An-Najah National University

Faculty of Graduate Studies

Characterization of Essential Oil from *Nepeta curviflora* and Evaluation of their Bioactivity

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Dedication

This thesis is dedicated to:

My loving parents for their encourgment, patience, and prayers

My beloved sisters and brothers for their love and support

My beloved nieces Sham and Ellien and my sister-in-law Walaa'

My dearset friends; particularly my friend Laila for her support throughout this whole process

I dedicate this work

Acknowledgments

Praise to the almighty Allah, who has enabled me to accomplish this work.

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I am eternally grateful to my family for their love, support, and assistance throughout my studies.

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Finally, special thanks to the chemistry department at my University.

الإقرار

انا الموقعة ادناه مقدمة الرسالة التي تحمل العنوان:

Characterization of Essential Oil from *Nepeta curviflora* and Evaluation of their Bioactivity

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Declaration

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

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Date: **التاريخ: 12/8/2021**

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Characterization of Essential Oil from *Nepeta curviflora* and Evaluation of their Bioactivity

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Abstract

For thousands of years, medicinal plants and herbs have been used to treat different kinds of illnesses and to improve people's healthcare. Many studies have shown that essential oils from plants have a variety of beneficial properties in medicine, industry, and cosmetics. *Nepeta curviflora* is a naturally growing plant in the eastern parts of the Mediterranean region, including Palestine. It has a pleasant minty smell and has been used in folk medicine as a remedy for the treatment of many diseases. On account of the wild distribution of this plant and its medicinal use, this study was designed to investigate the chemical composition of *N. curviflora* essential oil (from leaves and flowers) and its stem extracts and to assess *in-vitro* antioxidant, porcine pancreatic lipase, α-glucosidase, and α-amylase inhibitory activities of *N. curviflora* EO. Additionally, it was designed to investigate the antioxidant and antimicrobial properties of the extracts from the stems of *N. curviflora*.

N. curviflora EO was obtained using microwave ultrasonic-assisted extraction, and the chemical constituents of the EO were determined qualitatively and quantitatively using GC-MS. The antioxidant activity of the EO and the stem extracts was assessed by inhibition of DPPH free radicals.

The anti-lipase activity was evaluated using PNPB. The anti-amylase activity was assessed using DNSA. In addition, *α*-glucosidase inhibitory activity was evaluated using PNPG. Methanol extracts of the stems of *N. curviflora* were subjected to a sequence of flash column chromatography using different eluents with various polarities. The purity of the isolated compounds was monitored by TLC. Moreover, functional groups of the compounds were detected using IR. The antimicrobial activity of *N. cuviflora* stem extracts was examined using the microdilution method.

Twenty compounds were identified from *N. curviflora* EO, representing 100% of the total EO, of which 1,6-dimethyl spiro[4.5]decane **55** (27.51%), caryophyllene oxide **56** (20.08%), and *β*-caryophyllene **38** (18.28%) were the major compounds. *N. curviflora* EO has exhibited antioxidant activity with an IC_{50} value of 6.3 ± 0.43 μg/mL in comparison with trolox (positive control). The obtained EO displayed *α*-glucosidase, *α*-amylase, and porcine pancreatic lipase inhibitory activities with IC_{50} values of 26.3 ± 0.57, 45.7 ± 0.26, and 54.9 ± 0.34 μg/mL, respectively, in comparison with the positive controls orlistat and acarbose. Furthermore, three extracts of *N. curviflora* were separated (labeled as A, B, and C). All of the extracts exhibited antioxidant activity with IC_{50} values of 78.53 ± 1.09, 43.365 ± 0.165, and 171.48 ± 1.57 μg/mL for A, B, and C, respectively. Among the three extracts, extract B showed a moderate antibacterial effect against *S. aureus* with a MIC value of 156.3 μg/ml. However, all of the extracts had no activity against the *Candida albicans* fungi strain.

Chapter One

Introduction

Nowadays, in a time where maintaining health is the most important thing for everyone, manufactured medicines and drugs play a huge role in reaching this goal. With this in mind, many people are turning back to the main source that has started it all, the medicinal plants and herbs. To explore the huge power that these plants hold, many efforts have been poured into investigating them. Plants have been used since ancient times for many purposes, including coloring, production of food, treatment of diseases, flavoring, and even cosmetic aims [1].

The intriguing part about these medicinal plants is that they are readily available, have fewer side effects, and cheaper prices compared to manufactured drugs. This has sparked the interest of many people in both Eastern and Western cultures in the treatment of a range of diseases [2,3].

1.1 A brief history of the use of medicinal plants

Our ancestors have been using plants as medicines for their health since ancient times, even though there was not enough information about illnesses, what caused them, or even how to cure them at the time. They have used their instincts to treat diseases as such instinctive use was practiced on animals as a source of food [4]. The oldest evidence that proves the use of medicinal plants as drugs was approximately 60000 years ago [5] and the

first written medicinal records were found on clay tablets written by Sumerians from Nagpur in 5000–3000 BCE [6]. The preserved body of an iceman called *Ötzi* that was killed accidentally in the Alps mountains between 3400 and 3100 BCE is another evidence as medicinal plants were discovered in his body, which indicates that at that time they were aware of the benefits of medicinal plants [7].

Several cultures have utilized medicinal plants due to their healing properties. This knowledge was inherited from ancestors over the centuries and evolved through personal experiences. Examples of these cultures include Greek civilization, Chinese culture, which is well-known for Traditional Chinese Medicine (TCM), one of the earliest systems of treatment and therapies, and Ayurveda or Traditional Indian Medicine, which is the oldest health care system on earth that combines both physiologic and holistic medicine to work together as a natural healing process. Moreover, about 25,000 formulations based on plants were used in traditional Indian medicines [8-12].

The Arabs have made enormous efforts in both science and medicine. Many Arab and Muslim scholars were among the first to practice basic pharmacy. For example, Ali Ibn Rabban Al Tabri (782-855 AD) in his book '*Firdous Al Hikmat'*, which focused on drugs and poisons [13]. Ibn al-Baitar has described in his book '*Compendium on Simple Medicaments and Foods'* the properties of over 1400 plants [14]. In addition, nearly 700 herbal medicines have been documented going back to 1500 BC in Egypt [15].

1.2 Medicinal herbs and drug discovery

For many years, natural products have played a crucial role in the challenging task of drug discovery due to their great therapeutic agents. According to the World Health Organization (WHO), about 80% of the world population depends on medicinal plants and traditional medicine as a major source for treating different kinds of ailments and for improving health care [16,17].

For the time being, studies show that more than 50% of the available drugs are derived by one way or another from medicinal plants, including the early famous drugs like Aspirin **1** (derived from willow bark trees), which is used to reduce pain, fever, or inflammation, Morphine **2**, a potent analgesic (isolated from the Opium poppy), Digoxin **4**, a medication used to treat various heart conditions (isolated from Foxglove) and Quinine **3** used to treat malaria (derived from *Cinchona calisaya*) [18,19]. These medicinal and aromatic plants showed their efficiency in treating difficult diseases like cancer, AIDS/HIV, and other contagious diseases [20].

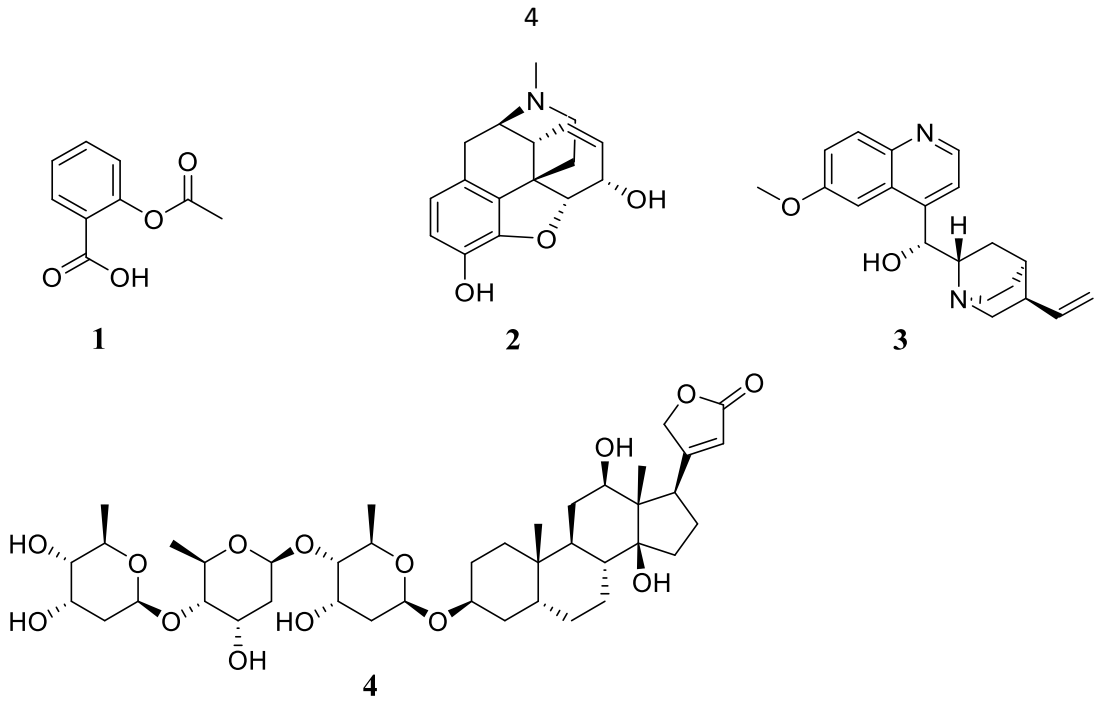


Figure 1.1. Structures of the famous drugs: Aspirin 1, Morphine 2, Quinine 3, and Digoxin 4.

Currently, the entire world is suffering from the novel coronavirus (COVID-19) that has led to a dramatic loss of human life all around the world and forced many countries into lockdown. This virus damages the respiratory system in humans and it's a very contagious disease [21]. Many medicinal plants have been tested to help treat or manage COVID-19 symptoms. According to research, the important bioactive components of these plants, such as *Andrographis paniculata*, *Citrus spp.*, and *Cuminum cyminum*, can help relieve symptoms such as fever and cough [22].

1.3 A Palestinian perspective

Palestine is one of the richest plant habitats. Being located on the eastern shores of the Mediterranean and at the crossroads of three continents: Asia, Africa, and Europe. Even with its limited area, which is 28292 km^2, Palestine

holds a wide range of biodiversity to the point where its flora consists of 137 plant families, including 2652 identified plant species [23,24]. All of this has made the exploration of herbs and medicinal plants a huge part of Palestinians' lives due to their antioxidant and antimicrobial advantages, although many plants are yet to be investigated [25,26].

1.4 Medicinal plants' secondary metabolites

The term 'Medicinal plants' can be defined as any plants that have therapeutic properties and compounds with a beneficial pharmacological effect on the human body to maintain health, either in traditional medicine or in modern medicine [27-29]. These herbs are packed with organic compounds, called phytochemicals. The role of these phytochemicals lies in giving the plants protection against herbivores and diseases, also giving them their color, aroma, and flavor [30,31].

