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COMPOSITION ANALYSIS OF GARLIC EXTRACT AND EVALUATION OF THE ANTIMICROBIAL, ANTIOXIDANT, DNA PROTECTIVE AND PHOTOLUMINESCENCE PROPERTIES

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ANAS ALTOUTANJI

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Supervisor

Prof. Dr. Hüseyin ZENGİN

by

Anas ALTOUTANJI

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Submitted by **Anas ALTOUTANJI** in partial fulfillment of the requirements for the degree of Master of Science in **Biochemistry Science and Technology, Gaziantep University** is approved by,

Prof. Dr. Çiğdem AYKAÇ
Director of the Graduate School of Natural and Applied Sciences …….....

Prof. Dr. Abuzer ÇELEKLİ
Head of the Department of Biochemistry Science and Technology …….....

Prof. Dr. Hüseyin ZENGİN
Supervisor, Chemistry
Gaziantep University …….....

Exam Date: 03 September 2024

Examining Committee Members:

Prof. Dr. Hüseyin ZENGİN
Gaziantep University …….....

Prof. Dr. İbrahim Halil KILIÇ
Gaziantep University …….....

Prof. Dr. Mehmet ASLANOĞLU
Harran University …….....

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Anas ALTOUTANJI

ABSTRACT

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ALTOUTANJI, Anas
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Many cultures globally have documented the medicinal and spicy properties of garlic (Allium sativum L.). Where recently Ajoene has been considered one of the main bioactive compounds present in garlic extracts, having extremely viable antifungal, antibacterial, antiviral, and antiparasitic properties. This study focused on the ethanol extract of Ajoene derived from raw garlic, and its prospective application in addressing bacterial infections resistant to antibiotic treatments. Emphasis was placed on evaluating the antioxidant properties. The antioxidant capabilities were scrutinized *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenger assay, while antibacterial activities were assessed through the disc diffusion method. Additionally, spectroscopic analyses were performed on the Ajoene extract to assess its photoluminescence properties, and investigations into potential DNA protective activities were also investigated.

Key Words: Garlic Extract, Ajeone, Antibacterial Activity, Antioxidant Activity, DNA Protective Activity, Photoluminescence.

ÖZET

SARIMSAK ÖZETİNİN BİLEŞİM ANALİZİ VE ANTİMİKROBİYAL, ANTİOKSİDAN, DNA KORUYUCU VE FOTOLÜMİNESANS ÖZELLİKLERİNİN DEĞERLENDİRİLMESİ

ALTOUTANJI, Anas
Yüksek Lisans Tezi, Biyokimya Bilimi ve Teknolojisi
Danışman: Prof. Dr. Hüseyin ZENGİN
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Dünya çapında pek çok kültür sarımsağın (Allium sativum L.) tıbbi ve baharatlı özelliklerini belgelemiştir. Son zamanlarda Ajoene, sarımsak ekstraktlarında bulunan ve son derece etkili antifungal, antibakteriyel, antiviral ve antiparazitik özelliklere sahip ana biyoaktif bileşiklerden biri olarak kabul edilmiştir. Bu çalışma, çiğ sarımsaktan elde edilen Ajoene'nin etanol ekstraktına ve bunun antibiyotik tedavilerine dirençli bakteriyel enfeksiyonlara yönelik potansiyel uygulamasına odaklandı. Antioksidan özelliklerin değerlendirilmesine vurgu yapıldı. Antioksidan özellikler, 2,2-difenil-1-pikrilhidrazil (DPPH) temizleyici deneyi kullanılarak *in vitro* incelenirken, antibakteriyel aktiviteler disk difüzyon yöntemiyle değerlendirildi. Ek olarak, fotolüminesans özelliklerini değerlendirmek için Ajoene ekstraktı üzerinde spektroskopik analizler yapıldı ve potansiyel DNA koruyucu aktivitelerine ilişkin araştırmalar da araştırıldı.

Anahtar Kelimeler: Sarımsak Ekstraktı, Ajeone, Antibakteriyel Aktivite, Antioksidan Aktivite, DNA Koruyucu Aktivite, Fotolüminesans.

"Dedicated to my family"

My mother Hifaa, my father Shukri, my dear Wife Alaa and our precious daughter Malak.

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LIST OF SYMBOLS

α	Alfa
β	Beta
γ	Gamma
π	Pi
λ	Wavelength
ε	Molar absorptivity coefficient
ϕ_f	Quantum yield
τ_f	excited-state lifetime

LIST OFABBREVIATIONS

DNA	Deoxyribonucleic acid
UV-Vis	Ultraviolet Visible Spectrophotometer
PL	Photoluminescence Spectrophotometry
FT-IR	Fourier Transform Infrared
GC-MS	Gas Chromatography – Mass Spectroscopy
cm	Centimeter
mm	Millimeter
ml	Milliliter
g	Gram
mg	Milligram
µg	Microgram
KJ	Kilo joule
Kcal	Kilo calorie
DPPH	2,2-diphenyl-1-picrilhydrazyl
H_2O_2	Hydrogen peroxide
Fe^{+2}	Ferrous ion
AGE	Aged garlic extract
OSCs	Organosulfur compounds
H2S	Hydrogen sulfide
FGE	Fermented garlic extract
ACE	Angiotensin-converting enzyme
Na^+	Sodium ion
K^+	Potassium ion
Ca^{2+}	Calcium ion
pH	Potential of hydrogen

CHAPTER 1
INTRODUCTION

1.1 Motivation of Study

Garlic (*Allium sativum L.*) and many of its extracts (including Ajoene extract) have been used for their properties and therapeutic effects for many thousands of years, recent research and studies for its mechanism of action indicated that garlic has wide spectrum of properties; not limited to the antimicrobial activity (including the antibacterial, antifungal, antiviral, and antiprotozoal activities), but has also a considerable actions on the cardiovascular, immune, and digestive systems.

This research study has prioritized Turkish garlic among other species because it is considered one of the higher vegetables rich in protein contents, (even among the garlic's other species as will be described later), where the motivation of this study aims to:

1- Identify the composition of Turkish garlic in comparison with other available sample species of garlic.
2- Review the beneficial utilization of Turkish garlic and its Ajoene extract to provide a contribution for any additional studies of its antimicrobial properties, by investigating its antibacterial activity against the resistant strain to continue the efforts of Prof. Dr. Thomas Wirth as he indicated that "it is believed that Ajoene can be used as a novel drug in fighting antibiotic resistance." By examining the antimicrobial activity of Ajoene against bacterial-resistant strains such as *Stenotrophomonas Maltophilia*.
3- Exploring the other potential beneficial effects of the Ajoene extract including the DNA protective activity on the Bacteria's DNA (plasmid).
4- Measuring its antioxidant, photoluminescence, and UV preventative activities.

1.2 The Classification of Garlic

The classification of garlic is given in Table 1.1 which is indicate the Kingdom, Clade, Order, Family, Genus that garlic is belonging to with other important sub classification.

Table 1.1 The classification of garlic adapted from [1]

Classification of Kingdom	*Plantae*
Classification of Clade	*Tracheophytes*
Classification of Sub-Clade 1	*Angiosperms*
Classification of Sub-Clade 2	*Monocots*
Classification of Order	*Asparagales*
Classification of Family	*Amaryllidaceae*
Classification of Subfamily	*Allioideae*
Classification of Genus	*Allium*

1.3 Botany of Garlic

Garlic is a long-living plant, related to the *Amaryllidaceae* (amaryllis family), it is one of the species of the bulb-shaped blooming plant under the genus *Allium*. Within sight of the same genus (*Allium*), the relatives of garlic are the leek, chive, onion, shallot [2], welsh, and Chinese onion [3]. The plants of garlic normally raise in tall up to 2 feet (60 cm) [4]. The leaf looks plane, linear, rigid, and between 1.25–2.5 cm in width, with a sharp head. regarding the variety, usually, the short, stiff stem above the bulb is where the lengthy leaves come from, or raise from the pseudo-stem of leaf sheaths that interlock, cloves which are edible bulblets, are 10 up to 20 enclosed within the membranous skin of the bulb, which are asymmetric in shape, with an exception of those closest to the center [4].

The bulb generally is odorous and incorporates layers, an internal sheath that envelopes the clove, and outer layers of skinny sheathing leave encircling it [4].
The following Figure 1.1 is showing the garlic's clove within the garlic and alone without being peeled.

Figure 1.1 Garlic cloves [5]

The following Figure 1.2 shows the garlic flower head in the garlic plant in the blooming season.

Figure 1.2 Garlic flower head [6]

The blooming of the green-white or pinkish flowers is the initiation for the bracts that were initially covering the spheral flower cluster to split open. These pink or purple blooms appear in the middle to end of summer (from July to September) [3].

Occasionally small bulbils (secondary bulbs that grow in place of blooms) and sterile blossoms appear on flower stems [3]. Cloves or top bulbils can be planted to propagate garlic, which is typically grown as an annual crop [3]. Garlic seeds may also be utilized. Where Garlic may be cultivated in mild temperatures and is simple to grow [3]. Garlic has types (subspecies), the most common types are the hard-neck and soft-neck varieties.

1.4 Varieties

Central Asia is the primary source of the biodiversity of garlic cultivars with more than a hundred and twenty cultivars [3]. The hard-neck and soft-neck varieties are the most common varieties of garlic [3]. Within these two groups, ten primary genetic kinds or types exist [3]. Climate can tremendously affect flavor and the range of production [3].

1.4.1 Hard-neck varieties (*Allium sativum var ophioscorodon*)

Hard-neck cultivars, also known as bolting or topsetting varieties, generate a flower stalk (scape) [3]. Most of the time, the created flowers die and turn into bulbils [3]. These little aerial cloves share the same genetic makeup as the parent plant [3]. A bulb will develop if the aerial cloves are utilized for reproduction, but it will be small and take two to three years for the bulb to grow to a size that can be sold [3]. Rigid necks have hard flower stalks that prevent braiding and storage difficulties are frequently encountered as their roots and cloves dry out slowly, usually taking several months [3].

"Rocambole," "Purple Stripe," and "Porcelain" are examples of similar hard-neck kinds [3]. There are also "Asiatic," "Creole," and "Turban" varieties [3]. Hardneck cultivars can be just as productive as soft-neck varieties in relatively low temperatures and cold locations [3].

1.4.2 Soft-neck varieties (*Allium sativum var sativum*)

Soft-neck types typically don't develop a floral stalk [3]. Due to their little flower stalk and bulbil proliferation [3]. Usually make the soft-neck type more prolific because all of their energy is directed toward growing a bulb instead of the scapes that are produced by hard necks [3]. These cultivars are the most widely utilized for commercial cultivation [3]. Some soft necks can generate a small portion of a flower stalk, and bulbils will develop immediately on top of the bulb [3].

Soft-neck types often last up to six to eight weeks in storage and have a more extended time of use than hard-neck kinds [3]. Soft necks have ten to forty cloves per plant and are similarly simple to braid [3]. "Artichoke" and "Silverskin" are kinds with soft necks [3]. The Figure 1.3 presents the two main varieties of garlic, the soft-neck and the hard-neck types of garlic.

Figure 1.3 Two main types of *Allium sativum*, (garlic) [7]

1.5 Origin of Garlic

The plant has been grown in England since 1540 and is believed to be native to South and Central Asia, Siberia, and the Himalayan region to the west [8]. Earlier than that, Garlic is thought to have been introduced to China by the Sumerians, from whence it eventually migrated to Korea and Japan [9]. The Sumerians were actively using garlic's medicinal properties from 2600 to 2100 BC [9]. The spread of garlic most likely started in the old world and continued in the new. Nevertheless, some historians continue to assert that China is where garlic first appeared [9].

Since 2700 BC, garlic has been one of the most utilized cures in the old history of China [10]. Then, because of its warming and energizing properties, it was placed in yang (the idea of Yin and Yang, which states that in the bad there is good, and in the good there is bad) [10]. The use of garlic has been recommended for those with depression [10]. Garlic has not been incorporated into Japanese Buddhist tradition because of these stimulating effects [10]. Garlic is also not a favorite ingredient in Japanese cuisine [10].

In spite of the fact that people who had eaten garlic were banished admittance into the sanctuaries and were alluded to as "rank roses", the Ancient Greeks also valued it [11]. Garlic bulbs ranging from 1850–1400 BC were found during the archaeological exploration on the Greek island of Crete at the Knossos Palace [11]. Before major wars, early Greek army commanders fed their armies garlic [11]. Garlic was a highly valued treatment during the middle Ages, in the Arabic medical school [11]. Arabic physicians made a significant contribution to the growth of the use of garlic as a medicine during the Middle centuries [11]. Retrograde Western Europe at the same time was ignorant and unaware of garlic [11]. Due to the sterility of numerous garlic cultivars, which prevents cross-testing with wild relatives, it is challenging to identify the common garlic's wild parent [11]. Garlic intently looks like the *Allium longicuspis* species, grown successfully in the southwestern and mid-Asia regions [11]. It has been noted that *Allium sativum* is unlikely derived from its ancestor *Allium longicuspis*, as it is primarily a sterile plant [11].

1.6 Composition of Garlic

The long chains of carbohydrates, saponin-containing compounds, phenol-containing compounds, and organic sulfur-containing compounds are a variety of biologically active substances that exist in *Allium sativum* [12]. The main content of the garlic has been listed in Table 1-2 indicating the amounts of each main group of biological compounds for the garlic plant in units of grams.

Table 1.2 The composition of 100 g raw garlic adapted from [13]

Contents	**Amount (Units of Grams)**
Carbohydrates	33.06
Protein	6.36
Dietary fiber	2.1
Fat	0.5
Contents	**Amount (Units of Kilo Joule)**
Energy	623
Contents	**Amount (Percentage of total)**
Water (H_2O)	59%

Although the moisture content detection was lower in Turkish garlic, it was also reported to have a protein level that was markedly larger than that of other vegetables [13]. The Table 1-3 contains the main vitamins that are present in the garlic plant with their indicated amounts in units of grams.

Table 1.3 The main vitamins existing in 100 g raw garlic adapted from [14]

Vitamin	**Amount (Units of Milligrams)**
(B1) Thiamine	0.2
(B2) Riboflavin	0.11
(B3) Niacin	0.7
(B5) Pantothenic	0.595
(B6) Vitamin	1.23
(B9) Folate	0.003
Ascorbic acid	31.2
Choline	23.2

The main minerals found in the garlic plant are listed in Table 1-4, and their amounts are given in grams.

Table 1.4 The main minerals existing in 100 g raw garlic adapted from [14]

Mineral	**Amount (Units of Milligrams)**
Potassium (K)	401
Calcium (Ca)	181
Phosphorus (P)	153
Magnesium (Ma)	25
Sodium (Na)	17
Iron (Fe)	1.7
Manganese (Mn)	1.67
Zinc (Zn)	1.16

In addition, Selenium and Germanium are additionally tracked down in garlic [14]. However, their sums rely upon the items in the minerals present in the dirt [14]. The composition of different garlic from different countries shows difference in the contents of minerals as compered in Table 1-5 [15].

Table 1-5 The environment influences over garlic's mineral content in mg/Kg adapted from [15]

Minerals	**Turkish garlic**	**Tunisian Garlic**	**Indian Garlic**
Fe	52.91	5.90	0.39
Cu	9.12	1.61	0.3
Ma	1056	25	0.77
P	6009	140	4600

Turkish garlic contained 22 different fatty acids, with 49.3 percent of lauric acid, and 20.4 percent of linoleic acid being the most abundant [15]. The hydro-distilled oil that was produced had a yield of 0.14 percent [15]. The total phenol content was 43.6 g, while the total flavonoid content was 13.2 mg [15].

An enzyme known as Alliinase and the amino acid known as Alliin are fresh garlic's most active components [16]. Allicin, which is accountable for the strong smell of garlic, is formed when these compounds combine when a clove of garlic is chewed, chopped, bruised, or cut [16]. In a matter of hours, Allicin disintegrates into various

sulfur compounds [16]. Where at least 17 amino acids, 33 sulfur-containing compounds, and many enzymes are present in *Allium sativum* [16].

The primary active ingredients of *Allium sativum* include organic-sulfur-containing compounds including *E/Z*-Ajoene, SAC, and Alliin, as well as Allicin [16].
Figure 1.4 shows examples of all the compounds discussed above as well as additional ones.

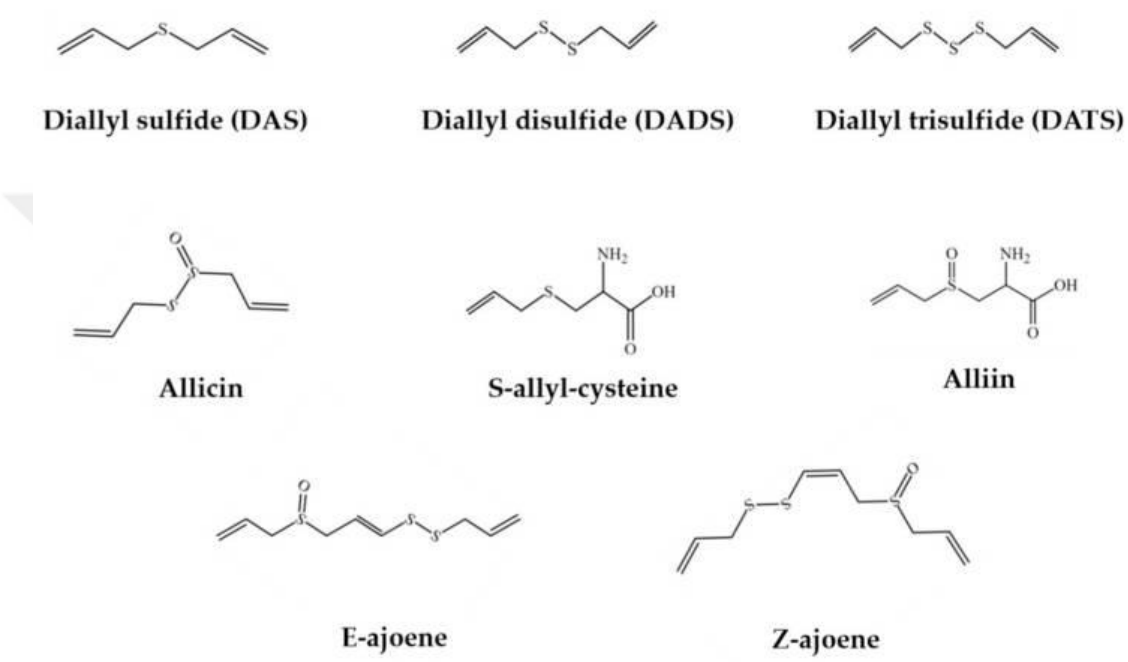


Figure 1.4 Chemical composition of major organosulfur compounds in garlic [17]

The saponin compounds discovered uniquely in garlic (purple) include proto-desgalactotigonin, voghieroside D1, sativoside B1-rhamnose, and sativoside R1 [18]. When compared to garlic (white), the overall quantity of saponins detected in garlic (purple) was about forty times greater [18]. Additionally, garlic has higher concentrations of more than twenty phenolic compounds than several important vegetable crops [18].

β-resorcylic acid was the predominant phenolic component, trailed by gallic acid, protocatechuic acid, then quercetin [19]. Additionally, it was reported that eighty-five percent of fructose, fourteen percent of glucose, and one percent of galactose were shown to be present in garlic polysaccharides [20].

1.7 Properties of Garlic

Allicin, diallyl polysulfides, S-allylcysteine, Ajoene, and vinyldithiins are sulfur-containing compounds that are produced in raw or smashed garlic. Along with non-sulfur-containing substances like enzymes, flavonoids, and saponins [21]. Whenever the cells of the plant are destroyed, the phytochemicals that give garlic its pungent flavor are created [21]. So when a cell's wall is damaged by cutting or crushing, the cell vacuoles which are containing enzymes will be free in the cytosol which results in the breakdown of several sulfur-containing compounds [21]. The resulting chemicals are what give the garlic its pungent or spicy flavor and potent aroma [22]. As some of the compounds are reactive, they keep interacting longer [22].

Garlic is significantly more effective than onion, shallot, or leeks amongst *Alliums* because it has by far the highest quantities of preliminary reaction compounds [22]. These substances are thought to be evolved as a defense technique to prevent bugs, worms, and bird species from damaging the plant [23]. Garlic's flavor and odor are principally produced by sulfur substances [23]. The chemical ingredient most closely linked to the "hot" sensation of raw garlic has been identified as Allicin [23]. This substance activates the thermo-transient receptor potential channels that give food its burning sensation of heat [23]. Garlic's pungency is reduced by cooking because Allicin is eliminated [23]. The distinctive garlic smell is primarily caused by Allicin and its derivative products: DAD and DAT along with Ajoene and Vinyldithiins which are considered as other Allicin-derived compounds [2]. Due to the powerful odor that garlic is frequently produced it is called the "stinking rose" [2]. Even the next day, garlic breath and sweat may be strongly recognizable if have been eaten in large quantities [2]. Because of the formation of allyl methyl sulfide (sulfur compounds) resulted of have a strong odor in garlic, which cannot be broken down further [2]. So, the allyl methyl sulfide (AMS) enters the bloodstream, and it travels to the lungs and skin before being excreted [2]. Consuming garlic may have a long-lasting effect because the digestion process of the garlic takes many hours and the release process of AMS from the human body takes many more hours as well [2]. Garlic's abundance of sulfur compounds is also what causes it to turn green or blue when picked and cooked [2].

CHAPTER 2
LITERATURE REVIEW

2.1 Previous Research on Garlic Activities

As one of the oldest agricultural harvests, garlic is still used as food and medicine all over the globe, its history dates back to 800 BC [24]. *Allium sativum* is the most common garlic species, which has been extensively studied for its antifungal, antiprotozoal, antibacterial, antiviral, and anticancer activities [25]. In addition to being acknowledged as a therapeutic treatment, garlic has also been shown to have significant medicinal value [25]. Garlic has been utilized as food and medicine by ancient Indians, Chinese, Egyptians, Greeks, and Romans for many thousands of years [25]. The middle ages witnessed one of the most widely recognized uses for garlic since it was considered to be very beneficial against the plague [25]. Louis Pasteur first formally investigated and documented the antibacterial effects of garlic in 1858 [25]. Garlic was utilized as a therapy for battlefield wounds during both World Wars before antibiotics were frequently used [25].

2.1.1 Antioxidant Activity

The dynamic compounds in garlic, like phenols and saponins, have several oxidation-prevention properties [26]. The oxidation-prevention properties of garlic were likewise influenced by various handling techniques [26]. Typically, cooked garlic has less antioxidant activities than raw garlic [26]. Garlic has potent antioxidant effects, this was revealed by much previous research, where scientists assessed raw and cooked garlic's antioxidant properties [26]. They discovered that more antioxidant properties are possessed by raw garlic using the β-carotene method, it was also reported that stir-fried garlic possessed more antioxidant properties [26]. According to the results of an additional study, ethanolic concentrate made from garlic sprouts showed more grounded cell support practices than ethanolic concentrate made from unrefined garlic [27]. Additionally, scavenging with H_2O_2

and other chemicals showed that fresh garlic has less antioxidant features than aged garlic [28].

