An-Najah National University

Faculty of Graduate Studies

Characterization of Polyphenol-Containing Polar Extracts from *Bassia Arabica* and *Nepeta Curviflora* Boiss and Evaluation of their Bioactivity

By

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This thesis was defended successfully on 16/6/2021 and approved by:

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– Dr. Ahmad khasati / External Examiner	.....
– Dr. Derar smadi / Internal Examiner	.....

Dedication

To my parents

To my brothers and sisters

To all my friends

I dedicate this work

Acknowledgments

Praise be to God who honored me with the completion of this thesis. I extend my thanks and gratitude at the beginning to my supervisor, Dr. Nawaf Al-Maharik, who was supportive of me with his knowledge and experience throughout my work, and to Dr. Nidal Jaradat, for his efforts and support. I also want to thank my parents for their support and love, and my thanks to my brothers, sisters and friends.

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Finally, I thank all the staff in the department of chemistry at An-Najah University, as well as, Mr. Nafez Dwikat for all his efforts.

الإقرار

انا الموقعة ادناه مقدمة الرسالة التي تحمل العنوان:

Characterization of Polyphenol-Containing Polar Extracts from Bassia Arabica and Nepeta Curviflora Boiss and Evaluation of their Bioactivity

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Declaration

The work provided in this thesis, unless otherwise referenced, is my research own work, and has not been submitted elsewhere for any other degree of qualification.

Students name: ولاء فهمي حلمي عوده **اسم الطالبة:**

Signature: **التوقيع:**

Date: 16/6/2021 **التاريخ:**

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Characterization of Polyphenol-Containing Polar Extracts from *Bassia Arabica* and *Nepeta Curviflora* Boiss and Evaluation of their Bioactivity

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Abstract

Around the world, medicinal plants are widely used to treat a wide range of human illnesses and diseases. Palestine's geographic position at the crossroads of Eurasia and Africa enhances the country's biodiversity in terms of fauna and flora. Despite its small size, Palestine supports over 2,700 plant species. The research aims to study the chemical composition and pharmacological effects of *Bassia arabica* and *Nepeta curviflora,* as well as their antibacterial and antioxidant activities. After the powdered plants were suspended in methanol, it was exhaustively extracted with ethyl acetate and fractionated using various chromatographic techniques.

Ethyl acetate extracts of both *B. arabica* and *N. curviflora* were subjected to a sequence of silica gel column chromatography using different eluents with various polarities. The purity of the isolated fraction was conducted by thin-layer chromatography (TLC) and confirmed using high-performance liquid chromatography (HPLC). Moreover, functional groups of the pure fractions were detected using infrared spectroscopy. In addition, the radical scavenging capacity of plant extracts was assessed by DPPH assay, and antibacterial activity of *B. arabica and N. curviflora* fractions was evaluated

using broth micro-dilution method based on the estimation of minimum inhibitory concentration (MIC).

Fraction A3 of *B. arabica* display antibacterial activity against all types of bacteria, with MIC values ranging from 0.195 to 6.25 mg/mL. Ethyl acetate extract of *B. arabica* had the best antioxidant potential (IC_{50}= 4.91 ± 0.03µg/mL). As a result, the water extract of *N. curviflora* had the strongest antioxidant capacity (IC_{50}= 2.83± 0.25µg/mL).

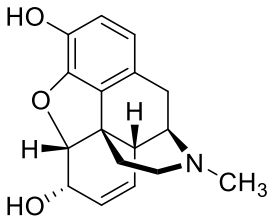
Chapter One

Introduction

1.1 History of Medicinal Plant

Many diseases have been treated with medicinal herbs. They are also called alternative medicine, ethnic medicine, folk medicine and complementary medicine. Traditional medicine is based on people's perceptions and understanding of various cultures, which they use to diagnose and cure diseases [1]. Many ancient cultures, such as the Egyptian, Chinese, Indian, and North African civilizations, used traditional medicine. In traditional medicine, natural plants are regarded as one of the most essential sources of medicines. Traditional medicine is still used in many countries today, according to studies [2].

Morphine **1** was derived from the poppy plant and was used as a pain reliever for acute and chronic pain. It was one of the first bioactive materials isolated and extracted from natural sources. Another example is camptothecin **2**, a bioactive material that is used in traditional Chinese medicine to treat gastrointestinal tumors and was previously used to treat cancer.



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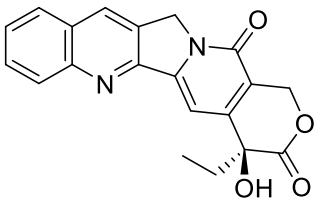

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1.2 Chemistry of Natural Plant

Medicinal herbs, which were first discovered by man in ancient times, are the most important sources of traditional drugs. Medicinal plants are used in the treatment of many common diseases. Man has always been racing to discover more chemical compounds occurring in medicinal plants, as well as ways to separate and extract them from these plants. Natural products (NPs) are biologically active substances that belong to a family of chemical compounds that have been extracted from various plants, animals and microorganisms [3]. Photosynthesis in plants is responsible for the production of thousands of natural products that are divided into primary or secondary metabolism. Few compounds are classified as primary metabolism and the vast majority of compounds are secondary metabolism [3].

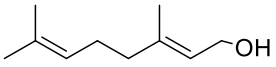
1.3 Classification of Secondary Metabolism

Secondary metabolism provides the plant with non-essential functions and does not directly impact the organism's development and reproduction as primary metabolism does. It does, however, play a key role in protecting plants from pathogenic microorganisms like viruses, fungi, and bacteria [4]. Secondary metabolites are divided into several groups. The most important are terpenoids, alkaloids, and phenolic compounds [5].

1.3.1 Terpenoids

Isoprenoids are another name for terpenes. They're natural products made from isoprene molecules. Terpenes are the most common and diverse natural

product group, with over 23,000 different structures [5]. Geraniol **3**, abscisic acid **4**, diosgenin **5**, and beta carotene **6** are examples of terpenes.



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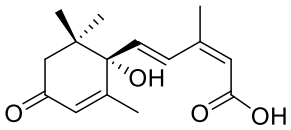

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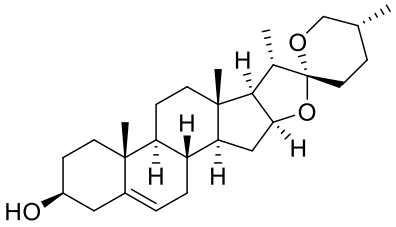

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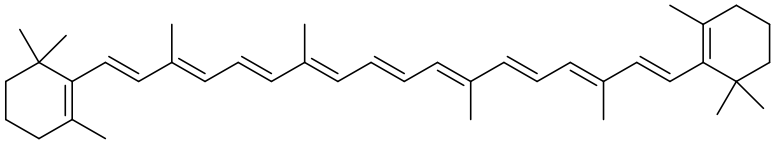

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Terpenoids have a wide range of pharmacological properties. They're used to treat diseases in both humans and animals. Terpenoids are used in the perfumery and spice industries because they have a distinct scent and taste. Some of the major groups of terpenoids, as well as their biological activities, are mentioned in Table 1.1 [6].

Table 1.1: Major classes of terpenoids.

Value of (n) or Number of isoprene units	Number of carbons	Molecular formula	Terpenoids classes	Biological activity
1	5	C_5H_8	Hemiterpenoid [5]	Anticancer
2	10	$C_{10}H_{16}$	Monoterpenoid [5]	Antibacterial activity
3	15	$C_{15}H_{24}$	Sesquiterpenoid [5]	Antimicrobial activity
4	20	$C_{20}H_{32}$	Diterpenoid [5]	Antimicrobial activity
5	25	$C_{25}H_{40}$	Sisterterpenes [5]	Antifungal activities
6	30	$C_{30}H_{48}$	Triterpenoid [5]	anti-inflammatory
7	35	$C_{35}H_{56}$	Meroterpens [5]	Antibacterial activity
8	40	$C_{40}H_{64}$	Tetraterpenoid [5]	Antioxidant activity

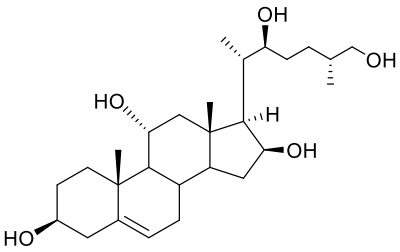
1.3.1.1 Monoterpenoid

Monoterpenes are the most basic natural terpenes, derived from essential oils extracted from various plant parts such as leaves, roots, and bark. Several studies have reported the efficiency of these oils in the treatment of tumors. They have antibacterial properties as well [7]. Acyclic, monocyclic, and bicyclic compounds are the three major classes of monoterpenoids [7].

1.3.1.2 Saponins

Saponins are glycosides with surface activity. They are classified into two classes based on their chemical structure: steroidal saponins **7** and

triterpenoid saponins **8** [8, 9]. At the C-3 position, both of these forms have a glycosidic linkage. They are obtained naturally from medicinal plants, marine organisms, and bacteria [8]. Saponins are toxic to cold-blooded animals, especially fish, and can cause hemolysis of red blood cells.



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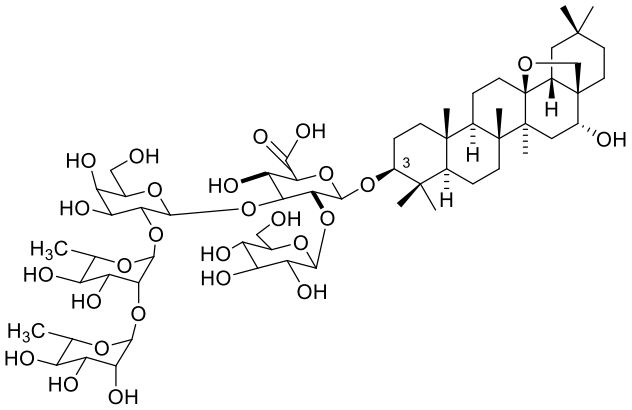

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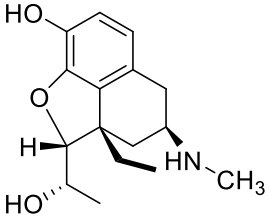
1.3.2 Alkaloids

The nitrogen atom is the atom that distinguishes this group of organic compounds. Other elements such as oxygen and sulfur are present, but phosphorous, bromine, and chlorine are uncommon [5]. Alkaloids are typically produced by plants that use the secondary metabolism process. Alkaloids have pharmacological effects, much like other natural products, and are used to treat hypertension [5]. The most common alkaloids are morphine **1** and caffeine **9**.



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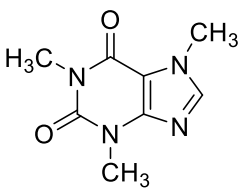

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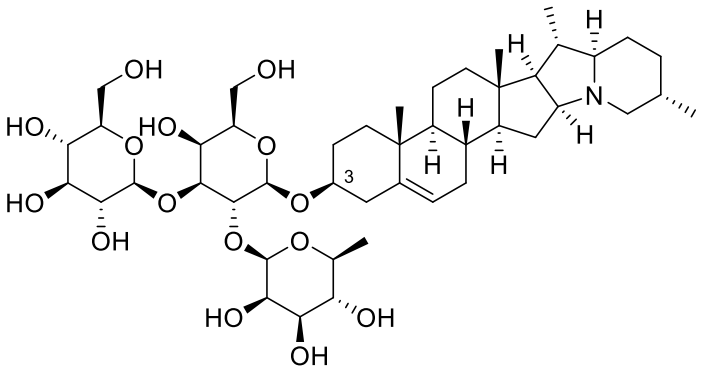
Solanine **10** is a glycoalkaloid found in nightshade plants, especially in potato species [9]. Potatoes can be used in a variety of ways. Women, for example, use potatoes to care for their hair. Additionally, potatoes contain secondary metabolites such as the glycoalkaloid poison solanine **10** [12].



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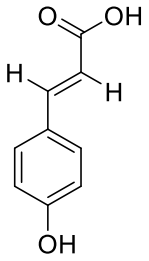
1.3.3 Phenolic Compounds

Benzoic molecules with one or more hydroxyl groups, as well as their derivatives, make up phenolic compounds. Phenolic phytochemicals have a wide range of biological effects, including antioxidant, anticancer, and antimicrobial properties [13]. They are divided into three categories: phenolic acid, tannins, and flavonoids [14].

1.3.3.1 Phenolic Acid

Phenolic acid is the first group of phenolic compounds. This is a family of aromatic acid compounds that can be found in plant foods (fruits, vegetables, and grains). Hydroxycinnamic **11** and hydroxybenzoic acid **12** are among the compounds found in this group. Plants require phenolic compounds for

growth and reproduction [15]. Caffeic **13** and ferulic acids **14** are the two most common hydroxyl cinnamic acids [16].



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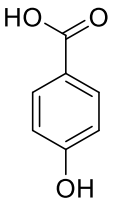

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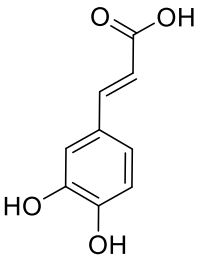

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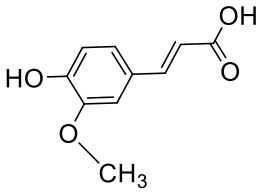

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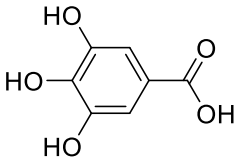
Hydroxybenzoic acid is a type of aromatic compound that can be found in abundance in fruits and nuts. The most common derivatives of these compounds are gallic acid **15** and ellagic acid **16** [16]. Hydroxybenzoic acid **12** displays several properties, including anti-oxidant and anti-cancer properties [16].



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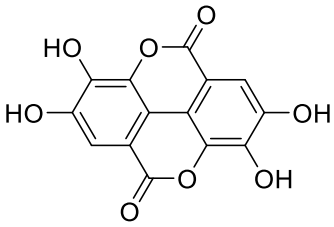

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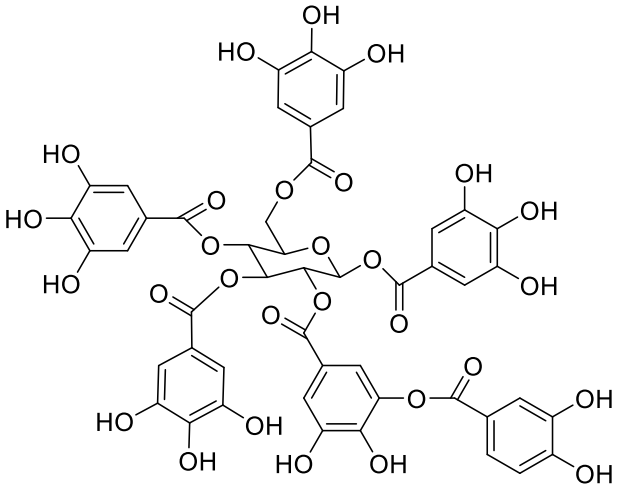
1.3.3.2 Tannins

Tannins are polyphenols, water-soluble, and can bind to tissue proteins. Tannins are divided into two categories based on their chemical composition: hydrolyzable tannin **17** and condensed tannin **18** [17].



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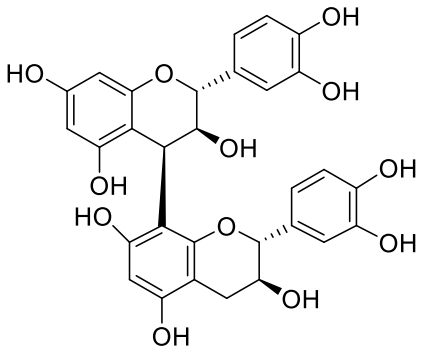

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Gallic **15** and ellagic acids **16** are produced when tannins are hydrolyzed by acid or enzymes [18]. Nonhydratable tannins or condensed tannins are resistant to hydrolysis. They are flavonol polymers, which are formed by the condensation of flavonoids [17].

1.3.3.3 Flavonoids as Natural Products

Flavonoids are formed by plants' secondary metabolism and can be found in a variety of plant sections, including leaves, stems, and roots. Flavonoids are pigments that give plants their color, flavor, and aroma. In addition to their essential properties in plants, flavonoids exhibit a wide range of bioactive properties such as antioxidants, antibacterial properties, cardio defensive factors, anti-cancer properties, and skin defense from ultraviolet rays [19].

1.4. Classification of Flavonoids

Flavonoids contain 15 carbon atoms, and are composed of two phenyl rings linked by a three-carbon chain. The (C_6-C_3-C_6) scheme can be used to

describe the basic skeleton of flavonoids (Figure 1.1) [20]. Flavonoids are classified as flavones, flavanones, flavonols, flavanonols, isoflavones, and anthocyanins based on differences in their basic structure [20].

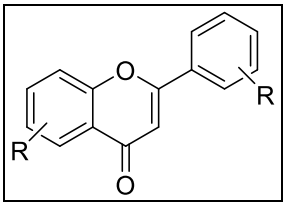
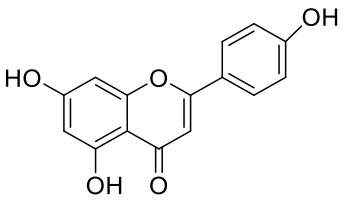


Figure 1.1: Basic skeleton of flavonoid.

1.4.1 Flavones

2-Phenylchromen-4-one is the common name for flavones. The presence of a ketone at position C-4 and a double bond between positions C-4 and C-3 of the C ring distinguishes them. Flavones can be found in the stems, leaves, and roots of plants [21]. They're also present in 70 different plant families around the plant kingdom [20]. Apigenin **19** and luteolin **20** are the most representative flavonoids in this subclass. Apigenin **19** is present in onions, parsley, and fruits [22]. Furthermore, compound **19** is a non-toxic flavonoid that reduces the rate of cell oxidation by scavenging free radicals [16].



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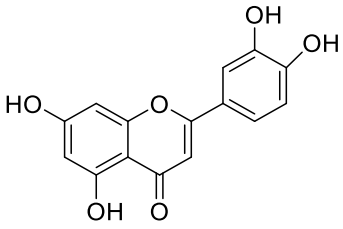

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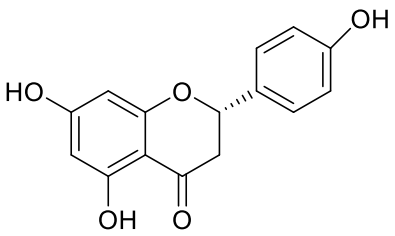
1.4.2 Flavanones

Flavanones are called dihydroflavones. They are characterized by the absence of a double bond between C-3 and C-4 in the C ring. Naringenin **21**, hesperetin **22,** and hesperidin-7-rutinoside **23** are the major representative compounds belonging to this class of flavonoids [20]. Compound **21** has been isolated from citrus, fruits, seeds, and beans [16]. Like most flavonoids, naringenin **21** displays a wide range of biological health effects, such as anti-inflammatory and antioxidant activities [23]. Compound **23** is characterized by the rutinose (disaccharide) attached to the aglycone, hesperetin [24]. Hesperidin-7-rutinoside **23** was isolated from the peel pulp of citrus fruit [25]. Hesperidin **22** exhibited anticoagulant, cardioprotective effects against oxidative stress, anti-allergic action, and anti-inflammatory [25].