Plant metabolites are organic compounds that take part as the intermediates and products of metabolism. The term 'metabolite' has been used to refer to small molecules [32]. These metabolites are generally classified into two major categories: primary and secondary metabolites. Primary metabolites are the compounds that are directly involved in the primary metabolic processes of an organism and are necessary for its growth, development, and reproduction [33]. They are produced during the growth phase, as a result of the growth mechanism in the organism. Examples of primary metabolites include proteins, enzymes, carbohydrates, lipids, vitamins, ethanol, lactic acid, etc. On the other hand, secondary metabolites are organic compounds

that are not necessary for an organism to live, but rather involved in ecological functions and long-term impairment of the organism's survivability, productivity, or even aesthetics [34]. They also play a huge role as a defense mechanism against herbivores and other interspecies defenses [35,36].

Secondary metabolites are biosynthetically derived from primary metabolites and are produced in small quantities. These natural products range in quality and quantity depending on the plant species and their growing locations [32]. Nowadays, with advances in research and technology, the importance of secondary metabolites has increased, which has resulted in their extensive use in the drug and pharmaceutical industry [37].

1.4.1 Classification of secondary metabolites

Secondary metabolites can be classified in different ways according to their chemical structure (like having rings, containing sugar), composition (for example, containing nitrogen or not), their solubility in different solvents, or their biosynthetic pathways. Usually, secondary metabolites are classified depending on their biosynthetic pathways into three large molecule families: terpenoids, phenolic compounds, and alkaloids [38,39].

1.4.1.1 Phenolic compounds

Polyphenols and phenolic compounds are natural metabolites that are primarily produced by the shikimate/phenylpropanoid pathway. Their structure consists of an aromatic ring, containing one or more hydroxyl

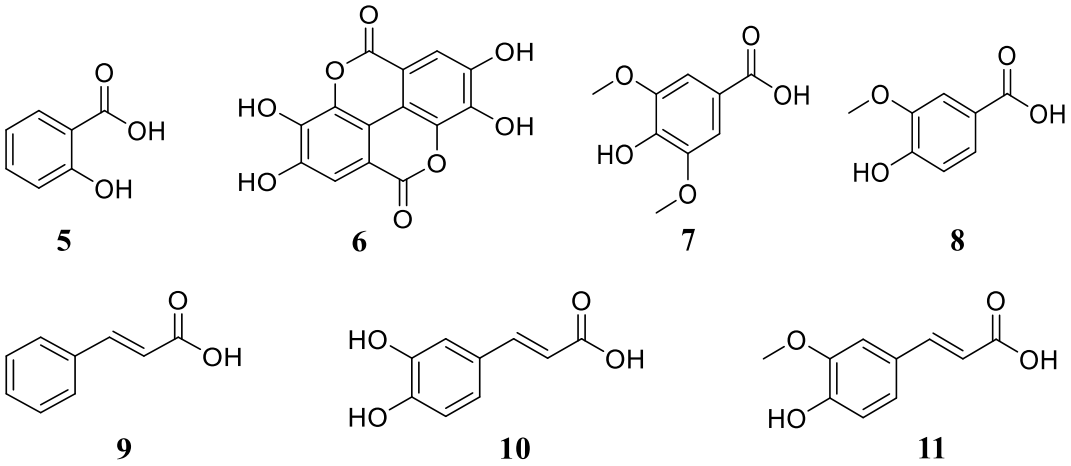
substituents, and they can range from simple phenolic molecules to highly polymerized compounds [40]. They play an important role in plant responses to biotic (such as pathogen and herbivore attacks) and abiotic stresses (such as radiation, temperature, humidity, and nutritional stress). Furthermore, they are involved in flower and fruit pigmentation and plant reproduction [41-43]. These compounds are highly valuable for their antioxidant and antiproliferative properties, in which the high dietary intake decreases the risk of cardiovascular and cancer diseases [44,45].

There is a wide range of phenolic compounds that occur in nature as a result of the diversity of their structure. These compounds can be categorized into several classes. The most important phenolic compound classes found in the human diet are phenolic acids, flavonoids, and tannins.

1.4.1.1.1 Phenolic acids

Phenolic acids include one-third of the constituents of most phenolic compounds. Chemically, these compounds are characterized by being formed by at least one aromatic ring where at least one hydrogen is substituted by a hydroxyl group [46]. Depending on positioning and the number of the hydroxyl groups on the aromatic ring, phenolic acids are classified as hydroxybenzoic acids (HBAs) and hydroxycinnamic acids (HCAs). Hydroxybenzoic acids are derivatives of benzoic acid with seven carbon atoms framework of C_6-C_1 structure. Some of the major hydroxybenzoic acids are salicylic acid **5**, ellagic acid **6**, syringic acid **7**, and vanillic acid **8**. Hydroxycinnamic acids (HCAs) are cinnamic acid

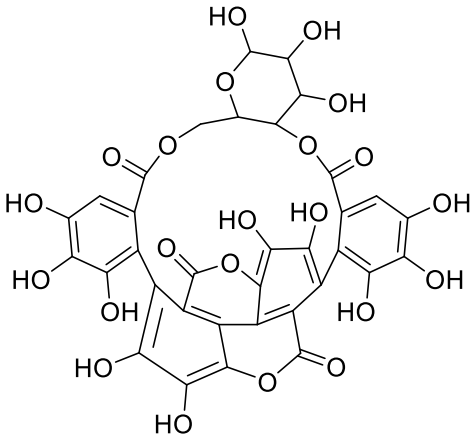
derivatives with the C_6-C_3 framework; for example, Cinnamic acid **9**, caffeic acid **10**, and ferulic acid **11** are common HCAs. These structural characteristics have attracted attention to phenolic acids due to their antioxidant, anti-inflammatory, immunoregulatory, anti-allergic, anti-atherogenic, anti-microbial, and anti-cancer activities, and anti-diabetic properties [47,48].



1.4.1.1.2 Tannins

Tannins are considered as one of the most important groups of secondary metabolites that act as a natural barrier against insects, pathogens, and animals, due to their ability to react with proteins and their antioxidant properties [49]. Chemically, they are divided into two classes: Hydrolyzable and nonhydrolyzable or condensed tannins. Hydrolyzable tannins (e.g. punicalin **12** [50]) are found in wood or stems, like in the stems of berries [51], whereas condensed tannins (e.g. procyanidin B2 **13** [52]) are mostly found in fruits and leaves, such as grapes and apples or tea leaves. Tannins are chemically reactive and can precipitate proteins, which is why they are

mainly used by the leather industry, which involves the production of leather from the vegetal source of tannins, i.e. the tanning process [53,54]. They can also be used in other industries and applications, including the mineral industry, wine production, and the oil industry [55].



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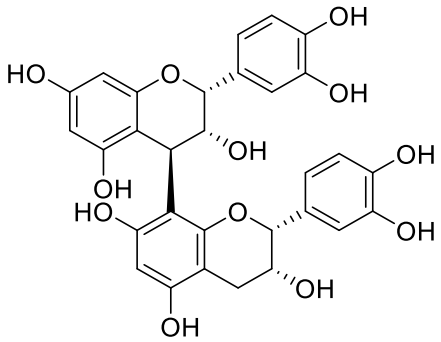

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1.4.1.1.3 Flavonoids

Flavonoids are natural polyphenolic compounds with a low molecular weight [56]. They are found everywhere in plant parts, including flowers, leaves, bark and seeds. So far, more than 8000 different compounds have been identified, yet many more are still to be found [57]. These compounds carry out important functions in plants, including protecting them from ultraviolet radiation, oxidative cell injury, pathogens, and predators. They also attract pollinators due to the coloration of the plants' flowers, as the flavonoids provide attractive color pigments such as yellow, red, blue, and purple [58-60].

The basic flavonoid structure consists of a diphenyl propane (C_6–C_3–C_6) skeleton with A, B, and C as the three main phenolic rings. Different classes

of flavonoids differ in the degree of oxidation of the C ring and the substituent patterns in the A and/or B rings. Such differences have led to structural diversity in these compounds [61]. Flavonoids can be grouped into various subclasses. The main six subclasses are flavanones **14**, flavonols **15**, flavones **16**, anthocyanins **17**, flavanols **18,** and isoflavonoids **19** [62]. This diversity between flavonoid subgroups has led to a myriad of different beneficial properties, like antioxidant, anti-inflammatory, antiallergic, anticancer, and antiviral properties [63-65]. Figure 1.4 shows the subclasses of flavonoids and examples of their dietary sources :

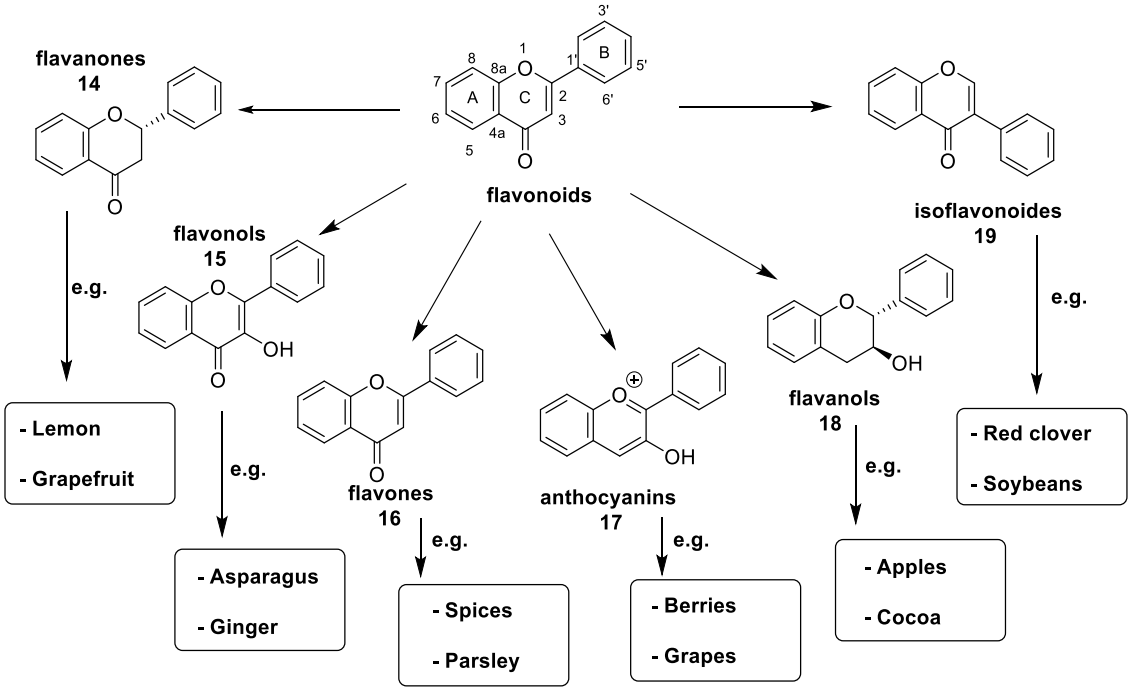


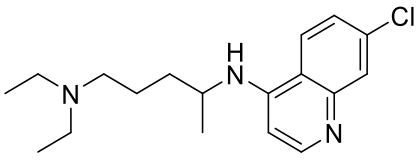
Fig. 1.2. The Subclasses of Flavonoids and Examples of their Dietary Sources.

1.4.1.2 Alkaloids

Alkaloids are nitrogen-containing heterocyclic chemical compounds that are abundant in nature, i.e., found in at least 25% of medicinal plants. They are

bitter-tasting chemicals and are often toxic [66]. These compounds are generally isolated from plants, but they could also be found in insects, animals, and marine invertebrates [67-69].