The antioxidant capability of single-clove garlic extract was higher than that of multi-clove garlic extract, it also has more concentration of phenolic compounds [29].

2.1.2 Anti-Inflammatory Activity

The anti-inflammatory activities are considerable activities that garlic and its bioactive extracts are demonstrated to possess. In research, the garlic extract (ethyl linoleate) is down-regulating the cyclooxygenase-2 (COX2) expression and inducible NO synthase (iNOS) in lipopolysaccharide-stimulated RAW 264.7 macrophages by decreasing the synthesis of prostaglandin *E*-2 and nitric oxide (NO) [30]. Additional research paper indicated that the garlic 14-kDa protein inhibits the transcription factor nuclear factor-*kappa B* (NF-κB) signaling pathway, by inhibiting inflammatory mediators such as NO, TNF-α, and interleukin (IL)-1β, this was shown in the lipopolysaccharide-stimulated J774A.1 macrophages [31]. Furthermore, AGE decreased inflammation in animals lacking the protein apolipoprotein *E* [32]. The result of an increase in the performance of Adenosine Monophosphate-activated Protein Kinase (AMPK) in the liver, and lower levels of Tumor Necrosis Factor (TNF-α) and IL-1 receptor-associated kinase 4 was due to the AGE treatment [32]. Additionally, by lowering resistance, taking garlic supplements helped obese or overweight patients who had osteoarthritis [33].

Garlic was able to reduce inflammation mostly by blocking inflammatory mediators like TNF-α, NO, and IL-1 in both *in vitro* and *in vivo* models [33]. Garlic also can treat arthritis due to its low or absence of toxicity [33]

2.1.3 Anti-Microbial Activity

It has been discovered that garlic extracts possess a various antifungal and antibacterial activities [34]. The biological and antimicrobial properties of garlic are thought to be influenced significantly by thiosulfinates, particularly Allicin [35]. However, due to its instability, Allicin is habitually changed into different compounds, including Diallyl polysulfides (DASn) and Ajoenes that have antimicrobial properties *in vitro* [36]. In addition, crude garlic treatment repressed *Helicobacter pylori* in the patients' stomachs with contamination of *Helicobacter pylori* in a clinical preliminary [37].

A search for "garlic antimicrobial" on PubMed returns more than three hundred fifty academic papers. This extensive body of literature consists of studies that examined the antibacterial effects of raw garlic preparations, different garlic extracts, and specific organosulfur components of raw garlic against different microorganisms, including Multidrug-resistant pathogens. The biological and chemical characteristics of the hydrophobic molecules found in garlic and its antimicrobial properties were discussed in this section.

2.1.3.1 Allicin

The most predominant and distinctive component that containing Sulphur molecule in crude garlic is Allicin. It is derived from Alliin [38]. Broad-spectrum antibacterial activity of Allicin against Gram positive and negative bacteria, including multidrug-resistant bacteria, has been demonstrated [39]. Allicin has also been demonstrated to have antiviral, antifungal, and antiparasitic properties [40]. The S-allylmercapto change of proteins that containing thiol molecule in microorganisms, which brings about destructive situations like a sharp decrease in glutathione levels, and the enlistment of protein conglomeration, has been reported as a mechanism by which Allicin exhibits antimicrobial activity [41]. In addition, it has been demonstrated that treating methicillin-resistant *S. aureus* (MRSA)'s skin infection with Allicin applied topically improves outcomes [42]. Allicin, however, has been demonstrated to degrade or be metabolized in the blood within a few seconds, indicating that it is unstable [40]. Due to its instability, Allicin may only be used in direct inhalation or as an external medicine [43].

2.1.3.2 Vinyldithiins

From a single Allicin molecule, vinyldithiins with 3-vinyl-4H-1, 2-vinyl-4H-1, 3-dithiin, and 2-dithiin are converted [44]. These substances are typical compounds that containing Sulphur existed in goods made from garlic oil solubilize [2]. Numerous biological effects of Vinyldithiins are reported to be present, including the ability to prevent obesity [45], nevertheless, they lack antimicrobial effects [46].

2.1.3.3 Ajoenes

The Sulphur-containing compounds known as Ajoenes (which have to *E*–*Z* notation: *Z*-Ajoene and *E*-Ajoene) are also typical components found in garlic oil macerate products. Three Allicin molecules are transformed into both Ajoenes [38]. Numerous groups have evaluated Ajoenes' antimicrobial activity. Yoshida et al investigated the Ajoenes' activity against Gram negative and positive bacteria, with MIC values that range from 5 to 20 g/ml for Gram-positive bacteria and 100 to 160 µg/ml for Gram-negative bacteria. Additionally, they indicated that *E*-Ajoene was slightly less active than *Z*-Ajoene [47].

Ajoenes' antibacterial properties against three strains of *H. pylori* were examined further, and it was determined that the *Z*-form and the *E*-form had similar antibacterial effect for the both *Z* & *E* forms at concentrations of 15-20 g/ml and 25 g/ml, respectively [48]. *Aspergillus Niger* and *Candida albicans* are only a couple of the fungi that Ajoenes are effective against [49]. As a result, Ajoenes appear to be effective antimicrobial substances; however, like with Allicin, these substances quickly vanish when mixed with blood [43].

2.1.3.4 DASn

When garlic oil is processed using the steam distillation method, DASn, which are important components of garlic oil, are generated from Allicin [50]. DASn in Garlic oil's Sulphur atom numbers range from one to nine subjected to the generation terms. Mostly, tri- and tetra-Sulphur compounds are plentiful [50].

Garlic oil could suppresses the reproduction of *S. aureus*, *E. coli*, *B. subtilis* due to its antibacterial properties [51]. The mechanism of entering cells, damaging the structure of the cell, and causing cytoplasmic and macromolecule leakage was found to be the inhibition mechanism of the fungus *Penicillium funiculosum* by garlic oil [52]. The interference mechanism with the typical metabolism of *Candida albicans* by garlic oil was also found to be the linkage to the activation of important genes that participated in formation of adenosine triphosphate (ATP), the cell life cycle, and protein synthesis in the cell organelle [53].

Briefly, according to previously mentioned researches, garlic oil is the key antibacterial component that breaks down the structure and metabolism of bacterial cells. While the types of garlic and the ways they are processed affect their antibacterial properties.

The antimicrobial activity of DASn compounds against drug-resistant bacteria and Gram-positive bacteria is limited [54]. Diallyl sulfide (DAS1) is followed by diallyl tetrasulfide (DAS4), diallyl trisulfide (DAS3), and diallyl tetrasulfide (DAS4) in the order of their antimicrobial properties according to the Sulphur atoms' number of in the molecules [55]. As a result, DASn with more than five Sulphur atoms are more likely to have more effective against bacterial pathogens.

2.1.3.5 Turkish Garlic Against Chinese Garlic

The antimicrobial activity of two distinct *Allium sativum L*. samples from Turkey (TR) (Taşköprü, Kastamonu, Turkey) and China (CN) was compared. The antibacterial activity of 17 species of Gram negative and positive bacteria, including *Bacillus, Enterobacter, Escherichia, Klebsiella, Listeria, Pseudomonas, and Staphylococcus* was tested [56]. The antifungal activity of the compounds against *Candida albicans* was also examined by three distinct procedures—chopping, freezing, and slicing by disk diffusion— were used to test the antimicrobial activity. The findings demonstrated that compared to Chinese garlic, Turkish garlic had greater antimicrobial activity [56].

2.1.3.6 Anti-Viral Activity

Garlic has been a component of traditional herbal treatments for a variety of communicable diseases, such as the common cold and other infections caused by viruses [57]. The purposes behind garlic's ethnic medicinal usage in different viral diseases have been affirmed by late pharmacological researches of different garlic separates and their secluded Organic Sulfur Compounds [57].

The viruses that have been shown to have antiviral activity are listed in Table 2-1 under their species and family names, along with garlic extract and its organosulfur compounds as follows.

Table 2.1 Lists of virus against the garlic extracts that have anti-viral activity

Virus' Name	**Family of Virus**	**Virus' Syndrome or Common infection**	**References**
Adenovirus-3/ Adenovirus-41	Adenoviridae	Infection of respiratory tract	[58]
Coronavirus	Coronaviridae	Infections of respiratory tract (human & animal)	[59]
Dengue virus	Flaviviridae	Hemorrhagic fever	[60]
Herpes simplex virus-1; Herpes simplex virus-2; Human cytomegalovirus	Herpesviridae	Infections of genital tracts, herpes, and cold sores	[61]
Influenza A virus-H1N1/Influenza B virus;	Orthomyxoviridae	human and animal Flu	[62]
Human rhinovirus-2		Infections of respiratory tract (human)	[63]
Hepatitis A virus		Infectious of the liver (human)	[64]
Measles virus; Newcastle disease virus; Parainfluenza virus-3	Paramyxoviridae	Infections of respiratory tract (human)	[65]
Porcine Rotavirus (PRV); Rotavirus SA-11	Reoviridae	Infections of Gastrointestinal and diarrhea (human)	[66]

2.1.4 Modulating Immune System

The immune system is benefiting from numerous bioactive compounds found in garlic. Where the outflows of IL-6/10, TNF-α, and interferon-γ in Crude 264.7 macrophages are being managed by the polysaccharides that are present in garlic which also have an immunomodulatory impact [67]. Polysaccharides that have a greater capacity for immunomodulation are found in fresh garlic more than in black garlic [67]. The fructan components break down during processing which is believed to be the reason for that [67].

In a study conducted *in vivo* (on Wistar rats), it was found that an important equilibrium between the T-helper 1 versus T-helper 2 response was made by dual treatment of levamisole and garlic oil [68]. Additionally, the immune-boosting properties of garlic polysaccharides are considerably enhanced by selenylation modification, Moreover, selenizing the polysaccharides in garlic encourages lymphocyte multiplication, raises the serum antibody titer in hens that are 14 days old, improves Interleukin 2 and interferon-γ [69]. Also, Consuming AGEs has been shown to boost immune system function and reduce the severity and frequency of colds and the flu in humans [70]. Finally, the primary immune-modulating compounds in garlic seem to be the polysaccharides.

2.1.5 Cardiovascular Protection

Recent studies on the powder of garlic have indicated that it successfully lowers blood pressure, total cholesterol as well as other cardiovascular disease risk factors, which makes garlic one of the most promising choices in the rising industry of products made from natural ingredients that preserve the cardiovascular system [71].

2.1.5.1 Antihypertensive Activity

Garlic was reported to be able to bring down blood pressure by restraining the angiotensin changing over protein, increment the creation of NO and hydrogen sulfide (H_2S), and diminish oxidative pressure [72]. S-1-propylenecysteine was also found to be the AGE's most important antihypertensive compound. In spontaneous hypertension rats, it was indicated that S-1-propylenecysteine reduced systolic pressure and enhanced peripheral circulation without affecting control rats' systolic blood pressure [73].

Some other analyses revealed that the nitrites inside the fermented garlic extract (FGE) reduced pulmonary hypertension in rats with monocrotaline-induced pulmonary

hypertension by boosting the production of PKG and eNOS and lowering the vascular endothelial cell bonding MMP-9 expression [74]. Additionally, captopril's inhibition of ACE and hypertension in rats was enhanced by the combination of garlic and Alliin [72]. Enzymatically browning processed garlic was given to 44 hypertensive patients in a placebo-controlled study, where it decreased significantly their diastolic and systolic blood pressures [75].

2.1.5.2 Anti-hyperlipidemic Activity

In both animal and human studies, garlic has been shown to lower blood lipid levels [76]. A study found that processing garlic at high temperatures and pressure effectively reduced total cholesterol, and triglyceride levels in some rats who had been taken a high-cholesterol food [76].

In an in-depth research, the consumption of 300 mg of garlic daily for eight weeks increased HDL levels while lowering cholesterol and LDL levels [77]. However, garlic had no effect on triglyceride levels in diabetic dyslipidemic patients [77].

In addition, 41 patients with hypercholesterolemia found that taking an aged garlic supplement for thirteen weeks decreased the F2-isoprostanes concentrations in the patient's urine and plasma [78]. Moreover, the outcomes of raw garlic were lower than those of aged garlic [78].

2.1.5.3 Heart Protection

The heart can be protected by garlic. In rats, it has been indicated that garlic increases the Na^+/K^+-ATPase protein levels and inhibits isoproterenol-induced heart hypertrophy and remodeling [79].

Through triggering the sirtuin 3-manganese superoxide dismutase pathway and deacetylating manganese superoxide dismutase, garlic extract was reported to protect the heart function of streptomycin-induced diabetic mice [80]. Additionally, in obese rats that are resistant to insulin, garlic extract improved cardiac and mitochondrial dysfunction and had preservative properties on the variability of heart rate [81].

Additionally, in rats with chronic renal failure induced by gentamicin, it was reported the improvement of tissue of the heart, reduction of oxidative pressure, and management of Ca^{2+} and Na^+/K^+ ATPase activities, with the treatment of garlic extract [82]. In addition, Allicin was quickly changed over into natural diallyl polysulfide with the existence of thiols, which effectively supplied H_2S to keep the heart safe [12].

2.1.5.4 Other Cardiovascular Protective Effects

There are further cardiovascular benefits of garlic. It has been observed that garlic inhibits the aggregation of platelets, which could be associated with garlic's antioxidant properties and the antioxidant substances it possesses [83].

Additionally, AGE treatment decreased serum levels of thromboxane B-2 and C-reactive protein, increased the activity of AMPK in the livers of mice with *E*-knockout apolipoprotein, and decreased protein levels of kinase 4 associated with TNF-α and IL-1 receptors [32]. Additionally, in mice with E-knockout apolipoprotein, AGE prevented early stages of atherosclerosis from progressing due to lipid deposition and vascular inflammation, and in humans, AGE prevented the formation of coronary artery calcification [84].

2.1.6 Anticancer Activity

Natural vegetables have been shown to have anticancer properties, including garlic's active components which have also been shown to protect against a variety of cancers in recent studies, worldwide, cancer is acknowledged as the leading factor of death [85].

2.1.6.1 Controlling the Metabolism of Carcinogenic Substances

According to a study, Garlic's sulfur-containing components can lessen the release of agents that cause cancer, lowering the incidence of developing cancer when people are exposed to a variety of carcinogens every day [86]. Additionally, the organic allyl sulfides in garlic have the ability to prevent the production of nitrosamines, a type of cancer-causing agent that is created during food making and storage [87]. Further, the allyl sulfides in garlic can prevent DNA alkylation, a precursor to nitrosamine carcinogenesis [86].

2.1.6.2 Reducing Cell Proliferation and Growth

The cancerous cell has an endless capacity for reproduction. The proliferation of cancer cells in humans, including cancer cells of the HepG2 in the liver, Caco2 in the colon, PC-3 in the prostate, and MCF-7 in the breast, was reported to be suppressed by crude garlic extract [88]. DATS, one of bioactive compounds in garlic, is capable of inhibiting the cell cycle in the G2/M phase and suppressing the proliferation of SGC-7901 gastric cancer cells [89]. Additionally, a specific human epithelial ovarian cancer cell (A2780) can be stopped in the G1/S phase of the cell cycle by S-allyl

cysteine [90]. The growth inhibition of pancreatic adenocarcinoma cells in humans and inducing an arrest of the cell cycle in the G2/M phase was reported to be a property of a SAC analog called S-propargyl-l-cysteine (SPRC) [91].

S-allylmercaptocysteine (SAMC), which comes from garlic, slowed the cell cycle and stopped hepatocellular carcinoma cells from growing. It increased the G0/G1 phase percentage while decreasing the S phase percentage [92]. Furthermore, it has been proven that Allicin stifles the growth of adenocarcinoma cells in the stomach. It prompted cell cycle capture in the S-stage, without influencing typical gastrointestinal cells (INT-407) [93]. Also, it was demonstrated that Ajoene inhibited the expansion of glioblastoma multiform cancer stem cells, and breast cancer cells in humans [94]. In another study, the growth of prostate cancer and multiple myeloma cells *in vitro* was suppressed by ethanolic garlic extract. *In vivo*, ethanolic garlic extract also suppressed the proliferation of mammary tumor cells by increasing endoplasmic reticulum stress [95].

2.1.6.3 Inducing Apoptosis

By up-regulating cytochrome C and down-regulating the Bax/Bcl-2 protein, Alliin was found to decrease the mitochondria's membrane potential and induce apoptosis in adenocarcinoma cells in the stomach through producing ROS [93]. Moreover, S-propargyl-l-cysteine also activated the JNK pathway, inducing human pancreatic ductal adenocarcinoma cells to undergo apoptosis and preventing the proliferation of tumors in the Panc-1 xenograft model *in vivo* [91].

2.1.6.4 Reducing the Unfavorable Impacts of Anticancer Treatments

Allium sativum has been reported to lessen the side effects of many cancer treatments [96]. In research, adult male rats' cisplatin-induced renal histological, and biochemical modifications, such as hemorrhaging, necrosis, and degeneration, were alleviated by AGE [97]. Furthermore, tamoxifen's anticancer activity in mice was enhanced by Allicin, and tamoxifen treatment-induced liver damage was minimized. Also, alkaline phosphatase, glutathione, alanine aminotransferase, and aspartate aminotransferase levels were less affected by tamoxifen in the presence of Allicin [98]. In addition, patients receiving chemotherapy for hematological malignancies in the lower-risk febrile neutropenia subgroup showed that garlic prevented febrile neutropenia [99]. In

addition, Garlic reduced febrile neutropenia in patients undergoing chemotherapy for hematological malignancies in the lower-risk cohort [99].

2.1.6.5 Other Anti-Cancer Actions

The BALB/c mice's immune reactions to fibrosarcoma were improved and cancer development was inhibited by AGE, which increased the proportion of CD4+/CD8+ on embedded fibrosarcoma growths and increased *in vitro* creation of IF-γ in splenocytes [100]. Additionally, it was demonstrated that lemon and garlic extract stimulates the immune system by raising IF-γ, IL-2 and 4 levels, in front of implanted breast cancer in mice [101]. In addition, DADS protected FVB/N mice from developing colon tumors caused by dextran sulfate and azoxymethane [102]. Decreased inflammation by inhibiting NF-κB nuclear localization and glycogen-synthase kinase-3β which are also benefits of the therapy of DADS [102].

2.1.7 Hepato-protective Activity

Numerous studies have demonstrated that garlic, among other natural products, protects the liver. Black garlic extract inhibited apoptosis, lipid peroxidation, and inflammation in rats [103]. A different study found that garlic decreased the liver damage that alloxan caused in rats and enhanced the biochemical blood indicators for the functions of the liver including aspartate transaminase, alanine transaminase, urea, and creatinine [104]. Additionally, in albino mice, the mixture of garlic and vitamin C defended against the damage of Cd-induced liver [105]. Moreover, male rabbits' acute liver injury caused by CCl4 was better protected by single-clove garlic than by multi-clove garlic [29]. In addition, it was demonstrated that garlic oil had a defensive impact against liver injury by expanding the initiation of hepatic cell antioxidant enzymes and decreasing the apoptosis process in rats' hepatic cells [106].

Furthermore, it was reported that S-methyl-l-cysteine, and DAS/DADS, which are active compounds in garlic, could heal and protect against liver damage, including ethanol-induced liver damage, both acutely and chronically [107].

The nonalcoholic fatty liver disease that was placed on through a diet with high-fat content in rats, was found to be prevented by DADS in garlic essential oil, by inhibiting cytochrome P450 2E1 expression, DADS significantly decreased pro-inflammatory cytokines released from the liver and improved the antioxidant properties [108].

On the other hand, a study on animals demonstrated that consuming too much garlic diet has a negative effect on the liver morphologically [109]. In Wistar albinism rats, fresh garlic extract at different concentrations was injected with a tube straight into the stomach, thirty days later, resulting in bleeding, increased weight, and nodular swelling on the liver's exterior surface [109].

In general, garlic can support the treatment of acute or chronic liver damage, but excessive consumption of garlic can have negative side effects. The dosage of garlic use must be evaluated.

2.1.8 Protection Effects on Digestive System

It has been indicated that garlic can be used to treat gastric tissue damage. The gastric ulcer duration, the volume of total gastric acid, the count of microbes, and changes in histopathology were all reduced in a study that used garlic and cabbage extract as a treatment [110]. The rats' gastric juice's pH value also improved as a result of this treatment [110]. In addition, in male rats, it was demonstrated that mucosal injury in the stomach, caused by indomethacin, has been healed by the oral intake of AGE with a remarkable reduction in the total amount of bacteria in the stomach [111]. AGE also prevented ulcers caused by indomethacin in rats through a decrease in oxidative stress and an increase in glutathione, prostaglandin E-2, and NO in the stomach’s tissue [112].

The bioactive substances in garlic are also crucial for keeping the digestive system safe. through different mechanisms such as suppressing the phosphorylation of p38 and JNK, and (ERK1/2)-(PPAR)-γ [113]. Allicin was shown to suppress launching of the AP-1/NF-B/(STAT-1) in mice with ulcerative colitis [113]. DAS has an inhibiting effect on the NO and STAT-1 expression in interferon-γ-triggered intestinal cells, as well as decreases interferon-inducible protein-10 and IL-6 [114]. Furthermore, dinitrobenzene sulfonic acid-induced colitis in mice was ameliorated by DAS and DADS [114]. Raw garlic consumption has also been demonstrated to lower H. pylori and bacterial urease activity in the stomachs of fifteen participants [37].

In general, Garlic and its bioactive components contribute to better gastrointestinal functioning and treat colitis, gastric ulcers, and other digestive issues through reducing oxidative stress, controlling inflammation, and eliminating *H. pylori*.

2.1.9 Anti-Diabetic Activity

In 1st type diabetic-rats induced by streptomycin, garlic was reported to heal the injuries of pancreatic cells and decrease pathological changes as well as oxidative stress [115]. Additionally, rats with diabetes were protected from diabetic retinopathy by garlic [116]. Following 49 days of gastric gavage with raw garlic extract in rats, the glucose-ratio in the blood, the total body weight, and morphological changes in the retinal tissue were getting better for all the group members which got treatment with garlic [116]. Furthermore, in rats with streptomycin-induced diabetes, AGE exerted an anti-diabetic effect depending on the dose [117].

According to additional research, garlic supplements effectively decreased fructosamine and glycosylated hemoglobin in many randomized controlled trials which included more than seven hundred patients with type 2 diabetes mellitus. This study reported that taking supplements containing garlic helped manage type 2 diabetes [118]. As a result, the treatment of diabetes complications may benefit from garlic's bioactive components.