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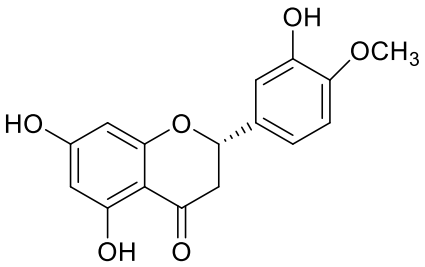

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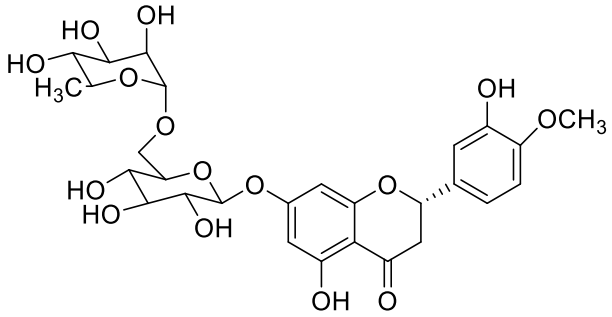

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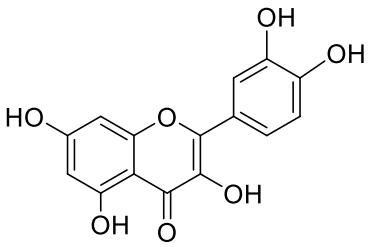
1.4.3 Flavonols

Flavonols have a unique structure due to the presence of a hydroxyl group at the C-3 of the C-ring. This type of flavonoid can be found in fruits and vegetables such as onions, lettuce, apples, and grapes [21]. Some examples of flavonols are quercetin **24**, fisetin **25**, myricetin **26,** and kaempferol **27**.



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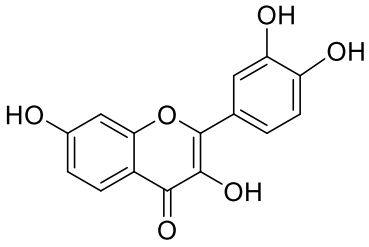

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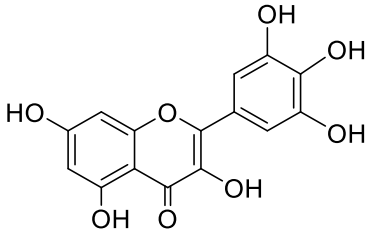

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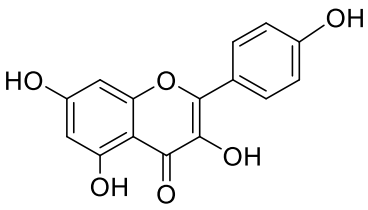

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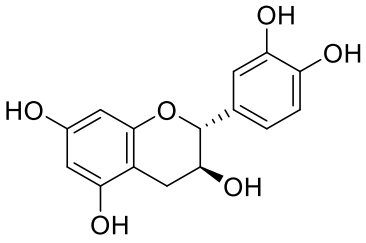
Several studies have shown that fisetin is responsible for inhibition of DNA damage, free radical scavenging, inhibition of induced tumors and improving traditional chemotherapy by combining it with cisplatin [26]. In addition, quercetin plays an important role as an allergic, antiviral activity and anti-inflammatory agent [26].

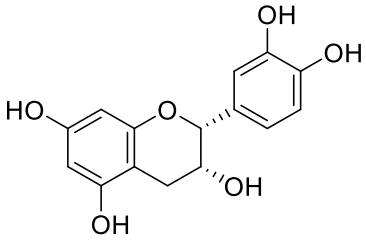
1.4.4 Flavanonols

Flavanonols (dihydroflavanols) are called catechins **28**. Catechins (flavan-3-ol) are found mainly in vegetables, fruits, and plant-based drinks, and the

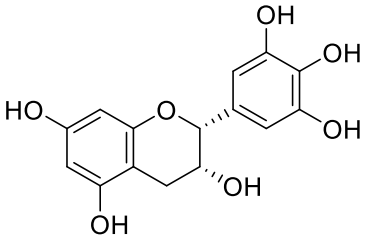
leaves of the tea plant [27]. Consumption of foods containing catechins is often associated with the reduced incidence of cancer and heart diseases, and with the ability to remove free radicals [27]. Epicatechin (EC) **29**, epigallocatechin (EGC) **30**, epigallocatechin gallate (EGCG) **31** are the most abundant catechins in food [27].



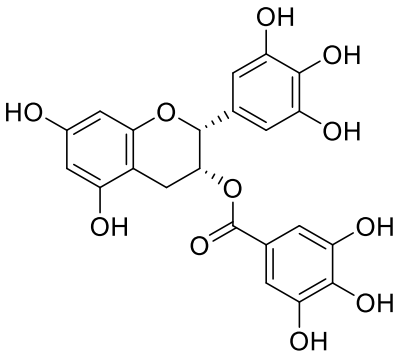
(+)-Catechins **28**



(-)-Epicatechin (EC) 29



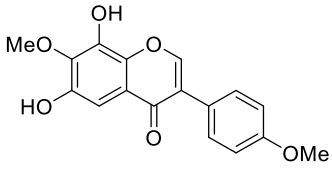
(-)-Epigallocatechin **30**



(-)-Epigallocatechin gallate **31**

1.4. 5 Isoflavones

In plants, isoflavones, as well as flavonoids, are found in three forms: simple isoflavones (aglycone) **32**, isoflavones glycosides **33,** and complex isoflavones (prenylated) **34** [28].



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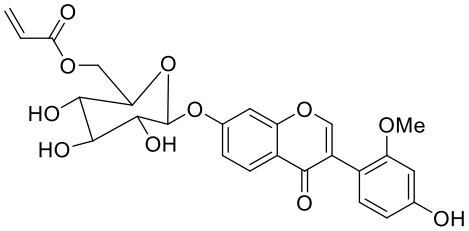

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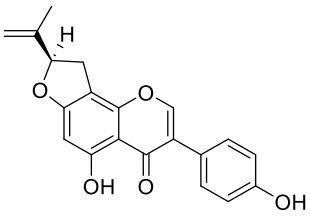

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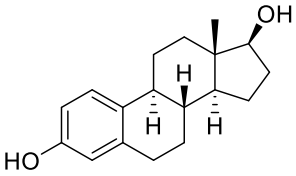
Phytoestrogens are a class of non-steroidal plant-derived compounds that have a structural and functional similarity to estradiol **35** and bind to estrogen receptors. Phytoestrogens can be found in a variety of foods, where soybeans are the main source of isoflavones. Genistein **36** and daidzein **37** are the isoflavones with the most estrogenic activity [29].



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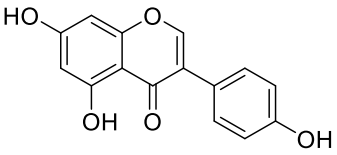

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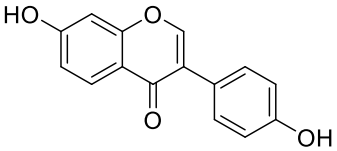
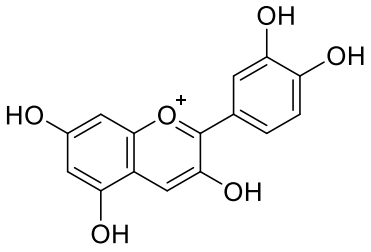


37

The most common source of genistein is soy products, and it is effective in scavenging superoxide and peroxynitrite from both enzymatic and non-enzymatic systems [16]. Soybeans contain isoflavones that have benefits against many diseases such as atherosclerosis, osteoporosis, and cardiovascular disease [16].

1.4.6 Anthocyanins

Anthocyanins are naturally occurring compounds present in the form of glycosides [16]. They are responsible for giving color to the flowers and fruit of plants depending on the pH. More than 600 anthocyanidin molecular structures have been identified [16]. Anthocyanins are of interest due to their biological effects, including antioxidants, anticancer and anti-inflammatory activities [16]. They are also used in the food industry [20,21]. Some examples of such compounds are cyanidin **38** and delphinidin **39.**



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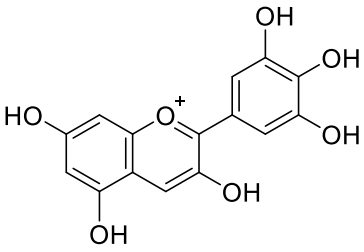

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1.5 Metabolism of Flavonoids in Humans

It is well established that flavonoids undergo thorough metabolism before entering the systemic circulation. The absorbed flavonoids are bound to albumin and transferred to the liver through the portal vein, where they undergo multiple biotransformations that result in different conjugated types of flavonoids. Additionally, the intestinal mucosa, kidney, and other tissues are also implicated in flavonoid metabolism. Oxidation, reduction, hydrolysis, and conjugation with sulfate, glucuronate, or O-methylation are the most important metabolic transformation reactions of flavonoids. It has been hypothesized that these transformations have a significant impact on

flavonoids' antioxidant function and interactions with proteins. Conjugation reactions with glucuronic acid and/or sulfate appear to be the most frequent form of the flavonoid metabolic pathway. The small intestine is the primary organ involved in the glucuronidation of several flavonoids through uridine-5‘-diphosphate glucuronosyltransferases (UGTs). Sulfation and methylation both occur in the cytosol by sulphotransferases (SULTs) and Catechol-*O*-methyl transferases [30].

Flavonoids are metabolized in the liver after digestion and intestinal metabolism. Some flavonoid glucuronides can be hydrolyzed and either re-glucuronidated at different positions or conjugated with sulfate. Microflora enzymes metabolize unabsorbed flavonoid fractions that reached the colon. As seen in Fig. 1.2, scission of the flavonoid structure will occur at the C-ring, depending on their hydroxylation patterns. The most common dietary flavonoids, catechin **28**, and quercetin **24**, both of which have a 5,7,3‘,4‘-hydroxylation sequence, will increase C-ring opening after hydrolysis. Enzymes from intestinal microorganisms are responsible for the initial ring fission of flavonoids, as well as the demethylation and dihydroxylation of the subsequent phenolic acids.

The hydroxylation patterns of flavonoids can cause scission of their structure, as seen in Figure 1.3 [30].

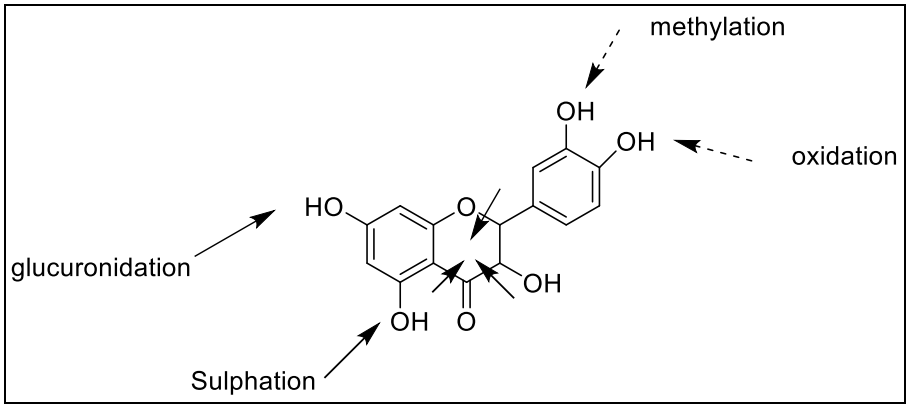


Figure 1.2: Potential sites of biotransformation and ring cleavage of flavonoids.

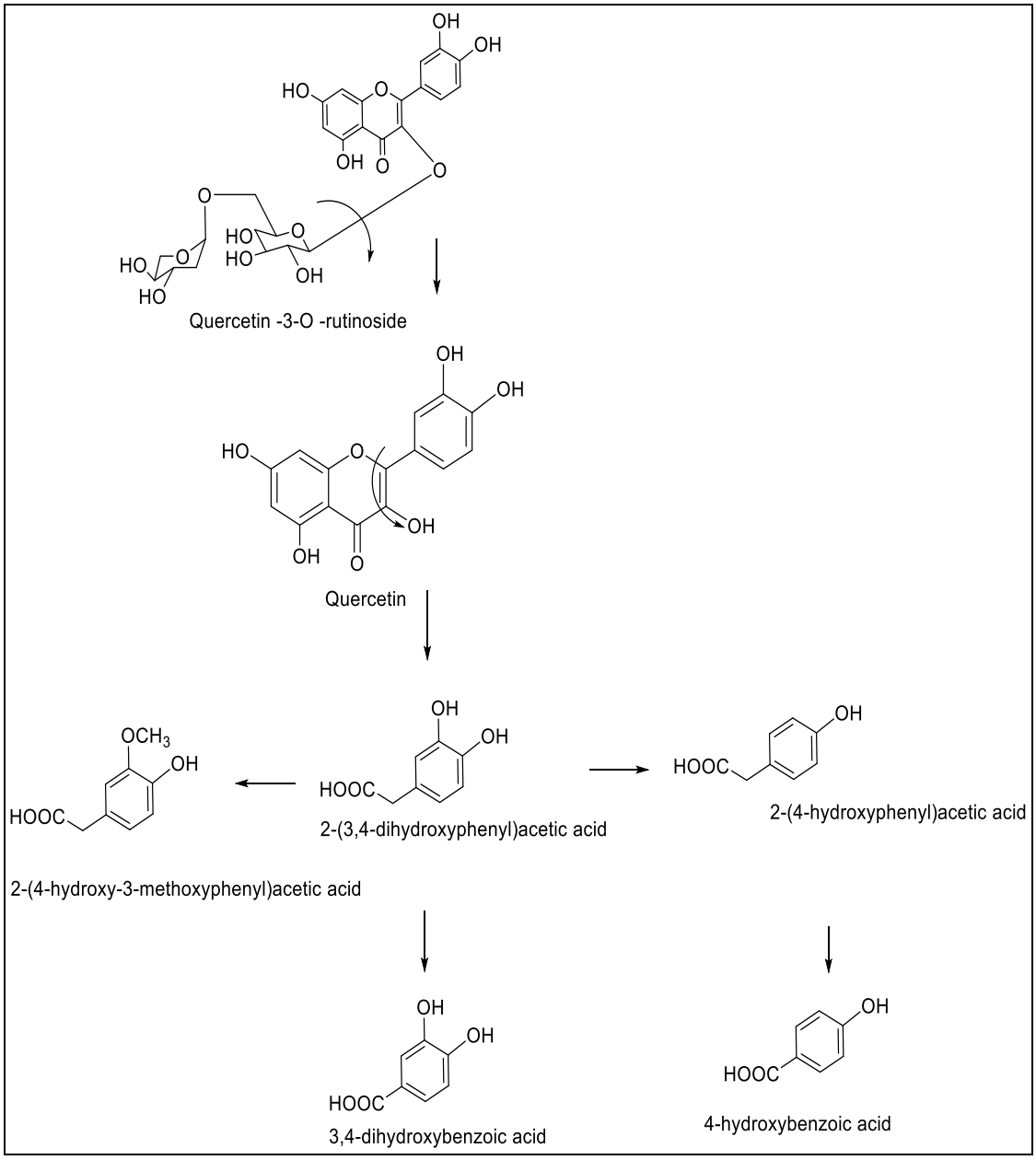


Figure 1.3: Metabolism pathway of quercetin [31].

1.6 Effects of Plant Flavonoids and other Phenolic Compounds on Human Health

Flavonoids and other phenolic compounds have a wide range of biological activities, such as antioxidant, anticancer, and antimicrobial effects [13, 14].

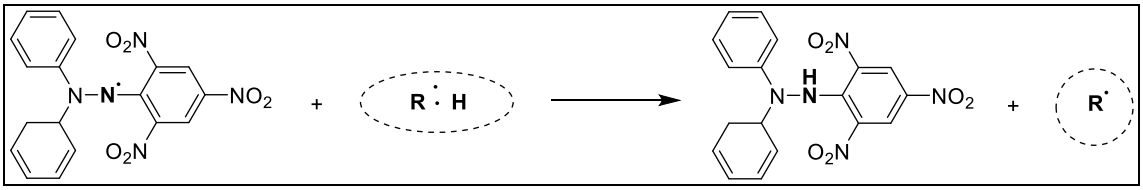
1.6.1 Antioxidant Effects

One of the most important biochemical properties of flavonoids and other phenolic compounds is their antioxidant activity. Oxidative stress in a biological system is defined as an imbalance of antioxidants and oxidizing substances. It is caused by a decrease in antioxidant activity or an increase in the number of free radicals [32]. Oxidation reactions are one of the most important basic reactions that occur inside the human body. The body needs it to perform many bioprocesses such as oxidation of food, but these reactions produce free radicals [33]. Antioxidants are molecules that can scavenge free radicals that are harmful to the organism. There are three types of antioxidants. First, enzymes, also called natural antioxidants, such as catalase, are found in all aerobic organisms and catalyze the conversion of H_2O_2 to water and oxygen. The second type of antioxidant is vitamins such as vitamin C, vitamin A and vitamin E. Our bodies do not produce vitamins, instead, they are obtained from a vitamins rich diet. Flavonoids enter our bodies through the consumption of a flavonoid-rich diet, such as soybeans and flavonoid products. And the third type is plant compounds that are naturally present in most foods [32].

Many free radicals are produced during the metabolism process in the cells of the body. These free radicals divide and destroy cell molecules through a series of reactions. They also destroy lipids, proteins, enzymes and nucleic acids in the cell, which makes our bodies susceptible to many infections, viruses, and cancers [33]. Flavonoids and other phenolic compounds act as

antioxidants by inhibiting enzymes, chelating trace elements involved in generating free radicals and scavenging free radicals [34].