Alkaloids exhibit defense functions in plants. They work as natural herbicides against predators due to their general toxicity [70,71]. The use of plants that contain alkaloids can be traced back almost to the beginning of civilization, as people used them as drugs or remedies to treat diseases or even as a poison [70,72,73]. Alkaloids from plants are applied in the pharmaceutical industry to a great extent due to their health benefits as anti-malarial agents (quinine **4** and chloroquine **20**), anticancer agents like vincristine **21**, taxol **22**, and vinblastine **23** [74,75]. Alkaloids can also be found in coffee and tea (caffeine **24**) and some dietary supplements, like the ones that contain the alkaloid ephedrine **25** that leads to weight loss [76,77].



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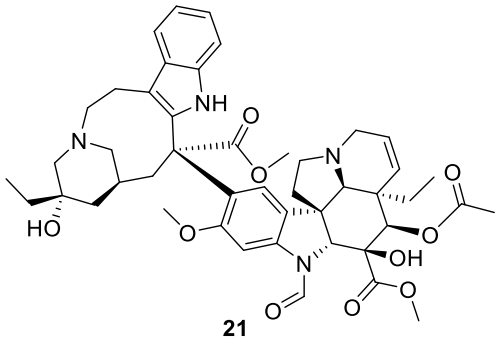

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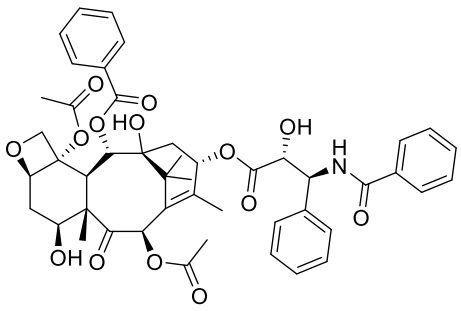

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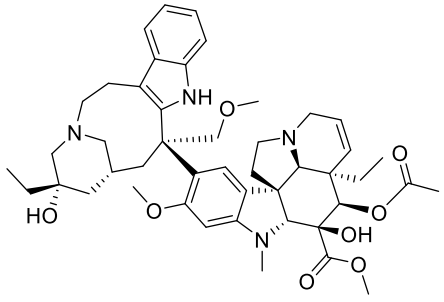

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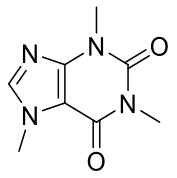

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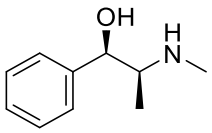

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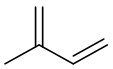
1.4.1.3 Terpenoids

Terpenoids (or isoprenoids) are well-known natural chemicals that make up the biggest and most diversified class of natural goods, with over 80,000 distinct compounds estimated [78]. Terpenoids are the oxygenated derivatives of hydrocarbon terpenes. The term terpenes refer to a compound that consists of an integral number of five-carbon isoprene **26** (2-methylbuta-1,3-diene) units [79]. From a structural point of view, terpenes are simple hydrocarbons, while terpenoids are one of the structurally modified classes of terpenes with different functional groups and an oxidized methyl group moved or removed at different positions [80,81]. Nevertheless, both the terms “terpene” and “terpenoid” are often used interchangeably [82].

Terpenoids are mostly obtained from natural sources like plants, bacteria or yeast or petrochemical sources [83,84]. Terpenoid structures vary, providing them with antifungal, antiparasitic, antimicrobial, antiallergenic, antiviral, immunomodulatory, and chemotherapeutic properties [83]. They are used in medical applications like anticancer drugs and antimalarial drugs such as artemisinin **27**. These compounds are also applied in food fields like artificial flavors and spices, and other fields, including vitamins, hormones, and cosmetics, as these compounds have a nice aroma [83,85,86].



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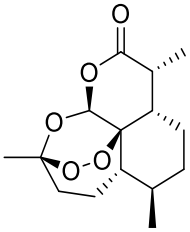

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Terpenoids are usually classified according to the number and structural organization of isoprene (C_5) units present in the molecule as hemiterpenoids (C_5), monoterpenoids (C_{10}) sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and polyterpenoids ($C_{>40}$ higher-order terpenoids). **Table 1.1** shows the classification of terpenoids based on the number of isoprene units and their molecular formula.

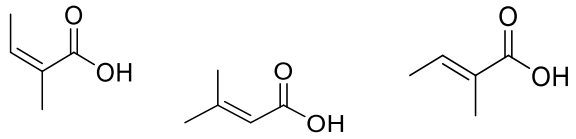
Table 1.1 Classification of terpenoids and their molecular formula.

Terpenoids	**No. of isoprene**	**Molecular formula**
Hemiterpene or isoprene	1	C_5H_8
Monoterpenoids	2	$C_{10}H_{16}$
Sesquiterpenoids	3	$C_{15}H_{24}$
Diterpenoids	4	$C_{20}H_{32}$
Sesterterpenoids	5	$C_{25}H_{40}$
Triterpenoids	6	$C_{30}H_{48}$
Tetraterpenoids	8	$C_{40}H_{64}$

1.4.1.3.1 Hemiterpenoids

Hemiterpenoids are the simplest and smallest known terpenoids. The most famous and prominent hemiterpene is the volatile hydrocarbon isoprene **26,** which is released from the leaves of many trees, including poplars, oaks, willows, and conifers [87]. Isoprene is a valuable polymer and a potential

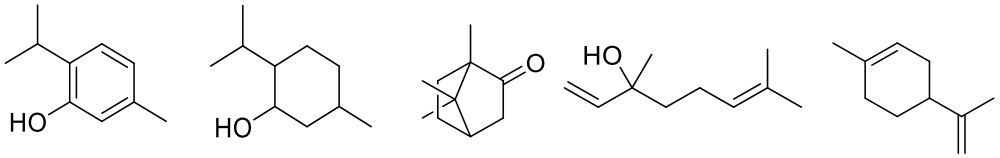
biofuel that has great potential as a building block in the synthetic chemistry industry [88]. Other examples of hemiterpenoids that are found in plants are angelic **28**, senecioic **29**, and tiglic acids **30** [89].



28 **29** **30**

1.4.1.3.2 Monoterpenoids

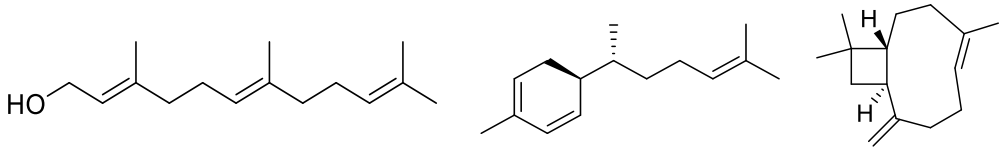
Monoterpenoids are a class of terpenoids that consist of a 10 carbon backbone (2 isoprene units) structure. They could be both plant-derived or synthetic compounds [90]. Monoterpenoids can be divided into three main subgroups: acyclic, monocyclic, and bicyclic. The compounds belonging to this class are usually the components of the essential oils extracted from many plants and they contribute to the characteristic odor and aroma of these plants. They are widely used in pharmaceutical companies, agricultural, food, and cosmetic industries as they have relatively low molecular weights and special fragrances which are useful for making perfumes [90-92]. Some common examples of monoterpenoids are thymol **31** from thyme and menthol **32** from mint (both are monocyclic), camphor **33** from camphor trees (bicyclic), and linalool **34** from lavender (acyclic), which is an antidepressant, sedative, analgesic, and antipsychotic compound [91-93]. Another famous example of monoterpenoids is limonene **35** (monocyclic), which is extracted from essential citrus fruit oils like lemon, lime, orange, and grapefruit [94].



31 32 33 34 35

1.4.1.3.3 Sesquiterpenoids

Sesquiterpenoids are the most diverse and predominant group of terpenoids. They include three isoprene units and have the formula $C_{15}H_{24}$. They are divided according to the number of carbon rings into acyclic, monocyclic, bicyclic, and tricyclic [95]. Like monoterpenoids, these compounds are also a major component of essential oils. The most characteristic examples of sesquiterpenoids are farnesol **36** (acyclic), which is found in many essential oils, for example, lemon grass, citronella, and rose. This compound is known for its anti-inflammatory, anti-tumor, and chemopreventive properties [96]. Other examples of sesquiterpenoids are *α*-zingiberene **37** (monocyclic) from ginger, and *β*-caryophyllene **38** (bicyclic) from clove oil, which is widely used in many food products, like spice blends, and cosmetics such as lotions [94,97].

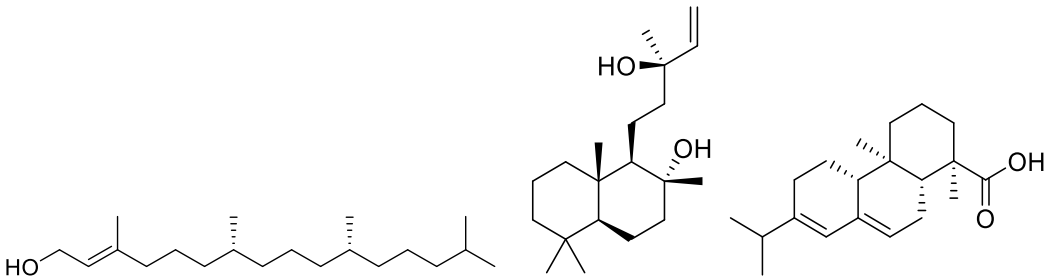


36 37 38

1.4.1.3.4 Diterpenoids

Diterpenoids are a very eminent class of natural terpenoids with a C_{20} carbon skeleton and four isoprene units having the molecular formula of $C_{20}H_{32}$. The main sources of diterpenoids are plants and fungi, but they can also be found

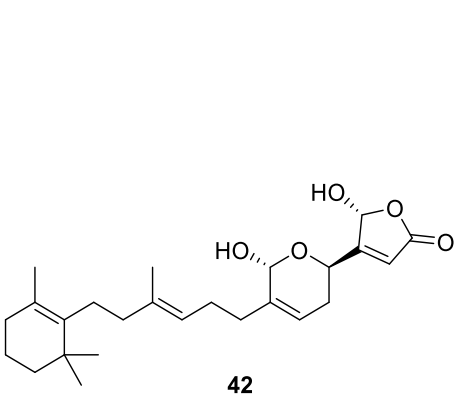
in insects and marine organisms [98,99]. Depending on their skeletal core, these compounds can be divided into linear, bicyclic, tricyclic, tetracyclic, pentacyclic, or macrocyclic diterpenes. This class of compounds has shown significant biological and pharmacological activities, including anti-inflammatory, anticancer, antimicrobial, cardiovascular, and many others [100,101]. Examples of these compounds include phytol **39** (acyclic), sclareol **40** (bicyclic), and abietic acid **41** (tricyclic) [94].



39 **40** **41**

1.4.1.3.5 Sesterterpenoids

Sesterterpenoids are a small group of terpenoids that consist of 25 carbon atoms with 5 isoprene units. They are generally isolated from insects, bacteria, fungi, and various marine organisms, mainly sponges [102,103]. Sesterterpenoids have many biological activities, including anti-inflammatory, antifeedant, antiprotozoal, and anticancer activities [102]. They are divided into linear, monocyclic, bicyclic, tricyclic, tetracyclic, and macrocyclic frameworks [104,105]. Some of the typical examples of sesterterpenoids are manoalide **42** (monocyclic), leucosceptrine **43** (bicyclic), and heliocide H1 **44** (tricyclic) [94].