2.1.10 Neuroprotection

In murine BV-2 microglial cells activated by the lipopolysaccharide, a research paper found that garlic extract can reduce inflammation of the neurons by managing the manufacturing of many oxidative stress-related receptors and reducing the synthesis of NO [119]. In a different study, organic compounds that contain sulfur in BV2 microglia cells triggered by lipopolysaccharide were linked to garlic's anti-neuritis activity [120].

It has been demonstrated that garlic therapy reduces lead-levels in the brain, blood, and partially protects rats' developing hippocampus neurons from Pb-induced neuronal death during pregnancy and lactation [121].

Using the (BBB) scoring system, the neurological effects of AGE on spinal cord rats (I/R model) were assessed, where the AGE group's BBB level was much higher than the I/R group, proving that AGE has a significant protective effect on the neurons [122]. Interestingly, AGE decreased the damage to rats' working memory by raising the amount of glutamate decarboxylase and glutamate transporter 1in the hippocampal region and reducing the loss of cholinergic neurons [123]. It was also proven that the garlic ethanol extract improved memory. Glutamate synthetase, (Na/K) ATPase, and

Ca^{2+} ATPase were all activated by garlic in the diabetic Wistar rat's hippocampus [124]. Additionally, the damage caused by monosodium glutamate was successfully stopped by the fermented garlic ethanol extract of working memory [125]. It was also indicated that *Z*-Ajoene prevented delayed neuronal death in the CA1 area of the hippocampus and decreased the level of lipid peroxidation [126]. Moreover, by decreasing acetylcholinesterase activity, inflammation of the neurons, and astrocyte proliferation, SAC helped rats with cognitive impairment [127]. Finally, the mentioned researches conducted previously indicated that garlic has remarkable neuroprotective activities and primarily affects the hippocampus. The importance of organic Sulphur compounds in neuroprotection has been demonstrated.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials and Methods Used Overview

The extraction procedure is a crucial step in isolating biologically active compounds from biological sources like plants, or other living tissues. A variety of crucial factors influence the type and quantity of compounds that are produced. These factors can be explained as below:

Choice of Solvent: Different solvents have varying polarities and extraction efficiencies. The selection of a suitable solvent or a solvent mixture is essential to ensure it can dissolve the target compounds effectively. The choice depends on the properties of the substances being separated and their solubility characteristics.

Extraction Method: Various extraction techniques exist, such as maceration, Soxhlet extraction, solid-phase extraction, etc. Each method has its advantages and is suited for different compound types. For instance, Soxhlet extraction is a continuous extraction method.

Extraction Parameters: Parameters such as extraction time, temperature, and pressure can also influence the efficiency of compound extracted. Longer extraction times or higher temperatures may increase the yield but could also degrade sensitive compounds.

Chemical Diversity: Different parts of the organism may contain distinct compounds. For example, the roots, stems, leaves, and flowers of a plant may contain different active compounds, so selecting the appropriate part for extraction is critical.

Purity and Isolation Techniques: Following extraction, purification and isolation techniques like chromatography (e.g., HPLC, TLC), crystallization, distillation, etc., are employed to separate and obtain pure compounds.

Understanding these factors and optimizing the extraction procedure is essential for obtaining a comprehensive profile of bioactive compounds from biological sources.

Researchers often need to fine-tune these parameters to isolate specific compounds of interest or explore the diverse array of chemicals produced by living organisms.

3.2 Equipment, Materials and Supplies Used

3.2.1 Glass Tools

Sterile bottles, graduated cylinders, round-bottomed, conical, and volumetric flasks, beakers, glass funnels, and graduated glass pipettes.

3.2.2 Chemicals

Ascorbic acid (Vitamin C), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), pure methanol, 96% ethanol, ethyl acetate, hydrogen peroxide (H2O2), dichloromethane, 9,10 diphenylantracene, distilled water (dH2O), TAE (Tris-acetate-EDTA) buffer, oxybenzone.

3.2.3 Used kits

Agar (Mueller-Hinton), blank antimicrobial dicks, susceptibility antibiotic discs (Sulfamethoxazole 25 µg/ml), pBR322 DNA (Vivantis). bacteria (Stenotrophomonas Maltophilia).

3.2.4 Equipment and Supplies

1.5% Agarose gel, plates of agar, sterile wooden stick, filter papers (muslin cloth 6 mm in diameter), caliper to measure the inhibition zone, 5 ml, 2 ml, 1 ml pipettes, sterile cotton, aluminum foil, gas burner.

3.2.5 Devices

Rotary evaporator (Bauchi Rotavapor R-210), Autoclave (OT 40L), Incubator (Heraeus thermo scientific), Weighing balance (Metter toledo), Spectrophotometry (Biochrom Libra S60 B, England), UV transluminator (DNR-IS), gel documentation system (DNR-IS, MiniBIS Pro), Electrophoresis apparatus.

3.3 Collection of Plants and Preparation of Extracts

3.3.1 Used Plant

Plants of *Allium sativum* belonging to the hard-neck species Taşköprü sarımsağı were randomly collected in Feb-Mar 2022, from a local market in Gaziantep - Turkey. The plant was botanically and chemically characterized in the Departments of Biology and Chemistry at Gaziantep University. It was verified to be fresh and free of any chemical treatment.

3.3.2 Preparation of Ethanol Extract

Yoshida et al. 1987's method was used to prepare the Ajoene-rich extract [128]:
The Turkish garlic bulbs were shade dried and peeled off, then garlic cloves that had been peeled and weighed for 200 g were crushed in a homogenizer using cold distilled water, as demonstrated in the Figure 3.1.

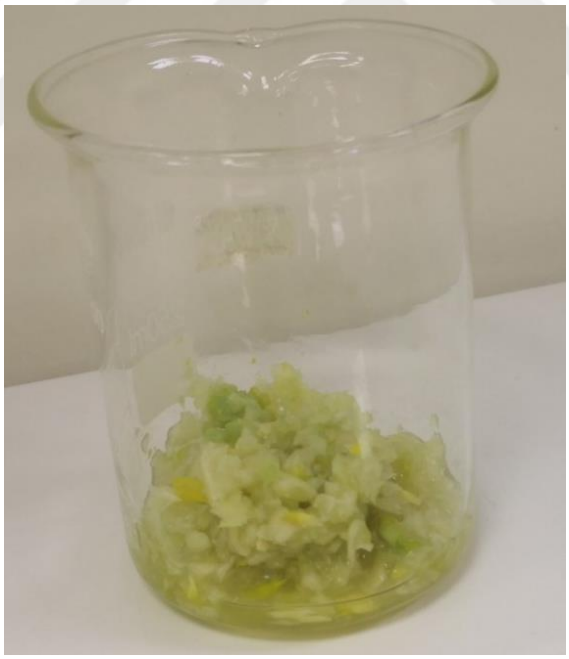


Figure 3.1 200 grams of garlic cloves that had been peeled and crushed in a homogenizer using cold distilled water

Then, a sample of 50 grams from the homogenized garlic cloves with cold distilled water was weighed as demonstrated in the Figure 3.2.

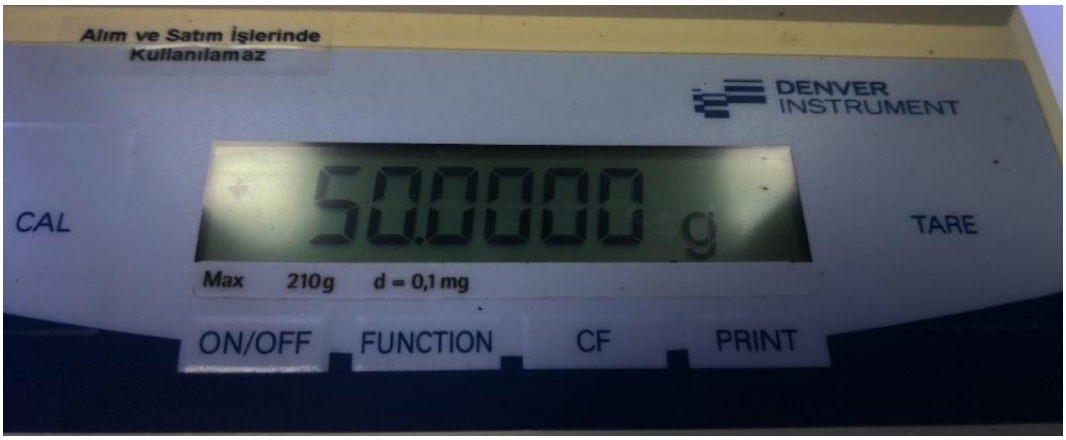


Figure 3.2 50 grams sample from the homogenized mixture with distilled water

A 100 ml of 96% ethanol was used to reflux the homogenized mixture for 2 h, as demonstrated in the Figure 3.3.

Figure 3.3 The sample of garlic under reflux with ethanol

After that, a muslin cloth was used to filter the garlic paste, as demonstrated in the Figure 3.4.

Figure 3.4 The sample is being filtered using muslin cloth

In a separating funnel, the filtrate was mixed with amount of ethyl acetate (120 ml), and the mix was shaken, then sample was being separated using a separating funnel as demonstrated in the Figure 3.5.

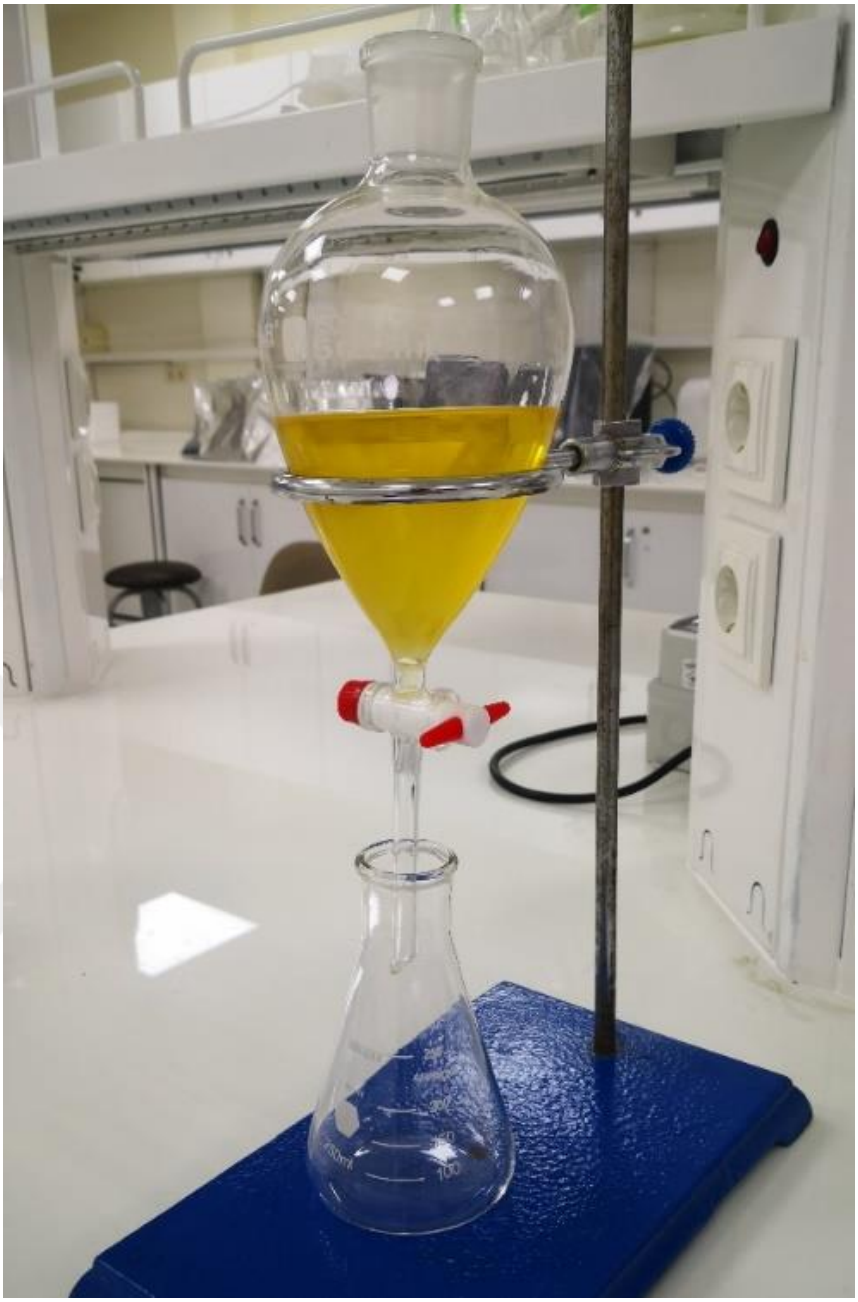


Figure 3.5 The sample is being separated using separating funnel

A semi-solid extract was produced by collecting the upper layer that contain ethyl acetate and evaporating ethyl acetate at temperature of the room. This was the Ajoene-rich fraction of the extract, as demonstrated in the Figure 3.6.

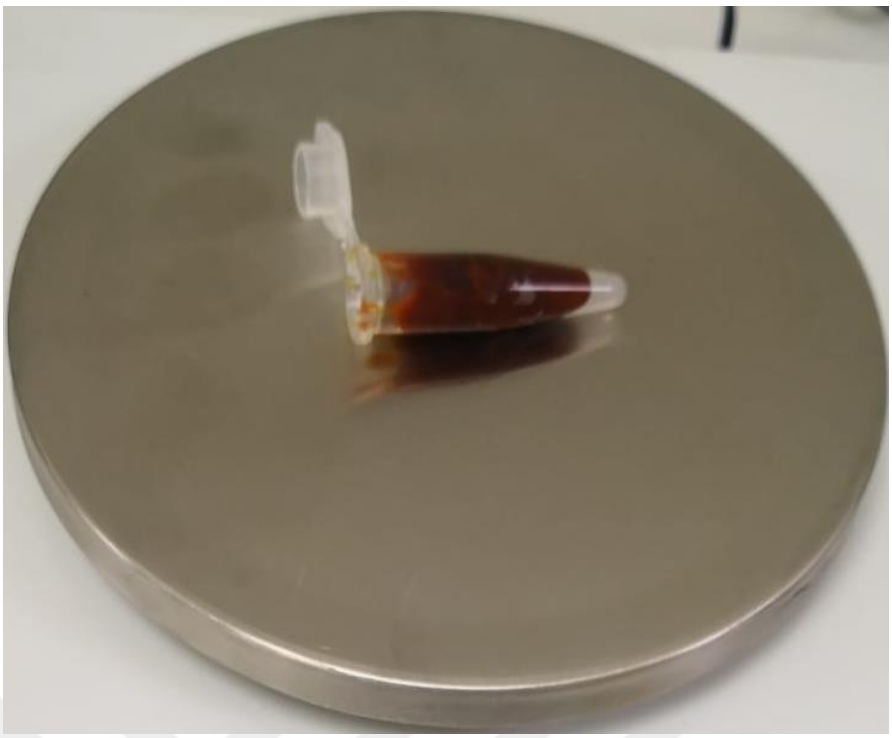


Figure 3.6 Ajoene-rich fraction of the garlic extract

3.4 Analytical Methods Used

The complexity of Ajoene extracts necessitates a multi-faceted approach utilizing various analytical techniques to comprehensively identify and characterize the compounds present. Combining these methods provides a more comprehensive knowledge of the elements and characteristics of Ajoene extract, facilitating future studies and possible utilization in medicine and health-related fields.

3.4.1 Gas Chromatography – Mass Spectroscopy GC-MS

This method has granted identifying and measurement of each compound's quantities within the Ajoene extract mixture. Where the high temperature was applied to 0.01g of the Ajoene-rich fraction extract, after that, when the extract was introduced into the GC device, it was transformed into the gaseous phase. Within the GC-MS device, a capillary column (30 x 0.25) m x 0.25 mm was equipped and helium gas was utilized as carrier gas with a flow rate of 1 ml/minute and a split ratio of 1:10.

The temperature settings are part of the detailed protocol for the gas chromatography-mass spectrometry (GC-MS) analysis. The breakdown of these temperature parameters and their significance in the analysis are as follows:

Isothermal Hold at 50°C for Five Minutes: Initially, the oven temperature of the gas chromatograph was set to 50°C and maintained for five minutes. This period is known as the "initial hold" or "isothermal hold." During this time, any volatile compounds with lower boiling points are separated and eluted first.

Temperature Ramp to 280°C at 10°C per Minute: After the isothermal hold, the temperature was ramped up from 50°C to 280°C at a rate of 10°C per minute. This ramping-up phase is called the "temperature program." Increasing the temperature at this rate facilitates the separation of a wider range of compounds based on their varying boiling points and volatility.

Hold at 280°C for Fifteen Minutes: Once the temperature reached 280°C, it was held constant for fifteen minutes. This period, known as the "final hold" or "isothermal hold," allows for the completion of the separation process and ensures sufficient time for the detection of compounds that may elute later due to higher boiling points or slower migration through the column.

Mass Spectrometer Interface Temperature (230°C) and Injector Port Temperature (290°C): These temperatures are specific settings for the mass spectrometer interface and injector port, respectively. They are optimized to ensure efficient ionization, vaporization, and transfer of the separated compounds into the mass spectrometer for analysis.

The careful control of temperatures in GC-MS analysis is crucial for achieving optimal separation and detection of compounds present in the sample. The temperature program, including initial holds, ramps, and final holds, is designed to effectively resolve compounds based on their thermal characteristics, aiding in their identification and quantification using mass spectrometry.

These temperature settings, when coupled with chromatographic separation and mass spectrometric detection, contribute to a comprehensive analysis of the compounds present in the Ajoene-rich fraction, allowing for potential identification and further understanding of its chemical composition.

This analysis was performed at Harran University, Science and Technology Application and Research Center, for performing the GCMS aromatic components analysis (to detect the active compounds present in the Ajoene extract).

3.4.2 Fourier-Transform Infrared Spectroscopy FTIR

This method is applied to identify functional groups in both carbon-containing and non-carbon-containing compounds by analyzing their absorption of infrared energy across a range of wavelengths. It involves using an interferometer to create an interferogram of the sample's infrared signal, which is then processed with a Fourier transform to produce the FTIR spectrum. Samples were directly placed on the ATR crystal without mixing in KBr plates. The FTIR measurements were conducted at room temperature using a straight ATR device. Infrared spectra were collected using a PerkinElmer 100 FTIR with a Gladia ATR Sampling attachment, operating at a resolution of 1 cm-1 and averaging over 16 scans.

3.4.3 Ultraviolet-Visible Spectroscopy UV-VIS

PG Method Equipment Ltd. utilized the T80+ UV/VIS Spectrograph for the analysis, employing dichloromethane as the solvent. Also, using an optical quartz cuvette with a path length of 1 cm to record the UV-Vis spectrum within the wavelength range of 190 to 1100 nm. This setup allowed to analyze the absorption and transmission of light by the sample across this specific wavelength range.

3.4.4 Analysis of the Ajoene Extract by Photoluminescence Spectroscopy

The Perkin-Elmer LS55 Fluorophotometer was utilized to investigate the photoluminescence characteristics. A 1 cm path length cuvette made of optical quartz was used to analyze the entire samples after dilution in dichloromethane. Measurements were taken at 276 nm wavelength for the specimens at multiple concentrations levels. Photoluminescence quantum efficiencies were measured using 9,10-diphenylantracene as the reference compound. The instrument used for fluorescence spectroscopy is depicted in Figure 3.7 of the experimental setup. This process allowed for the examination of the emitted light by the samples at specific wavelengths and the determination of their photoluminescence characteristics and efficiencies.

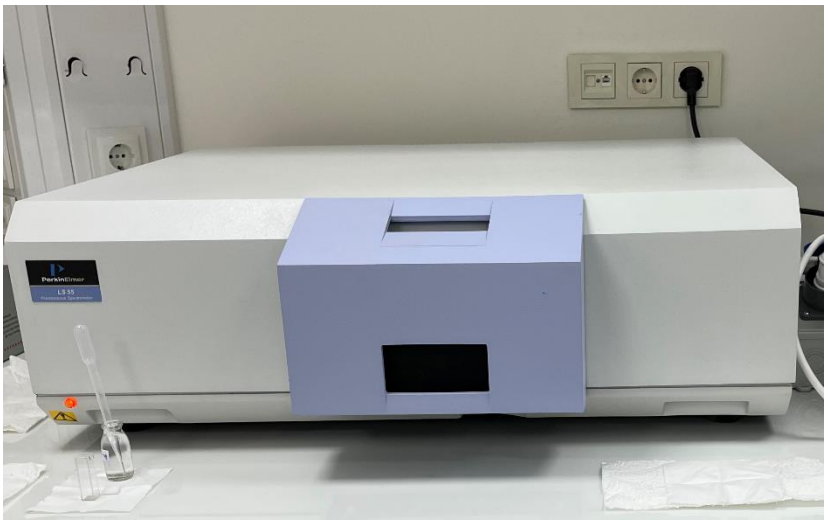


Figure 3.7 Fluorescence spectroscopy instrument photograph, taken from the laboratory

3.5 Biological Evaluation

3.5.1 Determination of *in vitro* Antioxidant Properties

The evaluation of the samples' capability to scavenge free radicals was conducted by assessing the decreasing of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) concentration in methanol. This was achieved using a UV-visible spectrophotometer. The reduction in DPPH concentration signifies its reaction with antioxidant compounds present in the samples. UV-visible spectrophotometry allowed for the measurement of changes in absorbance at specific wavelengths, where in this experiment the absorbance was used to measure at 517 nm, indicating the degree of scavenging activity against free radicals present in the samples. Ascorbic acid (Vitamin C) and the test samples were both dissolved in methanol to prepare their individual solutions.

To initiate the assay, a 100 ml volumetric bottle containing 4 mg of DPPH solution was measured, and pure methanol was added to make it up to the mark. To acquire (0.012 mg/mL) fresh DPPH solution.

The Ajoene ethanolic extract that will be tested was made by weighing 0.5 grams of the Ajoene extract as demonstrated in Figure 3.8.

