assay.

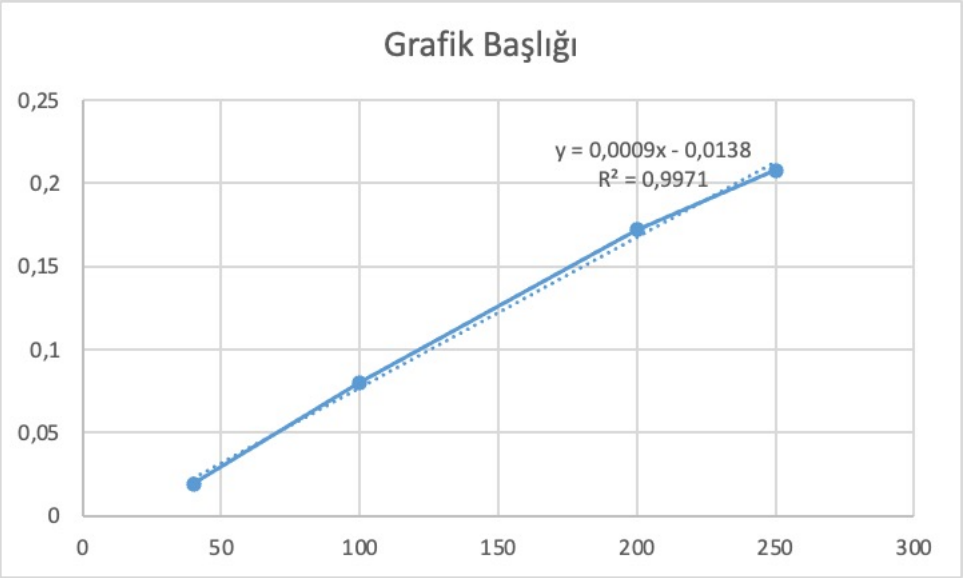

Figure A. 3 Calibration curve of gallic acid for the determination of the antioxidant activity through DPPH assay.