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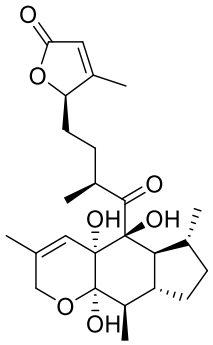

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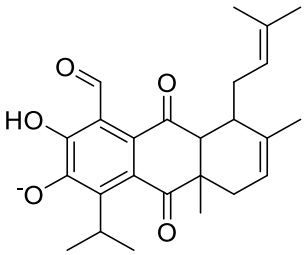

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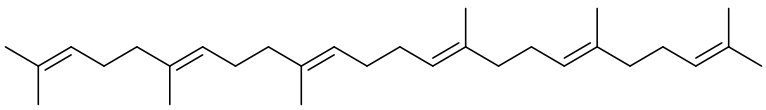
1.4.1.3.6 Triterpenoids

Triterpenoids are compounds that consist of six isoprene units and have the molecular formula of $C_{30}H_{48}$. Triterpenoids are biosynthesized from the acyclic C_{30} hydrocarbon, squalene **45**, which is a linear triterpene [106]. These compounds have a diversity of complex structures and approximately more than 20,000 triterpenoids have been identified in nature [107]. They are potent phytochemicals that are utilized for preventing many diseases, especially different kinds of cancer [107-109]. Examples of triterpenoids include ursolic acid **46** that is derived from bilberries, apples, lavender, cranberries, oleanolic acid **47** from garlic and java apple, and betulinic acid **48** from white birch [108].



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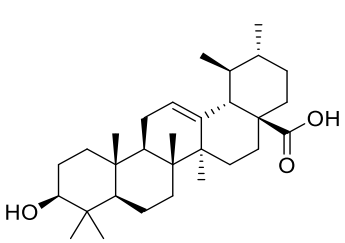

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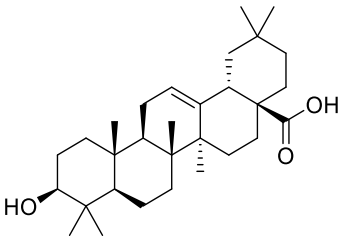

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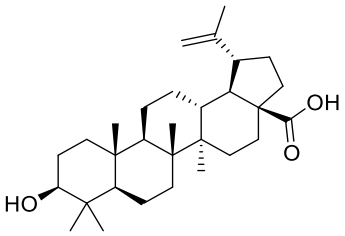

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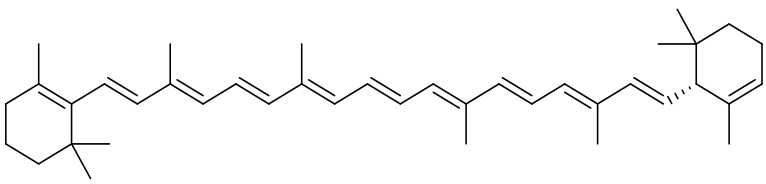
1.4.1.3.7 Tetraterpenoids

Tetraterpenoids are a class of terpenoids that are produced from 8 isoprene molecules. They have the molecular formula $C_{40}H_{64}$. The most common tetraterpenoids are carotenoids. Carotenoids are natural fat-soluble pigments that paint plants and animals in yellow, red, or orange [110,111]. These compounds are used commercially as food colorants and they have many vital biological functions due to their antioxidant and anti-inflammatory activities [103]. Many carotenoids have been reported to be anti-carcinogenic and their dietary intake is associated with the decreased risk of several cancers. Examples of these compounds are α-carotene **49**, β-carotene **50**, lycopene **51**, fucoxanthin **52**, and canthaxanthin **53** [112,113].



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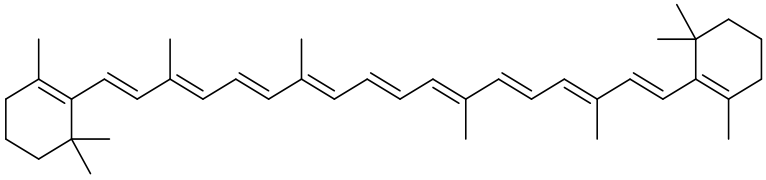

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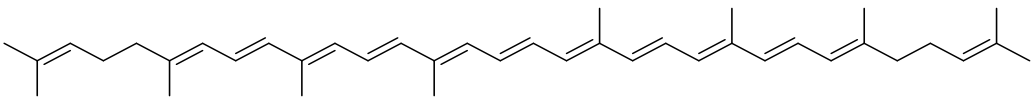

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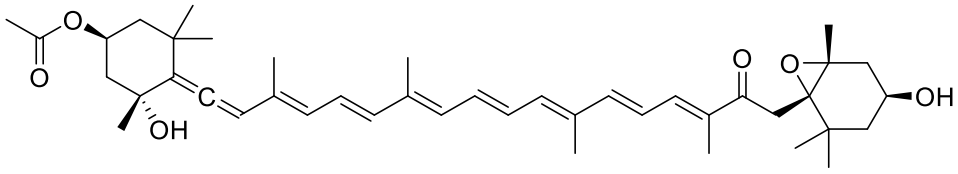

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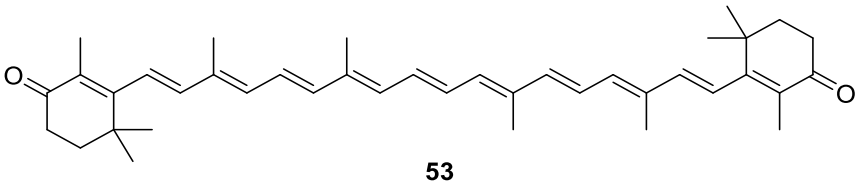

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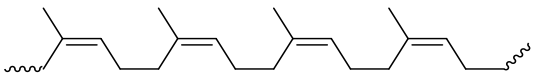
1.4.1.3.8 Polyterpenoids

Polyterpenoids are terpenoids that comprise more than forty carbons with more than eight isoprene units. These compounds are polymeric isoprenoid hydrocarbons. The most widely known example of these compounds is the natural rubber **54** (*cis*-configuration) molecule, which is a high-molecular-weight polymer with all of the C-C double bonds that are in a *cis*-configuration. Rubber has been found to provide protection against herbivores and a mechanism for wound healing in long vessels called laticifers [94,114].



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1.4.2 Essential oils

Essential oils (EOs), also known as volatile oils, are secondary metabolites of plants that are defined as concentrated aromatic hydrophobic oily and volatile liquids stored in a variety of plant materials such as wood, bark,

roots, flowers, buds, seeds, leaves, twigs, rhizomes, herbs, peels, and tissues of a single botanic species [115-117]. They are labeled as “essential oils” due to the presence of the “essence” in these plants. They have a strong odor and are mostly derived from plants. However, a few are produced by other animals and microorganisms too [118]. EOs are typically colorless, but some are orange (Citrus *sinensis*, Sweet orange), green (Citrus *bergamia*), and pale yellow (Yellow mandarin) [119]. In nature, EOs are considered great allelopathy agents as they play a crucial role in plant defense against herbivores and insects as well as their important role in signaling processes owing to their aroma characteristics [120]. In the last three decades, EOs have attracted the attention of researchers and scientists worldwide, especially in aromatherapy and phytomedicine, due to their biological activities, such as antiviral, antifungal, antibacterial, antitumor, and antioxidant activities [121-122]. They also have a significant potential to act as natural preservatives due to their antimicrobial properties, which has piqued the interest of the cosmetic and food industries, as well as a natural marketing image and strong fragrant properties [123]. Additionally, they are used in food technology as flavors, and in agriculture as insecticides [124].

In general, EOs are very complex natural mixtures of different components (20–60) at quite different concentrations, and they can consist of a few to more than 100 substances [125]. For the most part, EO components include lipophilic terpenoids as the most characteristic and frequent constituents, phenylpropanoids, or short-chain aliphatic hydrocarbon derivatives of low molecular weight [126]. Nonetheless, the extracted components of EOs can

be different even for the same plant species, according to many factors, including soil composition, climate, age, vegetative cycle stage, and plant organ [127,128].

1.4.2.1 Extraction methods for essential oils

Essential oils are gaining a huge interest in the pharmaceutical, cosmetic, medicinal, agricultural, sanitary, and food industries. Due to this huge demand for EOs, many extraction and production technologies are utilized to improve the overall yield of the EOs extracted from their raw materials. The chosen extraction technique hugely affects the chemical composition of EOs [129]. Inappropriate extraction techniques may destroy phytochemicals present in the EO, and affect the quality and the production yield of EO [130]. In general, extraction techniques can be categorized into two categories: classical methods and innovative methods.

1.4.2.1.1 Classical methods

These are the conventional and the most frequently used methods applied to extracting plant EOs, like hydro-distillation, steam distillation, and solvent extraction.

1.4.2.1.1.1 Hydro-distillation

This is the oldest and simplest method for EO extraction. This method is based on the principle of isotropic distillation. The whole plant material is soaked in water inside a vessel and the whole mixture is boiled by a heating source, then the vapor is converted into a liquid inside a condenser. Finally,

a decanter collects the condensate (essential oils with water) [131]. Although this method is simple, it has the disadvantage of high susceptibility to hydrolysis of the obtained compounds and the obtained oil from this method is relatively low [132].

1.4.2.1.1.2 Steam distillation

Steam distillation is considered to be the broadest technique used for EO extraction. About 93% of the EOs are extracted by this technique [133]. This method is based on steam (from a steam generator) as both the heating agent and the carrier for the components extracted from the plant material [134]. The main advantage of this technique is that the plant material is only in contact with steam, not boiling water. The yield of the extracted oil from steam distillation is higher than in the hydro-distillation method [132].

1.4.2.1.1.3 Solvent extraction

This technique is used for the extraction of EOs from fragile or delicate flower materials by using organic solvents like petroleum ether, acetone, hexane, and methanol, as these plant materials can’t be extracted by the means of heat or steam [130]. This procedure includes mild heating of the mixture consisting of the plant sample and the solvent, followed by filtration and evaporation of the solvents. There are many drawbacks to this method, including long extraction time, loss of low molecular weight species during solvent evaporation, consumption of large amounts of solvents, and more expensive than the other methods [135-137].

1.4.2.1.2 Innovative techniques of EO extraction

Since the classic extraction methods have many disadvantages, including the huge amounts of CO_2 emissions, solvents that are used, energy costs, and the fact that the EOs may undergo chemical alteration such as oxidation and hydrolysis, the development of new green extraction techniques has received a lot of attention as these techniques have improved the quality of the extracted EOs and lowered the time, energy, carbon dioxide emissions, and enhanced the production yield [138,139].

1.4.2.1.2.1 Supercritical fluid extraction (SFs)

It is a process to extract plant materials (especially EOs, lipids, and flavors) from the matrix using a supercritical fluid (as the extracting solvent), which is a solvent that has been subjected to pressure and temperature above its critical point [140]. CO_2 is the most chosen supercritical solvent for the extraction of EOs due to its plentiful attractive properties: being non-toxic, highly soluble, safe, nonflammable, thermodynamically stable, available in high purity at relatively low cost, relatively low critical pressure (74 bars) and temperature (32°C), non-corrosive, higher selectivity for desired compounds, inertness, and easy removal from the extract [141-143]. Extraction of EOs by SFs is a faster process with less damage to the environment as it provides products free of toxic waste, especially in the presence of environmentally damaging conventional organic solvents, and this method is more selective for the compounds to be extracted, which improves the quality of the extracted EOs [144-146].