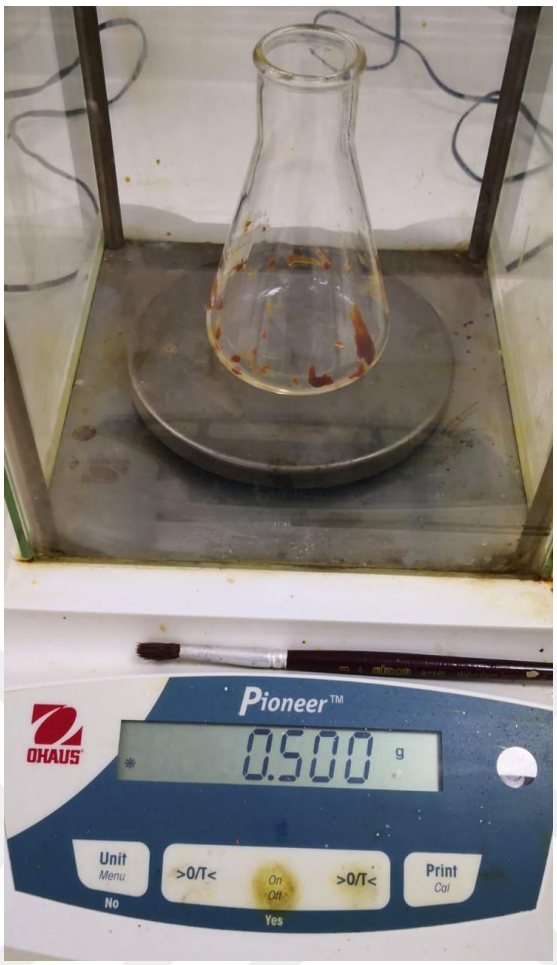


Figure 3.8 The sample of the Ajoene extract weighted for the antioxidant experiment

After that, 30 ml of MeOH were added to the 0.5 g sample to make the stock solution. The stock solution was then mixed in series with up to 10 ml of MeOH to make several solutions of varying concentrations (all amounts in ml unit) as demonstrated in Table 3-1.

Table 3.1 The contents of the samples mixtures with their quantities in ml

	Number 1	**Number 2**	**Number 3**	**Number 4**	**Number 5**
Stock Solution	2	4	6	8	10
MeOH	8	6	4	2	0
Total	10	10	10	10	10

The series of the stock solutions without MeOH are given in Figure 3.9.

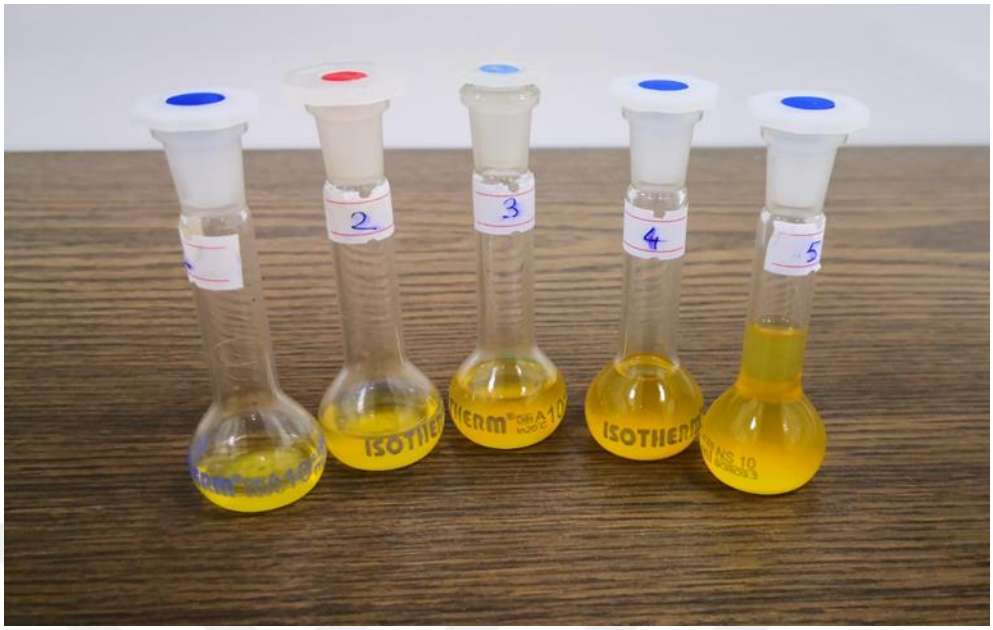


Figure 3.9 The series of stock solutions without MeOH

The series of the stock solutions after adding MeOH are given in Figure 3.10.

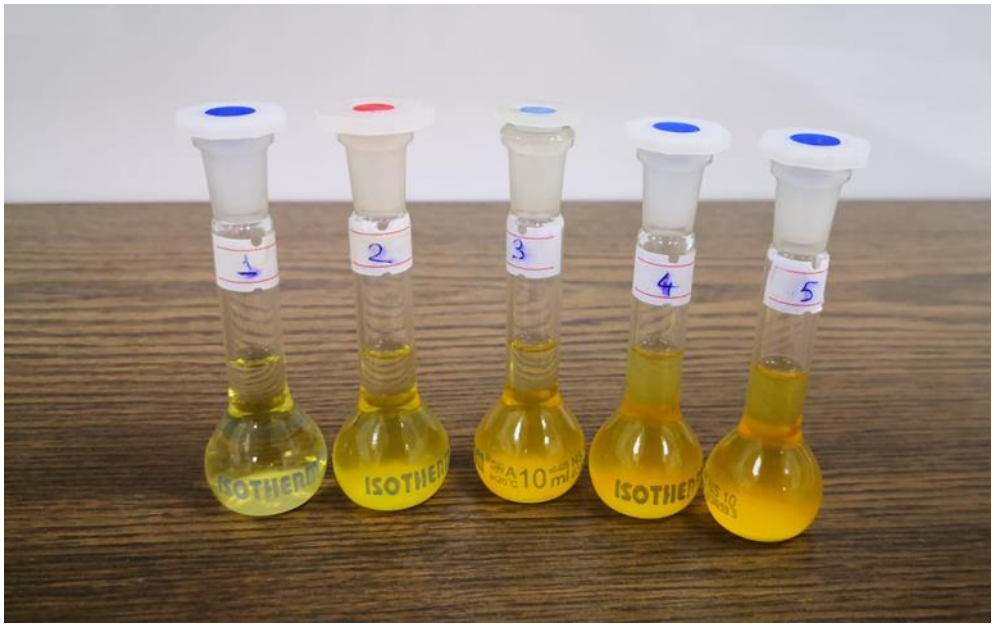


Figure 3.10 The series of stock solution with MeOH

Then, 9 ml of solution made from DPPH was combined with 1 ml of each sample. And put in another container as shown in Figure 3.11.

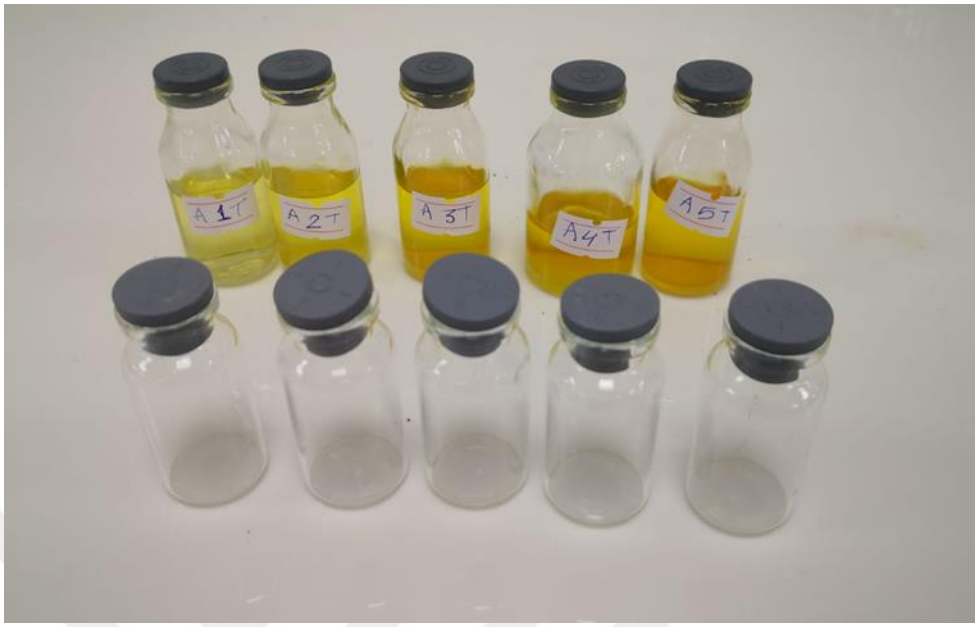


Figure 3.11 The samples after adding with 9 ml of DPPH solution

Ten ml of DPPH (0.012 mg/mL) produced in unadulterated methanol served as the negative control while the ascorbic acid served as the positive control.

After stirring properly, this mixture was kept 30 minutes away from the light in a dark place to ensure no effect of light on the DPPH as this compound is very sensitive to the light and it can decomposed very fast, anther parameter was considered for the covered samples is to be set at room temperature (250C) as the high temperature could also affect the reaction of the DPPH with the sample, it is believed that high temperature could faster the reaction between the DPPH and sample that could result in false reading in the spectroscopy. The Figure 3.12. Is showing the well covered samples after adding the DPPH.

Figure 3.12 The covered samples after adding with 3 ml of DPPH solution

Using the formula described in Table 3-2, the inhibition percentage of the DPPH oxidative effect was measured to evaluate the antioxidant activity of various concentrations obtained from the Ajoene extract.

Table 3-2 The calculation description for all the samples

Sample No.	**Calculation Description**
Number 1:	30 ml 0.5 g (500 mg); $M_1.V_1=M_2.V_2 \Longrightarrow$ 33.3 mg. 1 ml= M_2. 10 ml 2 ml X mg $M_2 = \frac{33.3\ mg\ X\ 1\ ml}{10\ ml}$ = 3.33 mg/ml X= $\frac{2\ ml\ X\ 500\ mg}{30\ ml}$ = 33.3 mg/ml = C_1
Number 2:	30 ml 0.5 g (500 mg); $M_1.V_1=M_2.V_2 \Longrightarrow$ 66.6 mg. 1 ml= M_2. 10 ml 4 ml X mg $M_2 = \frac{33.3\ mg\ X\ 1\ ml}{10\ ml}$ = 6.66 mg/ml X= $\frac{4\ ml\ X\ 500\ mg}{30\ ml}$ = 66.6 mg/ml = C_2

Number 3:	30 ml 0.5 g (500 mg); $M_1.V_1=M_2.V_2 \Longrightarrow$ 100 mg. 1 ml= M_2. 10 ml 6 ml X mg $M_2 = \frac{100\ mg\ X\ 1\ ml}{10\ ml}$ = 10 mg/ml X= $\frac{6\ ml\ X\ 500\ mg}{30\ ml}$ = 100 mg/ml = C_3
Number 4:	30 ml 0.5 g (500 mg); $M_1.V_1=M_2.V_2 \Longrightarrow$ 133.3 mg. 1 ml= M_2. 10 ml 8 ml X mg $M_2 = \frac{133.3\ mg\ X\ 1\ ml}{10\ ml}$ = 13.3 mg/ml X= $\frac{8\ ml\ X\ 500\ mg}{30\ ml}$ =133.3 mg/ml = C_4
Number 5:	30 ml 0.5 g (500 mg); $M_1.V_1=M_2.V_2 \Longrightarrow$166.6 mg. 1 ml= M_2. 10 ml 10 ml X mg $M_2 = \frac{166.6\ mg\ X\ 1\ ml}{10\ ml}$ = 16.6 mg/ml X= $\frac{10\ ml\ X\ 500\ mg}{30\ ml}$ =166.6 mg/ml = C_5

The simultaneous observation of the yellowish reduced form (DPPH/H+) of the stable violet DPPH radical was detected as demonstrated in Figure 3-13.

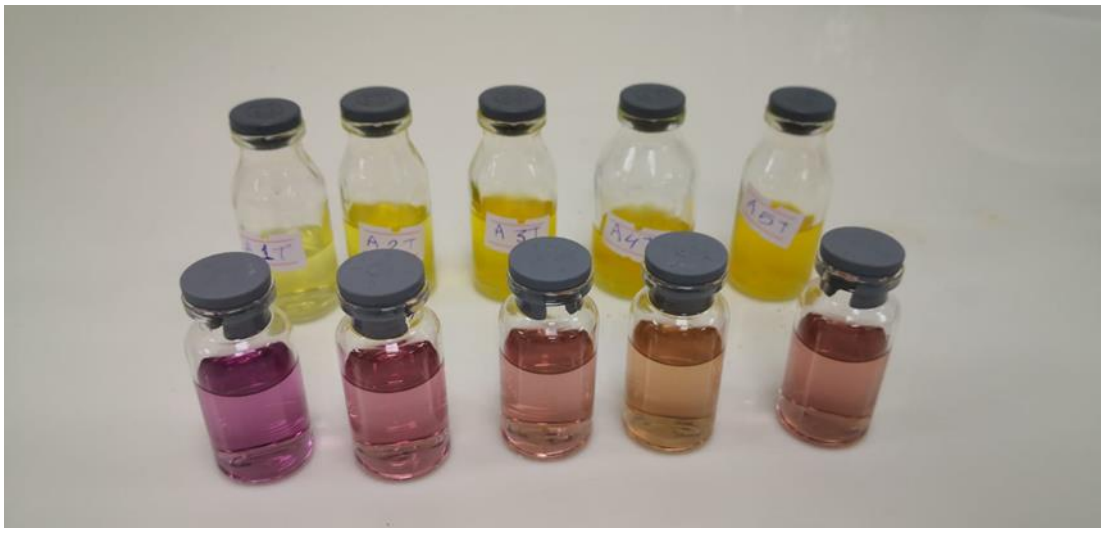


Figure 3.13 The yellowish reduced form the stable violet DPPH radical of the Ajoene extract samples

Figure 3.13, depicts a series of extract concentrations alongside DPPH. This visual aid represents a set of experimental samples or concentrations of the test extracts and their interaction with DPPH. It can show how the absorbance or other measurements change concerning different concentrations of the test samples when mixed with the DPPH solution, supporting the assessment of antioxidant activity.

The free radical scavenging activity was calculated using the following formula, which gives the proportion of DPPH free radicals that have been scavenged:

Radical scavenging activity (%) = [(Absorbance control − Absorbance) / Absorbance control] × 100

This formula represents a method to quantify the antioxidant activity of test samples against DPPH free radicals. It involves comparing the absorbance measurements of a control (DPPH solution without any antioxidant) with those of the test samples (DPPH solution with the antioxidants being tested). This comparison is expressed as a percentage, reflecting the reduction in absorbance caused by the test sample's ability to neutralize or scavenge the DPPH free radicals.

3.5.2 Determination of Antibacterial Activity

Garlic is a strong antibiotic that functions well facing the microorganisms that are impervious to pharmaceutical antibiotics. Ten bacteria- and yeast-resistant species were subjected to garlic extract testing in Europe in the 1970s [129].

The Ajoene extract was subjected to determine the antibacterial activity against the Bacterial resistant strain (*Stenotrophomonas maltophilia*), as it’s a natural Bacteria antibiotic-resistant strain, where the bacteria strain was obtained from Microbiology Laboratory, Faculty of Medicine, Gaziantep University.

The Ajoene extract's antibacterial properties obtained from the ethanol extract of the Turkish garlic plant was evaluated using the method of disk diffusion. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria were taken into account regarding the disc diffusion test used in the evaluation of antimicrobial activity determination.

3.5.2.1 Preparation of Discs of Ajoene Extract

The Ajoene extract obtained from the ethanol extract of the garlic plant was prepared as 5 mg/ml, and the suspensions were prepared by absorbing 20 µl into 6 mm diameter sterile blank discs. *Stenotrophomonas maltophilia* bacterial strain obtained from Gaziantep University, Faculty of Medicine, Microbiology Laboratory.

The prepared samples were inoculated into an MHA medium and heated at 37 °C in an incubator.

After 24-48 h of incubation, the strains formed on the petri dish were taken with the help of a swab and suspended in tubes containing spinal fluid (SF).

Taking into account the EUCAST's criteria, the *Stenotrophomonas maltophilia* bacterial strain was incubated at 37 °C in MHA media for 24 h to ensure its growth, and a fresh culture was obtained. Then, this fresh culture was taken with the help of a loop and transferred to tubes containing SF, and a homogeneous suspension was obtained by vortexing as shown in Figure 3.14.

Figure 3.14 The vortexing process to obtain homogeneous suspension

Another suspension was prepared from this obtained suspension with the help of a McFarland (McF) device at a density corresponding to 0.5 McF in the Barium Sulfate Turbidity Standard.

100 µl of this suspension at 0.5 McFarland density was transferred to MHA Petri dishes to be used for disc diffusion, and the Petri dishes were prepared by spreading it to cover the entire petri dish with the help of a swab stick.

Sterile blank discs of 6 mm diameter prepared with Ajoene extract at different concentrations using the serial dilution methods (1:2) starting from (2 mg/ml, 1 mg/ml till 0.0625 mg/ml), then extending the samples by adding (3 mg/ml, 4mg/ml, and 5 mg/ml) where all these concentrations were placed in Petri dishes cultivated with *Stenotrophomonas maltophilia* with the help of sterile forceps as shown in the Figure 3.15.

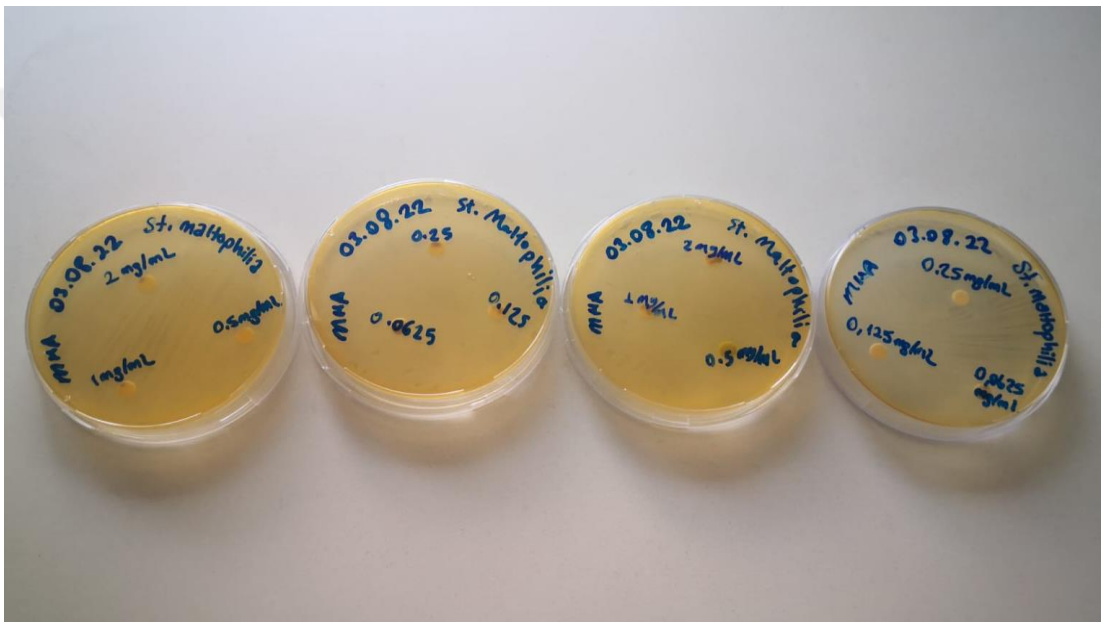


Figure 3.15 The petri dishes with different concentrations of Ajoene extract

In order to clearly measure the zones that may occur during incubation, the discs were placed in the petri dish, taking care to be 22 mm between the discs and 14 mm from the edge of the petri dish. In the study, the Petri dishes on which the discs were placed were incubated at 35 °C for 24 h. After the incubation, the diameters of the zones formed in the Petri dishes were measured with the help of a ruler and recorded for evaluation. The positive control used was Sulfamethoxazole 25 µg/ml.

3.5.3 DNA Protective Activity

The DNA protective activity of Ajoene extract was investigated through DNA electrophoresis experiment, which is a typical scientific method for dividing up DNA fragments according to size. It is widely used in molecular biology and biochemistry for various purposes, including DNA analysis, genotyping, and DNA fragment purification.

Here's an outline of a basic DNA electrophoresis experiment:

1. Assembling the agarose gel:
   - Measure and weigh the right quantity of agarose powder, in accordance with the intended gel concentration (usually between 0.5% to 2% agarose).
   - In a flask filled with TAE buffer, add the agarose powder.
   - Utilizing an open flame, warm the mixture until the agarose is fully dissolved.
   - Allow the agarose solution to cool slightly before pouring it into a gel mold.
   - Insert a comb to create wells for sample loading.
   - Allow 20 to 30 minutes for the gel to harden at room temperature.
2. Prepare the DNA samples:
   - Mix each DNA sample with gel loading dye in a 1:5 ratio (e.g., 5 µL of DNA sample + 1 µL of loading dye), then gently vortex the samples to ensure mixing.
3. Load the DNA samples:
   - Withdraw the comb from the gel with caution.
   - After adding TAE buffer to the electrophoresis chamber, place the gel inside.
   - To provide a size reference, load the DNA ladder into a single well.
   - Using a micropipette, carefully load the DNA samples into individual wells.
4. Run the electrophoresis:
   - Connect the electrodes to the power supply and set the appropriate voltage (typically 80-120V, according to the concentration of the gel and desired separation).
   - Depending on the required separation resolution and the size of the DNA fragments, run the electrophoresis for a duration of 30 minutes to two hours.
5. Visualize the DNA:
   - After electrophoresis, the gel should be gently taken out of the chamber.
   - Then the gel should be placed on a UV transilluminator and turn on the UV light.
   - DNA molecules will fluoresce under UV light due to the presence of ethidium bromide or other DNA-specific stains.

- Use a gel documentation system or a smartphone camera to capture an image of the DNA bands.

6. Analysis:

- Analyze the DNA bands based on their size and intensity.
- Compare the bands with the DNA ladder to estimate the size of the DNA fragments in the samples.
- Interpret the results based on the experimental objectives.

3.5.3.1 Preparation of Controls and Extracts

The different Ajoene concentrations samples, as well as the Positive and negative controls were prepared as the following equations as demonstrated in the Table 3-3.

Table 3.3 List of the Ajoene extract samples as equations

Sample 1: X + 5 mg/mL AE (5 µl) + Y
Sample 2: X + 4 mg/mL AE (5 µl) + Y
Sample 3: X + 3 mg/mL AE (5 µl) + Y
Sample 4: X + 2 mg/mL AE (5 µl) + Y
Sample 5: X + 1 mg/mL AE (5 µl) + Y
Sample 6: X + 0.5 mg/mL AE (5 µl) + Y
Sample 7: X + 0.25 mg/mL AE (5 µl) + Y
Sample 8: X + 0.125 mg/mL AE (5 µl) + Y
Sample 9: X + 0.0625 mg/mL AE (5 µl) + Y
Positive Control: Plasmid DNA (3 µl) + dH_2O (6 µl)
Negative Control: Plasmid DNA (3 µl) + dH_2O (6 µl) + UV+ H_2O_2 (1 µl)
Oxybenzone: Plasmid DNA (3 µl) + Oxybenzone (5 µl) + UV+ H_2O_2 (1 µl)

X: Plasmid DNA (3 µl), AE: Ajoene Extract, Y: UV+ H_2O_2 (1 µl)

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Chemistry of the Ajoene

Ajoene is a sulfur-containing compound found in garlic (*Allium sativum*) that exhibits various biological activities, including antibacterial, antifungal, antiviral, and anticancer properties. Its chemical structure is derived from the reaction of Allicin, another compound found in garlic, through a process called thiolation. Here's a brief overview of the chemistry of Ajoene.