Studies have shown that flavonoids inhibit many enzymes such as protein kinase and xanthine oxidase lipoxygenase, cyclooxygenase, NADPH oxidase, and glutathione S-transferase, which are responsible for the production of free radicals [33]. The DPPH assay is an economical, easy, and fast method used to evaluate the non-enzymatic antioxidant activity of plant extracts. 2,2-Diphenyl-1-picrylhydrazyl- (DPPH) is an organic compound classified as a stable free radical, because it contains an unpaired electron on the N-atom. In methanol solution, this compound is characterized by a purple color, which in the presence of antioxidants turns yellow, as shown in scheme 1.1. DPPH is a commonly used in vitro (laboratory) assay for the evaluation of phenolic compound antioxidant activity [35]. At room temperature, a Double-Beam UV-Vis Spectrophotometer was used to record the absorbance at about 517 nm.



DPPH DPPH-H

Purple Color (yellow) Colorless

Scheme 1.1: Reaction mechanism of DPPH with antioxidant.

R:H = represents antioxidant, R•: represents antioxidant radical.

DPPH can interact as a free radical with various antioxidants by many mechanisms. The first proposed mechanism is single electron transfer (SET) as indicated in scheme 1.2. In the SET mechanism, a single electron is transported from the nucleophile to the substrate and a radical intermediate is produced. Then formed anion ($\mathbf{A}^-$) removes one proton ($\mathbf{H}^+$) from the antioxidant ($\mathbf{B{\bullet}H}^+$) and the antioxidant produces a radical ($\mathbf{B}^{\bullet}$) [35].

$$\mathbf{A}^{\bullet} + \mathrm{B:H} \xrightarrow[\text{SET -step 1}]{-e^-} \mathbf{A}:^- + \mathrm{B{\cdot}H}^+ \xrightarrow[\text{SET- step 2}]{-H^+} \mathrm{A:H} + \mathbf{B}^{\bullet}$$

Scheme 1.2: Reaction mechanism of antioxidants with free radicals.

A•: represents free radical, B: H: represents an antioxidant

The second mechanism hydrogen atom transport (HAT) (scheme 1.3) and the last mechanism is a combination of the HAT and the SET mechanism [35]. In the HAT mechanisms, free radicals remove one hydrogen atom ($\mathbf{H}^{\bullet}$) from antioxidant and antioxidant produced as a radical.

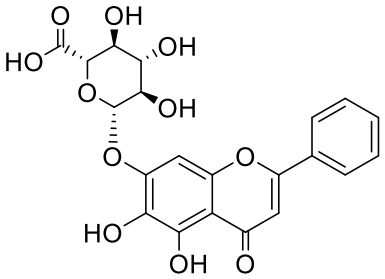
$$\mathbf{A}^{\bullet} + \mathrm{B:H} \xrightarrow[\text{HAT- step 1}]{-H^{\bullet}} \mathrm{A:H} + \mathbf{B}^{\bullet}$$

Scheme 1.3: Reaction mechanism of antioxidants with free radicals (HAT).

1.6.2 Antimicrobial Effects

Flavonoids are used to treat bacterial, protozoan, and fungal infections for two reasons; to destroy bacteria or fungal cells, and to prevent the spread of bacterial toxins and their effects [36]. Flavonoids have been reported to possess antibacterial mechanisms of action based on their structure-activity

relationships, such as inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism [34]. The flavonoids apigenin **19**, baicalin **40**, and galangin **41** have been studied for their inhibitory activity against sensitive and antibiotic-resistant strains of *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [34].



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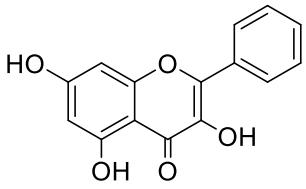

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To evaluate the in vitro antimicrobial activity of a plant extract, a number of laboratory techniques have been devised, including disk diffusion and broth dilution. The dilution method is used to assess microorganisms' ability to produce visible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing dilutions of the antimicrobial agent [37].

Broth dilution is a procedure in which a known number of bacteria is injected into containers containing equivalent quantities of broth with an antimicrobial solution at incrementally (typically geometrically) increasing concentrations. The performance of the broth dilution test on microdilution plates with a capacity of ≤500 µL per well is referred to as broth Microdilution [37].

The lowest concentration of antimicrobial agents that prevents the growth of microbes is known as MIC, and it is usually expressed in mg/mL or µg/mL [38].

1.7 Medicinal Plant in Palestine

Despite its small size (27,000 km2), there are over 2,700 plant species in Palestine. The phytochemical composition of the majority of them has not been studied [39]. Most popular plant families were *Compositae, Gramineae, Leguminaceae, Crucifera, Labiatae,* and *Liliaceae* [39]. Just about 800 of these plants are classified as rare [39]. Three *Bassia* species (*B. arabica* (Figure 1.4), *B. eriophora, and B. muricata*) are found in Palestine, spreading to the Negev desert, as well as in Jericho, near the Dead Sea. It was reported that flavonoids were isolated and identified from ethanoic extracts of *B. eriophora* aerial parts grown in Aljouf/SA [40].

Figure 1.4: *Bassia arabica* plant.

1.7.1 *Amaranthaceae (Chenopodiaceae)* Family

Many studies in the past considered the *Chenopodiaceae* as a sister family of the *Amaranthaceae* family, but recently, these two families have been considered as one family [41]. *Amaranthaceae* (*Chenopodiaceae*) is a large family of angiosperms, presenting a cosmopolitan distribution with more than 175 genera and 2000 species. It is distributed all over the world with high economic and medical importance [42]. Several studies have shown that *Amaranthaceae* plants are a rich source of phytochemical compounds that display a wide range of biological activities such as hypolipidemic, antidiabetic [42]. Terpenoids and phenolic compounds are considered the most important active compounds that were isolated from this family. It has been demonstrated that the active compounds present in herbal plants from the *Amaranthaceae* display antifungal and antiviral effects [42].

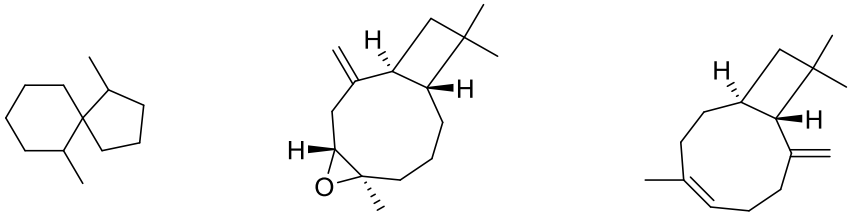
Bassia is a genus of flowering plants belonging to the family *Chenopodiaceae* (*Amaranthaceae*) with only 20 species distributed in the world, especially in the Irano-Turanian Region. Several *Bassia* plants possess high nutritional and medicinal values [43]. For instance, *Bassia muricata* is used in traditional medicine for the treatment of renal and rheumatic illnesses, and possesses diversified degrees of anti-inflammatory, analgesic, and antipyretic, as well as antispasmodic activities [44]. Furthermore, *B. Eriophora* has been used traditionally in parts of Iran as a remedy for Alzheimer's and hair loss. *B. latiofolia* has been reported to possess antitumor, analgesic, anti-estrogenic, hepatoprotective, anti-inflammatory, and wound healing activities. It is also

used traditionally for curing rheumatism, ulcers, bleeding, and tonsillitis [45].

1.7.2 *Lamiaceae* Family

Lamiaceae (*labiatae*) is a large family of flowering plants that includes approximately 258 genera and 7,193 species distributed all over the world with global economic and medicinal importance [46]. These plants are embodied by being alternatives to synthetic products in the treatment of diseases such as carcinogenesis, cardiovascular, and inflammation diseases [46, 47]. Peppermint, marjoram, thyme, and rosemary are the most common species belonging to the *Lamiaceae* family [46]. Many bioactive chemical compounds were isolated and identified from the *Lamiaceae* plants. The most important of these are phenolic compounds [47]. These phytochemical compounds possess natural antioxidant, antibacterial, and antifungal effects [47]. *Nepeta* is a flowering plant genus in the *Lamiaceae* family that is widely distributed in Europe, North Africa, North America, and Asia [50]. *Nepeta curviflora* is a 30-40 cm tall perennial herbaceous montane plant [51]. In the mountains of Palestine, Jordan, Syria, Lebanon, and Turkey, this plant germinates wildly [50]. In addition to their enormous importance in the cosmetics and nutraceutical industries, essential oils extracted from plants play an important role in medicine. Jaradat et al (Jaradat et al., 2020) studied the in-vitro antioxidant, porcine pancreatic lipase, α-glucosidase, and α-amylase inhibitory activities of the essential of *N. curviflora* oil obtained by microwave ultrasonic-assisted extraction, in addition to the identification

and quantification of the phytochemical composition of *N. curviflora* essential oil (figure 1.5) [50]. Twenty compounds were identified, representing 100% of the total EO. The most abundant components of *N. curviflora* EO were found to be 1,6-dimethyl spiro [4.5] decane **42** (27.5%), caryophyllene oxide **43** (20.08%), and β-caryophyllene **44** (18.28%).



42 **43** **44**

It's important to note that the elevated caryophyllene oxide **43** and β-caryophyllene **44** were identified with higher concentrations in the essential oil of *N. curviflora* growing in Palestine than in *N. curviflora* EO growing in Lebanon and Jordan [50].

Figure 1.5: *Nepeta curviflora* plant.

1.8 An introduction of Techniques Used in Natural Products Study

Living organisms of all kinds, animals, plants and microorganisms, produce compounds called natural products that have a major and important role in biological reactions. These reactions are divided into two types. The first type is primary reactions from which different compounds are produced, including proteins, fats, and acids, which are responsible for the production of secondary metabolite compounds such as terpenoids, alkaloids, and phenolic compounds.

Pretreatment, extraction, isolation, and purification are the four main methods for studying chemical composition and extracting natural products from plants [51]. Maceration, homogenization, grinding, and drying are the most important steps in the pretreatment process [51].

1.8.1 Extraction Techniques

Extraction is the first step in separating natural products. Several methods were used in this process, including solvent extraction, distillation, pressure, and sublimation. Solvent extraction is the most effective separation method for flavonoids and other phenolic compounds [52]. This method requires a solvent with a low boiling point, insoluble in water, has a medium polarity, and higher affinity for plant extracts than water. Soxhlet extraction, microwave-assisted extraction, sonication-assisted extraction, pressurized fluid extraction, and subcritical fluid extraction are the main methods used to extract plant phytochemicals [51].

1.8.2 Purification Techniques

Some of the most important methods used in the isolation and purification process are solid-phase extraction, acidity-based fractionation, and column chromatography [51]. Filtration, precipitation, and removal of chlorophyll, waxes, and tannins from the extracted analytes are examples of pre-purification methods [53].

1.8.3 Chromatographic Techniques

Chromatography is a technique used for separating a mixture of components. The separation is determined by the distribution of the mixture components into two phases: mobile and stationary [52]. Twist was the first to introduce the principle of chromatography adsorption at the beginning of the twentieth century. Initially, it was only used to separate pigments, which was later expanded to provide compound analysis and purification in phytochemistry [54]. The most popular method for purifying natural products is column chromatography. Due to the high capacity, simplicity, and low cost of silica gel adsorbents [52].

1.8.3.1 Thin layer Chromatography

Thin-layer chromatography (TLC) is a type of liquid chromatography technique used to separate nonvolatile mixtures of substances into their components. This technique is characterized by its speed, resolution, and sensitivity [54]. The solvent used is the mobile phase and the stationary phase is usually silica gel, aluminum oxide, or cellulose. TLC is an ideal

method used for biological and chemical analysis, and for determining qualitative results [54]. The principle operation of pH. TLC technique is simple. First, a sample of the material to be used is placed on the plate as a spot, then placed into a chamber containing the mobile phase (solvent or mixture of solvents) [55]. The mobile phase flows through the stationary phase and carries the mixture of components at different rates [55]. Before the mobile phase reaches the end of the stationary phase, a sample is removed from inside the chamber. The spots are visualized by ultraviolet light.

1.8.3.2 Preparative Thin Layer Chromatography

Preparative Thin Layer Chromatography (PTLC) is a useful technique used to separate and purify small quantities of materials or samples. It can use large amounts of samples, up to 300 mg, depending on the separation [56]. The sample of the material to be analyzed is placed in the form of long lines on a layer whose silica gel thickness ranges between (.05-2.0 mm) and is coated on glass panels. The separation is carried out with an appropriate solvent, then the material is scraped to be returned [56].

1.8.3.3 High-Pressure Liquid Chromatography

One of the most useful techniques for separating organic matter is high-performance liquid chromatography (HPLC), which has been applied to the separation, purification, and identification of natural products, especially flavonoids [55]. In the stationary phase of HPLC, the column C18 packing

material is used, and the mobile phase is (methanol-water). There are two types of HPLC techniques based on the mode of separation, normal phase chromatography and reverse-phase chromatography. In the normal phase, the stationary phase is polar and the mobile phase is non-polar, while the stationary phase of the reverse phase is non-polar and the mobile phase is polar [57]. Reverse-phase chromatography is the most widely used for the separation of polar compounds [57].

1.8.3.4 Gas Chromatography–Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) is an analytical technique that combines the separation properties of gas-liquid chromatography with the detection systems of mass spectrometry to isolate various constituents in the tested sample.

The analyte will have a significant vapor pressure between 30 and 300°C f or the GC to act [58]. The analyte which will be identified by GC-MS is fragmented depending on its mass [58].

1.9 Structure Identification Techniques

Several structural elucidation techniques are used for organic compound determination and identification isolated from natural products. The most important of these techniques are UV-visible, mass spectroscopy (MS), infrared spectroscopy (IR), nuclear magnetic resonance (NMR), and crystallography.

1.9.1 Infrared Spectroscopy (IR)

Infrared spectroscopy, also known as vibrational spectroscopy, is an analytical technique for determining function groups of organic and inorganic compounds [59]. The Infrared spectroscopy technique is based on absorbing infrared radiation with a wavelength range of 4000-400 cm^{-1}, which molecules of compound give vibration [59].

1.9.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy (NMR) is the most important method for the identification and quantification of unknown organic compounds. Using a small amount of sample for analysis by the NMR spectroscopy method enables us to determine the types and number of atoms and chemical structure of the compound of the sample to be analyzed [60]. The nucleus of atoms with a single mass or odd atomic number has magnetic properties known as "spin" that makes them active in NMR spectroscopy. Examples of these atoms are the nuclei of each H^{1}, C^{13}, P^{31}, N^{15} and F^{9} [61]. The source of energy in NMR is the long-wavelength radio frequency [59].

1.9.3 Mass Spectrometry

Mass Spectrometry (MS) is considered as an analytical tool used to characterize the composition of unknown compounds and to calculate their m/z values [61]. The most important steps of this technique are ionization, mass analysis, and detection. When the sample is inserted into a mass spectrometer, it will ionize and then the molecular mass is calculated [62]. There are many types of mass analyzers used in MS technology, such as the

quadrupole ion trap, sector block, and time flight analyzer. MS detectors generate either an induced current or a charge produced by ions [63]. The detector is an important component of mass spectrometry [63].

1.10 Aims of the Study

1. Study of the chemical composition and pharmacological effects of *B. arabica* and *N. curviflora*.
2. Evaluation of the antibacterial and antioxidant activities of *B. arabica* and *N. curviflora* of the methanol extract and of the fractions.
3. The antioxidant properties of both types will be evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) antioxidant assay.
4. Polar extracts, as well as fractions will be tested against a range of bacteria.

Chapter Two

Material and Method

2.1 Chemicals

All chemicals used were purchased from Sigma Aldrich chemical company and were used without further purification**.** The chemicals that have been used are: (Methanol, ethyl acetate, dichloromethane, ether, distilled water, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox, Mueller-Hinton broth, and dimethyl sulphoxide (DMSO).

2.2 *Bassia arabica* (Collection of Plant and Extraction)

The plant (*B. arabica*) was collected in January 2020, from Jericho, around 100 m away from the Dead Sea (31° 30` N 35° 30` E). The botanical identification (Pharm-PCT-360) was approved at the Pharmacognosy Laboratory, An-Najah National University by the pharmacognosist, Dr. Nidal Jaradat.

It was dried in the shadow for 2 weeks at room temperature. Air-dried stems of *B. arabica* (814.45 g) were grinded and suspended in methanol (2L) for 24 hours. The green suspension was stirred with a mechanical stirrer, then the methanol extract was filtered, and the solvent was removed using a rotary evaporator under reduced pressure.

2.2.1 Separation by Column Chromatography Technique

TLC was used to find a suitable eluent to separate the *B. arabica* into pure components by a column chromatography technique. Several formulations of

mobile phase were used to determine the number of compounds in the extract and these solvents were (dichloromethane 100%), (dichloromethane/ethyl acetate 9:1), (dichloromethane/ethyl acetate 8:2), (dichloromethane/ethyl acetate 7:3), (dichloromethane/ethyl acetate 6:4), and (dichloromethane/ethyl acetate 5:5).

Column chromatography technique was selected to separate components of ethyl acetate extract of *B. arabica* by using silica gel. The mobile phase consists of dichloromethane/ethyl acetate. The separation process was done with 100% dichloromethane and gradually increased its polarity by adding ethyl acetate until (dichloromethane/ethyl acetate 1:1) was reached. Identical fractions were collected depending on thin layer chromatography tests as shown in table 2.1.

Table 2.1: Results of separation by column chromatography of ethyl acetate extract.

DCM %	Ethyl acetate %	# Fractions before collection	# Fraction after collection	Weight of fractions in mg
100	0	-	-	-
90	10	1-6	A1	40 mg
90	10	7-8	A2	70 mg
90	10	9-11	A3	400mg
90	10	13-16	A4	70mg
90	10	17-19	A5	30 mg
90	10	20-23	A6	10 mg
90	10	24-26	A7	310 mg
90	10	27-30	A8	190 mg
80	20	31-33	A9	110 mg
80	20	34-35	A10	40 mg
80	20	36-39	A11	50 mg
80	20	40-45	A12	90 mg
70	30	46-50	A13	110 mg
60	40	51-54	A14	40 mg
			14 fractions	Total = 1560mg g (1.56 g)

2.2.2 Fraction A8

Fraction A8 (190 mg) contains three fractions that showed one spot on the TLC with very similar polarities that were separated using a PTLC technique with dichloromethane/ethyl acetate (9.5:0.5) as eluent. Three fractions were obtained, namely, $A8_1$, $A8_2$, and $A8_3$.

2.2.3 Fraction A9

Fraction A9 (110 mg) contains two fractions that showed one spot on the TLC with very similar polarities that were separated using a PTLC technique with dichloromethane/ethyl acetate (9:1) as eluent. Two fractions were obtained, namely, $A9_1$, $A9_2$.