1.4.2.1.2.2 Ultrasound-assisted extraction (UAE)

The principle of this method is to extract EOs from the plant material by subjecting ultrasounds (ultrasound frequency of about 20 KHz) to the sample and the solvent which is in direct contact with the plant material, leading to the formation of cavitation bubbles throughout the solvent and then followed by the compression of these bubbles, which changes the pressure and the temperature, increasing the rate of mass transfer of analytes to the solvent [147,148]. On top of that, the UAE can facilitate the separation of EOs by acting as an emulsifier that disperses the lipophilic molecules in water [149]. This method has many advantages, including shortening the extraction time, simplicity, and requiring less solvent; it also improves the quality of the extracted EOs because it is performed at room temperature, which prevents the natural products from decomposing and oxidizing [148,150].

1.4.2.1.2.3 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a technique that uses microwave radiation as the heating source of the solvent in contact with a sample in the solvent–sample mixture. Molecules are heated through dipole rotation and ionic conduction due to the effect of microwave radiation [151]. The main advantage of MAE is the rapid heating of the extraction solvent, which enhances the dispersion of the solvent into the sample and accelerates the partition of the analytes from the sample into the solvent [152]. The extraction solvent must be polar to be able to absorb microwave energy, like water, methanol, and ethyl acetate [153]. MAE has been used as an

alternative to conventional methods because it reduces the extraction time, the required solvent volume, is more selective and helps in minimizing the environmental impact by lowering CO_2 in the atmosphere [154].

1.4.2.1.2.4 Ultrasonic microwave-assisted extraction (UMAE)

A new innovative extraction technology that is based on combining both the microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), and utilizing the advantages of both microwave and sonochemistry to improve the efficiency and rate of extraction, achieving higher yields of the extracted EOs without the destruction of the wanted compounds, this technique is ultrasonic microwave-assisted extraction (UMAE).

1.4.2.2 Quantitation and qualification analysis of EOs

The increasing demand for EOs by different production companies has led to cases of EO adulteration, in which EOs are mixed with cheaper oils to get a higher profit while using low-cost synthetic materials [155]. To ensure the quality of EOs, many analytical techniques are used for the separation and identification of their components. One of the famous methods is gas chromatography-mass spectrometry (GC–MS). GC–MS is an analytical technique used for the classification and characterization of EOs, due to the volatile nature of EO components [156].

GC works on the principle of separating the volatile (also semi-volatiles) and thermally stable components in a sample mixture when heated. The heated gases are then passed through a column containing an inert gas, and when the separated substances exit the column, they are subjected to mass

spectrometry (MS), which identifies the compounds based on the analyte mass [157,158]. In addition to its sensitivity and good detection limit, GC-MS has provided an easy, faster separation at a low cost and in a shorter time [159]. This technique has many applications, including biological and pesticide detection, forensic and criminal cases, and environmental monitoring [157].

1.5 Biological activity

Bioactivity is how a material will respond after being exposed to a particular biological environment or substance. It could include a physiological response (e.g. antioxidant, anti-bacterial, etc.), tissue uptake, or metabolism [160,161].

1.5.1 Anti-oxidant activity

Free radicals (or reactive oxygen species (ROS)) are defined as molecules or molecular fragments that possess one or more unpaired active electrons in the valence shell (hydroxyl HO, nitric oxide NO, etc.) [162,163]. These chemical species are very reactive, uncharged, and short-lived molecules that result from aerobic metabolic processes and are beneficial for the human body as they work with the immune cells to kill microbes like bacterial cells. However, uncontrolled generation of free radicals, or gaining more of them from outside sources, could lead to damage to many vital biological components and result in many diseases, like cancers, heart and neurodegenerative diseases, and autoimmune diseases, etc. [164,165]. This causes a disturbance in the equilibrium between free radicals and the

endogenous defense mechanisms and leads to the production of oxidative stress [166]. Herein comes the role of antioxidants, which are chemicals that neutralize free radicals by stopping them from damaging biological cells and improving their overall health.

Some antioxidants are produced by the human body and are known as endogenous antioxidants, whereas exogenous (or dietary antioxidant) antioxidants come from outside the body. There are two sources of antioxidants: Synthetic antioxidants, which include phenolic compounds like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ) [167]. The second source is natural antioxidants such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), flavonoids, phenolic acids, and carotenoids. Hence, supplying the body with antioxidants from external sources, especially from plants that serve as a great source of natural antioxidants, will help in the treatment of many oxidative stress-linked diseases [168].

1.5.2 Anti-microbial activity (bacterial and fungi)

Infectious diseases that are caused by bacteria and fungi are the cause of many ailments and deaths all around the world. Pathogenic organisms such as bacteria and fungi are causing a global threat to humans and animals due to the evolution of multidrug-resistant (MDR) pathogens. This is a result of the misuse of antibiotics, poor hygiene, increased international travel, and the availability of antibiotics over-the-counter, followed by releasing their residues to the environment [169,170]. According to the World Health

Organization (WHO), about 700,000 people are killed every year globally due to antibiotic-resistant bacteria [171].

The popular antibiotic-resistant bacteria are *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, *methicillin-resistant S. aureus* (MRSA), *Klebsiella pneumoniae (K. pneumoniae)*, and *Pseudomonas aeruginosa*. Fungi species, particularly those belonging to the *Candida* family (e.g. *Candida albicans* (*C. albicans*)), are the leading cause of life-threatening systemic infections, particularly among HIV/AIDS patients [172].

Hence, extensive efforts have been devoted to research to find alternatives to synthetic antibiotics and to look for non-toxic bio-molecules that can work as both antimicrobials themselves and enhance the accessibility of the existing antimicrobials. Medicinal plants' phytochemicals are the best candidates as they have proved through the years their ability to work as remedies for various diseases and have improved human health [173].

1.6 *Nepeta curviflora* Boiss

Nepeta is one of the largest genus of the "Lamiaceae" (Labiatae) family, which consists of around 300 genera and almost 7,000 species distributed in Europe, Asia, North Africa, North America, India, and the Middle East [174,175]. The name *Nepeta* was created after the name of the ancient Italian city "Nephi" [176]. In folk medicine, *Nepeta* species are widely used as antiseptic, expectorant, anti-asthmatic, sedative, anti-tussive, antiviral, fungicidal, antipyretic, and antispasmodic agents. Different types of secondary metabolites and EOs isolated from different *Nepeta* species have

been used for the treatment of toothache and gum disease, many heart problems such as angina pectoris and the weakness of the heart, for liver and kidney diseases, and they have also been used against scorpion and snake bites [177-180]. They also show unusual behavioral effects on cats (e.g. *N. cataria L.* (catnip)) [181]. Furthermore, *Nepeta's* EOs are mainly used as a perfume or fragrance and for food flavoring purposes. Many biological studies have revealed that they are antifungal, antibacterial, insecticide, cytotoxic, repellent, antioxidant, anti-inflammatory, analgesic, and anticonvulsant [182,183].

The major constituents of the genus *Nepeta* species are nepetalactone, different classes of terpenoids including monoterpenes, sesquiterpenes, and cyclopentanoid iridoid derivatives. They also possess flavonoids, and phenolic compounds [184].

Nepeta curviflora Boiss, commonly known in Arabic as "na'na' elher" (or Syrian catnip), is a perennial herbaceous plant that grows up to 60 cm in the mountains. It is characterized by its bluish-violet flowers and small leaves. This aromatic plant grows in the wild mountains of Palestine, Jordan, Syria, Lebanon, and Turkey [185]. In Palestine, this wild herb is reported to grow in a variety of locations, including Ramallah, Nablus, and Galilea.

In folk medicine, *N. curviflora* is used to treat many diseases, like Alzheimer's disease, and it is also used as an antipyretic agent [186].

Additionally, this herb has been utilized as a healing remedy for fever, bronchitis, flu, diarrhea, colds, colic, and cough [187].

Previously conducted studies on the EOs extracted by the hydro-distillation technique from the aerial parts of *N. curviflora* in Lebanon and Jordan have been reported [188,189]. The essential oil of *N. curviflora* collected from Lebanon was rich in *β*-caryophyllene **38**, which was detected at a higher concentration as compared to that of the oil of *N. curviflora* growing in Jordan (50.20%, 11.53%, respectively). In addition, 4a*α*,7*α*,7a*α*-nepetalactone, the main oxygenated monoterpene identified in the oil of Jordanian *N. curviflora* (43.85%), was not observed in the oil of *N. curviflora* growing in Lebanon. Another two studies conducted in Lebanon on the EOs of *N. curviflora* [190,191] have also indicated that the main constituent of the EO is *β*-caryophyllene **38** (41.6, 50.2% respectively).

1.7 Objectives of the Study

This study is divided into two parts. The first part is concerned with the extraction of the essential oil from the leaves and flowers of *N. curviflora*, then analyzing it using GC-MS, and assessing the bioactivity of this EO. The second part is focused on extracting the phytochemicals from the stem of *N. curviflora*, followed by isolation and characterization of these phytochemicals, and evaluating their biological activity. The specific objectives of this study are:

1. Analyze the chemical composition of *N. curviflora* EO from the leaves and flowers using the GC-MS technique.
2. Investigating the anti-oxidant properties of the EO of *N. curviflora.*
3. Assessing the enzymatic properties of *N.curviflora* EO, including antilipase, antiamylase, and anti-glucosidase activities.
4. Isolate and identify the active components in the stem of *N. curviflora* using various techniques.
5. Investigate the antioxidant, antibacterial, and antifungal activities of the extracts from the stems of *N. curviflora*.

Chapter Two

Experimental Part

2. Materials and methods

2.1 Chemicals and Reagents

The materials that were used in this research were of analytical grade and were used without further purification.

DPPH (2,2-Diphenyl-1-picrylhydrazyl) was purchased from (Sigma-Aldrich, D9132-1G, Germany), Trolox ((s)-(-)-6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (was purchased from Sigma-Aldrich, 391921-1G, Denmark), Dimethyl sulfoxide (DMSO) (Riedel-de-haen, Q4199, Germany), 3,5-dinitro salicylic acid (Sigma-Aldrich, USA), Methanol (Loba Chemie, India), RPMI-1640 medium (Roswell Park Memorial Institute-1640 medium) (Sigma-Aldrich, R0883, UK), Tris− HCl buffer (SDFCL, India), porcine pancreatic lipase (Sigma-Aldrich, USA), orlistat (Sigma-Aldrich, Germany), *p*-nitrophenyl butyrate (from Sigma-Aldrich, Germany), porcine pancreatic α-amylase (Sigma-Aldrich, India), acarbose (Sigma, USA), PBS (phosphate buffer saline) (Sigma-Aldrich, 79383, Germany), and *p*-nitrophenyl α-D-galactopyranoside (Sigma-Aldrich, USA).

2.1.1 Chemicals used for the extraction of *N. curviflora* stems

Solvents used (methanol, dichloromethane (DCM), ethyl acetate (EtOAc), hexane, and diethyl ether) were purchased from Sigma Aldrich (Germany). Silica gel (100–200 and 200–300 mesh) for flash column chromatography (FCC) and GF254 silica gel for thin-layer chromatography (TLC) were purchased from Sigma-Aldrich (USA).