Formation

Ajoene is formed from Allicin (diallyl thiosulfinate), which is produced when garlic is crushed or chopped. Allicin undergoes a thiolation reaction, the general mechanism involves the following steps:

1. Allicin, which is produced when garlic is crushed or chopped, contains two sulfhydryl (SH) groups (-SH) in its structure.

2. Under various conditions such as heating or exposure to air, Allicin undergoes oxidation reactions. These reactions can involve the loss of hydrogen sulfide (H_2S) and the formation of a sulfonic acid intermediate.

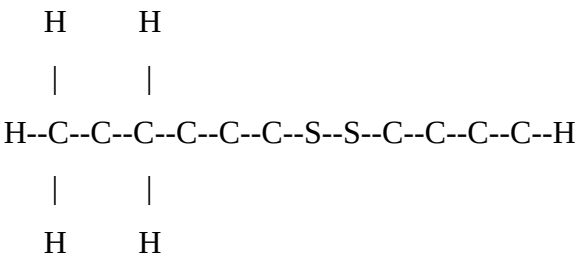
3. The sulfonic acid intermediate can further react with another Allicin molecule to form Ajoene. This process involves the formation of a disulfide bond (S-S bond) between the two Allicin molecules, resulting in the formation of Ajoene [130].

Molecular Weight

248.5 g/mol [131].

Chemical Structure

The chemical structure of Ajoene is $C_9H_{14}OS_3$ [131], as follows:



Every "C" in this structure stands for a carbon atom.

A hydrogen atom is represented by each "H".

The "S" represents a sulfur atom.

The bonds between atoms represent single bonds, except for the sulfur-sulfur bond, which represents a double bond.

The double bond between the two sulfur atoms forms a disulfide linkage, which is characteristic of Ajoene.

Two isomeric forms of Ajoene are known to exist: Z-Ajoene (Cis form) of chemical formula 1 and E-Ajoene (Trans form) of chemical formula 2 as demonstrated in Figure 4.1.

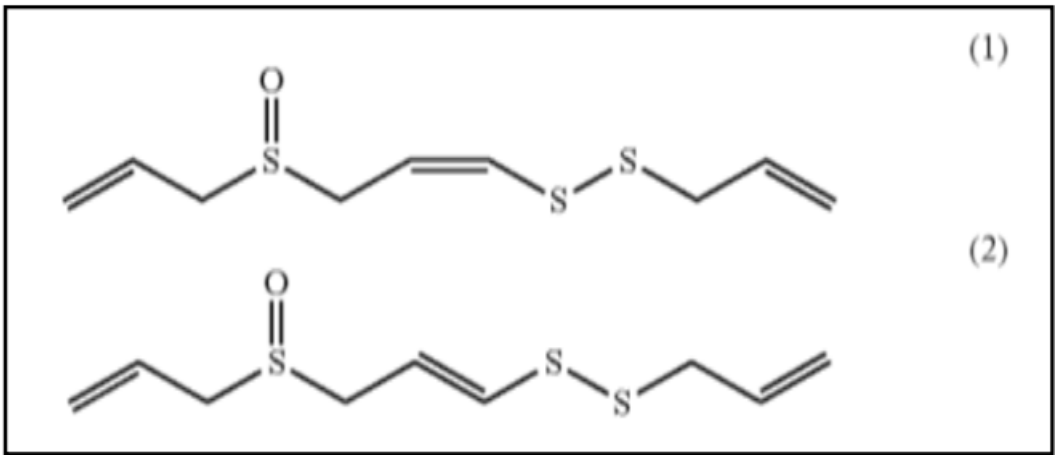


Figure 4.1 *E* and *Z* Isomers of Ajoene [132]

Properties

- Ajoene is a lipophilic compound, meaning it dissolves well in lipid-based environments.

- It has a characteristic odor and taste, which are typical of garlic-derived compounds.

- Ajoene is relatively stable under acidic conditions but can undergo hydrolysis in alkaline conditions.

Biological Activities

As explained in chapter II, and according to previous studies Ajoene was found to:

- Demonstrates antibacterial efficacy against a variety of pathogens, including as viruses, bacteria, and fungi. It disrupts microbial membranes and inhibits enzyme activity.

- It has antithrombotic properties, meaning it can inhibit blood clot formation.

- Ajoene has possible anticancer effect by causing cancer cells to undergo apoptosis or programmed cell death, and by preventing tumor development.

- It has been investigated for its potential in treating cardiovascular diseases and as an anti-inflammatory agent.

Overall, Ajoene represents an important bioactive compound derived from garlic with promising potential for various medical and therapeutic applications. To completely comprehend its modes of action and therapeutic potential, further investigation is recommended.

4.2 Yield of Ethanol Extract

The total yield of the Ajoene extract was 2.84 g which is count of 5.68 % of the total sample. This yield was obtained through extraction from the 50 g sample of freshly peeled cloves of Turkish garlic (Taşkopru sarımsağı) according to Yoshida et al. method of preparing the Ajoene-rich extract semi-solid fraction [128], as well as considering The preparation method of the garlic extract with enhanced Ajoene content of the Korean patent [133], which indicated a very good yield when compared to the Ajoene content of the garlic extract obtained through the standard extraction process which is count of 0.45%, this preparation method yielded a very high amount of Ajoene. Figure 4.2 shows the total yield of Ajoene after the extraction.

Figure 4.2 Yield of ethanol extract, upon taring

4.3 Gas Chromatography (GC) Measurements

The GCMS aromatic component analysis results of Ajoene extract done by the University of Harran, Science and Technology Application and Research Center (HUBTAM), was as presented in Figure 4.3, while the interpretation of this figure as follows:

The X-Axis: Retention Time:

The x-pivot of the gas chromatogram typically shows how long it takes the analytes to travel through the section and get to the mass spectrometer location. The peaks that are demonstrated indicate the time at which each component reaches the detector.

The Y-Axis: Concentration (Intensity Counts):

The amount of a given analyte is usually shown on the region of the peak, or the y-axis. The number of counts that the mass spectrometer detector at the site of retention makes when surveying a GC/MS chromatogram does not completely settle the area.

According to Table 4-1, GC-MS detection result was used to define the biological active compounds in Ajoene extract. A total of 70 compounds from Ajoene extract were found.

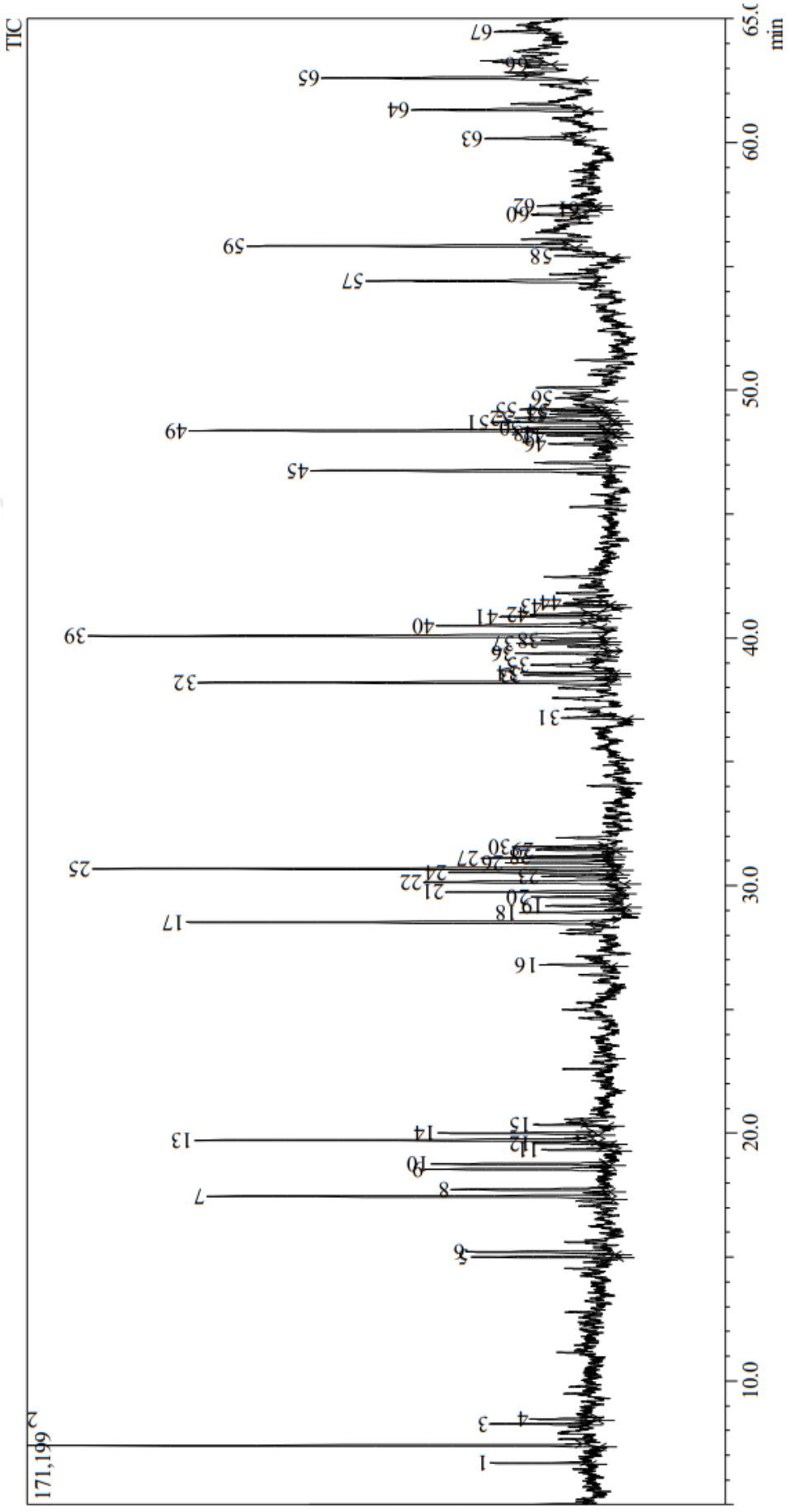


Figure 4.3 The GCMS aromatic component analysis result of Ajoene extract

Table 4.1 The Bioactive compounds of the GC-MS analysis result

Peak#	R.Time	Area	Area%	Name
1	6.689	60093	0.60	Nonane
2	7.392	354289	3.55	Acetate <octyl->
3	8.265	66405	0.67	Nonane
4	8.460	55904	0.56	Isothiocyanate <phenylethyl->
5	15.002	131143	1.31	Dodecane
6	15.211	145619	1.46	Dodecane
7	17.456	354309	3.55	Tridecane
8	17.733	146638	1.47	Tridecane
9	18.551	158116	1.58	Dihydrocitronellol
10	18.758	145425	1.46	Dihydrocitronellol
11	19.337	57704	0.58	Tridecane
12	19.596	65499	0.66	Tridecane
13	19.723	325740	3.26	Dodecane
14	20.006	139212	1.39	Tridecane
15	20.354	52574	0.53	Tridecane
16	26.804	58688	0.59	Tetradecane
17	28.523	396910	3.98	Octadecane
18	28.917	95580	0.96	Octadecane
19	29.177	84264	0.84	Heneicosane

Table 4.1 is continuing…

Peak#	R.Time	Area	Area%	Name
20	29.541	80236	0.80	Octadecane
21	29.746	163204	1.64	Tridecyl alcohol
22	30.139	252849	2.53	Tridecyl alcohol
23	30.376	58716	0.59	Heneicosane
24	30.537	172026	1.72	Pentadecanol
25	30.686	494435	4.95	Octadecane
26	30.921	94058	0.94	
27	31.108	137614	1.38	Heptadecane
28	31.180	55352	0.55	Octadecane
29	31.453	78617	0.79	Eicosane
30	31.567	88085	0.88	Octadecane
31	36.774	67410	0.68	Nonadecane
32	38.205	408754	4.10	Pentacosane
33	38.506	89307	0.89	
34	38.599	91180	0.91	Pentacosane
35	38.919	82860	0.83	Phenylacetate <methyl-, para-tert-butyl->
36	39.377	80595	0.81	Docosane
37	39.774	90027	0.90	Heptadecyl alcohol

Table 4.1 is continuing…

Peak#	R.Time	Area	Area%	Name
38	39.904	62306	0.62	Pentacosane
39	40.078	633083	6.34	Pentacosane
40	40.497	194407	1.95	Tetracosane
41	40.860	106709	1.07	Eicosane
42	40.930	65871	0.66	Neodene
43	41.285	52153	0.52	Heneicosane
44	41.416	48391	0.48	Tetracosane
45	46.757	310851	3.11	Pentacosane
46	47.836	65155	0.65	Pentadecanol
47	48.184	74428	0.75	Heptadecyl alcohol
48	48.280	71678	0.72	Pentacosane
49	48.374	447403	4.48	Pentacosane
50	48.489	79635	0.80	Pentacosane
51	48.710	116608	1.17	Pentacosane
52	48.886	163388	1.64	Heptadecyl alcohol
53	49.034	54219	0.54	Tetracosane
54	49.165	50914	0.51	
55	49.234	74092	0.74	Pentacosane
56	49.690	69382	0.70	Heptadecyl alcohol

Table 4.1 is continuing…

Peak#	R.Time	Area	Area%	Name
57	54.418	278318	2.79	Pentacosane
58	55.437	49595	0.50	Pentacosane
59	55.826	322752	3.23	Pentacosane
60	57.101	59589	0.60	Pentacosane
61	57.305	54149	0.54	
62	57.446	45557	0.46	Pentacosane
63	60.169	88786	0.89	Pentadecanolide
64	61.326	191898	1.92	Pentacosane
65	62.616	262609	2.63	Pentacosane
66	63.150	80368	0.81	
67	64.480	51319	0.51	Pentacosane
68	67.640	57057	0.57	Pentacosane
69	68.792	155374	1.56	Pentacosane
70	68.998	61885	0.62	Pentacosane
		9979366	100.00	

As can be seen from Figure 4.3 and Table 4.1, gas chromatography-mass spectrometry (GC-MS) analysis of the Ajoene extract revealed the presence of various bioactive components, with alkanes and phenols being the major constituents. A total of 70 chemicals were identified from the GC section of the Ajoene extract. Among these compounds, several significant ones were highlighted, including Nonane, Acetate <octyl->, thiocyanate <phenylethyl->, Dodecane, Dihydrocitronellol, Tridecane, Octadecane, Heneicosane, Tridecyl alcohol, Heptadecane, Pentadecanol, Pentacosane, Eicosane, and Phenylacetate.

Here's a summary of the major compounds identified along with their properties and potential applications:

The primary and predominant active ingredient in Ajoene extract was Pentacosane, with 6.34% and 40.078 Real Time, and it was detected in 21 areas with total sum up to 38.38%. Pentacosane, known as CH_3-$[CH_2]_{23}$-CH_3, belongs to the class of alkanes [134]. With the general formula C_nH_{2n+2}, they are acyclic branched or unbranched hydrocarbons made up only of hydrogen atoms and saturated carbon atoms [134]. The hydrocarbon lipid pentacosane molecule is almost insoluble in water due to its extreme hydrophobicity, and relatively neutral. It has a role as a semiochemical and a plant metabolite [134]. It has numerous benefits, including Antioxidant, and Antibacterial and Antifungal Activity [135].

The second biggest peak 4.95 % and 30.686 Real Time is the Octadecane, or CH_3-$[CH_2]_{16}$-CH_3, is an alkane [136]. These hydrocarbons can be branched or unbranched acyclic, and they all include hydrogen atoms and saturated carbon atoms, with the general formula C_nH_{2n+2} [136]. Octadecane is a molecule that is comparatively neutral, very insoluble in water, and extremely hydrophobic [136].

Consequently, octadecane is classified as a hydrocarbon lipid molecule [136]. Solvent, lubricant, transformer oil, and anti-corrosion compounds have all been reported to utilize it. Along with being a chemical intermediary in organic synthesis, it is also utilized in paraffin [137].

It's worth noting that alkanes are hydrocarbons consisting entirely of hydrogen and saturated carbon atoms. They are known for their hydrophobic nature and are often used in various industrial applications, as described for pentacosane and octadecane.

Phenols, another class of compounds identified, are aromatic compounds containing a hydroxyl group attached directly to a benzene ring. They are known for their

antioxidant and antimicrobial properties and are commonly found in natural products with medicinal potential [138].

Overall, the presence of these compounds in the Ajoene extract suggests its potential for various applications, including antioxidant, antibacterial, antifungal, and industrial uses, as highlighted by the properties and applications of the identified compounds.

Some of the most significant compounds have been recognized to have significant biological properties against certain diseases or bacterial infections, illustrated in Table 4-2 with references from the available research. These compounds may be responsible for the antibacterial activity and other properties of the Ajoene extract.

Table 4.2 Ajoene extract's identified compounds and their biological activity as reported in the researches

Name of the compounds (Acid)	**Biological properties**	**Available research**
Dodecanoic	Antibacterial, Antioxidant	[139,140]
Tetradecanoic	larvicidal properties	[141]
Pentadecanoic	Antioxidant	[135]
Hexadecanoic	Antibacterial	[142]
Octadecanoic	Antiviral activity	[143]

4.4 Fourier Transform Infrared (FTIR) spectroscopy Measurements

The FT-IR spectra of Ajoene extract were used in this investigation to analyze and validate the chemical structures of the chemicals present. Figure 4.4 (a) displays the Ajoene extract's FT-IR spectrum. In accordance with the Ajoene extract's chemical structure, all of the predicted peaks were identified. The chemical structures of Ajoene extract can be understood by interpreting these peaks as follows. The FT-IR shows distinct critical zones for each of the components in Ajoene extract. The first area spans 3716-3001 cm^{-1}. This is the O-H stretching zone from phenols, polysaccharides, and any other molecules containing OH, including Alliin. In this area, Ajoene extract has a very strong and wide band absorption. The primary, highly intense absorption peak may be found at 3269 cm^{-1}. The second range is from 2879 to 3000 cm^{-1}. This is the N-H stretching of the amino acid Alliin, which is generated from cysteine and other substances that include amine groups. At 2928 cm^{-1} is where the primary strong absorption peak is found. The range of the third area is 2881–2726 cm^{-1}. In aliphatic

alkenes, such (*E,Z*)-Ajoene and other aliphatic olefinic structures in the total compounds, this is the C-H vibrational stretching. At 2849 cm^{-1} is where the primary strong absorption peak is situated. The range of the fourth area is 2386-1933 cm^{-1}. This is the aliphatic alkane chain's C-H stretching peak, or -CH_2-. The faint peaks at 2287, 2084 and 1982 cm^{-1} are linked to the overall compounds' C-H stretching peak in the -CH_2-aliphatic alkane chain. The range of the fifth area is 1784–1512 cm^{-1}. In amino acids like Alliin and other molecules containing carbonyl groups, this is the vibrational stretching of the C=O carbonile group. The vibrational stretching of the C=O carbonile group in amino acids like Alliin and other chemicals containing carbonyl groups is linked to the prominent peak at 1611 cm^{-1}. The sixth area spans 1587–1475 cm^{-1}. In aliphatic complete olefinic structures, this is the C=C vibrational stretching. The C=C vibrational stretching in complete aliphatic olefinic structures is linked to the prominent peak at 1444 cm^{-1}.

The seventh area spans 1550–1397 cm^{-1}. In an aliphatic alkyl or alkane chain, this is the C-H bending mode. The faint peak at 1481 cm^{-1} is part of the total compounds' aliphatic alkyl or alkane chain's C-H bending mode. The eight area spans 1340–1130 cm^{-1} and 1370–1305 cm^{-1}. This is the stretching mode of the S=O vibration found in sulfones and sulfoxides. The molecular asymmetric stretching vibrations are demonstrated by the strong peak at 1401 cm^{-1}, and the S=O molecular symmetrical stretching vibrations are demonstrated by the medium strength peak at 1117 cm^{-1} in the structure of the molecule containing the sulfones and sulfoxides group. Region nine spans 1410–1215 cm^{-1}. This is how molecules containing hydroxyls bend O-H. The prominent peak at 1362 cm^{-1} is associated with the hydroxyl bending mode in substances that contain hydroxyl groups. The tenth area spans 1370–1190 cm^{-1}. This is the amine and amide compounds' C-N stretching peak. The C-N stretching peak of the amine compound Alliin and other compounds containing amine groups is linked to the medium strength and weak peaks at 1332 and 1251 cm^{-1}. The range of the eleventh area is 1158–952 cm^{-1}. This is the carboxylic acid's C–O asymmetric stretching vibration. The amino acid Alliin and other chemicals containing carboxyl groups are linked to the C–O asymmetric stretching vibration, which is responsible for the medium strength and strong peaks observed at 1115, 1103, and 1046 cm^{-1}. The range of the twelfth area is 975–565 cm^{-1}. This is the O-H bonds' out-of-plane bending. The out-of-plane bending of the O-H bonds is indicated by the strong and medium-strong peaks at 991, 924 and 817 cm^{-1}. The range of the thirteenth area is 885-710

cm^{-1} This represents the C-S stretching peak in compounds that contain C-S bonds. Allicin and (*E,Z*)-Ajoene, as well as other aliphatic alkenes, have a C-S stretching peak that is linked to the medium strength peaks at 852 and 761 cm^{-1}. Region fourteen spans 898–561 cm^{-1}. In 1* and 2* amines and amides, this is the N-H bending mode. The N-H bending mode in 1* and 2* amine and amide compounds is represented by the strong peaks at 672 and 598 cm^{-1}. The fifteenth area falls between 740 and 490 cm^{-1}. This is the peak of S-S stretching in compounds that contain S-S bonds. Strong peaks at 519 and 507 cm^{-1} correspond to the S-S stretching peak found in compounds Allicin and (*E,Z*)-Ajoene, which are aliphatic alkenes. The final area spans 690–430 cm^{-1}. This stretching vibration is that of an alkyl halide. Throughout the entire complex, the alkyl halide stretching vibration is responsible for the faint peaks at 495, 465, 427 and 415 cm^{-1}.