2.3 *Nepeta curviflora (*Collection of Plant and Extraction)

The leaves and stems were collected from Palestine's Berzeit mountains. The botanical identification (Pharm-PCT-1633) was approved at the Pharmacognosy Laboratory, An-Najah National University by the pharmacognosist, Dr. Nidal Jaradat.

Air-dried stem, leaves of *N. curviflora* were grinded powdered and suspended in methanol (2L) for 24 hours. The green suspension was stirred with a mechanical stirrer, then methanol extract was filtered and the solvent removed using a rotary evaporator, washed with deionized H_2O, and then exhausted with hexane (200mL).

2.3.1 Separation by Column Chromatography Technique (*Nepeta curviflora*)

The column chromatography technique was selected to separate the components of ethyl acetate extract (11.9 g) of *N. curviflora* by using silica gel as a stationary phase. The liquid mobile phase consists of dichloromethane/ethyl acetate. The separation process was done with (dichloromethane 100%), and gradually increased its polarity by adding ethyl acetate until (ethyl acetate/acetone 7:3) was reached. Identical fractions were collected (Table 2.2) depending on the TLC test.

Table 2.2: Fractions resulted from column chromatography technique.

# Fraction after collection	# Fractions before collection	Weight of fractions (mg)
N1	1-4	1260 mg
N2	5-16	270 mg
N3	17-19	920 mg
N4	20	160 mg
N5	21-24	2320 mg
N6	25-29	130 mg
N7	30-34	430 mg
N8	35-40	400 mg
N9	41-46	240 mg
N10	47-52	300 mg
N11	53-58	300 mg
N12	59	250 mg
N13	60	260 mg
N14	61	450 mg
N15	62,63	1130 mg
N16	64	80 mg
N17	(washing with methanol)	320 mg
Total = 17 Fractions		Total = 10390 mg (10.39 g)

2.3.2 Fraction N5

Fraction N5 (2.32 g) was subjected to silica gel column chromatography using dichloromethane and ether (9.5: 0.5 v\v). TLC test was used to collect identical fractions. As a result, fractions were obtained, namely, $N5_1$ and $N5_2$.

2.4 Identification of the Phytochemical Composition of Plant Extracts

2.4.1 IR Spectroscopy

Functional groups of the fractions isolated from both *B. arabica* and *N. curviflora* plants were characterized using the FT-IR spectrometer (NICOLIT iS5 from Thermo Fisher Scientific).

2.4.2 HPLC Technique

The purity of obtained fractions was checked by TLC plates and confirmed using HPLC analysis (Fig a.3-a.13). A qualitative analysis was conducted using HPLC-DAD Water 1525, with a C18 column (5µm, 4.6×250 mm cartridge). The mobile phase consists of solvent A (water) and solvent B (methanol). The HPLC separation was achieved using binary-solvent gradient elution, which began with 100% of solvent A and 0% of solvent B and increased to 0% of A and 100% of B. The injection volume was 20 µL with a flow rate of 0.7 ml/min. In this study, reverse phase chromatography was used to separate polar compounds from plant extracts. The stationary phase of the reverse phase is non-polar and the mobile phase is polar.

2.5 Antimicrobial Activity Test

The antibacterial activity of *B. arabica and N. curviflora* fractions was evaluated using the broth micro-dilution method based on estimation of the minimum inhibitory concentration (MIC) [64]. Stock solutions of the two plants were prepared in DMSO and two-fold serial dilutions were inserted in 96-well microliter plates.

2.5.1 Microorganisms Used

Six types of bacteria namely MRSA (clinical strain), *Staphylococcus aureus* (ATCC 25923), Klebsiella pneumoniae (ATCC 13883), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 8427), and *Pseudomonas aeruginosa* (ATCC 9027), also *Candida albicans* (ATTC 90028) were used, Fluconazole, ampicillin, ciprofloxacin antibiotics were used as positive controls.

2.5.2 Determination of (MIC) Against Different Microorganisms

The Solubility of fractions was examined in both distilled water and DMSO, then each of fractions was dissolved in H_2O or DMSO to prepare a stock solution of different concentrations. After that, each fraction was sterilized in an autoclave for 15 minutes. A spectrophotometer was used to measure the optical density of all solutions at 620 nm, with normal saline acting as a blank. To obtain a bacterial suspension with 1.5×10^8 colony forming units (CFU/mL), the turbidity of the bacterial suspensions was adjusted to the 0.5 McFarland turbidity standard (optical density of 0.08 to 0.12). The yeast *C.*

albicans' turbidity was also adjusted to equal that of a 0.5 McFarland solution (optical density of 0.12 to 0.15) with a concentration of 1 $\times 10^6$ – 5 $\times 10^6$ CFU/mL. Six colonies of bacteria and *c. albicans* were transferred to sterile tubes each containing 4-5 mL of normal saline. The bacteria prepared were transferred in to new sterile tubes by taking 100 µL of them and adding Mueller Hinton, completing the final volume of 10 mL. Therefore, 50 µL of the Mueller Hinton was added into each well in each column of rows from A to G in the bacteria microplates (Fig 2.1). Starting from the first column, 50 µL of each extract was added. The serial dilution method was used until the rest of the columns were completed up to column No. 10. Then, 50 µL of bacteria was added to each row from columns 1-11. The same steps were repeated for all extracts by using *C. albicans*. Plates were incubated at 37˚C for 24 hours. The first plate that contained no visible growing bacteria was considered as the MIC. For all bacteria tested in the current work, ciprofloxacin, ampicillin, and fluconazole were used as positive controls in the current study.

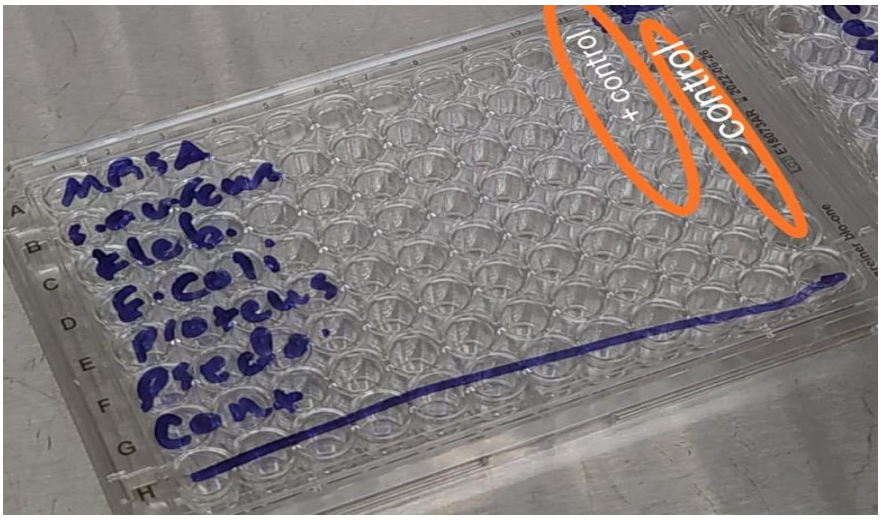


Figure 2.1: Microplates for the antimicrobial test.

For all tested bacteria, four controls were considered: positive control which contains media + bacteria (column # 11), negative control which contains media only (column # 12), Compound control (Compound+ media): to be sure there is no contamination & turbidity and changes are not due to the compound itself (so compounds were serially diluted in this control) and DMSO was tested for every microbe separately to check the effect on each one. Antimicrobial activity for DMSO was considered.

2.6 Antioxidant Activity Test

In the present study, a free radical scavenging activity test was used to study antioxidant activity for eight fractions of pure compounds from *B. arabica* and *N. curviflora* plants.

2.6.1 Free Radical Scavenging Activity

The antioxidant activity of *B. arabica and N. curviflora* extracts was evaluated based on the ability of the extracts to scavenge free radical diphenyl-1-picrylhydrazyl (DPPH). A stock solution (100 µg/mL) was prepared from *B. arabica* and *N. curviflora* extracts by dissolving 10 mg of each extract in methanol (100 mL), with serial dilutions to obtain different concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µg/mL). 1 mL of the diluted extracts and 1 mL of methanol were mixed with 1 mL of DPPH solution freshly prepared at a concentration of 0.002% w/v (2 mg per 100 mL methanol). Using a UV-visible spectrophotometer, the absorbance of the samples was measured at 517 nm against the control after 30 minutes of

incubation at room temperature in a dark place. By replacing the plant extract solution with neat methanol, a blank solution was formed. The inhibition percentage was determined using the equation below.

DPPH Inhibition Activity (%) = [(A_{blank} - A_{sample})/ (A_{blank}) ×100] [65]

A_{blank}: The absorbance of blank at 517 nm.

A_{sample}: The absorbance of the sample at 517 nm.

The (IC_{50}): half maximal inhibitory concentration for all fractions was measured. The same conditions were used for the evaluation of the inhibition percentage and IC_{50} of trolox as a positive control.

Chapter Three

Results

3.1 *Bassia arabica* Plant

3.1.1 Phytochemical of Polyphenolic Composition of *Bassia arabica*

Fourteen fractions of *B. arabica* were obtained by column chromatography. The purity of the obtained fractions was checked by TLC plates and confirmed using HPLC analysis. Due to the lack of NMR techniques at An Najah National University, as well as Covid-19, fractions were not sent abroad to run ^{1}H-and ^{13}C-NMR and 2D-NMR that help in identification the purified fractions.

The functional groups of fractions of high purity were identified infrared spectroscopy.

Fraction A5: IR analysis showed that fraction A5 of *B. arabica* had a carbonyl group (1745 cm^{-1}) (Fig a.14, appendix).

Fraction $A8_1$: IR analysis showed that fraction $A8_1$ of *B. arabica* had a ketone group (1707 cm^{-1}) (Fig a.15, appendix).

Fraction A 8_2: IR analysis showed that fraction $A8_2$ of *B. arabica* had a ketone group (1709 cm^{-1}) (Fig a.16, appendix).

Fraction $A8_3$: IR analysis showed that fraction $A8_3$ of *B. arabica* had a ketone group (1697 cm^{-1}), and carbon-carbon double bond group (1593 cm^{-1}) Fig, a.17, appendix).

Fraction A11: IR analysis showed that fraction A11 of *B. arabica* had a carbonyl group (1700 cm^{-1}) (Fig. a.18, appendix).

Fraction A13: IR analysis showed that fraction A13 of *B. arabica* had a ketone group (1687 cm^{-1}) (Fig. a.19, appendix).

Using the HPLC technique, the phenolic content of plant extract fractions was determined qualitatively, depending on trolox and gallic acid as a standard (Fig. a.1, a.2).

The retention time of fractions A8 (Fig. a.5), A9 (figure a.6), A11 (Fig. a.7), and A12 (Fig. a.8) of *B. arabica* (3.449 min, 3.45 min, 3.52 min, 3.44 min) respectively were found in the range of gallic acid. While fraction A3 retention time (14.39 min) was found in the range of trolox (10.631). This confirms that those fractions are phenols.

3.1.2 Antimicrobial Activity

In the antimicrobial test, two ethyl acetate fractions namely (A3 and A7) were tested for their antimicrobial activity. Broth dilution method was employed to evaluate in vitro antimicrobial activity. The MIC values (mg/mL) for all fractions are listed in the Table below.

Table 3.1: Antibacterial activity MIC values of *B. arabica* fractions and antibacterial and antifungal positive controls (mg/mL).

MIC values (mg/mL) / Sources	MRSA	*S. aureus*	*K. pneumoniae*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*
Ethyl acetate extract (50mg/mL)	3.125	1.562	R	R	R	R	R
Fraction A3 (50mg/mL)	0.195	0.390	0.390	0.781	0.390	6.25	0.390
Fraction A7 (50mg/mL)	R	R	R	R	R	R	R
Fluconazole (0.032mg/mL)	0	0	0	0	0	0	0.00165
Ampicillin (0.032mg/mL)	0	0.00312	0.001	0.00312	0.018	0	0
Ciprofloxacin (0.032mg/mL)	0.0125	0.00078	0.000125	0.00156	0.015	0.00312	0

R: Resistance

3.1.3 Antioxidant Activity

Samples of ethyl acetate extract, and three ethyl acetate fractions, namely, (A7, A11and A12) were tested for their antioxidant activity. scavenging DPPH free radical assay, percentage inhibition and IC_{50} values (µg/mL) of all fractions were measured and recorded in the following Table.

Table 3.2: DPPH Inhibition activity % for *B. arabica* and trolox.

	Inhibition %				
Concentration (µg/mL)	Trolox	Ethyl acetate extract	Fraction A7	Fraction A11	Fraction A12
1	19.27	31.12	44.40	22.27	16.80
2	16.80	41.93	44.53	22.40	23.44
3	45.44	41.28	44.53	49.61	24.22
5	54.95	41.80	45.70	49.74	29.30
7	87.11	41.80	54.95	50	29.30
10	95.83	58.95	55.86	54.17	29.69
20	95.83	71.48	58.98	54.30	33.59
30	97.14	84.38	59.38	66.80	33.59
40	97.53	87.89	59.77	71.22	37.89
50	97.14	95.70	59.77	74.35	36.98
80	97.14	96.09	56.64	89.97	45.05
100	97.14	96.09	56.90	91.41	45.31

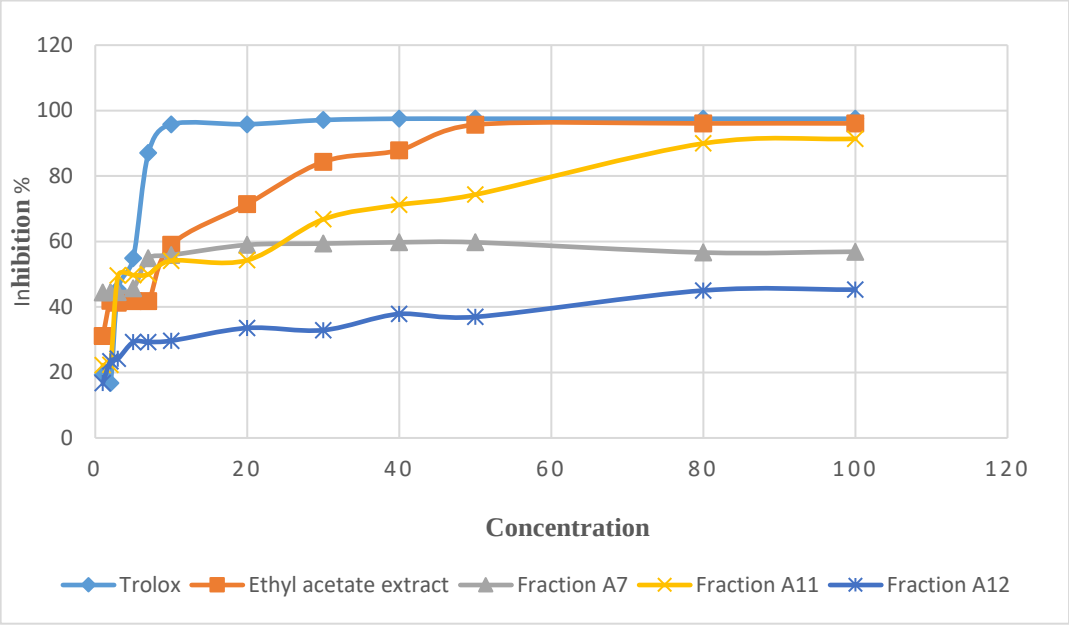


Figure 3.1: DPPH inhibitory activity by *B. arabica* fractions and trolox.

Table 3.3: IC_{50} values (µg/mL) of *B. arabica* fractions.

Fraction	IC_{50} (µg/mL) ± SD
Trolox sample	3.39 ± 0.72
Ethyl acetate extract	4.91 ± 0.03
Fraction A7	5.12 ± 0.11
Fraction A11	7.28 ± 0.07
Fraction A12	343.43 ± 37.43

SD: standard deviation

3.2 *Nepeta curviflora* Plant

3.2.1 Phytochemical of Polyphenolic Composition of *Nepeta curviflora*

Seventeen fractions of *N. curviflora* were obtained by flash chromatography. The purity of the obtained fractions was checked by TLC plates and confirmed using HPLC analysis.

The functional groups of fractions showing one spot on tlc and almost one major peak on HPLC were determined using infrared spectroscopy.

Fraction N4: IR analysis showed that fraction N4 of *N. curviflora* had a ketone conjugated group (1688 cm^{-1}) (Fig. a.20, appendix).

Fraction $N5_1$: IR analysis showed that fraction $N5_1$ of *N. curviflora* had a hydroxyl group as a broad band (3347.57 cm^{-1}), carbon-carbon double bond (1631cm^{-1}), and a carbonyl ketone (1723 cm^{-1}) (Fig. a.21, appendix).

Fraction $N5_2$: IR analysis showed that fraction $N5_2$ of *N. curviflora* had a carbonyl ketone (1706.60 cm^{-1}) (Fig. a.22, appendix).

Fraction N6: IR analysis showed that fraction 6 of *N. curviflora* had a hydroxyl group as a broad band (3328 cm^{-1}), and a carbonyl ketone (1711 cm^{-1}) (Fig. a.23, appendix).

Using the HPLC technique, the phenolic content of plant extract fractions was determined qualitatively, depending on trolox and gallic acid as a standard (Fig. a.1, a.2).

The retention time of fractions $N5_2$ (Fig. a.11) and N6 (Fig. a.12) of *N. curviflora* (4.518 min, 3.10 min) respectively is found in the range of gallic acid. This confirms that those fractions are phenols.

3.2.2 Antimicrobial Activity

Samples of ethyl acetate extract, water extract, and five ethyl acetate fractions were tested for their antimicrobial activity. The broth dilution method was employed to evaluate in vitro antimicrobial activity. The results of MIC values (mg/mL) for all fractions are listed in the Table below.

Table 3.4: Antibacterial activity MIC values of *N. curviflora* fractions and antibacterial and antifungal positive controls (mg/mL).

MIC values (mg/mL) / Sources	MRSA	*S. aureus*	*K. pneumoniae*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*
Ethyl acetate extract	R	R	R	R	R	R	R
Water extract (50mg/mL)	R	R	12.5	25	25	R	R
Fraction N5 (50mg/mL)	3.75	3.75	7.5	7.5	7.5	R	R
Fraction $N5_1$	3.125	1.562	6.25	6.25	6.25	R	1.562
Fraction $N5_2$ (30mg/mL)	1.562	1.562	3.125	3.125	3.125	6.25	1.562
Fraction N6 (50mg/mL)	3.125	1.562	6.25	6.25	6.25	6.25	3.125
Fraction N15 (50mg/mL)	R	R	R	R	R	R	R
Fluconazole (0.032mg/mL)	0	0	0	0	0	0	0.00165
Ampicillin (0.032mg/mL)	0	0.00312	0.001	0.00312	0.018	0	0
Ciprofloxacin (0.032mg/mL)	0.0125	0.00078	0.000125	0.00156	0.015	0.00312	0

R: Resistance.