2.2 Instrumentation

Equipment that were used in this study are:

Electronic balance (Wagl, AS 220/C/2, Radwag, Poland), microwave ultrasonic extractor (CW-2000, Hong Kong, China), laminar flow (MRC, BBS12HGs, Israel), 24-well plates and 96-well plates (Greiner bio-one, North America), shaking laboratory water bath (Lab Tech, BPXOP1001040, Namyangju, South Korea), micropipettes (Finnpipette, Finland), CO_2 incubator (ESCO, 2012-74317, Singapore), and spectrophotometer (Jenway, UV/Vis Spectrophotometer 6505, UK).

2.3 Plant material collection and preparing

The aerial parts of *Nepeta curviflora* were compiled at the end of May 2019 from the Birzeit mountains in Palestine. The botanical identification was approved and coded by Dr. Nidal Jaradat the pharmacognosist at the Pharmacognosy Laboratory, An-Najah National University. The fresh leaves and flowers were separated carefully from the stem of the plant, washed thoroughly with distilled water, then left to dry in the shade at humidity (55

± 5 RH) and temperature (25 ± 2 °C) for two weeks. The dried leaves and flowers were grinded separately from the stem using a mechanical blender and stored in paper bags for further isolation process.

2.4 Extraction of the *N. curviflora* essential oil

The extraction of *N. curviflora* EO was performed using the microwave-ultrasonic apparatus as described previously by Jaradat et al. [192]. One-liter round-bottom flask containing 50 g of the dried plant powder and 500 mL of distilled water was placed in the microwave-ultrasonic apparatus. The flask was connected to the Clevenger apparatus, which was also placed in the same apparatus. The microwave extractor apparatus power was adjusted at 1000 W for 15 min at 100 °C. The extracted EO was dried using calcium chloride and transferred into a small clean, well-closed glass bottle, labeled with the name of plant, date, code of the sample, covered with aluminum foil, and stored in the refrigerator at 2-8 °C. The isolated EOs yield average was 1.7% from the dried parts.

2.5 Identification of the phytochemical composition of *N. curviflora* EO by gas chromatography-mass spectrometry (GC–MS)

The separation and identification of *N. curviflora* EO components were accomplished by employing the Perkin Elmer Elite-5-MS fused silica capillary column (30 m × 0.25 mm, film thickness of 0.25 μm), where Helium was used as a carrier gas at a standard flow rate of 1.1 mL/min. The temperature of the injector was adjusted at 250 °C with an initial temperature

of 50 °C, the initial hold of 5 min, and a ramp of 4.0 °C/min to 280 °C. The total running time was 62.50 min and the solvent delay was from 0 to 4.0 min. MS scan time was from 4 to 62.5 min, covering the mass range from 50.00 to 300.00 m/z. The chemical constituents of *N. curviflora* EO were identified by comparing their mass spectra with the reference spectra from the MS Data Centre of the National Institute of Standards and Technology (NIST), and by matching their Kovats and retention indices with values described in the literature [193,194]. The EOs Kovats and retention indices values were compared with 20% of HPLC grade of reference EOs purchased from Sigma-Aldrich (Germany) [195]. Quantitative calculations were carried out by calculating percentage areas for each component from *N. curviflora* EO.

2.6 Free radical scavenging assay

The free 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of *N. curviflora* EO was adopted for determining the antioxidant potential of *N. curviflora* EO using the method of Sonboli and Jaradat et al. [196]. A solution of the EO in methanol (1 mg/mL) was prepared followed by preparing a solution of trolox (the reference product) in methanol (1 mg/mL). The stock solutions were then used to make a dilution series with six serial dilutions at concentrations of 2, 5, 10, 20, 50, and 100 g/mL. One mL of each EO dilution was mixed with 1 mL of 0.002 g/mL methanolic DPPH solution. Methanol (1 mL) was added to give a final working volume of 3 mL. Because of its sensitivity to light, the DPPH solution has to be made fresh. The blank

control of the series concentrations was prepared by dissolving DPPH in methanol in 1:2 ratios in the absence of the EO. All solutions were incubated at 25 °C under the exclusion of light for 30 min. A spectrophotometer was used to measure the absorbance at a wavelength of 517 nm.

The antioxidant activity of the corresponding *N. curviflora* EO was determined in terms of inhibition percentage for *N. curviflora* EO, with trolox using the following equation:

$$\boldsymbol{In\%} = \frac{\boldsymbol{A\ blank - A\ sample}}{\boldsymbol{A\ blank}} \times \mathbf{100}$$

Equation (2.1): Inhibition% of antioxidant activity [197]

Where A_{blank} and A_{sample} represented the absorption of the blank (all reagents without the sample) and the sample respectively.

2.7 Porcine pancreatic lipase inhibition assay

The porcine pancreatic lipase inhibition was assessed employing a previously reported procedure [198].

2.7.1 Preparation of stock and working solutions

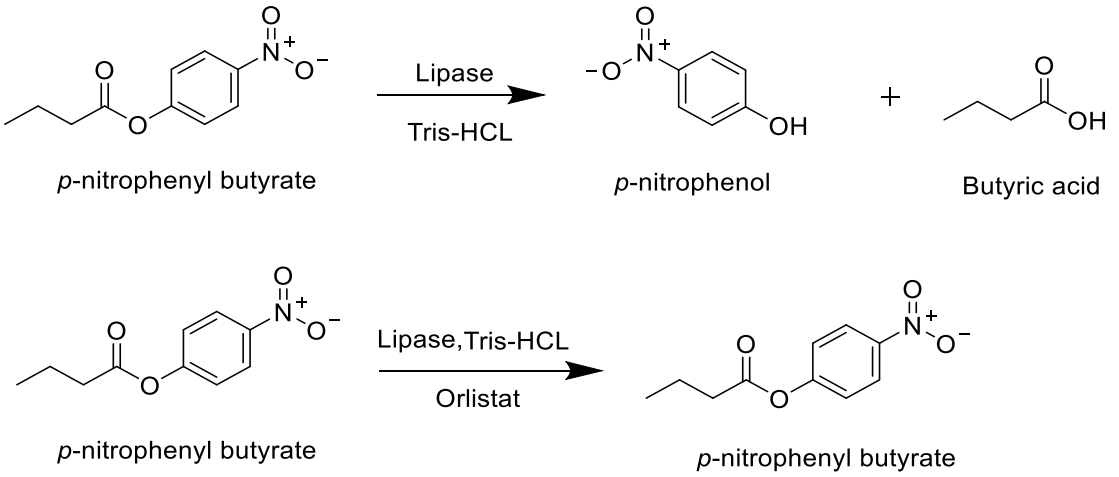
A stock solution of *N. curviflora* EO was prepared in 10% dimethyl sulfoxide (DMSO) (500 μg/mL), of which a dilution series of five different concentrations of 50, 100, 200, 300, and 400 μg/mL were produced. A fresh stock solution of porcine pancreatic lipase in the Tris− HCl buffer (1 mg/mL) and a stock solution of 20.9 mg of *p*-nitrophenyl butyrate (PNPB) in 2 mL of acetonitrile was prepared.

2.7.2 Assay of pancreatic lipase (PL) inhibition

For each working solution, 0.1 mL of porcine pancreatic lipase (1 mg/mL) was mixed in a separate test tube with 0.2 mL of *N. curviflora* EO from each of the dilution series. After that, Tris− HCl was added to bring the final volume of the working solutions to 1 mL. After a 15-minute incubation at 37 °C, each test tube was filled with a 0.1-mL solution of *p*-nitrophenyl butyrate in acetonitrile, and the resulting mixture was incubated for another 30 min at 37 °C. Pancreatic lipase activity was assessed by measuring the hydrolysis of PNPB into *p*-nitrophenolate using a UV spectrophotometer at 410 nm. The same experiment was repeated using orlistat (positive control). Percentage lipase inhibition by plant EO was calculated using the following equation:

Lipase inhibition% = $(A_B - A_{ts})/A_B \times 100\%$ (**Equation (2.2):** Pancreatic lipase inhibition**)**

where A_B is the blank solution's recorded absorbance and A_{ts} is the recorded absorbance of the tested sample solution.



Scheme (2.1): Hydrolysis of *p*-nitrophenyl butyrate (PNPB) with and without Orlistat [199]

2.8 *α*-Amylase inhibition assay

A standard method with minor changes was used to assess the *α* -amylase inhibitory efficacy of *N. curviflora* EO [200]. The essential oil (100 mg) was dissolved in a few milliliters of 10% DMSO, followed by 0.02 M Na_2HPO_4/NaH_2PO_4 and 0.006 M NaCl buffer solution (pH 6.9) to bring the volume to 100 mL, yielding a stock solution with a concentration of 1 mg/mL, of which the following dilutions 10, 50, 70, 100 and 500 µg/mL were prepared using 10% DMSO as the diluent. *N. curviflora* EO (0.2 mL) was mixed with porcine pancreatic *α*-amylase (0.2 mL, 2 units/mL). After a 10-minute incubation period at 30 °C, the tubes were filled with a freshly made 1% starch solution in water (0.2 mL) and incubated for at least 3 minutes. At this point, the reaction was quenched by adding 3,5-dinitro salicylic acid (DNSA), and the mixture was diluted with distilled water (5

mL), before being heated at 90 °C in a water bath for 10 min. After cooling the mixture to room temperature, the absorbance was measured at 540 nm. The blank control was made with the same quantities as the above but with buffer (0.2 mL) instead of *N. curviflora* EO. Acarbose (the commercial antidiabetic drug) was used as a standard reference following the procedure described above. The *α*-amylase inhibitory activity was calculated utilizing the following equation:

$$\alpha\text{-Amylase inhibition\%} = (A_B - A_T)/A_B \times 100\%$$

(Equation (2.3): *α*-Amylase inhibition**)**

where A_B is the absorbance of the blank sample and A_T = absorbance of the test sample.

2.9 *α*-Glucosidase inhibitory assay

The *α*-glucosidase inhibitory activity of *N. curviflora* EO was tested according to the standard protocol with minimal modifications [201]. A dilution series of *N. curviflora* EO was prepared, to obtain concentrations of 100, 200, 300, 400, and 500 μg/mL. The reaction mixtures contained *α*-glucosidase solution (0.1 mL of 1 U/mL) were mixed with 0.2 mL of the EO dilution and 0.5 mL of 100 mM phosphate buffer (pH 6.8). After that, the mixtures were incubated at 37 °C for 15 min, and 5 mM *p*-nitrophenyl-*α*-D-galactopyranoside (PNPG) was added to each of the reactions mixtures and incubated at 37 °C for another 20 min. The reactions were stopped by adding 0.1 M Na_2CO_3 and the absorbance was recorded at a wavelength of 405 nm

for the EO sample. Acarbose was used as a positive control at the same concentrations as the plant EO. The inhibition percentages were calculated according to the equation below:

α-Glucosidase inhibition% = $(A_B - A_s/A_B) \times 100\%$ **(Equation (2.4)**: α-Glucosidase inhibition**)**

where A_B is the absorbance without enzyme inhibitor and A_S is the absorbance in the presence of the enzyme inhibitor.

2.10 Data analysis

Data has been expressed as means ± SD. Statistical analysis was conducted using the Student t-test. Differences considered being significant at $p \leq 0.05$. Calculations of the inhibitory concentration 50% (IC_{50}) were obtained from the Prism dose-response curve (Prism Graph pad, version 7.0 of prism for Windows (GraphPad Software, San Diego, CA, USA).