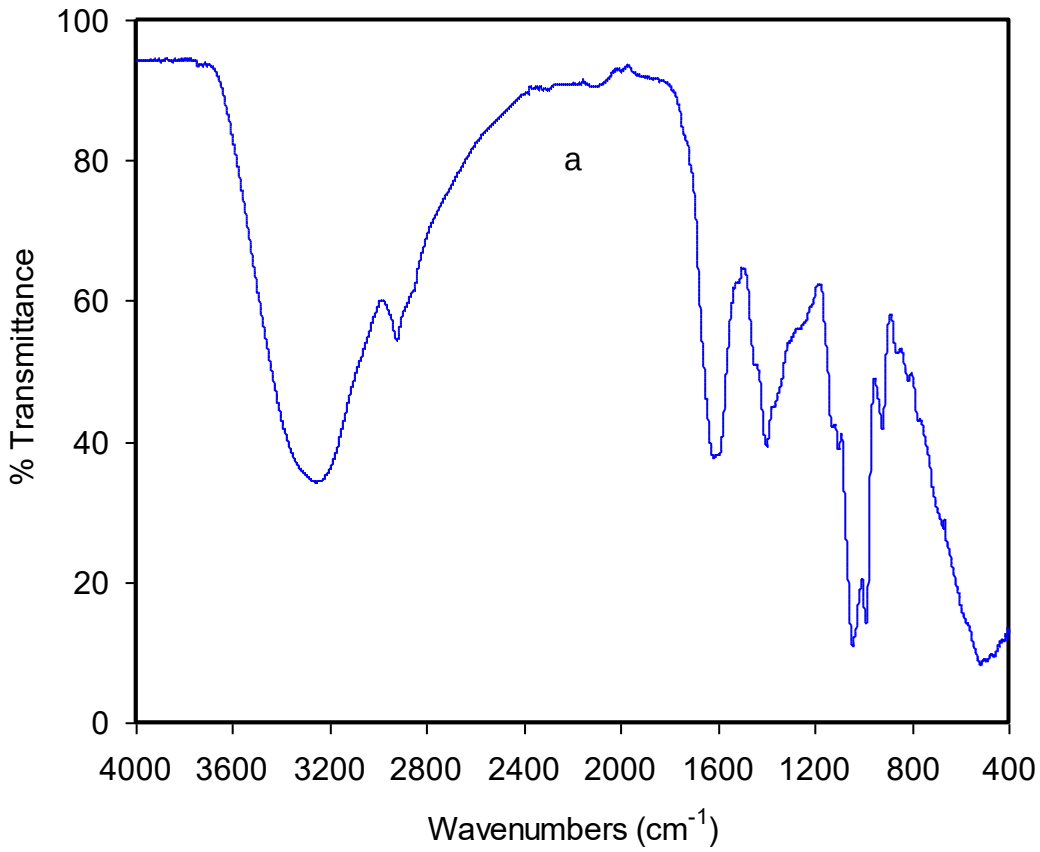


Figure 4.4 FT-IR spectra of (a) Ajoene extract

Table 4.3 provides a summary of all the FT-IR data for the Ajoene extract that has been seen.

Table 4.3 FT-IR data of Ajoene extract

Bond (Type of compound)	**Reference of Wavenumber (cm^{-1}) [144]**	**Observed Wavenumber (cm^{-1})**	**Intensity**
O-H band	3760-3010 (Stretch)	3716-3001 3269	s
N-H band (Amin, Amid)	3510-3900 (Stretch)	3000-2879 2928	s
C-H bond (aliphatic, alkene)	3050-2700 (Stretch)	2881-2726 2849	s
C-H bond (-CH_2- aliphatic, alkane)	2410-2000 (Stretch)	2386-1933 2287, 2084, 1982	w
C=O bond (carbonyl group)	1725-1580 (Stretch)	1784-1512 1611	s
C=C bond (aliphatic, alkene)	1685-1605 (Stretch)	1587-1475 1444	s
C-H bond (aliphatic, alkyl or alkane)	1480-1390 (Bend)	1550-1397 1481	w
S=O (sulfones, sulfoxides)	2 bonds: 1375-1300 ~1300 Asymmetric; 1350-1140 ~1150 Symmetric (Molecular vibration stretch)	1370-1305 1401 Asymmetric; 1340-1130 1117 Symmetric (Molecular vibration stretch)	s m
O-H bond	1420-1120 (Bend)	1410-1215 1362	s
C-N bond (amine, amide)	1385-1175 (Stretch)	1370-1190 1332, 1251	m,w
C-O bond	1205-955 (Asymmetric stretch)	1158-952 1115, 1103, 1046	m,s
O-H bond	960-660 (Out-of-plane bending)	975-565 991, 924, 817	s,m
C-S bond	900-700 (Stretch)	885-710 852, 761	m
N-H bond (1^* and 2^* amines, amides)	890-555 (Bend)	898-561 672, 598	s
S-S bond	750-500 (Stretch)	740-490 519, 507	s
Alkyl halide	700-450 (Stretch)	690-430 495, 465, 427, 415	w

s: strong, m: medium, w: weak

4.5 Ultraviolet-Visible Spectrophotometer (UV-Vis) Measurements

In this research, a spectrophotometric grade methanol (CH_3OH) solvent was used to analyse the UV-Vis spectra of an Ajoene extract and vitamin C as a standard.

The Ajoene extract's absorbance was identified at 286 nm in methanol (CH_3OH) solvent, which has a 5.1 polarity index. The extract chemical structures exhibit a π-$\pi*$ transition, which is responsible for the absorption observed at 286 nm in spectra. As a result, the existence of a single peak in the Ajoene extract CH_3OH solution indicates the presence of pure extract chemical structures. Along with the primary peak at 286 nm, the resulting Ajoene extract also displayed three prominent shoulder peaks at around 292, 323 and 445 nm.

The spectra of the Ajoene extract show that, as would be predicted, as the sample concentrations grew, so did the intensity of the π-$\pi*$ transition peak. Additionally, as the sample concentration rose, the maximum absorption wavelengths of the sample marginally moved to higher absorption wavelengths (from 282 nm to 286 nm). This is seen in Figure 4.5.

These findings suggested that the sample's maximum absorption wavelengths were somewhat influenced by its concentrations.

Figure 4.5 displays the UV-Vis spectra of the Ajoene extract at various concentrations.

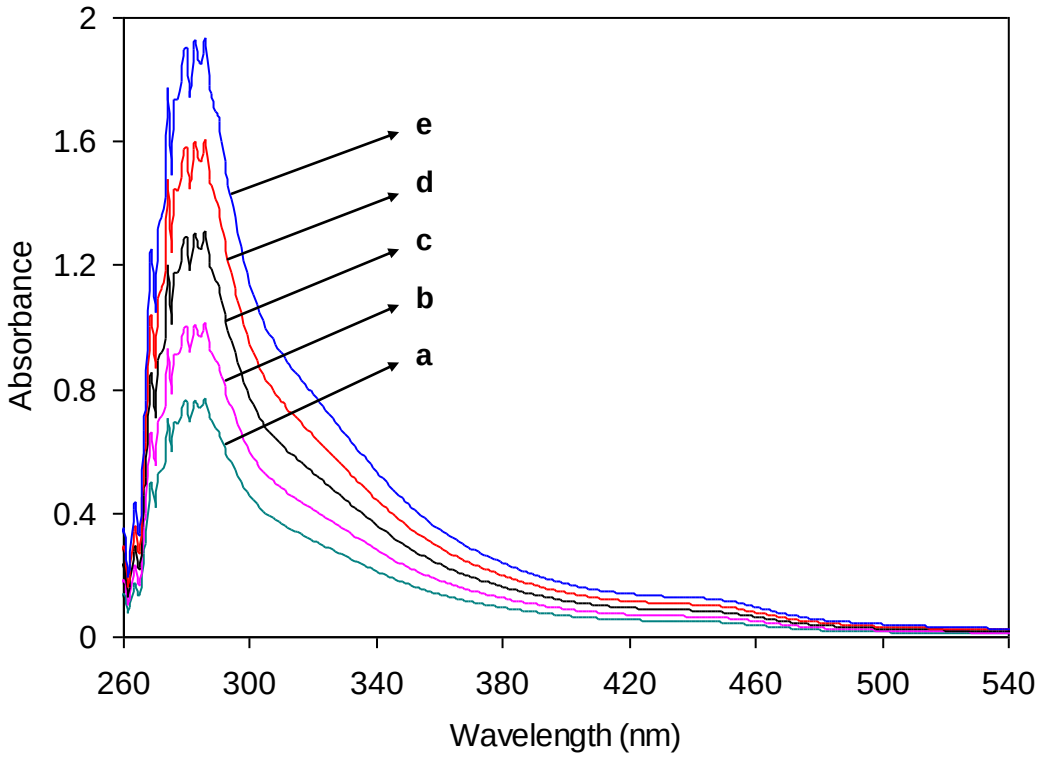


Figure 4.5 Ajoene extract's UV-Vis spectra at various concentrations: 83.30, 249.90, 416.50, 583.10 and 749.70 µg/mL

The UV-Vis data of Ajoene extract have been summarized in the Table 4-4 which includes the maximum absorption intensity, maximum absorption wavelength, and molar absorptivity coefficient of each tasted Ajoene extract samples' concentrations.

Table 4.4 Ajoene extract's UV-Vis data

Concentration (µg/mL)	**ε (mL µg^{-1} cm^{-1})**	**λ_{max} Abs (nm)**	**In Abs**
a) 83.30	9.05x10^{-3}	282	0.754
b) 249.90	3.98x10^{-3}	283	0.995
c) 416.50	3.08x10^{-3}	284	1.284
d) 583.10	2.74x10^{-3}	285	1.599
e) 749.70	2.57x10^{-3}	286	1.926

λ_{max} Abs: maximum absorption wavelength, In Abs: maximum absorption intensity, ε: molar absorptivity coefficient

Figure 4.6 displays the UV-Vis spectra of vitamin C as a standard at various concentrations. For conventional Vitamin C, an absorption may be identified at 272 nm in methanol (CH_3OH) solvent, which has a 5.1 polarity index. The π-π^* transition in the vitamin C molecular structure is the main cuase for the absorption at 272 nm in spectra. In the CH_3OH solution of vitamin C, the existence of a single peak so suggests the presence of pure compound chemical structures. The strength of the π-π^* transition peak went up as the sample concentrations increased, as predicted, according to the vitamin C spectrum.

Additionally, as the sample concentration went up, its maximum absorption wavelengths moved to higher absorption wavelengths (from 269 nm to 272 nm). This is seen in Figure 4.6. These findings suggested that the sample's maximum absorption wavelengths were somewhat influenced by its concentrations.

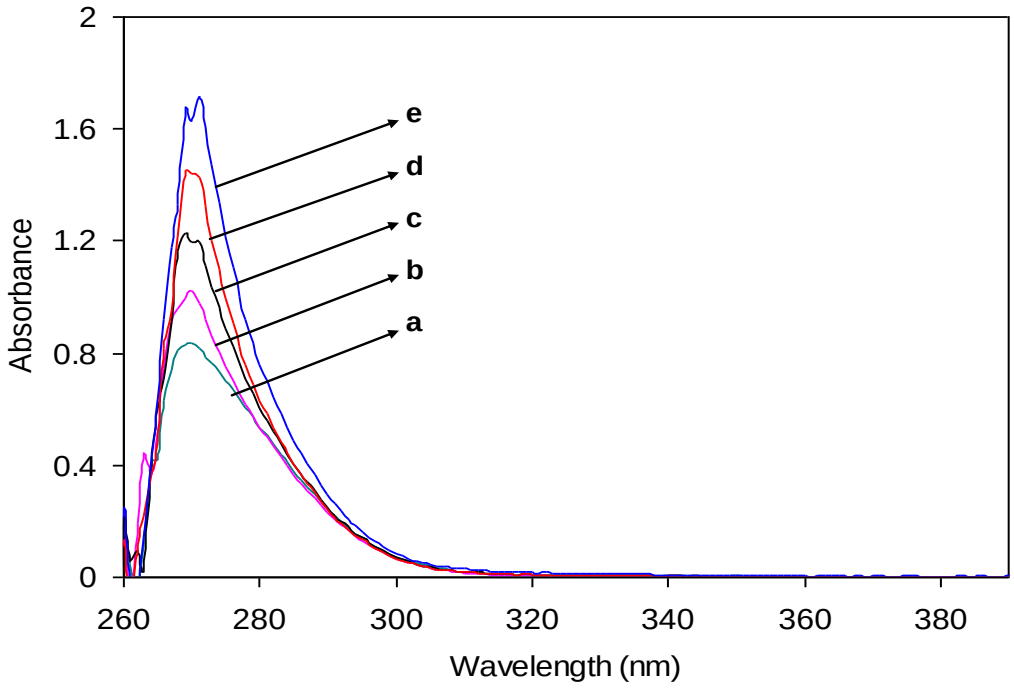


Figure 4.6 UV-Vis spectra of Vitamin C as a standard at different concentrations; a) 92.61, b) 185.22, c) 277.83, d) 370.44 and e) 463.06 µg/mL

In Table 4.5, the UV-Vis statistics for Vitamin C are compiled. As the sample concentrations increased the values of the molar absorptivity coefficient dropped. This decline can be attributed to the sample's molar absorption capacity being negatively impacted by the sample's concentration increase.

Table 4.5 Vitamin C's UV-Vis data as a standard

Concentration (µg/mL)	**ε (mL μg^{-1} cm^{-1})**	**λ_{max} Abs (nm)**	**In Abs**
a) 92.61	$8.97x10^{-3}$	269	0.831
b) 185.22	$5.52x10^{-3}$	270	1.023
c) 277.83	$4.42x10^{-3}$	270	1.227
d) 370.44	$3.92x10^{-3}$	271	1.451
e) 463.06	$3.71x10^{-3}$	272	1.716

λ_{max} Abs: maximum absorption wavelength, In Abs: maximum absorption intensity, ε: molar absorptivity coefficient

4.6 Photoluminescence Spectrometer (PL) Measurements

In medical science, photoluminescence is a vital instrument that is frequently employed. For potential applications in medical imaging and applications, the photo physical characteristics of the extracts were investigated. For the solutions of Ajoene extract (a), stimulated at 276 nm, the absorption and photoluminescence spectra were examined.

One noteworthy observation was that the Ajoene extract exhibited strong emission when exposed to UV light. Figure 4.7 displays the Ajoene extract (a) photoluminescence spectra in spectrophotometric grade methanol (CH_3OH). For the Ajoene extract (a), a strong maximum luminous intensity was detected at 373 nm, while the entire width at half maximum was recorded at 107 nm. Ajoene extract (a) demonstrated an excited-state lifetime of 3.73 ns and a photoluminescence quantum yield of 40%.

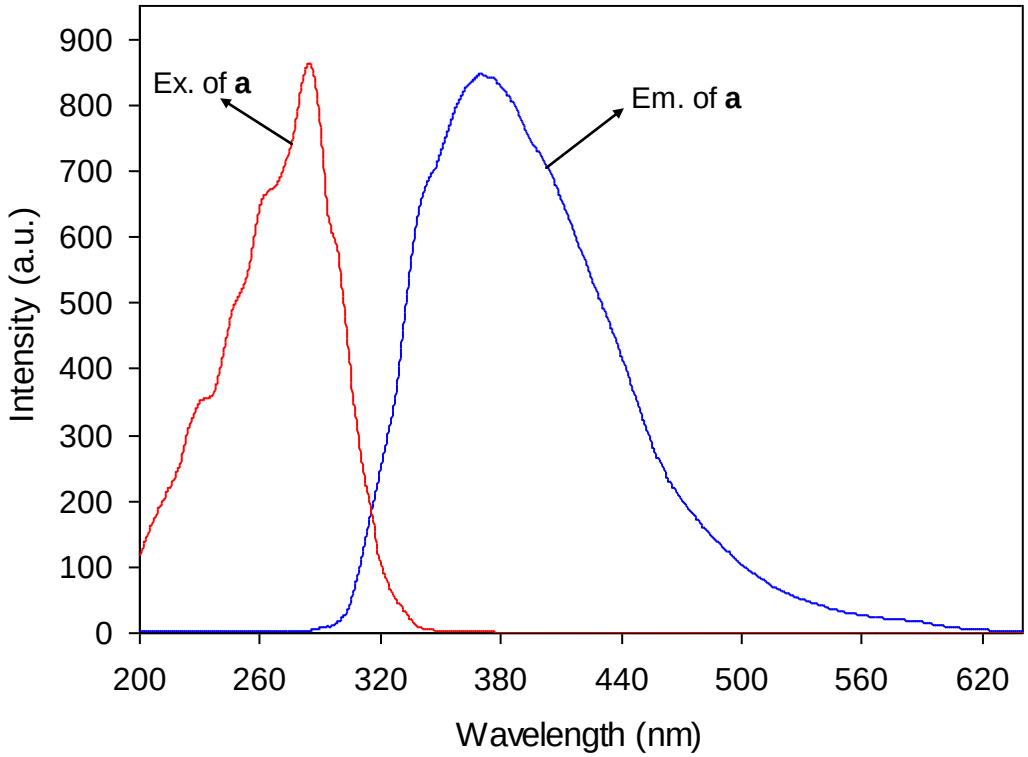


Figure 4.7 The samples were stimulated at 276 nm in order to obtain the photoluminescence spectra of the Ajoene extract (a) in spectrophotometric grade methanol (CH_3OH)

Additionally, various excitation wavelengths for the Ajoene extract's fluorescence behaviour were investigated. The observation that the Ajoene extract's fluorescence behavior was independent of the excitation wavelength provides evidence for the well-known Kasha's rule of excitation wavelength independence, that defines that emission originates from the initial electronic state's lowest vibrational level [145].

Figure 4.8 shows the emission spectra of an Ajoene extract (a) in CH_3OH at various excited wavelengths as an example.

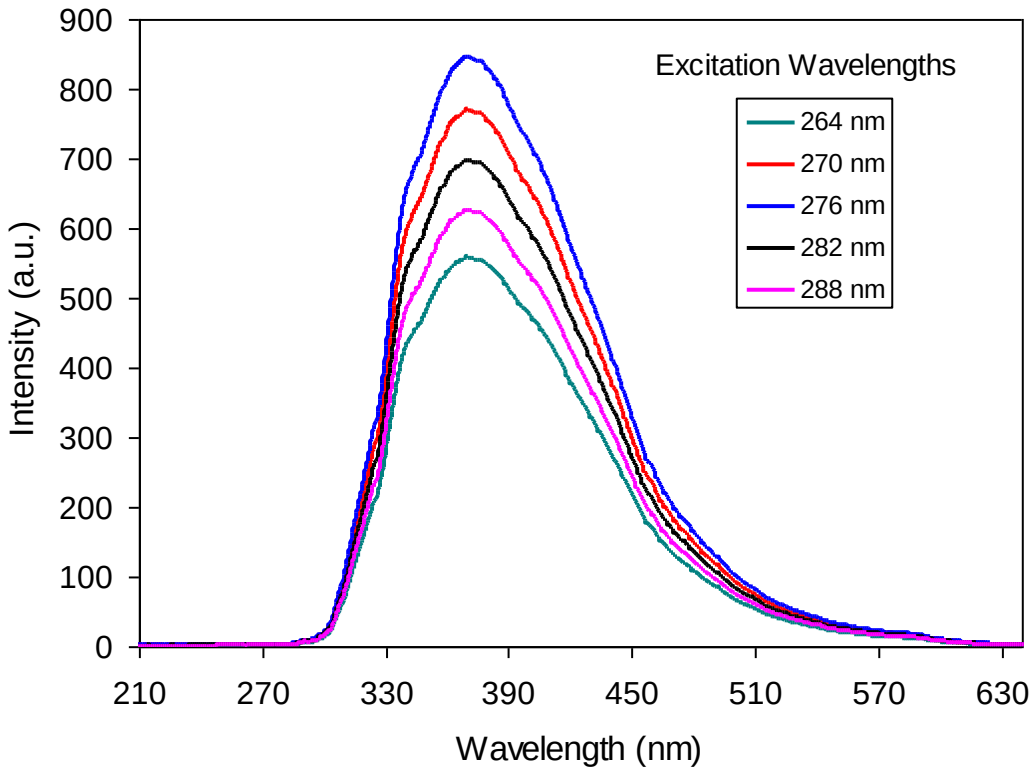


Figure 4.8 Ajoene extract's emission spectra at various stimulated wavelengths in spectrophotometric grade methanol (CH3OH) (c = 749.70 µg/mL)

Table 4.6 provides a summary of the photoluminescence data for the Ajoene extract (a). This extract's photoluminescent qualities may suggest that it has a lot of promise for use in photophysical and medical applications. Table 4.6 also provides the Stoke's shift values (ν_{ss}) for the Ajoene extract. Ajoene extract (a) has an emission maximum (λ_{max}) of 373 nm and a Stoke's shift (ν_{ss}) of approximately ~3741 cm^{-1}, or 87 nm. The extraction with high Stoke's shift value has been linked to intermolecular excimer production. This significant Stoke's shift indicates that the excited molecule's structural relaxation reflects the altered molecular conformation following excitation.

Table 4.6 Photoluminescence data for the Ajoene extract (a)

Compound	λ_{max} Ex (nm)	In Ex	λ_{max} Em (nm)	In Em	ϕ_f (%)	τ_f (ns)	ν_{ss} (cm^{-1})
a	286 (235;267;299)	854	373 (342;406;443)	842	40	3.73	3741

λ_{max} Ex: maximum excitation wavelength; In Ex: maximum excitation intensity;
λ_{max} Em: maximum emission wavelength; In Em: maximum emission intensity;
ϕ_f : quantum yield; τ_f : excited-state lifetime; ν_{ss}: Stoke shift.

4.7 Antioxidant Properties

The DPPH test was conducted to ascertain the Ajoene extract's ability to scavenge free radicals, using the standard ascorbic acid (Vitamin C). The percentage of anti-oxidant activity in the Ajoene extract produced by extraction was demonstrated in the DPPH test at concentrations of 83.30, 249.90, 416.50, 583.10 and 749.70 µg/mL (Figure 4.9). The anti-oxidant activity of the Ajoene extract produced using Yoshida et al. [128] method of preparing the Ajoene-rich extract varied from 11.24 ±1.05% to 93.94 ±1.03%. At a concentration of 749.70 µg/mL, the extraction of Ajoene yielded the best anti-oxidant activity. Based on regression analysis, the IC_{50} value of the Ajoene extract was 414.91 ± 2.17 µg/mL (Figure 4.9).