The antimicrobial activity of DMSO was tested separately for each microbe (Table 3.5).

Table 3.5: Antimicrobial activity of DMSO 50%.

Microorganism	DMSO 50% # of well	Final result MIC (mg/mL)
MRSA	1	12.50
S. aureus	1	12.50
K. pneumoniae	1	12.50
E. coli	1	12.50
P. vulgaris	1	12.50
P. aeruginosa	1	12.50
C. albicans	3	3.125

3.2.3 Antioxidant Activity

Samples of water extract, and three ethyl acetate fractions, namely ($N5_1$, $N5_2$ and N10) were tested for their antioxidant activity. Using the scavenging DPPH free radical assay method, the percentage inhibition and IC_{50} values (µg/mL) of all fractions were measured and recorded as follows in the table below:

Table 3.6: DPPH Inhibition activity % for *N. curviflora* and trolox.

Inhibition %					
Concentration (µg/mL)	Trolox	Water extraction	Fraction $N5_1$	Fraction $N5_2$	Fraction N10
1	19.27	50	41.05	46.88	14.71
2	16.80	50	44.14	47.01	14.71
3	45.44	50	41.14	47.14	14.84
5	54.95	51.95	47.79	47.27	16.80
7	87.11	51.43	54.30	47.27	16.80
10	95.83	53.91	49.09	48.05	22.14
20	95.83	60.81	49.22	48.44	27.34
30	97.14	62.37	50	49.96	35.42
40	97.53	62.37	50	49.09	42.45
50	97.14	69.14	55.08	49.22	58.07
80	97.14	77.34	58.85	50	76.82
100	97.14	81.51	58.98	54.17	76.825

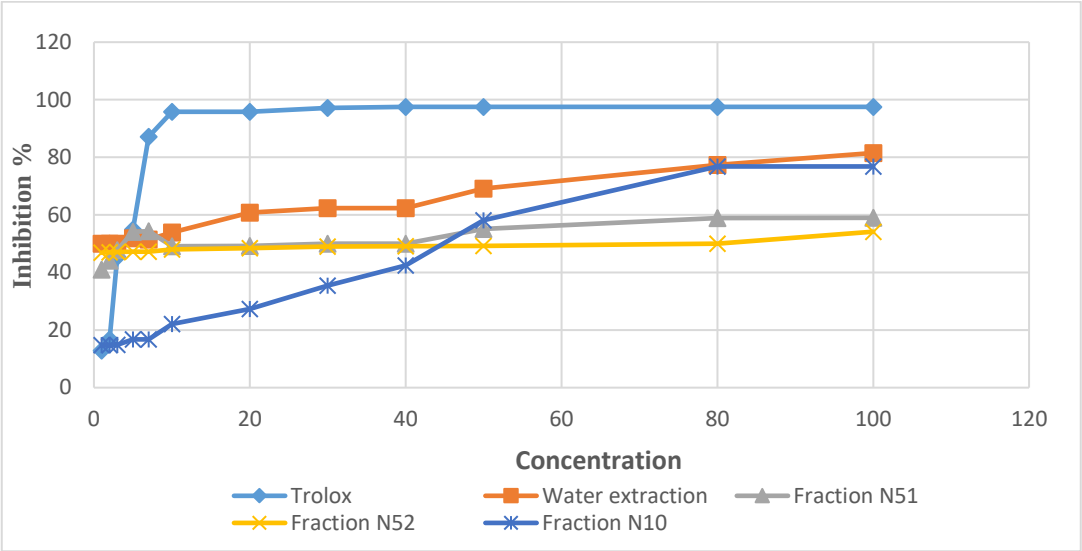


Figure 3.2: DPPH inhibitory activity of *N. curviflora* fractions and trolox.

Table 3.7: IC_{50} values (µg/mL) of *N. curviflora* fractions.

Fraction	IC_{50} (µg/mL) ± SD
Trolox Sample	3.39 ± 0.72
Water extract	2.83 ± 0.25
Fraction $N5_1$	8.91 ± 0.21
Fraction $N5_2$	45.23 ± 2.76
Fraction N10	38.32 ± 1.07

SD: standard deviation

Chapter Four

Discussion and Conclusion

4.1 *Bassia arabica* (Phytochemical of Polyphenolic Composition)

Medicinal and aromatic plants, such as *Bassia* species (*B. arabica*, *B. eriophora*, and *B. muricata*), growing in the Negab desert and Jericho, are abundant in Palestine (very close to the Dead Sea). *Bassia*, a genus of the *Amaranthaceae* family, is a rich source of phytochemical compounds with a wide range of biological activities, including hypolipidemic and anti-diabetic properties [42]. According to recent pharmacological studies, extracts from *Amaranthaceae* plants exhibit antioxidant, antidiabetic, anticancer, antibacterial, and anti-inflammatory properties. The most important active compounds that have been isolated from this family are terpenoids and phenolic compounds [42]. *B. arabica* has never been studied in terms of polyphenolic composition, antioxidant and antibacterial effect.

4.1.1 Antimicrobial Activity

As mentioned earlier, fractions from *B. arabica* were separated using a column chromatography technique. The MIC assay was employed to evaluate antimicrobial activity using the broth dilution method. The in vitro antibacterial activities of *B. arabica* extracts were evaluated against one type of fungus; *C. albicans* and six bacteria isolates, which includes four gram-negative bacteria namely *k. pneumoniae*, *E. coli*, *P. vulgaris* and *P.*

aeruginosa, in addition to two gram-positive bacteria, namely MRSA (clinical strain) and *S. aureus.*

Fraction A3 of *B. arabica*, one spot on the TLC, displayed antibacterial activity against all tested microorganisms as compared to other fractions of the plant. MIC values of fraction A3 were found in the range of 0.195 to 6.25 mg/mL. Among bacterial strains, MRSA was the most sensitive strain to fraction A3 (MIC of 0.195 mg/mL), while *P. aeruginosa* was the most resistant one (MIC of 6.25 mg/mL).

Alsaggaf (2018) found that ethanol extract from the *B. eriophora* plant had an MIC value of 0.075 mg/mL against *S. aureus* [66], which was better than in the current study.

4.1.2 Antioxidant Activity

Antioxidant results showed that ethyl acetate extract of *B. arabica* had a high antioxidant potential (IC_{50} = 4.91 ± 0.03 µg/mL), which was higher than the antioxidant activity displayed by fraction A11 (IC_{50} = 7.28 ± 0.07µg/ml). Ethyl acetate extract had the most value close to the trolox with IC_{50} of 3.39 ± 0.72.

Khalil et al. (2017) discovered that *B. eriophora* ethyl acetate extract had antioxidant activity with an IC_{50} value of 19.2 g/mL [67], whereas in this study, the methanol extract *B. arabica* as well as all tested fractions showed higher antioxidant activity than *B. eriophora*, with ethyl acetate extract having the highest antioxidant activity with an IC50 value of 4.91±0.03 4.91

µg/mL. Djahra et al (2018) reported that aqueous extract of *B. muricata* display antioxidant activity with an IC_{50} value of 543.26 ± 39.62 µg/mL [68]. Al Nasr (2020) studied the antioxidant activity of a methanol extract from the *B. eriophora* plant and found radical scavenging activity with an IC_{50} greater than 100 µg/mL [69].

4.2 *Nepeta curviflora* plant (Phytochemical of Polyphenolic Composition)

Nepeta is a flowering plant genus in the *Lamiaceae* family. Many bioactive chemical compounds were isolated and identified from the *Lamiaceae* plants. The most important of these are phenolic compounds [47]. These phytochemical compounds possess antioxidant, antibacterial, and antifungal properties [47]. For instance, *N. curviflora* is used in folk medicine as a remedy for nervous disorders and high blood pressure. It possesses different degrees of anti-inflammatory, antioxidant and antibacterial properties [48].

4.2.1 Antibacterial Activity

MIC values for fraction $N5_2$ of *N. curviflora* were found to be in the range of 1.562 and 6.25 mg/mL. The lowest MIC of fraction $N5_2$ was 1.562 mg/mL, and it was effective against MRSA, *S. aureus*, and *C. albicans*. Finally, among all the tested fractions, fraction $N5_2$ from *N. curviflora* had antibacterial activity against all types of bacteria.

In general, all tested microorganisms, six types of bacteria, and one type of fungal were inhibited by several plant extracts in this study. The antibacterial activity of extracts from the plants *B. arabica* and *N. curviflora* is varied. Omar

et al (2013) *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *S. aureus* were all resistant to the ethanol extract of *N. curviflora* [70], while the various extracts of *N. curviflora* inhibited all kinds of bacteria in our study, with the highest effective MIC value of 1.562 mg/mL. According to Sharifi et al (2020) the *N. juncea* methanolic extracts display significant antibacterial activity against the tested bacteria, with MIC values ranging from 25 to 100 µg/mL [71].

4.2.2 Antioxidant Activity

The antioxidant results showed that water extract display a high antioxidant potential, as measured by the concentrations at which 50% of radicals were quenched (IC_{50} = 2.83 ± 0.25 µg/mL), which was higher than the antioxidant activity from fraction 5_1 (IC_{50} = 8.91 ± 0.21µg/mL). Water extract's antioxidant activity was found to be somewhat greater than that of trolox (IC_{50} = 3.39 ± 0.72 µg/mL).

The results obtained from the current study were not in agreement with the findings by Seladji et al (2014), who found that methanol extract leaves had the most potent of all *N. nepetella* extracts, with an IC_{50} of (0.247 ± 0.03 mg/mL) [72]. Abdulmelik et al (2016) found that *N. nuda subs* exhibited remarkable oxidant activities, which were displayed by a water extract with IC_{50} (30.7 µg/mL) and IC_{50} = 52.3 µg/mL for ethanol extract [73].

4.3 Conclusion

HPLC analysis confirmed the purity of ethyl acetate extract of both *B. arabica* and *N. curviflora*. All fractions for both plants displayed antioxidant

activity. For *N. currvflora* the order of activity is : water extract> $N5_1$ >N10> $N5_2$>, and in the order ethyl acetate extract > A7 >A11 > A12 for *B. arabica*. Fraction A3 of *B. arabica* display antibacterial activity against all types of bacteria, with MIC values ranging from 0.195 to 6.25 mg/mL. Ethyl acetate extract of *B. arabica* had the best antioxidant potential (IC_{50}= 4.91 ± 0.03µg/mL). As a result, the water extract of *N. curviflora* had the strongest antioxidant capacity (IC_{50}= 2.83± 0.25µg/mL).

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Appendix

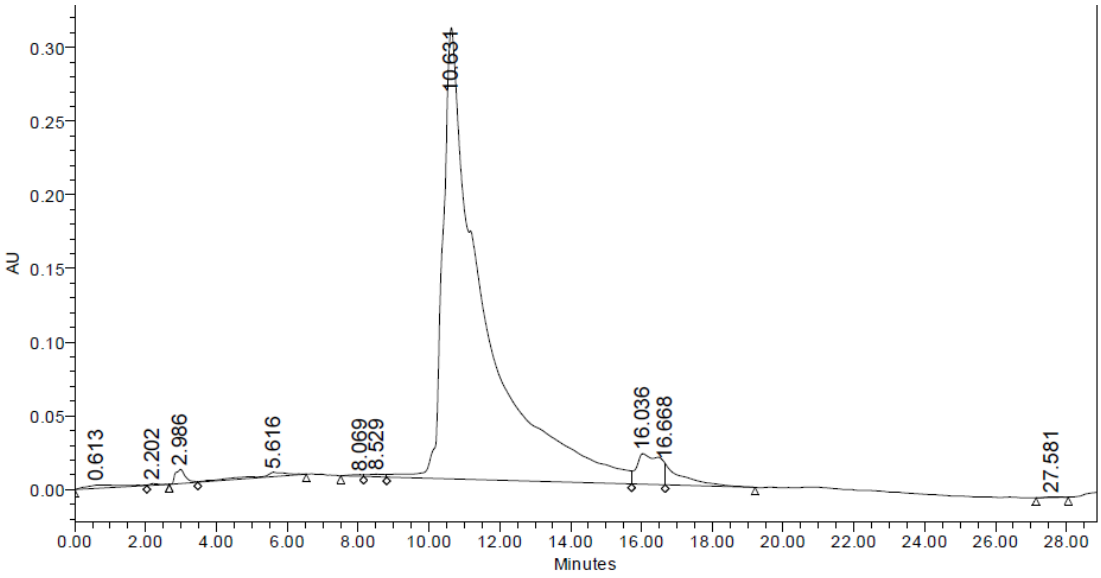

Figure a.1: HPLC chromatogram for trolox compound.

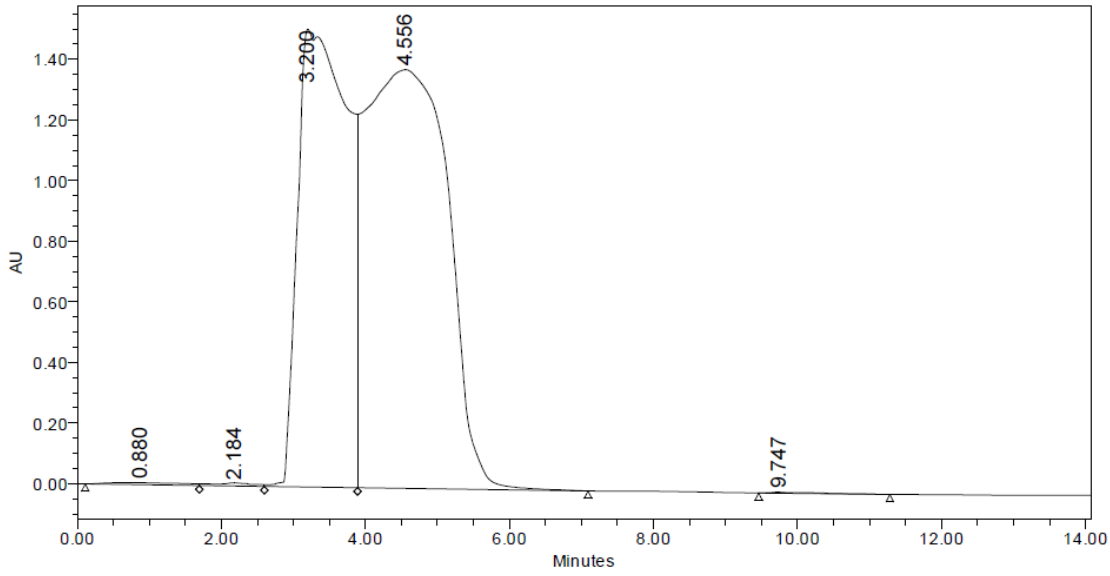

Figure a.2: HPLC chromatogram for gallic acid compound.

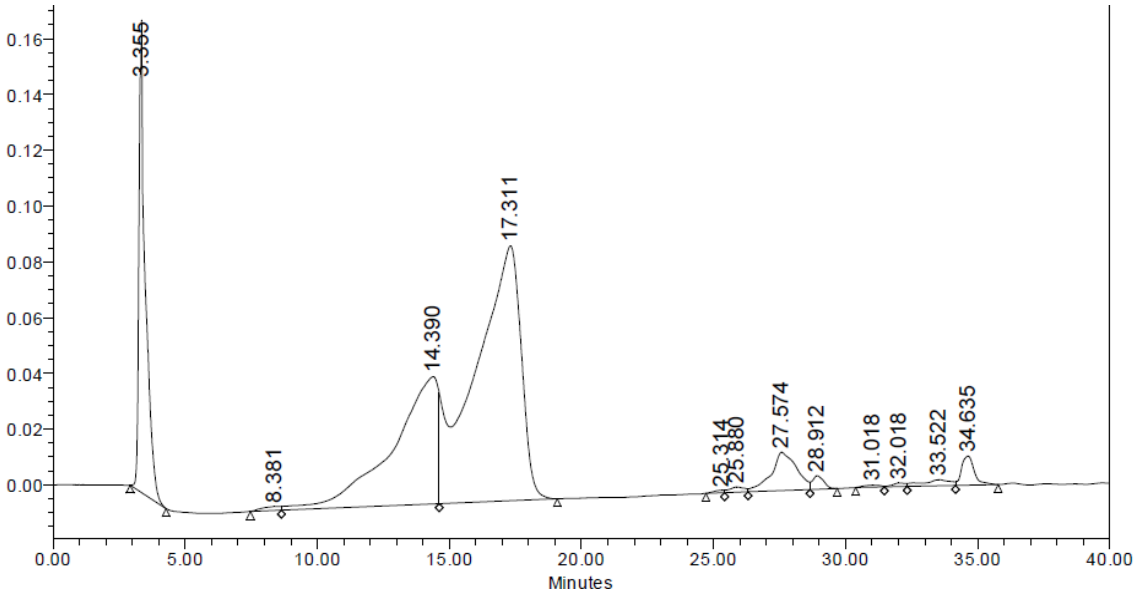

Figure a.3: HPLC chromatogram for fraction A3.

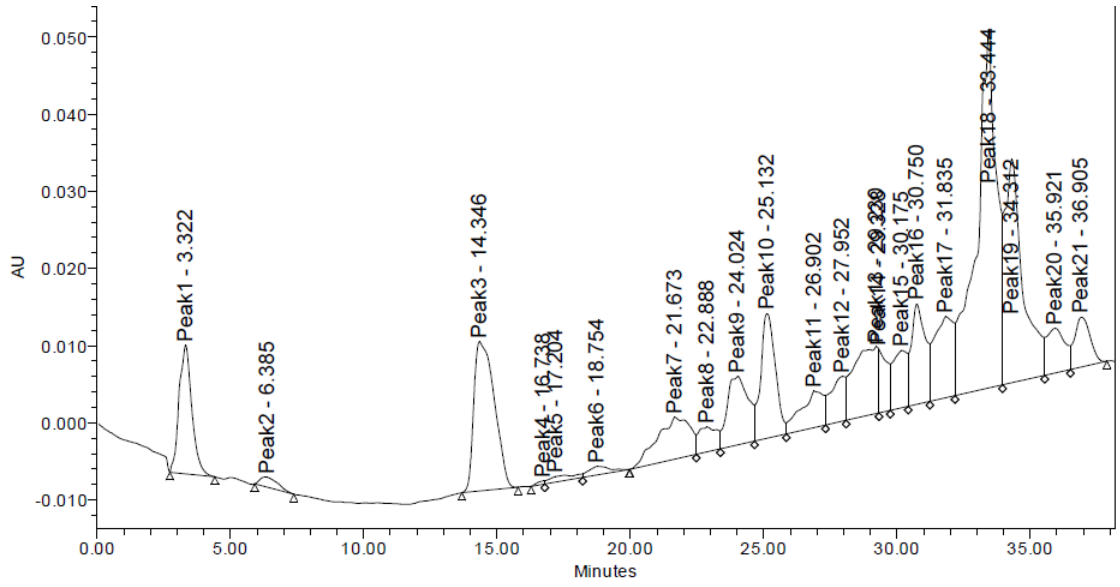

Figure a.4: HPLC chromatogram for fraction A7.