2.11 Extraction of phytochemicals from the stem of *N. curviflora*

The dried stems from *N. curviflora* (766 g) were powdered using a mechanical blender, then macerated with a sufficient amount of methanol (2L), followed by stirring using a mechanical stirrer for 24 hours. The resulting extract was filtered, and the remaining solid was subjected to extraction twice with methanol (0.5 L) under the same conditions. Afterward the filtration of the combined extract, methanol was removed under reduced pressure using a rotary evaporator. Then the concentrated extract was washed successively with pentane to remove the fats, waxes, nonpolar

compounds, followed by dissolving with ethyl acetate to extract the flavonoids and other polyphenols that are present in the extract, and then it was concentrated under reduced pressure at 40 °C to remove the excess ethyl acetate, resulting in a dried extract (3.16 g).

2.12 Isolation

The dried extract (3.16 g) was dissolved using dichloromethane (DCM), followed by decantation of the filtrate into a separate flask which was then introduced to the first column to start the separation process, and the crude extract (655 mg) which didn't dissolve in DCM (labeled as A) was stored for further use.

The separation process of the extract (filtrate from the previous step) was initiated by the determination of the best eluent that could be used in flash column chromatography, and this step was achieved by the TLC (Thin Layer Chromatography) experiments.

The first flash column chromatography was initiated using dichloromethane (DCM) and ethyl acetate (EtOAc) (at a ratio of 9:1 v/v) as an eluent. During the first chromatographic run, the polarity of the eluent was increased to allow the elution of more polar compounds and ensure a better separation by passing through a solution composed of dichloromethane and ethyl acetate (8:2, 7:3, and finally, at 6:4 v/v) (gradient elution). A total of 23 test tubes were collected (100 mL each). To determine which test tubes should be pooled together, another set of TLC experiments was performed on each of these tubes using an eluent of DCM/EtOAc at a (7:3) ratio. All of the

compounds were visualized under UV light, and the tubes that produced the same results were combined and evaporated under reduced pressure at 40 °C using a rotary evaporator, yielding five major fractions. To be more specific, each fraction contained the following test tubes: Fraction 1 (F1) included tubes (1 and 2), Fraction 2 (F2) included tubes (3-6), Fraction 3 (F3) included tubes (7,8), Fraction 4 (F4) included tubes (9-12), and Fraction 5 (F5) included tubes (13-23). They were all kept in sealed vials for further purification. These fractions were also subjected to HPLC analysis.

Both the fractions F2 and F3 were subjected to a second flash column chromatography using ether and hexane (5:5 v/v) as eluent. The eluent ratio was increased after conducting TLC experiments to be (ether: hexane in 7:3 v/v ratio) then finally into (ether: hexane 9:1 v/v ratio). A set of five sub fractions were obtained at this step after removing the solvents by rotary evaporator at 40 °C. After TLC and HPLC analysis of these fractions, the results showed the presence of a lot of compounds in almost all of the fractions. This has indicated that it is very hard to separate these fractions using the available simple techniques. However, because both of the major fractions, F4 and F5, had the least complicated HPLC results (fig.a.6 and fig.a.7, Appendix), they were subjected to further flash column chromatographic separation. Based upon the results of TLC analysis, the most suitable eluent consisted of DCM and ethyl acetate in a (7:3 v/v) ratio, and the same steps from the first column were repeated. From the TLC results for the resulting fractions, the fractions that showed the same results were pooled together and the solvents were removed by a rotary evaporator

at 40 °C. These fractions were analyzed by HPLC and only two of them were almost pure. They were named compound B (51.9 mg) and compound C (46.05 mg).

2.13 Structure elucidation

2.13.1 HPLC analysis

An HPLC analysis was carried out to check the number of components present in each fraction and to detect the purity of these components. The analysis was conducted using HPLC-DAD Water 1525, with a C18 column (5µm, 4.6×250 mm cartridge). The chromatographic separation was performed using binary-solvent gradient elution with the mobile phase of solvent A (water) and solvent B (methanol). The gradient elution started with 100% of solvent A and 0% of solvent B until 0% of A and 100% of B, with a flow rate of 0.7 ml/min. The total run time was 35 minutes, and the injection volume was 20µL. The wavelength used to detect all extracts was 254 nm.

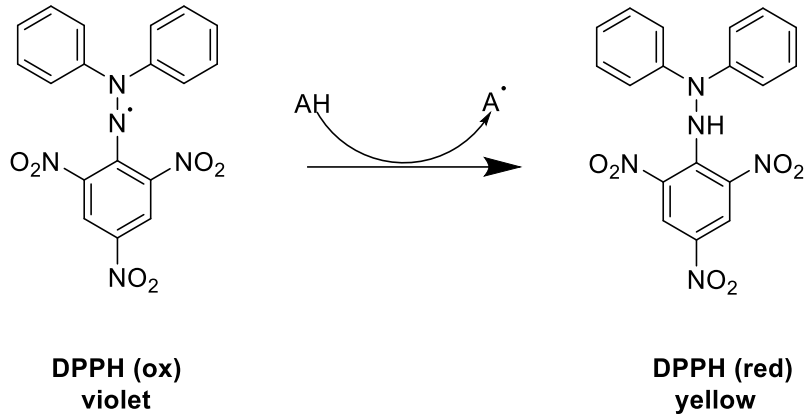
2.13.2 IR (Infrared spectroscopy) analysis

Functional groups of the extracted compounds were characterized using an FT-IR spectrometer (NICOLIT iS5 from Thermo Fisher Scientific).

2.14 Pharmacological screening for the extracts from the stem of *N. curviflora*

2.14.1 Antioxidant activity assay for the extracts

The scavenging activity of *N. curviflora* extracts was assessed using the same methods that were used for the free radical scavenging assay for *N. curviflora* EO [196]. The antioxidant activities of the plant's fractions (A, B, C,) and Trolox (positive control) were assessed by their ability to donate hydrogen atoms or electrons, which was identified by changing the deep violet color of the methanolic DPPH solution to colorless or pale yellow, as indicated in scheme 2.2:



Scheme (2.2): Principle of DPPH radical scavenging capacity assay [202].

Stock solutions at a concentration of 1 mg/mL in methanol and Trolox were prepared from *N. curviflora* extracts. Each one of these stock solutions was diluted in methanol to prepare 12 working solutions with the following concentrations: 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, and 100 μg/mL.

A freshly prepared DPPH solution (0.002% w/v) was mixed with both methanols and with each of the abovementioned working solutions at a 1:1:1

ratio. Furthermore, a negative control solution was prepared by mixing the DPPH solution with methanol at a 1:1 ratio. Afterward, all of these solutions were incubated at room temperature in a dark cabinet for 30 min. By the end of the incubation period, the optical density of these solutions was measured by a UV-Vis spectrophotometer at a wavelength of 517 nm using methanol as the blank solution.

The antioxidant activity of the corresponding plant compounds and Trolox standard were determined in terms of inhibition percentage of DPPH activity using the following equation:

$$\boldsymbol{In\%} = \frac{\boldsymbol{A\ blank - A\ sample}}{\boldsymbol{A\ blank}} \times \mathbf{100}$$

Equation (2.1): Inhibition% of antioxidant activity [197]

Where A_{blank} and A_{sample} represented the absorption of the blank (all reagents without the sample) and the sample respectively.

For this part specifically, the antioxidant half-maximal inhibitory concentration (IC_{50}) values for the studied plant fractions and Trolox standard solution, as well as their standard deviations, were determined from the graph plotted by the inhibition percentage against the concentrations of the plant fractions, using Microsoft Office Excel 2016.

2.14.2 Antibacterial and antifungal activity tests

2.14.2.1 Preparation of plant samples for testing

10 mg of each of the plant extracts (A, B, C) were dissolved in 0.5 mL of sterile dimethyl sulfoxide (DMSO), followed by dilution with 0.5 mL of

distilled water to obtain a concentration of 10 mg/ml. All of the extract's solutions were kept in the UV disinfection chamber for 20 minutes.

2.14.2.2 Preparation of growth media

The Mueller-Hinton Broth (MHB) media was prepared following the manufacturer's instructions, which were clearly labeled on the bottle. First, 8.4g of MHB powder was dissolved in 400 ml of autoclaved distilled water, then it was heated with stirring using a magnetic stirrer to the boiling point until the medium was completely dissolved. The resulting solution was autoclaved for 15 minutes at 121 °C, and it was cooled down to room temperature before using it.

2.14.2.3 Test microorganisms

a) **Bacterial strains:** Six reference bacterial strains obtained from the American Type Culture Collection (ATCC): four gram-negative bacteria strains: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883, UK), *Pseudomonas aeruginosa* (ATCC 9027, USA), *Proteus vulgaris* (ATCC 8427), and two gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538P, USA) and Methicillin-Resistant *Staphylococcus aureus* (MRSA). All the bacterial strains were sub-cultured on Mueller-Hinton agar.

b) **Fungal strain:** One fungal strain of *Candida Albicans* (ATTC 90028, USA) obtained from the American Type Culture Collection (ATCC) was used in this study. This strain was sub-cultured on Sabouraud Dextrose Agar.

2.14.2.4 Preparation of bacterial and fungal suspensions

All bacterial and fungal strains were cultured 24 hours before use. The bacterial suspension was formulated by the addition of a sterile specimen that was taken gently from the colony surface of each type of cultured strain and transferred to a separable sterile tube containing 5 mL of sterile normal saline. The optical density of all the solutions was measured by a spectrophotometer at ƛ = 620 nm, where normal saline was used as a blank. The turbidity of the bacterial suspensions was adjusted into 0.5 McFarland turbidity standard (optical density 0.08 to 0.1), to obtain a bacterial suspension with 1.5 x 10^8 colony forming units (CFU/mL). In addition, the turbidity of the yeast *Candida albicans* was adjusted to equal to 0.5 McFarland solution (optical density of 0.12 to 0.15) with a concentration of $1*10^6$ - $5*10^6$ CFU/mL. After that, the stock solutions of each bacterial and fungal strain were prepared by transferring 100 µL of the bacterial and fungal suspensions into 10 mL of Mueller Hinton Broth media, these stock solutions were further used in the experiment of the broth microdilution method.

2.14.2.5 Anti-microbial assay

The antimicrobial activity of the plant samples was assayed employing the broth micro-dilution method defined by the procedure as described in the literature with minor modifications [203,204] . Note that all the dilution processes were carried out under aseptic conditions.

a. Anti-bacterial assay

In the sterile 96 micro-well plates (figure 2.1) used to assess the antibacterial activities of the extracts, 50 µL of Mueller Hinton Broth

media was pipetted into all microplate wells except the last raw one (H) using a multichannel pipette. Then, excluding H raw, 50 µL of the first prepared solution of plant extract (A) was added to the wells of the first column. Afterward, 50 µL of the solution from well number 1 was transferred by multichannel pipette to wells number 2, which were mixed to obtain (2-fold) serial dilution. This process was replicated up to the well with the number 10. Then 50 µL from each type of the bacterial strains suspension was filled in its specific labeled row for the wells 1-11. The wells with number 11 contained only Mueller Hinton Broth media and the bacteria suspension without the plant extract, which was used as a positive control for microbial growth. Additionally, microwell number 12 contained only the growth media and was left uninoculated with any of the test microbes. It was labeled as a negative control. On top of that, G raw microwells contained only the extract solution to ensure that there is no contamination. The resulting turbidity in the G raw wells was not due to the extract itself. The same steps were repeated for the rest of the plant extracts (B and C). Finally, all the inoculated plates were incubated at 35˚C and the incubation period lasted for about 24 hours. The resulting turbidity in the microwells indicated bacterial growth.