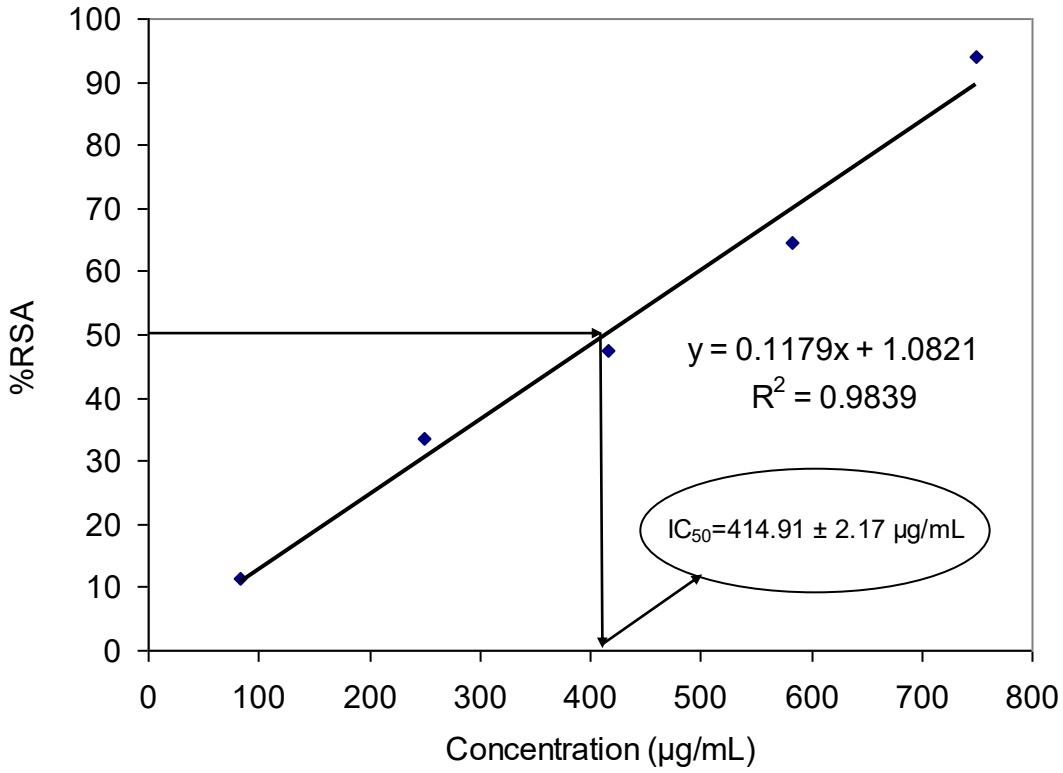


Figure 4.9 The free radical scavenging ability of the Ajoene extract at 83.30, 249.90, 416.50, 583.10 and 749.70 µg/mL concentrations, which was used to calculate the percentage of antioxidant activity. Using regression analysis and data from a separate experiment

UV-Vis spectroscopy was also used in this investigation to track the development of the antioxidant activity of the Ajoene extract at various doses. Figure 4.10 displays the UV-Vis spectral progression of the antioxidant activity of the Ajoene extract at various doses. Spectrum a in Figure 4.10 stands for a pure DPPH solution, while The reaction between the DPPH solution and the Ajoene extract at various doses is shown in spectra b–f.

Two absorptions for the DPPH solution can be identified at 329 and 515 nm in methanol (CH_3OH) solvent, which has a 5.1 polarity index. The stable violet DPPH (2, 2-diphenyl-1-picryl-hydrazyl) radical form is attributed to the absorption at 515 nm in this spectrum, whereas the yellowish reduced form ($DPPH/H^+$) is linked to the absorption at 329 nm. Consequently, the existence of two peaks in the Ajoene extract CH_3OH solution.

The Ajoene extract at varying doses reacts with the DPPH solution as demonstrated by spectra b-f in Figure 4.10 Simultaneously, there was a noticeable transformation of the stable violet DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical into a yellowish reduced form ($DPPH/H^+$). It is evident from the DPPH solution's spectrum that when sample concentrations rose, the peak at 515 nm's strength fell and the peak at 329 nm's intensity grew. These spectral data thus demonstrated the obvious transformation of the stable violet DPPH radical into a reduced state that is yellowish ($DPPH/H^+$).

Moreover, the spectra show that the Ajoene extract derived by means of the π-π^* transition peak at 286 nm was the source of its intensity.

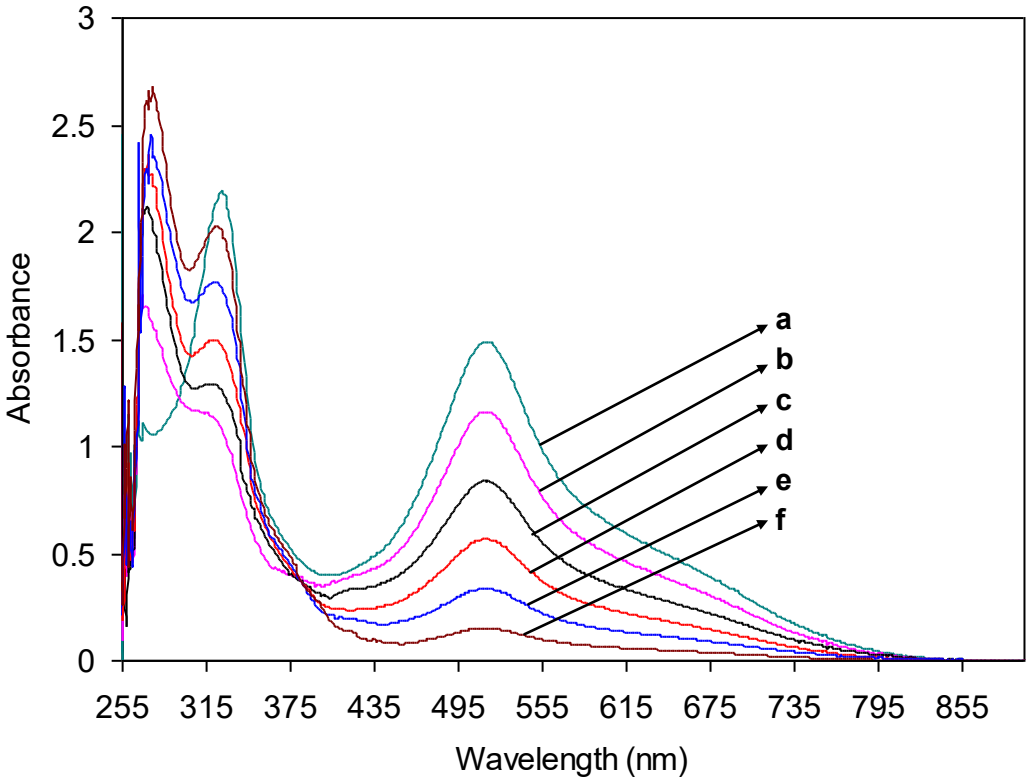


Figure 4.10 The antioxidant activity of the Ajoene extract at various doses of 83.30, 249.90, 416.50, 583.10 and 749.70 µg/mL was measured using the UV-Vis spectroscopy

The DPPH experiment was used to establish ascorbic acid's (Vitamin C) baseline free radical scavenging activity. Ascorbic acid (Vitamin C) demonstrated the percentage of anti-oxidant activity in the DPPH assay at concentrations of 92.61, 185.22, 277.83, 370.44 and 463.06 µg/mL. Figure 4.11 displays the ascorbic acid (Vitamin C) percentage of anti-oxidant action at various doses. the percentage of antioxidant activity was assessed. Using regression analysis and data from a separate experiment, the IC_{50} value was determined using MS Excel 2010. The mean ±SD of three independent experiments was used to express the values. The antioxidant activity of ascorbic acid (Vitamin C) (Standard), the positive control, ranged from 23.92 ±1.04% to 99.21 ±1.02%. At a concentration of 463.06 µg/mL, The vitamin C showed the greatest amount of antioxidant activity. Regression analysis yielded an IC_{50} value of 205.71 ± 1.13 µg/mL for ascorbic acid (Vitamin C) (Figure 4.11).

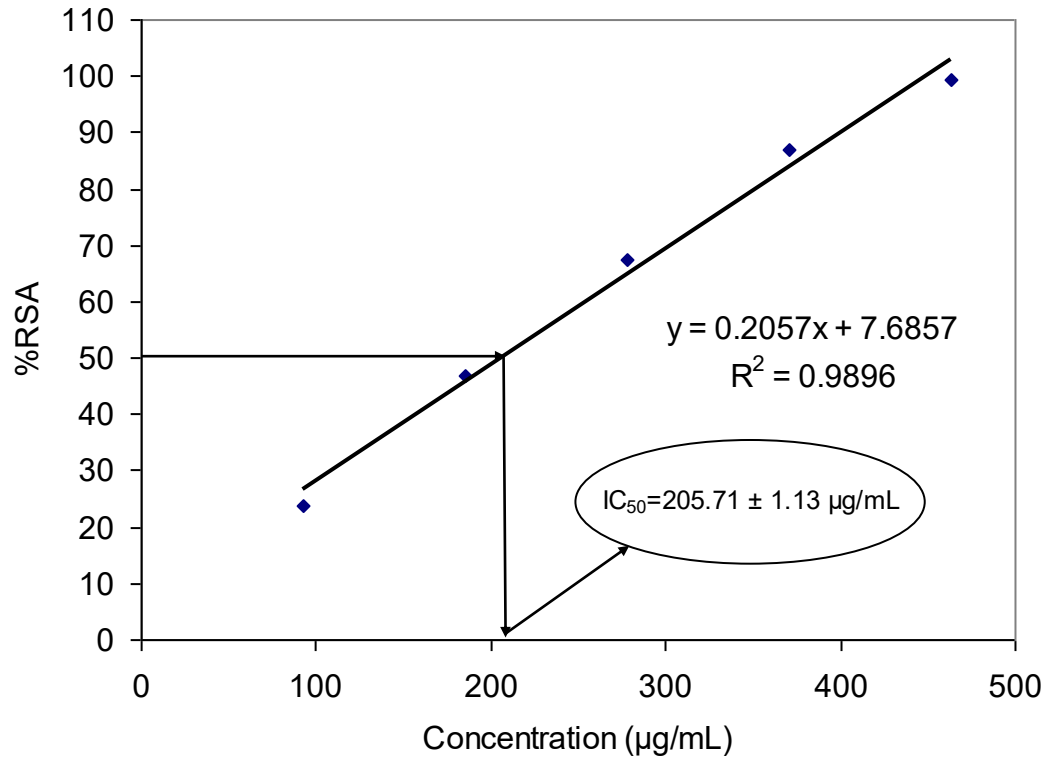


Figure 4.11 Using ascorbic acid (Vitamin C) as a standard at various concentrations of 92.61, 185.22, 277.83, 370.44 and 463.06 µg/mL

UV-Vis spectroscopy was also used in this investigation to track the development of ascorbic acid (Vitamin C) as a standard at various concentrations for antioxidant activity. Figure 4.12 displays the UV-Vis spectral progression of ascorbic acid (Vitamin C) anti-oxidant action at various doses. Ascorbic acid, or vitamin C, at varying doses, reacts with the DPPH solution, as demonstrated by spectra b–f in Figure 4.12. Spectrum a depicts the pure DPPH solution, and The reaction between ascorbic acid (Vitamin C) at various doses and the DPPH solution is represented by spectra b–f. Simultaneously, there was a noticeable transformation of the stable violet DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical into a yellowish reduced form (DPPH/H+). It is evident from the DPPH solution's spectrum that the peak at 515 nm had a decrease in intensity while the peak at 329 nm had a rise. Subsequently, these otherworldly outcomes showed the reasonable transformation of the steady violet DPPH revolutionary into a yellowish decreased structure (DPPH/H+). In addition, the spectra reveal that the intensity of the π-π^* transition peak at 272 nm, which belonged to vitamin C, gradually increased with sample concentration.

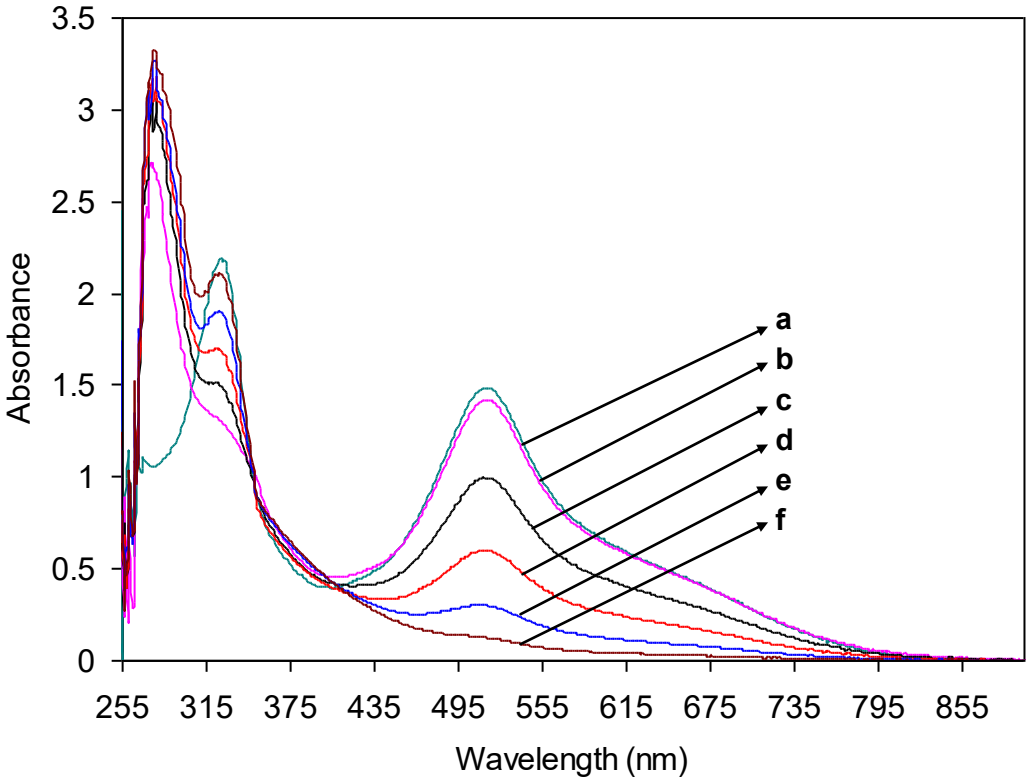


Figure 4.12 The anti-oxidant activity of ascorbic acid (Vitamin C) was measured using the UV-Vis spectroscopy at various concentrations: 92.61, 185.22, 277.83, 370.44 and 463.06 µg/mL

The present study's DPPH scavenging assay result showed that the extract of Ajoene had promising activity. The Ajoene extract's IC_{50} values were found to be 414.91 ± 2.17 µg/mL. Ajoene extract was indicated to possess the greatest activity as antioxidant, with a value of 93.94 ±1.03%, suggesting that it had greater antioxidant activity. Accordingly, the study's findings imply that chemicals found in Ajoene extract are able to give hydrogen to a free radical in order to remove an odd electron, which is what gives a radical its reactivity. This matches what was mentioned in research that the levels of antioxidant qualities in *E*- and *Z*-Ajoene vary, and they both have the ability to scavenge hydroxyl and DPPH radicals. The concentration may affect these antioxidative benefits [146]. The antioxidant activity of Ajoene extract has several positive effects on human health in general.

The DPPH radical scavenging activity results in a study comparing aged and non-aged garlic extracts, including distilled water, ethanol, and chloroform, indicated a declining trend according to the extraction solvent (distilled water > ethanol > chloroform) [147]. In comparison to the non-aged garlic group, the aged garlic group's DPPH radical scavenging properties were considerably greater when extracted using distilled water, ethanol, and chloroform [147]. The outcomes of the DPPH radical scavenging test and the ABTS radical cation scavenging activity were comparable [147]. In other words, the maximum activity was found in the distilled water extract of aged garlic, and the group of aged garlic exhibited higher activity than the non-aged garlic group [147]. Consequently, compared to the non-aged garlic group, the aged garlic group showed higher radical scavenging activities for DPPH and ABTS, with the aged garlic distilled water extract demonstrating the highest activity [147]. In comparison to fresh garlic, previous study data has indicated that aged garlic has higher concentrations of phenol, flavonoids, and other Sulphur compounds such S-allyl-(l)-cysteine (SAC, hydrophilic) and disulfide (hydrophobic). On top of that, SAC has strong radical scavenging properties [148, 149].

Another study on cooked garlic and antioxidant activity, conducted by Locatelli DA, showed that samples of cooked garlic demonstrated a certain level of antioxidant activity against all evaluated techniques [26]. In addition to iron ion reduction by hydrogen atom or electron transfer, the scavenging of free radicals (DPPH and $ABTS^{+}$) is the mode of action that best describes the antioxidant activity of raw garlic samples [26]. The pro-oxidant enzyme's primary mechanism of action for stir-fried samples was discovered to be its capacity to prevent radical chain propagation processes in addition to its inhibitory activity [26].

Table 4-7 summarises the data on antioxidant activity and the linear correlation coefficients determined by the samples' capacity to scavenge free radicals.

Table 4.7 The data on antioxidant activity and the linear correlation coefficients assessed based on the samples' ability to scavenge free radicals

Sample	Slope	Intercept	R^2	Anti-oxidant activity range	IC_{50} value
Ajoene	0.1179	1.0821	0.9839	11.24 ±1.05% - 93.94 ±1.03%	414.91 ± 2.17 µg/mL
Ascorbic acid	0.2057	7.6857	0.9896	23.92 ±1.04% - 99.21 ±1.02%	205.71 ± 1.13 µg/mL

Ajoene: Ajoene extract, and ascorbic acid (Vitamin C) as a standard.

4.7.1 Intermolecular and Chemical Bonding Interactions for Ajoene Extract as Antioxidant Agent

Utilizing a variety of radical scavenging effects, previous research examined the *E*- and *Z*-Ajoene prepared from garlic's antioxidative properties. The DPPH radical was lowered by the tested *Z*- and *E*-Ajoene in a concentration-dependent response. Compared to *E*-Ajoene, *Z*-Ajoene was more effective. The reduction of DPPH radicals could refer to the sulfinyl functional group. Of all the hydrogen atoms in Ajoene, those bound to the core double-bonded carbon atoms have the most positive excess charge (about +0.15e); in a proton transfer process, it is anticipated that these hydrogen atoms will leave the molecule first. This might have something to do with possible antioxidant action [150]. Ajoene was 5–11 times less effective than the more widely used antioxidant α-tocopherol (1 mM). The lack of reactivity of the sulfinyl functional group with the DPPH radical and/or the relative oxidation-reduction potentials of the alkenyl groups in comparison to the respective α-tocopherol may have contributed to the Ajoene's ineffectiveness in the DPPH radical quenching assay [151]. The inhibitory properties of *E*-and *Z*-Ajoene against OH^+ matched of those of the normal cell reinforcement α-tocopherol tried. The scavenging of OH^+ is based on the functional unit, as evidenced by the higher effectiveness of *E*- and *Z*-Ajoene (61 percent and 63 percent, respectively). Since OH^+ reacts with almost all organic compounds at or close to the rate of diffusion [152], it should come as no surprise that the components were evaluated for higher inhibitory effects. Based on the inhibition of NBT reduction, very little O_2 scavenging activity was observed for *E*- and *Z*-Ajoene. After 40 minutes of incubation, 1mM *E*- and *Z*-Ajoene reduced NBT by 1.9% and 4.8%, respectively. However, antioxidant activity was absent after 80 minutes of incubation. Ajoene may scavenge oxygen and produce oxygen. After incubation times of 80 and 120 minutes, no antioxidant effect was possibly observed. In Ajoene, there is a large positive charge improvement (about +1.4e) on the sulfur atom bonded to oxygen; the other two sulfur particles generally have less over abundance charge. Oxygen atom has a negative abundance charge of about - 0.8e, which is the main atom in the Ajoene having an alluring potential. Oxygen atoms in Ajoene may assume in significant part in the collaboration of Ajoene and its environment [150].

Research showed that *E*-and *Z*-Ajoene have different degrees of cell reinforcement properties, including rummaging exercises for DPPH revolutionaries and hydroxyl revolutionaries. These antioxidative impacts might fluctuate with the fixation. The

antioxidative activity generally increased with concentration within a certain concentration range. In addition, Ajoene with a low concentration scavenged more DPPH and OH^+ radicals than with a higher concentration. These *E*- and *Z*-Ajoenes could be used in the pharmaceutical industry, particularly in areas where garlic and its derivatives are utilized, as a potential food supplement, as a source of antioxidants, or both.

4.8 Antibacterial Activity Measurements

Ajoene has been extensively studied for its antimicrobial properties, including antibacterial activity. Researches suggests that Ajoene exhibits inhibitory effects against a variety of bacterial strains, making it a potential candidate for the development of novel antibacterial agents. Here are some key points regarding Ajoene's antibacterial activity:

Broad Spectrum Activity: Ajoene has been found to possess antibacterial activity against both Gram-positive and Gram-negative bacteria.

Mechanism of Action: The exact mechanism by which Ajoene exerts its antibacterial effects is not fully understood. However, studies suggest that it may interfere with bacterial cell membrane integrity, disrupt cellular processes, and inhibit enzyme activities crucial for bacterial survival.

Overall, Ajoene's antibacterial activity underscores its potential as a natural antimicrobial agent. This study may lead to the development of new strategies for combating bacterial infections, particularly those that are resistant to existing antibiotics. Where different Concentrations of Ajoene Extract prepared from Ethanol Extract of Garlic Plant were tested for their potential Antibacterial activity against *Stenotrophomonas maltophilia*, Figure 4.13 shows the various concentrations of Ajoene extract tested against the proliferation of *Stenotrophomonas maltophilia.*

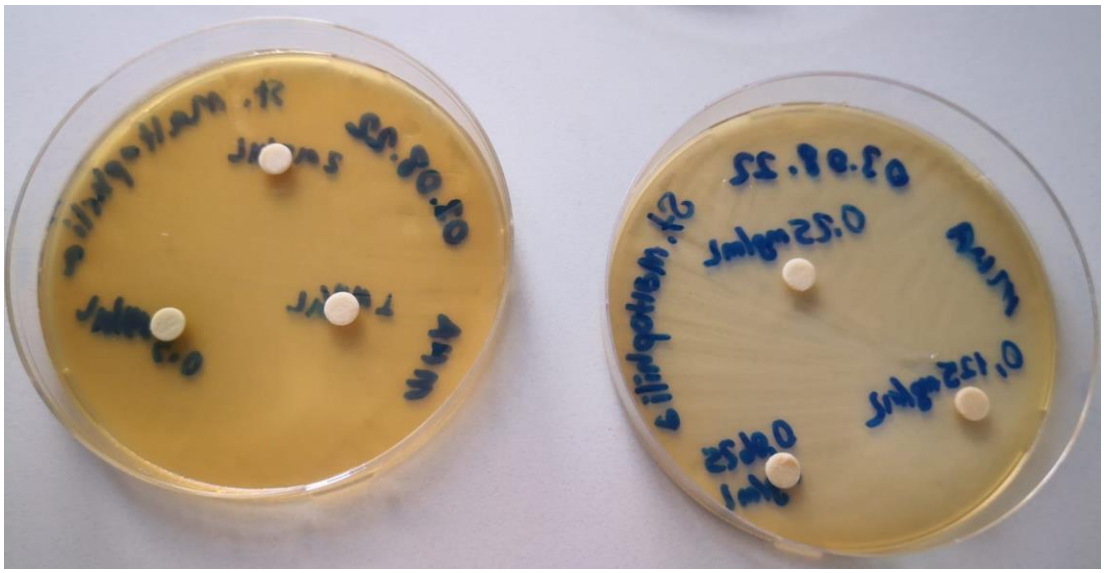


Figure 4.13 Different Concentrations of Ajoene Extract tested for Antibacterial activity against the proliferation of *Stenotrophomonas maltophilia*

The antibacterial activity against the proliferation of *Stenotrophomonas maltophilia* was observed at concentrations of 3, 4, and 5 mg/ml as Figure 4.14 indicated.