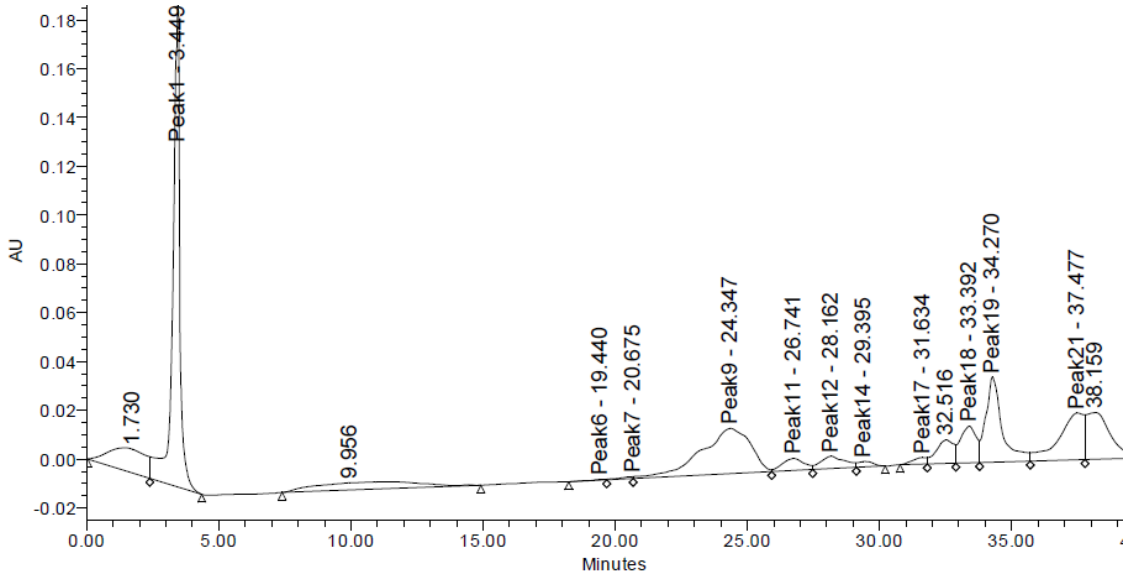

Figure a.5: HPLC chromatogram for fraction A8.

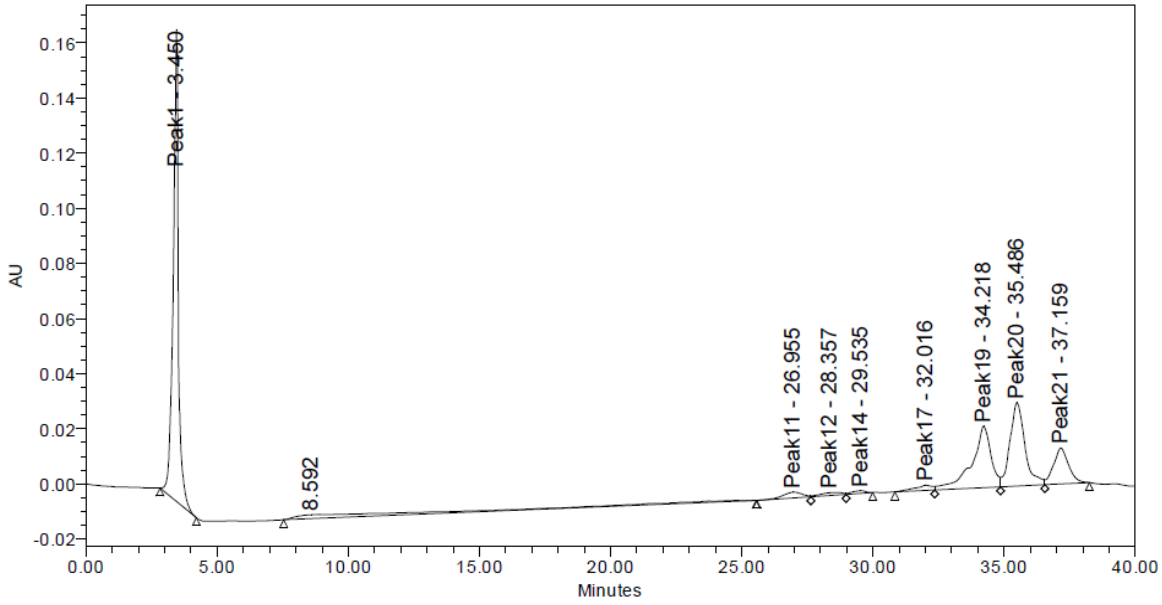

Figure a.6: HPLC chromatogram for fraction A9.

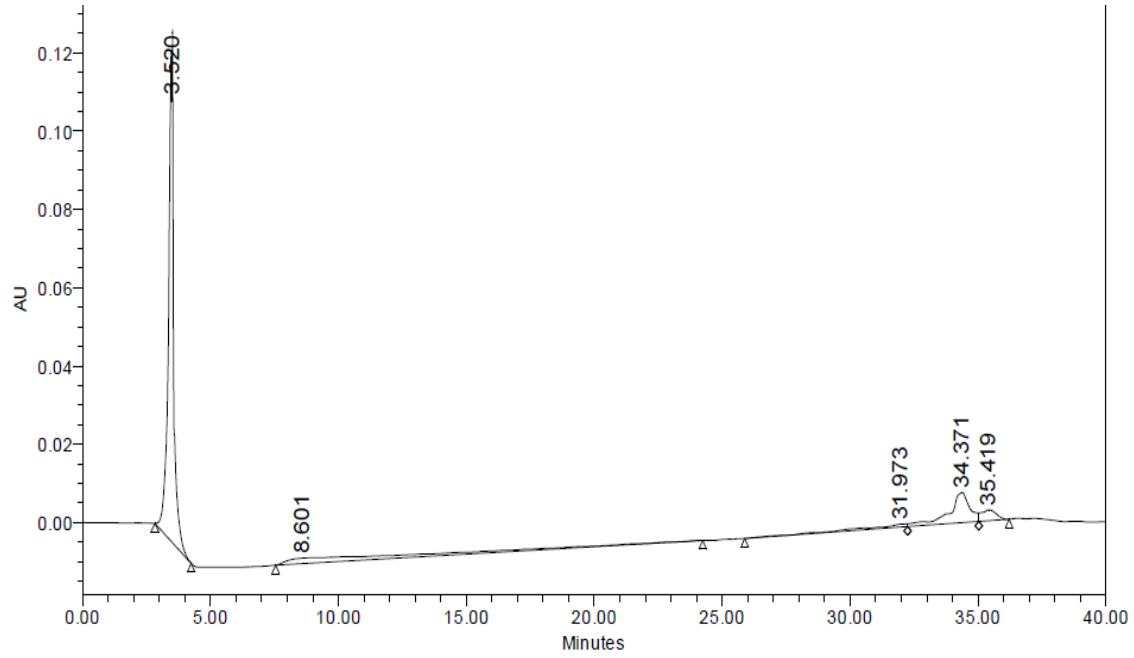

Figure a.7: HPLC chromatogram for fraction A11.

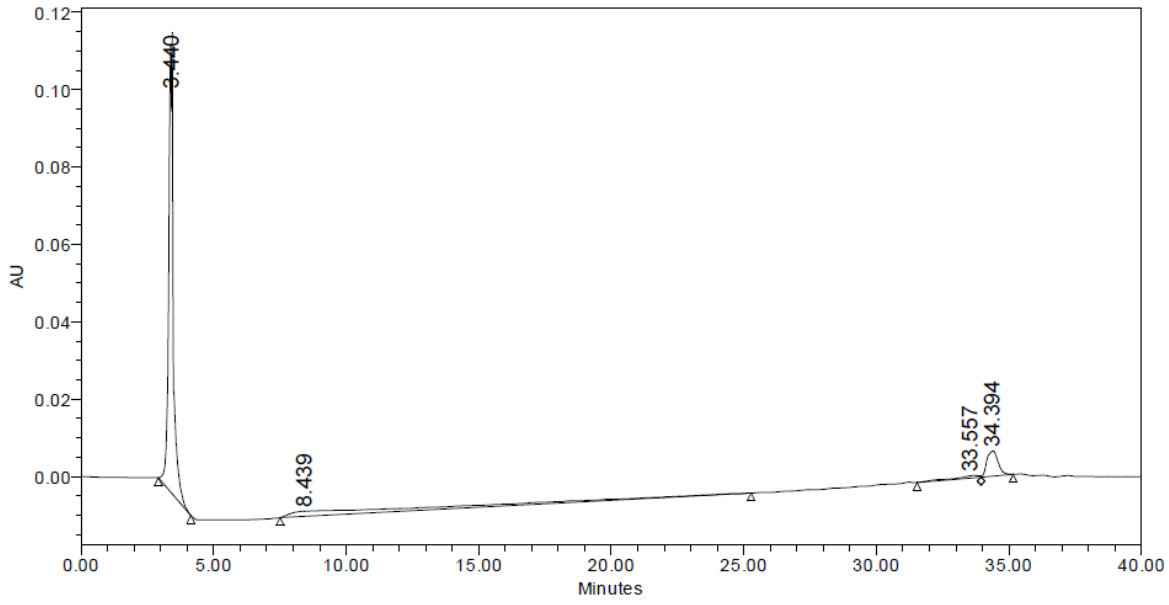

Figure a.8: HPLC chromatogram for fraction A12.

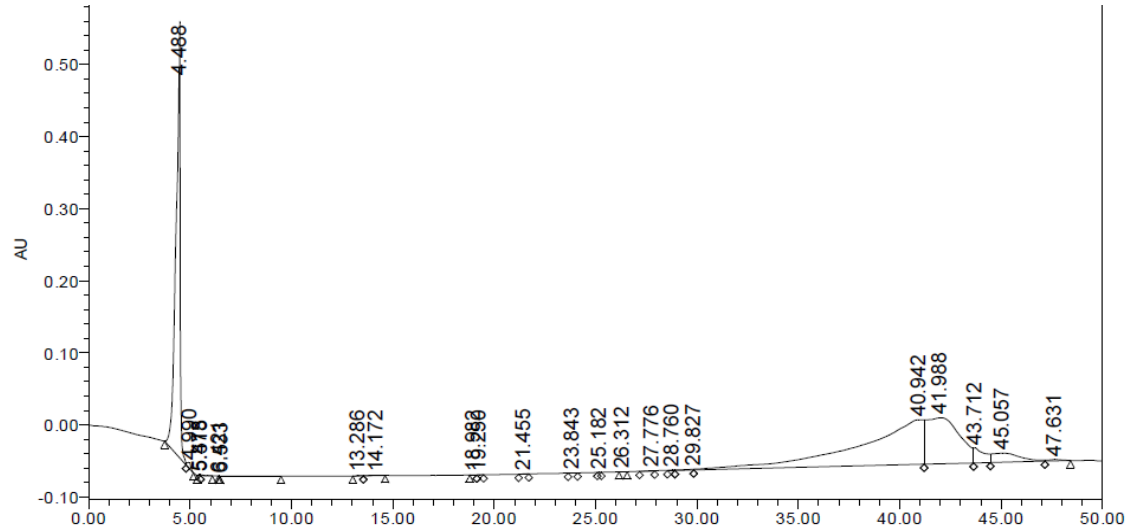

Figure a.9: HPLC chromatogram for fraction N5.

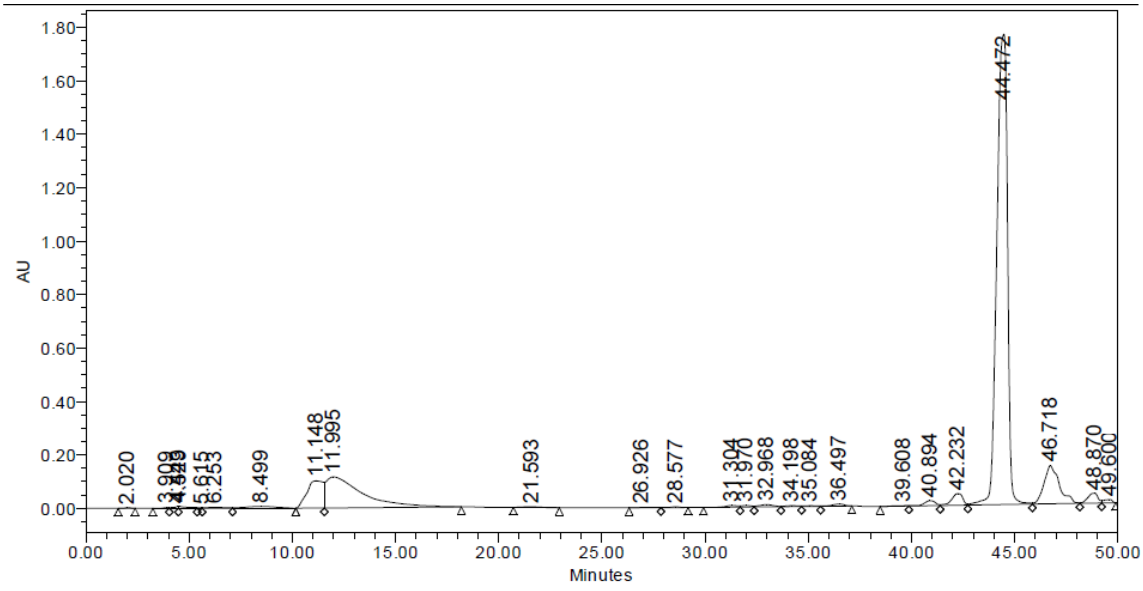

Figure a.10: HPLC chromatogram for fraction $N5_1$.

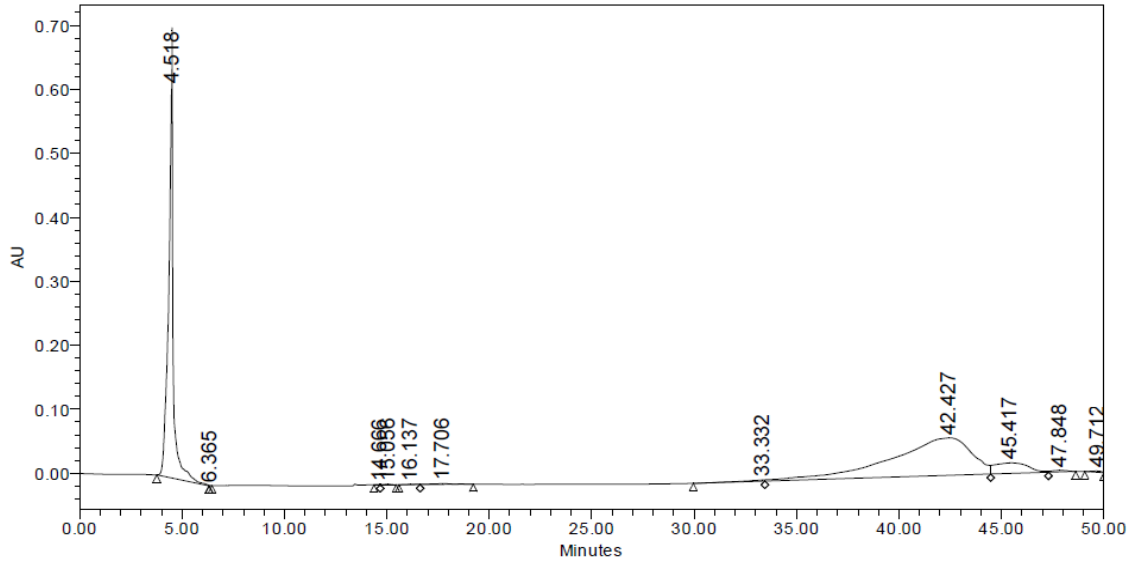

Figure a.11: HPLC chromatogram for fraction $N5_2$.

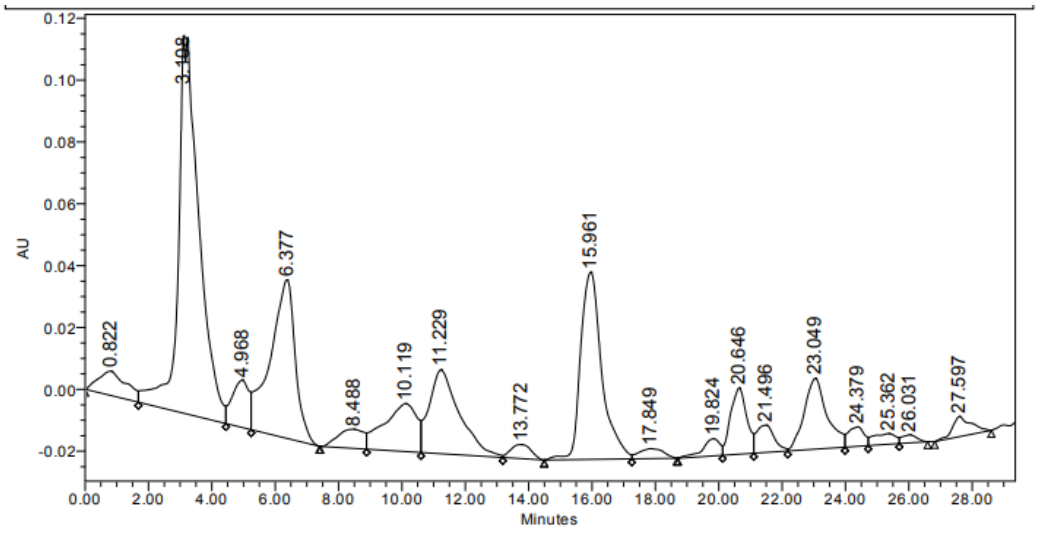

Figure a.12: HPLC chromatogram for fraction N6.

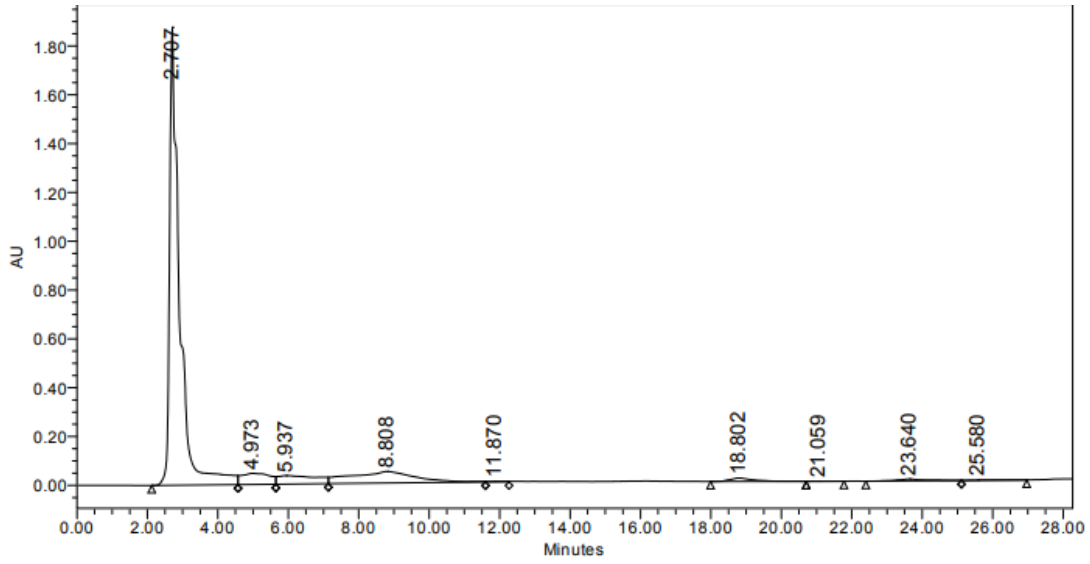

Figure a.13: HPLC chromatogram for fraction N15.

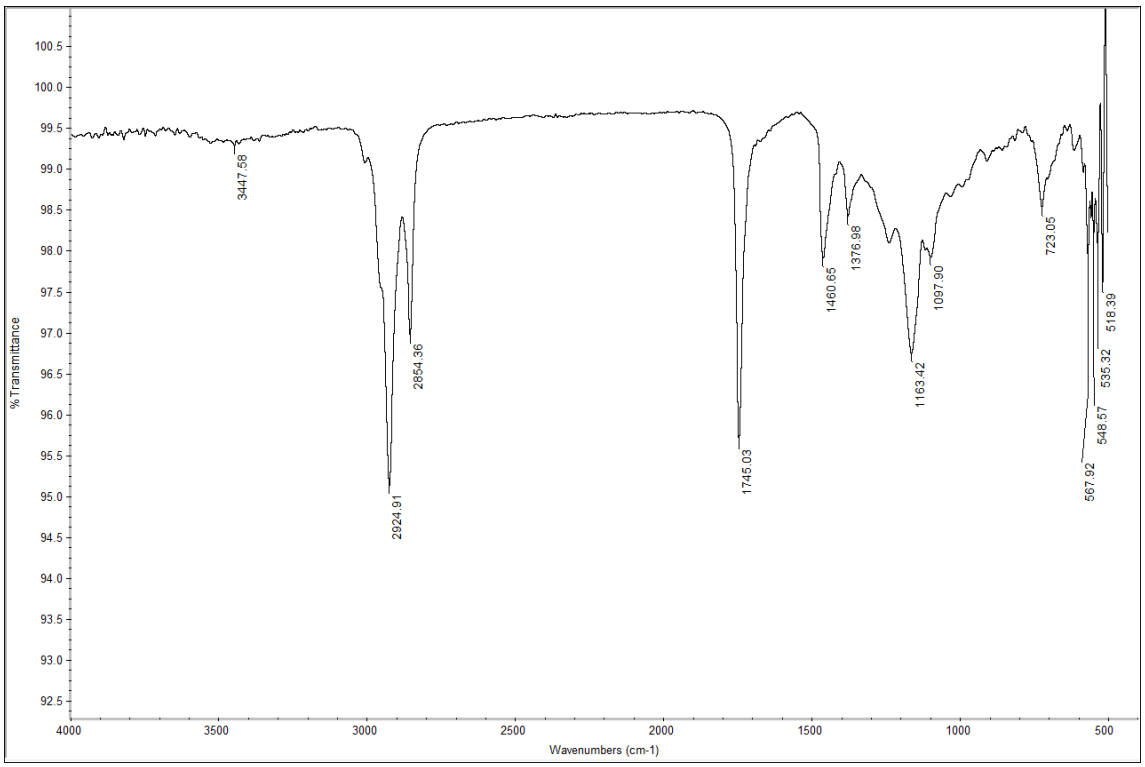

Figure a.14: IR spectra for fraction A5.

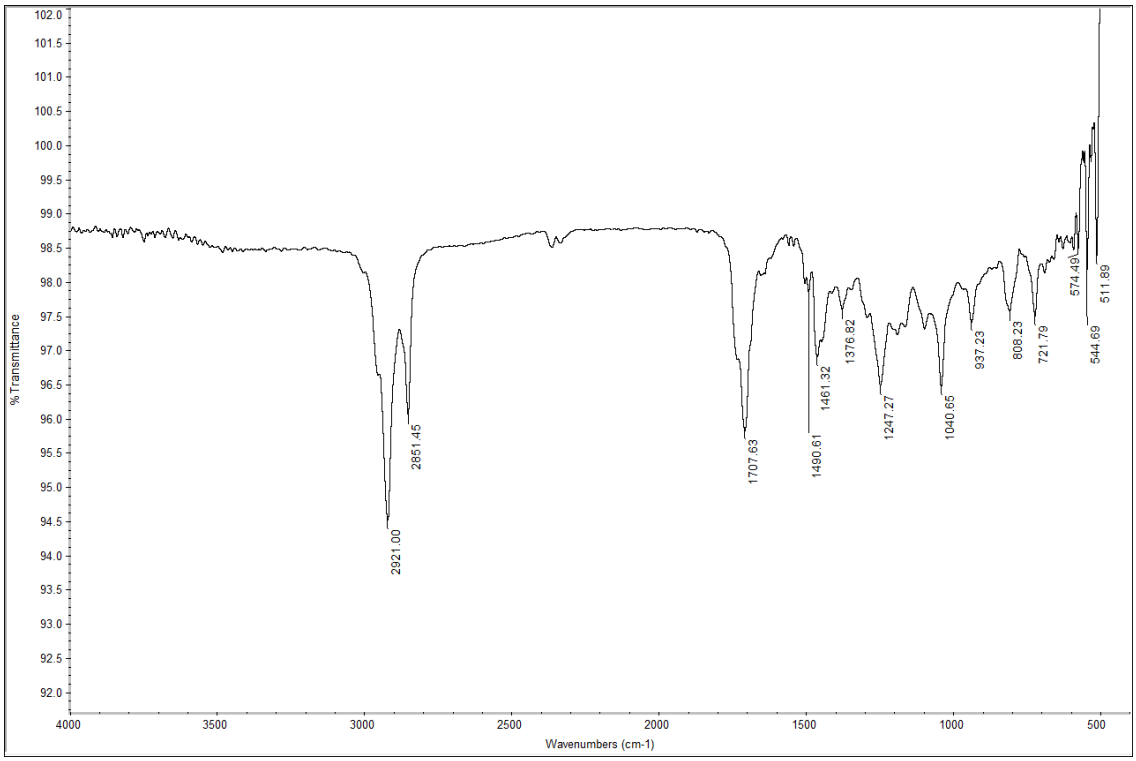

Figure a.15: IR spectra for fraction A8$_1$.

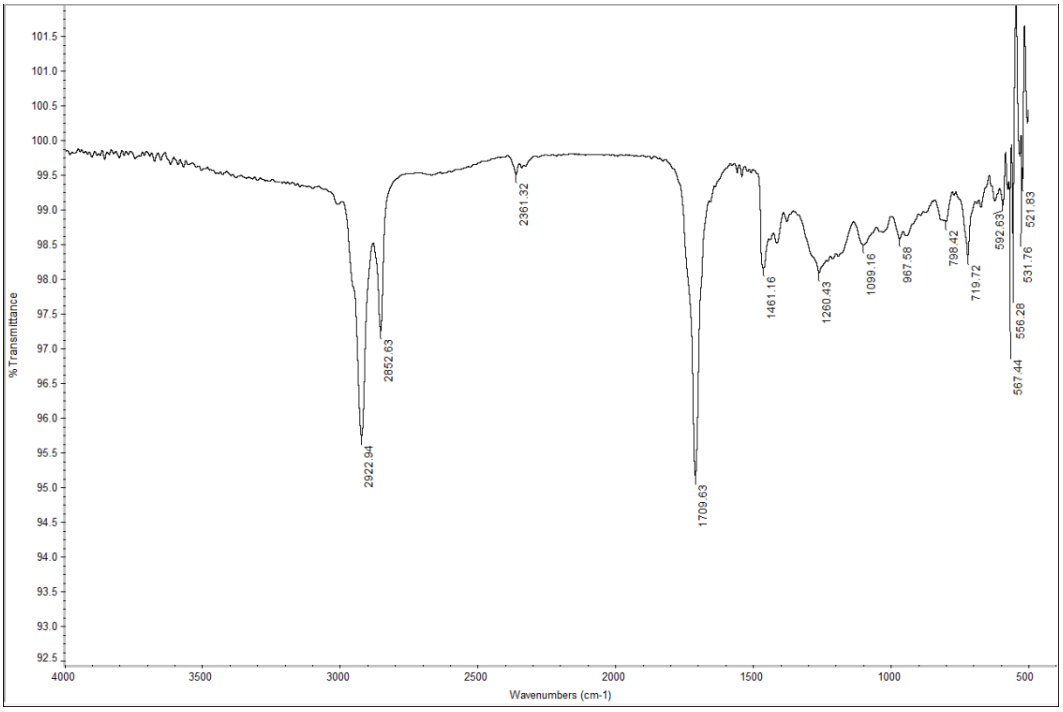

Figure a.16: IR spectra for fraction A8$_2$.

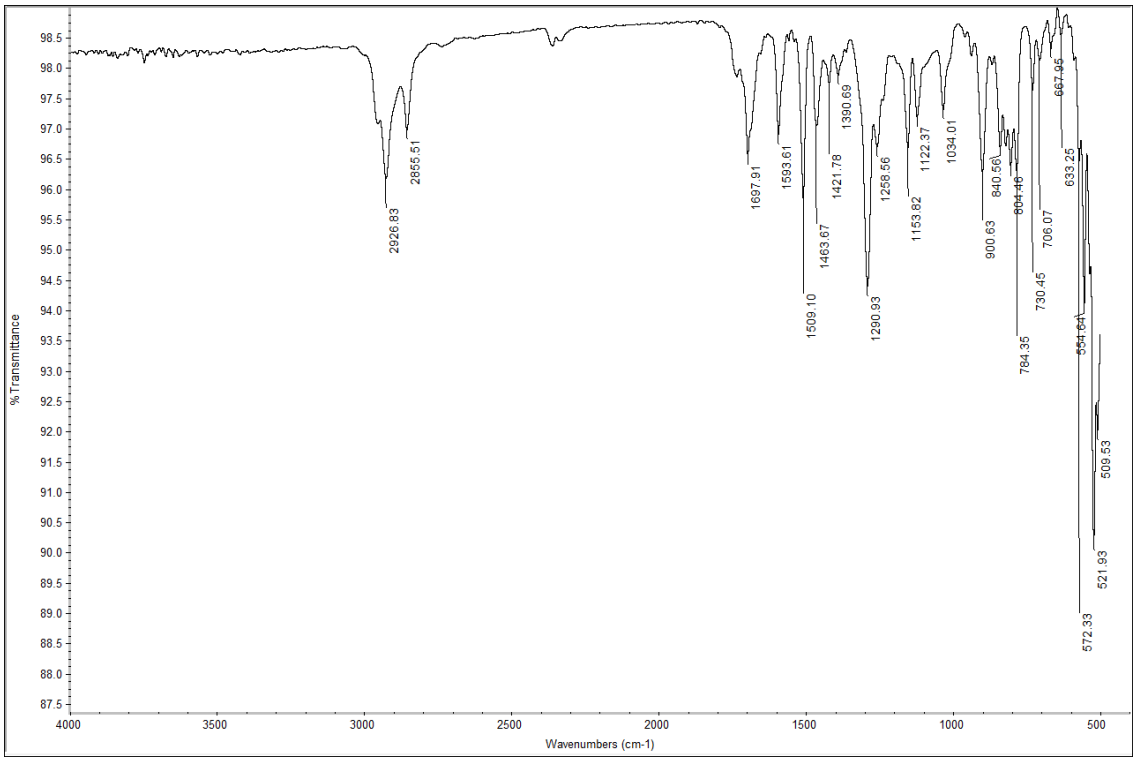

Figure a.17: IR spectra for fraction A8$_3$.

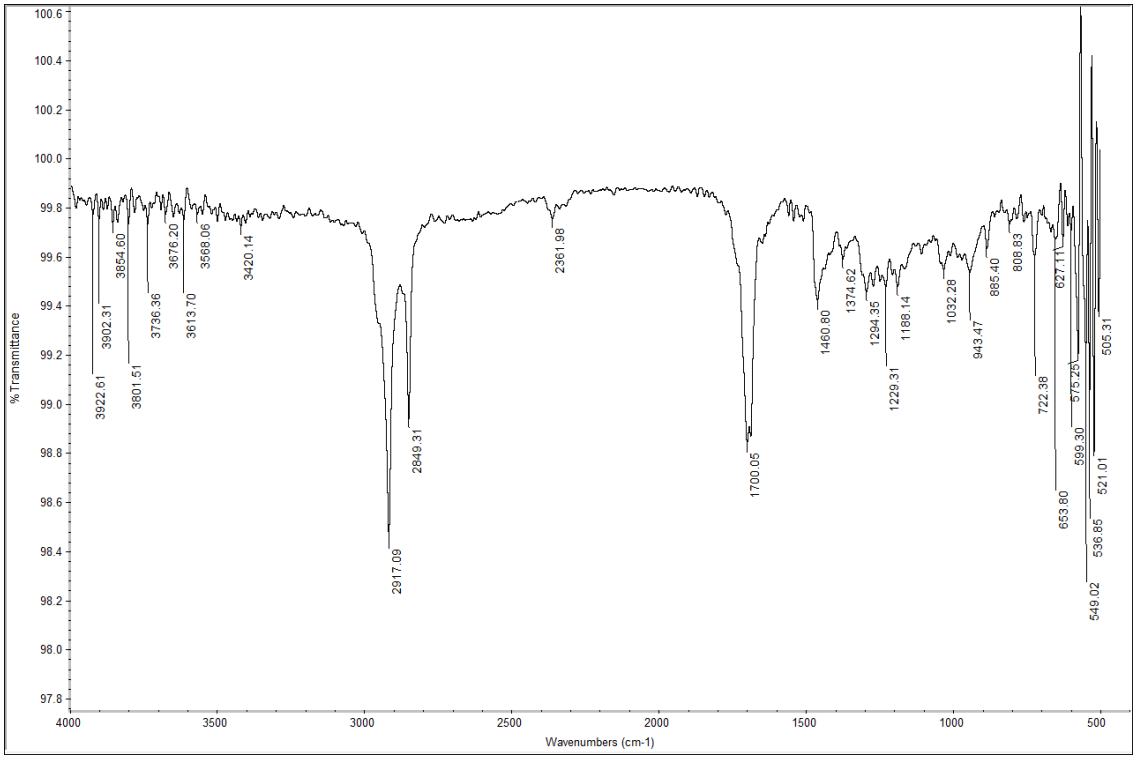

Figure a.18: IR spectra for fraction A11.

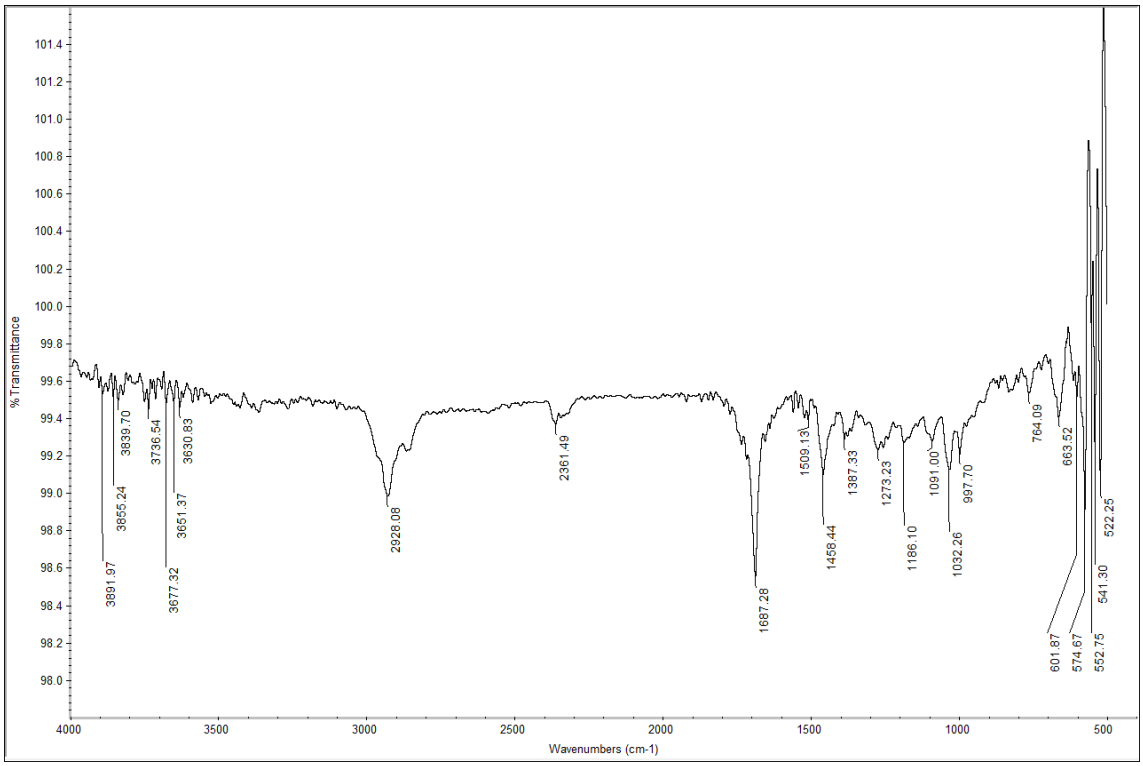

Figure a.19: IR spectra for fraction A13.