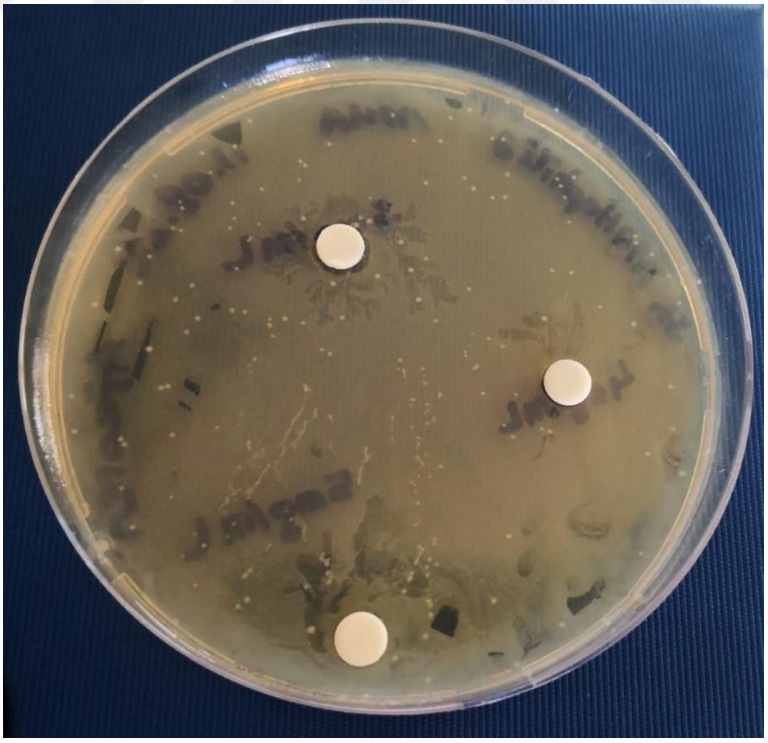


Figure 4.14 Positive indication for Antibacterial activity against the proliferation of *Stenotrophomonas maltophilia*

Where the whole antibacterial test result for all the concentrations of Ajoene extract was tested was demonstrated in details in Table 4.8.

Table 4.8 Antimicrobial Test Results

Microorganism name	Product	C (mg/ml)	Zone Diameter (mm)	Zone Diameter Rating (mm)
Stenotrophomonas maltophilia	Ajoene	5	8	>50 => S; <16 => R
		4	5	
		3	3	
		2	0	
		1	0	
		0,5	0	
		0,25	0	
		0,125	0	
		0,0625	0	
	Sulfamethoxazole	0.025	23	I

(C): Concentration, (S): Sensitive, (R): Resistant, (I): Variable.

As demonstrated in Table 4.8, this experiment showed that "Ajoene" ethanol extract tested at different concentrations starting from 0.0625 mg/ml up to 5 mg/ml at 37^{o} C, 18±2 hours after incubation, had some bactericidal effect on the hospital strain *Stenotrophomonas maltophilia*, where this activity was found at concentrations of 3, 4, and 5 mg/ml, with 8 mm diameter of inhibition zone at 5 mg/mL concentration, and it became limited at lower concentrations.

Ajoene, with its demonstrated antimicrobial activity against a broad spectrum of bacteria, including antibiotic-resistant strains, presents an attractive option for drug development. Its ability to inhibit the growth of bacteria and disrupt biofilm formation makes it a promising candidate for combating various infectious diseases caused by resistant pathogens.

Furthermore, the versatility of Ajoene in potentially serving as a precursor for the synthesis of novel antimicrobial agents adds to its appeal. By harnessing the chemical structure and properties of Ajoene, researchers can explore modifications and derivatizations to enhance its potency, improve bioavailability, and optimize pharmacokinetic properties.

Moreover, the use of natural compounds like Ajoene for drug development offers advantages such as reduced toxicity and side effects compared to synthetic drugs.

Additionally, the abundance and accessibility of garlic make Ajoene a cost-effective and sustainable source for pharmaceutical applications.

As research in this area progresses, further studies are needed to fully elucidate the mechanisms of action of Ajoene and optimize its therapeutic potential. Collaboration between researchers in various disciplines, including chemistry, microbiology, pharmacology, and clinical medicine, will be essential for translating the promising findings from preclinical studies into clinically relevant antimicrobial agents.

In conclusion, the exploration of Ajoene and other garlic-derived bioactive compounds as potential antimicrobial agents represents a promising avenue for addressing the global challenge of antibiotic resistance and improving public health outcomes.

4.8.1 Intermolecular and Chemical Bonding Interactions for Ajoene Extract as Antimicrobial Agent

The structures of various garlic-derived hydrophobic antimicrobial compounds are depicted in Figure 4.15 where Allicin is thought to be a key component in garlic's biological activity among these compounds. But Allicin is unstable compound and will in general be changed over into different mixtures, for example, Ajoene and diallyl polysulfides (DASn), which have been accounted for to display antimicrobial action.

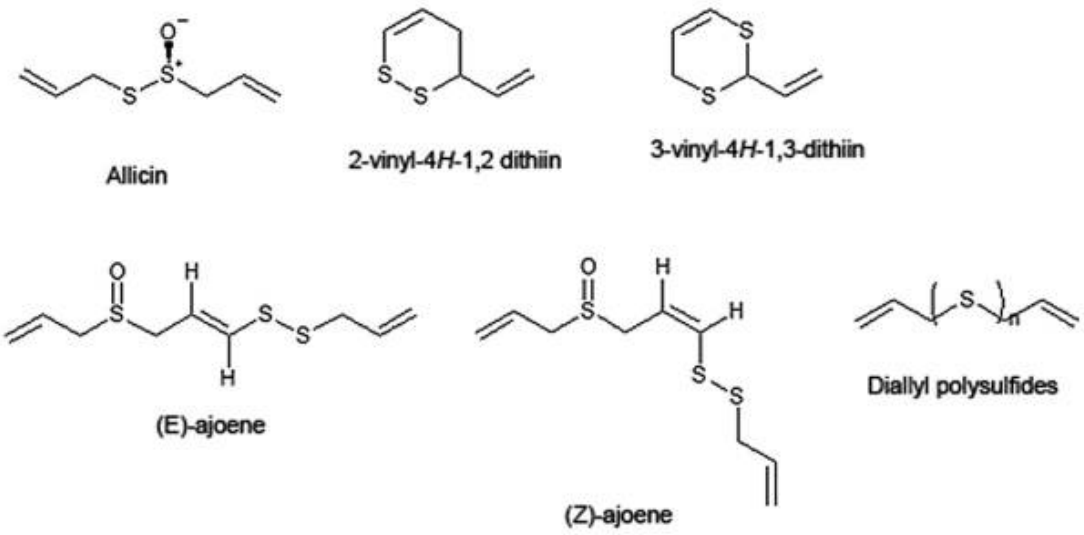


Figure 4.15 Hydrophobic compounds in garlic [153]

Ajoene, like Allicin, has an oxygenated sulphur bunch, and has been reported to hinder the expansion of Trypanosoma cruzi, conceivably by hindrance of

phosphatidylcholine biosynthesis [154]. In addition, phosphatidylcholine biosynthesis was recently demonstrated to be inhibited by Ajoene in the human pathogenic fungus Paracoccidioides brasiliensis [155]. The indicated inhibitory activity for Ajoene strongly implies that Allicin may potentially target other enzymes particular to microbes.

Ajoene's primary antimicrobial action stems from its chemical interaction with thiol groups of different enzymes, such as RNA polymerase, alcohol dehydrogenase, and thioredoxin reductase. These interactions can impact the vital metabolism of cysteine proteinase activity, which is linked to E. histolytica's pathogenicity [156]. Ajoene, at less than 20 ug/ml, reduced the development of Aspergillus niger and Candida albicans [128]. Therefore, it makes sense to draw the conclusion that Ajoene's broad range of antimicrobial activities comes from the numerous inhibitory effects it may have on different thiol-dependent enzymatic systems. Certain enzymes that seriously harm tissue include thiol proteases, can cause significant harm to the host tissues. The inhibition of these enzymes at low doses may not be fatal, but it is still enough to prevent the pathogenicity of the microorganism. Some enzymes, such thioredoxin reductase or dehydrogenases, may be impacted at somewhat greater doses. The microorganism may be killed off by even a partial blockage of these enzymes [156].

Past *in vitro* and *in vivo* examinations uncovered a critical restraint of P. aeruginosa Quorum sensing (QS) by rough garlic extract [157]. By bioassay-directed fractionation of garlic extricates, decided the essential QS inhibitor present in garlic to be Ajoene, a sulphur-containing compound with potential as an antipathogenic drug [157]. Through complete *in vitro* and *in vivo* examinations, the impact of manufactured Ajoene toward P. aeruginosa was clarified [157]. DNA microarray investigations of Ajoene-treated P. aeruginosa societies uncovered a focus subordinate lessening of a couple yet focal QS-controlled destructiveness factors, including rhamnolipid. In addition, treatment of *in vitro* biofilms with Ajoene demonstrated a distinct antimicrobial synergistic effect with tobramycin on the elimination of biofilms and the cessation of lytic necrosis in polymorphonuclear leukocytes [157]. In addition, in a mouse model of pulmonary infection, Ajoene-treated mice significantly cleared the infected P. aeruginosa compared to a control group that was not treated. This study is one more example of how QS-interfering compounds have the potential to treat bacterial infections [157].

Also, Ajoene is a condensation product of Allicin, appears to generally have more antiviral activity than Allicin. In a cell system infected with the human

immunodeficiency virus, it was discovered that Ajoene inhibited integrin-dependent processes [158]. It is interesting to note that the antiviral properties of garlic extracts are not effective against some viruses, such as the garlic plant virus X [159].

4.9 DNA Protective Activity Measurements

Some researchers suggest that Ajoene, possess DNA protective activity, which could have implications for its potential use in preventing or mitigating DNA damage-related diseases.

Here are some key points regarding Ajoene's DNA protective activity:

Antioxidant properties: Ajoene has been shown to exhibit antioxidant properties, which can help reduce oxidative stress and prevent DNA damage caused by reactive oxygen species (ROS). Oxidative stress is a key contributor to DNA damage and is linked to the occurrence of certain sickness, for example, cancer and aging-related disorders.

DNA repair enhancement: Some research suggests that Ajoene enhance the activity of enzymes involved in DNA repair processes. Maintaining genomic stability and avoiding mutations that might result in cancer and other genetic illnesses are important tasks for DNA repair systems.

Radioprotective effects: Ajoene has been investigated for its potential radioprotective effects, particularly against the damaging effects of ionizing radiation on DNA. Radiation exposure can induce DNA strand breaks and other forms of damage, and compounds with radioprotective properties could help minimize such damage.

Potential anti-carcinogenic activity: Given its ability to protect DNA and mitigate oxidative stress and inflammation, Ajoene have potential anti-carcinogenic effects. By preventing DNA damage and promoting DNA repair, Ajoene could help reduce the risk of cancer development and progression.

This study has investigated the Ajoene extract for its DNA protective activity through DNA electrophoresis experiment, where the pBR322 plasmid DNA (vivantis) was used to determine the efficacy of the Ajoene extract to protect DNA from UV and oxidative impact. For DNA protective activity testing, Ajoene extracts were sterile at 5, 4, 3, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml prepared in distilled water. Afterward, control and samples were prepared according to Russo et al [160].

Table 4.9 illustrating the Ajoene extract samples preparation method as equation for easy understanding.

Table 4.9 List of the Ajoene extract samples as equations

Sample 1:	X + 5 mg/mL AE (5 µl) + Y
Sample 2:	X + 4 mg/mL AE (5 µl) + Y
Sample 3:	X + 3 mg/mL AE (5 µl) + Y
Sample 4:	X + 2 mg/mL AE (5 µl) + Y
Sample 5:	X + 1 mg/mL AE (5 µl) + Y
Sample 6:	X + 0.5 mg/mL AE (5 µl) + Y
Sample 7:	X + 0.25 mg/mL AE (5 µl) + Y
Sample 8:	X + 0.125 mg/mL AE (5 µl) + Y
Sample 9:	X + 0.0625 mg/mL AE (5 µl) + Y
Positive Control:	X + dH2O (6 µl) + Oxybenzone (5 µl) + Y
Negative Control:	X + dH2O (6 µl) + Y

X: Plasmid DNA (3 µl), AE: Ajoene Extract, Y: UV+ H_2O_2 (1 µl).

5.0 µL of the prepared samples were put into the sample tubes. 3.0 µL of pBR322 plasmid DNA (172 ng.µL) was put into the tubes and 1.0 µL of 30% H_2O_2 was put into the tubes except for the positive control. Again, all tubes except the positive control were exposed to UV rays for 5 minutes and then loaded into 1.5% agarose gel by adding 2.0 µL of loading buffer. A UV transilluminator (DNR-IS) device, which produces light with a wavelength of 302 nm and an intensity of 8000 µW/cm at room temperature, was used as the light source. After 100 minutes of electrophoresis at 100 Volts, the gel was visualized in the documentation system (DNRIS, MiniBIS Pro) and its photographs were obtained. In this test system, pBR322 plasmid DNA without UV and H_2O_2 treatment were used as a control.

The data obtained as a result of the test performed on the Ajoene extract are given in Figure 4.15. While it is seen that DNA is broken in the negative control column in the

following Figure, it is evident that all three bands of plasmid DNA (ocDNA, LinDNA, and scDNA) are preserved in the first column, which is the positive control, can be seen, as expected, a DNA protective effect is observed in the oxybenzone column. According to the results, all Ajoene samples show DNA protective activity. It was observed that the DNA protective activity was higher, especially in the wells where the sample contained 0.5 mg/mL and lower concentrations.

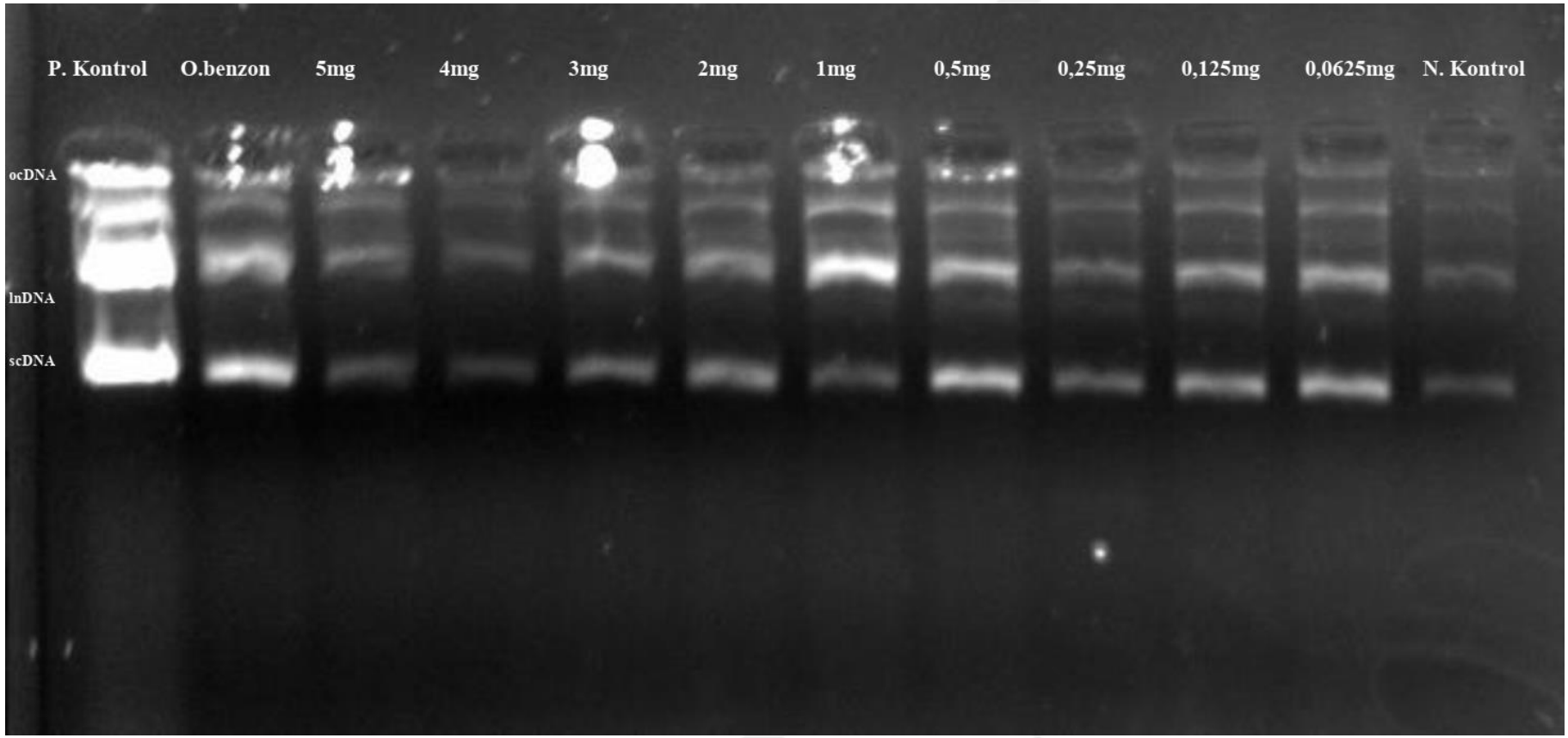


Figure 4.16 Electrophoresis image of Ajoene extract.

Overall, while further research is needed to fully understand the mechanisms underlying Ajoene's DNA protective activity and its potential therapeutic applications, existing evidence suggests that Ajoene has promising potential in this regard. Harnessing the DNA protective properties of Ajoene may contribute to the creation of innovative methods for the treatment or prevention of disorders linked to DNA damage, such as, cancer, cardiovascular diseases, and neurodegenerative disorders.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

This study was conducted to measure the bioactivity of Ajoene (Garlic extracted semi-solid fraction) in the areas of composition analysis, and biological properties such as Antioxidant, antibacterial (Drug Resistant strain), and DNA protective activities by employing techniques and devices available in the university laboratories, where findings were validated with other available evidence from other studies. Ajoene was extracted using the ethylation extraction process and Ajoene produced samples were examined using appropriate spectroscopic techniques, including UV-Vis, FTIR, GC-MS, and PL. The results of these spectrum measurements have been disclosed and analyzed. The chemical structures of Ajoene, yielded by the ethylation extraction process, were found to correspond with the expected compound configurations by FTIR spectroscopy. The compisition of Ajoene extract was determined through Gas Chromatography apparatus, where several bioactive components were found in the Ajoene extract, with phenols and alkanes being the main components. The GC analysis of the Ajoene extract yielded the identification of 70 compounds in total. Overall, the characteristics and uses of the discovered compounds indicate that the Ajoene extract implies its potential for a variety of uses, including antifungal, antioxidant, and antibacterial uses, for instant, Pentadecanoic and Octadecanoic which found in Ajoene extract, may be in charge of the antibacterial activity and other characteristics of the Ajoene extract. Turkish garlic exhibited a lower moisture content detection, but it was also shown to have a significantly higher protein content than other vegetables. It is worth mentioning that Turkish Garlic has the highest amount of Fe, Cu, Ma, and P in regard to the mineral contents in comparison with Indian & Tunisian garlic. In the area of Antibacterial activities, the hospital strain of *Stenotrophomonas maltophilia* was subjected to an antibacterial experiment, which demonstrated some bactericidal effects. Indeed, the potential of Ajoene and other bioactive compounds derived from garlic for drug development, is gaining attention in the scientific community. With the growing

concern over antibiotic resistance, there is a critical need for the development of alternative antimicrobial strategies.

Also, it has been demonstrated that Ajoene possesses antioxidant properties, which may contribute in mitigating oxidative stress and guarding against DNA damage brought on by reactive oxygen species. The DNA protective activity experiment result was also supporting the Antioxidant activity result, which evaluated three bands of plasmid DNA (ocDNA, LinDNA, and scDNA), all Ajoene samples show DNA protective activity. It was demonstrated that there was enhanced DNA-protecting action, especially in the wells containing 1 mg/mL and lower sample quantities. An observation revealed that Ajoene extract strongly emitted when exposed to UV light. Ajoene extract (a) showed a 40% photoluminescence quantum yield and an excited-state lifetime of 3.73 ns. The well-known Kasha's rule of excitation wavelength independence, which stipulates that emission comes from the lowest vibrational level of the initial electronic state, is supported by the discovery that the Ajoene extract's fluorescence behaviour was independent of the excitation wavelength. The photo luminescent properties of this extract may indicate that it has great potential for usage in photo physical and biomedical applications.

Given that absorption spectroscopy is complementary to fluorescence spectroscopy, the ultraviolet-visible (UV-VIS) spectrophotometry measurement was performed on the Ajoene extract. And the outcome demonstrates that the Ajoene compound is chromophore. Where this studay examine the Ajoene extract as a natural source for therapeutic usage as an antimicrobial, antioxidant, as well as DNA-protective active compound which can be an alternative to commercial antibiotics, antioxidants, and anticytotoxic agents.

At the end of this study, it is recommended to conduct additional researches to continue the current efforts in the areas of:

1- The relationship and biochemistry of Ajoene’s compounds and their harmonized mechanism of action.
2- Antibiotic synergy with Ajoene.
3- Role of Ajoene in decreasing the bacterial resistance to antibiotics.
4- Ajoene effects on anti-aging.
5- Anticancer property of Ajoene extract.
6- The effect of Consuming Ajoene extract on mental health.
7- The protective activity of Ajoene extract against Corona Viruses.

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CURRICULUM VITAE

Name and Surname: Anas ALTOUTANJI

EDUCATIONAL BACKGROUND

	Graduated School	Graduation Year
Bachelor (B.Sc.)	International University for Science & Technology (IUST)	2015

INTERNATIONAL CONFERENCES AND PROCEEDINGS

Huseyin Zengin, Anas Altoutanji, Gülay Zengin "Composition analysis of garlic extract and evaluation of the antimicrobial, antioxidant, DNA protective and photoluminescence properties" EurasianBioChem 2022 Conference, Ankara, 23-25 November 2022.

PUPLICATIONS

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