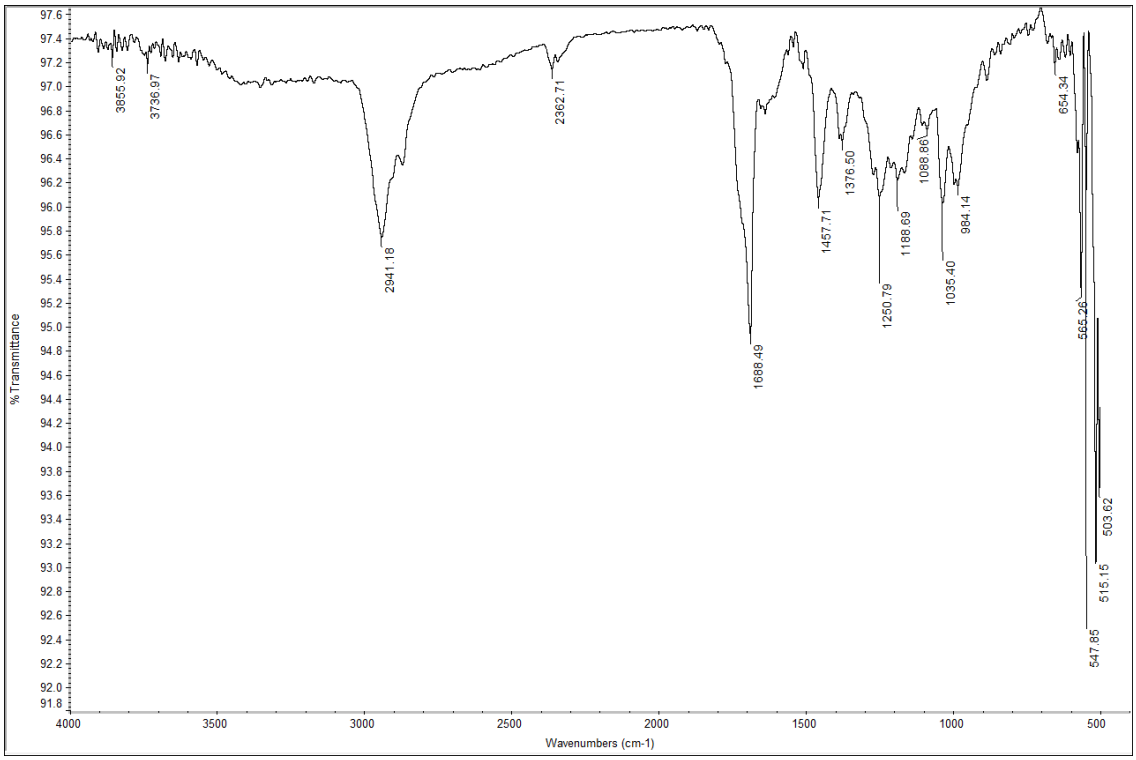

Figure a.20: IR spectra for fraction N4.

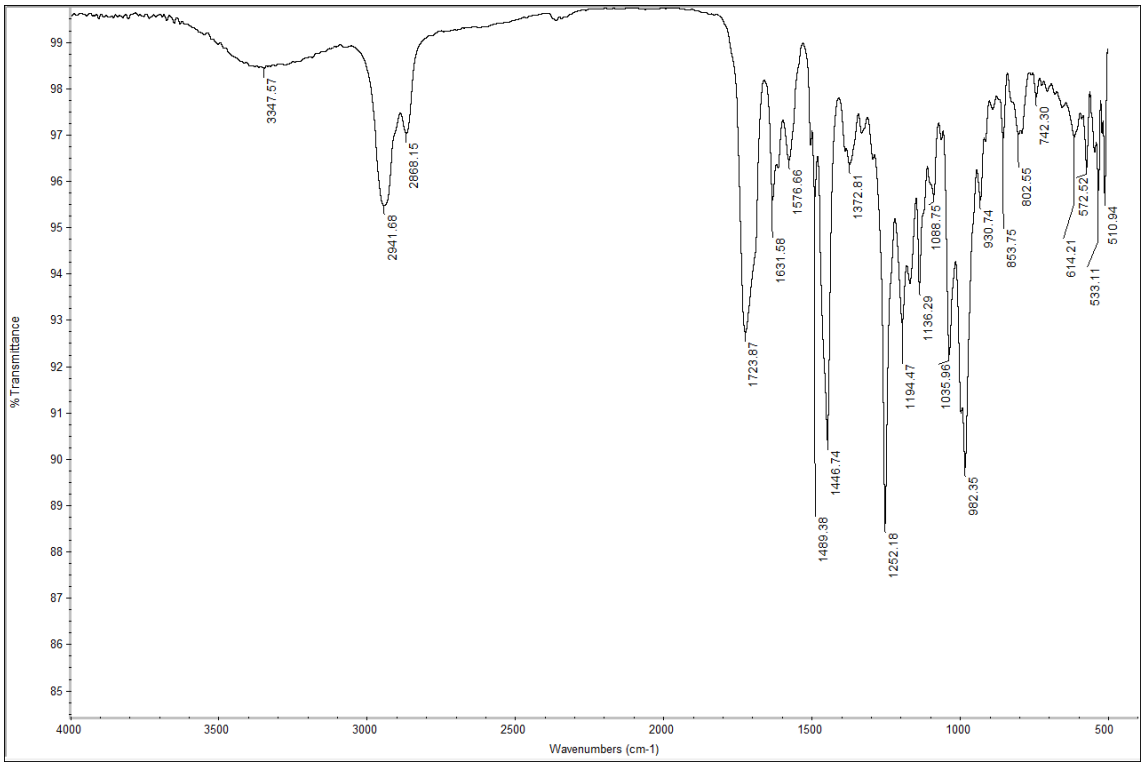

Figure a.21: IR spectra for fraction $N5_1$.

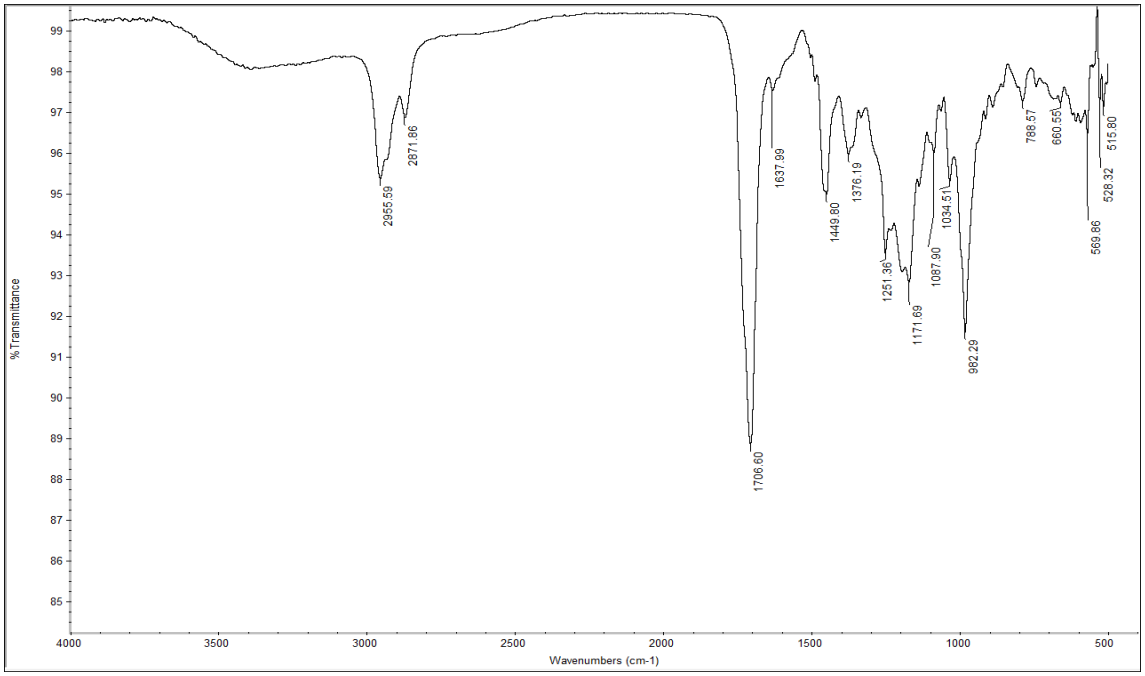

Figure a.22: IR spectra for fraction $N5_2$.

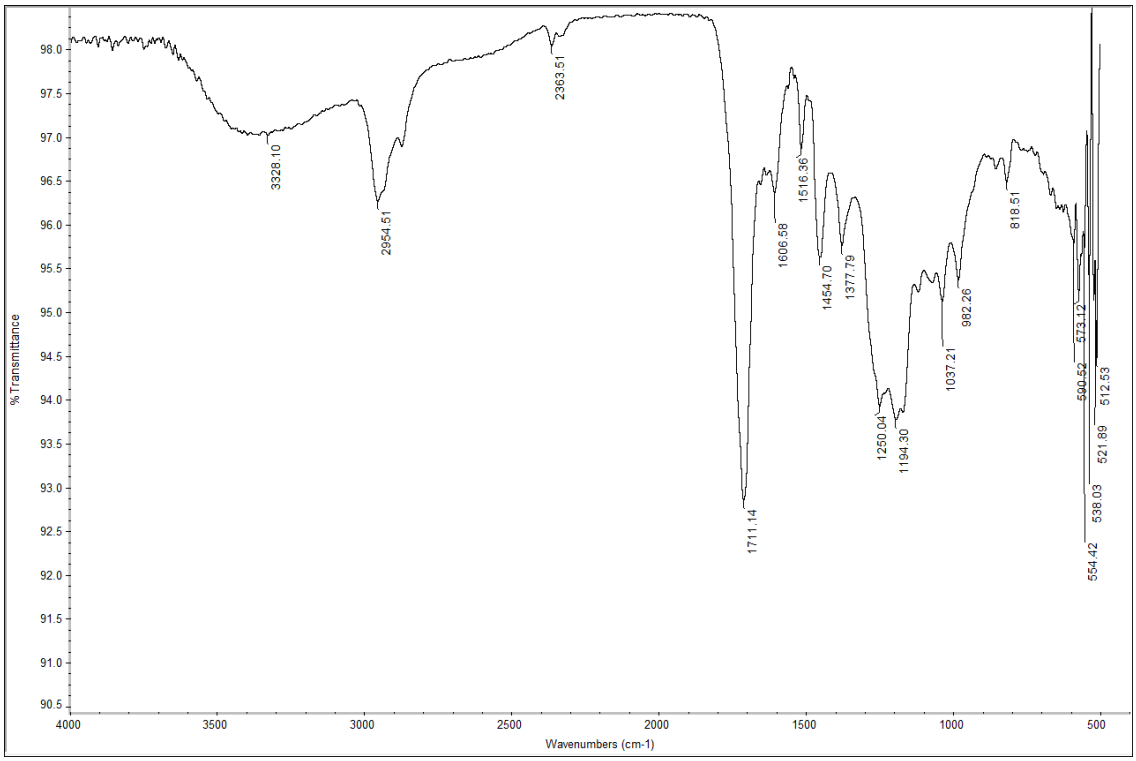

Figure a.23: IR spectra for fraction N6.

Table a.1: MIC values (mg/mL) for fraction A3.

Microorganism	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O	Trial 2 50 mg in 400 µL DMSO + 600 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	8	7.5	7	0.195
S. aureus	7	5	6	0.390
K. pneumoniae	7	5	6	0.390
E. coli	6	5	5	0.781
P. vulgaris	7	6	6	0.390
P. aeruginosa	2	3	2	6.250
C. albicans	6	6	6	0.390

Table a.2: MIC values (mg/mL) for fraction A7.

Microorganism	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O # well	Trial 2 50 mg in 400 µL DMSO + 600 µLH_2O # well	Trial 3 50 mg in 400 µL DMSO + 600 µL H_2O # well	Final result MIC (mg/mL)
MRSA	1	3.5	1	R
S. aureus	1	2.5	1	R
K. pneumoniae	1	3	1	R
E. coli	1.5	2.5	1	R
P. vulgaris	1	1.5	1	R
P. aeruginosa	1	1	1.5	R
C. albicans	R	3	3	R

Table a.3: MIC values (mg/mL) for ethyl acetate extract of *B. arabica*

Microorganism	Trial 1 50 mg in 1000 µL H_2O	Trial 2 50 mg in 1000 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	3	2	3	3.125
S. aureus	4	3.5	4	1.562
K. pneumoniae	R	R	R	R
E. coli	R	R	R	R
P. vulgaris	1.5	R	R	R
P. aeruginosa	R	R	R	R
C. albicans	R	R	R	R
100	45.31			

Table a.4: MIC values (mg/mL) for water extract.

Microorganism	Trial 1 50 mg in 1000 µL H_2O	Trial 2 50 mg in 500 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	R	R	R	R
S. aureus	R	R	R	R
K. pneumoniae	R	2	2	12.5
E. coli	R	1	1	25.0
P. vulgaris	R	1	1	25.0
P. aeruginosa	R	R	R	R
C. albicans	R	R	R	R

Table a.5: MIC values (mg/mL) for ethyl acetate extract of *N. curviflora*

Microorganism	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O	Trial 2 20 mg in 200 µL DMSO + 300 µL H_2O	Final result MIC (mg/mL)
MRSA	R	1	R
S. aureus	R	1	R
K. pneumoniae	R	1	R
E. coli	R	1	R
P. vulgaris	R	1	R
P. aeruginosa	R	1	R
C. albicans	R	1	R

Table a.6: MIC values (mg/mL) for fraction N5.

Microorganism	Trial 1 30 mg in 200 µL DMSO + 300 µL H_2O	Trial 1 30 mg in 200 µL DMSO + 300 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	2.5	3.5	3	3.75
S. aureus	2.5	3.5	3	3.75
K. pneumoniae	2.5	2	2	7.50
E. coli	2	2	2	7.50
P. vulgaris	2	3	2	7.50
P. aeruginosa	1	1.5	R	R
C. albicans	2	2	R	R

Table a.7: MIC values (mg/mL) for fraction $N5_1$.

Microorganisms	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O	# well	Final results MIC (mg/mL)
MRSA	3.5	4	3	3.125
S. aureus	4	4	4	1.562
K. pneumoniae	2	1.5	2	6.250
E. coli	2	1.5	2	6.250
P. vulgaris	3	2.5	2	6.250
P. aeruginosa	1.5	1.5	R	R
C. albicans	5	4	4	1.562

Table a.8: MIC values (mg/mL) for fraction $N5_2$.

Microorganism	Trial 1 50 mg in 400 µL DMSO + 600 µL H_2O	Trial 2 50 mg in 400 µL DMSO + 600 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	5	4	4	1.562
S. aureus	4	3.5	4	1.562
K. pneumoniae	3	2	3	3.125
E. coli	3	2	3	3.125
P. vulgaris	3.5	4	3	3.125
P. aeruginosa	2	2	2	6.250
C. albicans	4	4	4	1.562

Table a.9: MIC values (mg/mL) for fraction N6.

Microorganism	Trial 1 50 mg in 400 µL + 600 µL H_2O	Trial 2 50 mg in 400 µL DMSO + 600 µL H_2O	# well	Final result MIC (mg/mL)
MRSA	2.5	3	3	3.125
S. aureus	4	5	4	1.562
K. pneumoniae	1.5	2.5	2	6.25
E. coli	1.5	2	2	6.25
P. vulgaris	2	3	2	6.25
P. aeruginosa	1	2	2	6.25
C. albicans	2.5	3	3	3.125

Table a.10: MIC values (mg/mL) for fraction N15.

Microorganism	Trial 1 50 mg in 1000 µL H_2O	Trial 2 50 mg in 500 µL H_2O	Trial 3 50 mg in 500 µL H_2O	Final result MIC
MRSA	R	4	R	R
S. aureus	R	3.5	R	R
K. pneumoniae	R	2.5	R	R
E. coli	R	2.5	R	R
P. vulgaris	R	2.5	R	R
P. aeruginosa	R	1	R	R
C. albicans	R	4	1	R

جامعة النجاح الوطنية

كلية الدراسات العليا

توصيف المستخلصات القطبية المحتوية على مادة البولي فينول من نبتتي *Bassia Arabica* و*Nepeta Curviflora* Boiss وتقييم نشاطها الحيوي

إعداد

ولاء فهمي حلمي عودة

إشراف

د. نواف المحاريك

د. نضال جرادات

قُدِّمَت هذه الأطروحة استكمالاً لمتطلّبات الحصول على درجة الماجستير في الكيمياء في كلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

2021

توصيف المستخلصات القطبية المحتوية على مادة البولي فينول من نبتتي

Bassia Brabica **و** ***Nepeta Curviflora*** **Boiss**

وتقييم نشاطها الحيوي

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الملخص

في جميع أنحاء العالم، تُستخدم النباتات الطبية على نطاق واسع لعلاج مجموعة واسعة من الأمراض والأمراض التي تصيب الإنسان. يعزز الموقع الجغرافي لفلسطين على مفترق الطرق بين أوراسيا وأفريقيا التنوع البيولوجي في البلاد من حيث الحيوانات والنباتات. على الرغم من صغر مساحتها، فإن فلسطين هي موطن لأكثر من 2700 نوع نباتي. الهدف من هذا البحث هو دراسة التركيب الكيميائي والتأثيرات الدوائية

لنبتتي *B. arabica* و*N. curviflora* boiss بالإضافة إلى فحص نشاطهما المضاد للبكتيريا والأكسدة.

تم استخدام تقنيات كروماتوغرافية مختلفة لفصل مستخلصات الإيثل أسيتات لنبتتي *B. arabica* و *N. curviflora*وتم فحص النقاوة بواسطة كروماتوغرافيا الطبقة الرقيقة، علاوة على ذلك، تم الكشف عن المجموعات الوظيفية للكسور النقية باستخدام التحليل الطيفي بالأشعة تحت الحمراء.

تم اختبار فاعلية المستخلصات كمضاد للأكسدة من خلال تثبيط (2, 2-diphenyl-1-picrylhydrazyl). تم فحص فعالية المستخلصات كمضادات للميكروبات باستخدام طريقة (Microdilution) وتم اختبار ستة سلالات من البكتيريا وسلالة واحدة من الفطريات.

أظهر المستخلص (3) من نبتة *B. arabica* أ على نشاط بحد أدنى من التركيز(MIC) يساوي 0.195 µg/mL وذلك ضد نوع من البكتيريا methicillin-resistant staphylococcus aureus (MRSA).

أظهرت مستخلص الإيثل أسيتات لنبتة *B. arabica* أفضل قيمة مضاد تأكسد مع تركيز IC_{50} يساوي 4.91µg/mL و وكانت قيمة IC_{50} للمستخلص المائي تساوي 3.39µg/mL من نبتة *N. Curviflora*.