The lowest concentration of plant extracts, at which no visible bacterial growth in that microwell was observed, is considered as the minimum inhibitory concentration (MIC) of the examined plant extracts. All the established experiments were performed in triplicate, to control the sensitivity of the tested microorganisms.

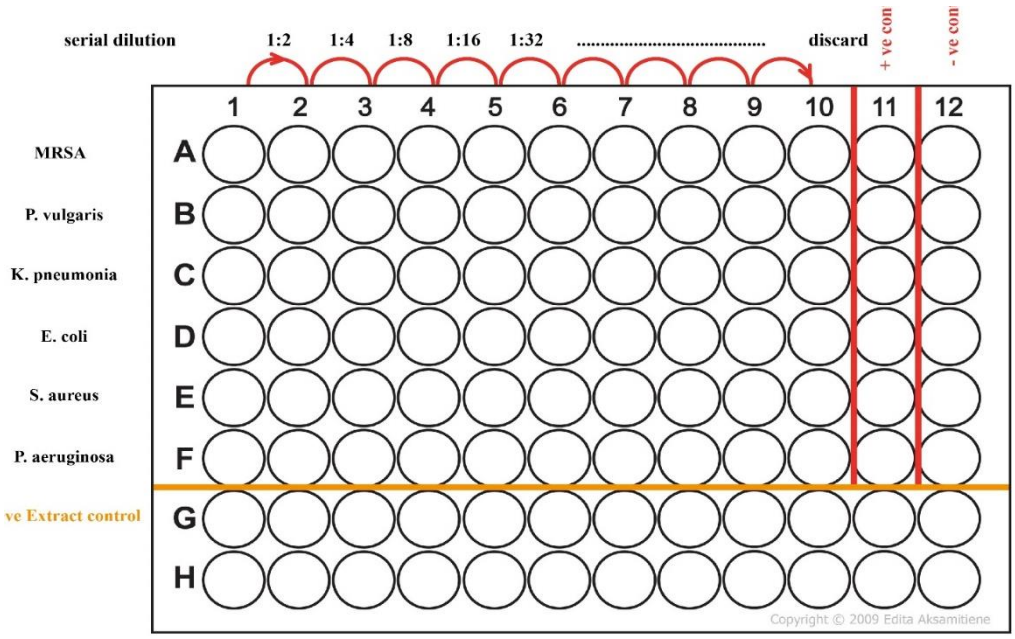


Fig. 2.1: 96 micro-wells plate for anti-bacterial assay

b. Determination of anti-yeast activity

The same procedure of broth microdilution assay that was used for bacterial strains was also used for the yeast *C. albicans* with minor modifications. As shown in figure 2.2, the plant extracts were added to microplate wells in duplicate; both raw A and B for the first extract (**A**), C and D for the second extract (**B**), E and G for the third extract (**C**). And the *C. albicans* suspension was added to the remaining raws A, C, and E. Then all the inoculated plates were incubated at 35˚C for 48 hours.

The lowest concentration of plant extracts, at which there is no visible *candidal* growth in that microwell was estimated as the minimum inhibitory concentration (MIC) of the examined plant extracts. All the established experiments were performed in triplicate, to control the sensitivity of the tested *C. albicans.*

In parallel, a control experiment was run to study the impact of the solvent alone (without plant extracts) on the growth of the seven test organisms. Dimethyl sulfoxide was diluted in a similar pattern with sterile MHB broth followed by inoculation and incubation.

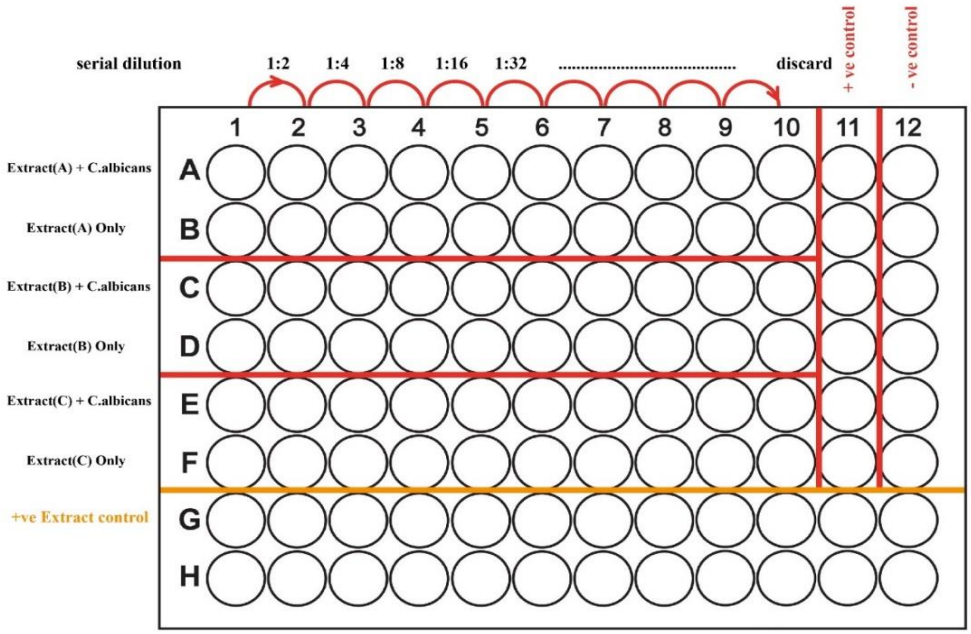


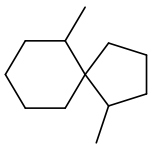
Fig. 2.2. 96 micro-wells plate for anti-yeast assay

Chapter Three

Results

3.1 GC–MS characterization of *N. curviflora* essential oil

The EO from the leaves and flowers of *N. curviflora* was quantitatively and qualitatively characterized by employing the GC–MS technique. Twenty compounds were identified, representing 100% of the total mass of EO. 1,6-Dimethyl spiro[4.5]decane **55** (27.5%), caryophyllene oxide **56** (20.08%), and *β*-caryophyllene **38** (18.28%) were revealed to be the most abundant components of *N. curviflora* EO. GC-MS analysis of the EO of *N. curviflora* was characterized by the richness of terpenoids, including Sesquiterpene hydrocarbon (38.49%), oxide sesquiterpenoid groups (20.08%), as well as considerable concentrations of spiroalkane (29.14%) as these compounds were found to be the major groups.



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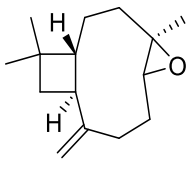

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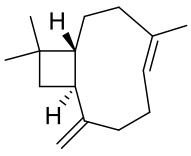

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It is worth noting that caryophyllene oxide **56**, which has high potential, was found in higher concentrations in EO *N. curviflora* growing in Palestine than in the studied plants vegetating in Lebanon and Jordan. The phytochemical composition, retention index (RI) and retention time (RT) with their

concentration (%) are represented in Table 3.1 and the GC–MS chromatogram is described in Fig. 3.3.

Table 3.1. The chemical composition of *Nepeta curviflora* essential oil. MW: molecular weight (g/mol); RI: retention index.

No.	Compounds	MW (g/mol)	RI	Identification	% Area
1	*trans*-Carane	138.25	808	MS, RI	0.07
2	*p*-Mentha-3,8-diene	136.23	772	MS, RI	1.58
3	1,3,4-Trimethyl-3-cyclohexenyl-1-carboxaldehyde	152.23	803	MS, RI	0.62
4	Limona ketone	138.21	731	MS, RI	1.58
5	1,6-Dimethyl spiro[4.5]decane	166.3	922	MS, RI	27.51
6	Decahydro-3a-methyl-6-methylene-1-(1-methylethyl)cyclobuta[1,2:3,4] dicyclopentadiene	230.39	787	MS, RI	1.97
7	Nepetalactone	166.22	840	MS, RI	5.38
8	cis, trans-1,9-Dimethylspiro[4.5]decane	166.3	875	MS, RI	1.64
9	*β*-Caryophyllene	204.36	924	MS, RI	18.28
10	*β*-Farnesene	204.35	792	MS, RI	6.12
11	*α*-Humulene	204.35	904	MS, RI	1.19
12	Aromadendrene	204.35	917	MS, RI	0.5
13	*β*-Cubebene	204.35	883	MS, RI	2.96
14	Humulen-(v1)	204.35	916	MS, RI	0.17
15	*γ*-Elemene	204.35	837	MS, RI	1.81
16	*γ*-Caryophyllene	204.35	830	MS, RI	4.95
17	*β*-Sesquiphellandrene	204.35	843	MS, RI	2.26
18	Argeol	220.35	734	MS, RI	1.07
19	4-Methylene-2,8,8-trimethyl2-vinylbicyclo[5.2.0]nonane	204.35	799	MS, RI	0.27
20	Caryophyllene oxide	220.35	732	MS, RI	20.08
	Total (%)				100

	Grouped components	%
1	Sesqueterene hydrocarbon	38.49
2	Alcoholic sesquiterpenoid	1.07
3	Oxide sesquiterpenoid	20.08
4	Monoterpene hydrocarbon	1.65
5	Aldehydic monoterpenoid	0.62
6	Ketonic monoterpenoid	1.97
7	Monoterpenoid lactone	5.38
8	Spiroalkane	29.14
9	Ketone	1.58
	Total identified groups (%)	100

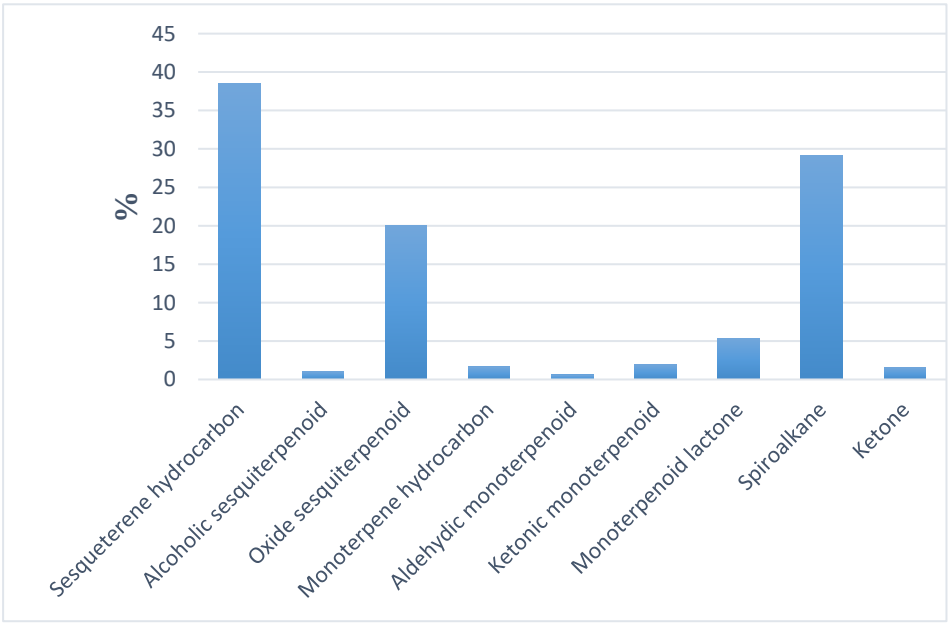


Fig. 3.2. Essential oil classes from *N. curviflora* aerial parts.

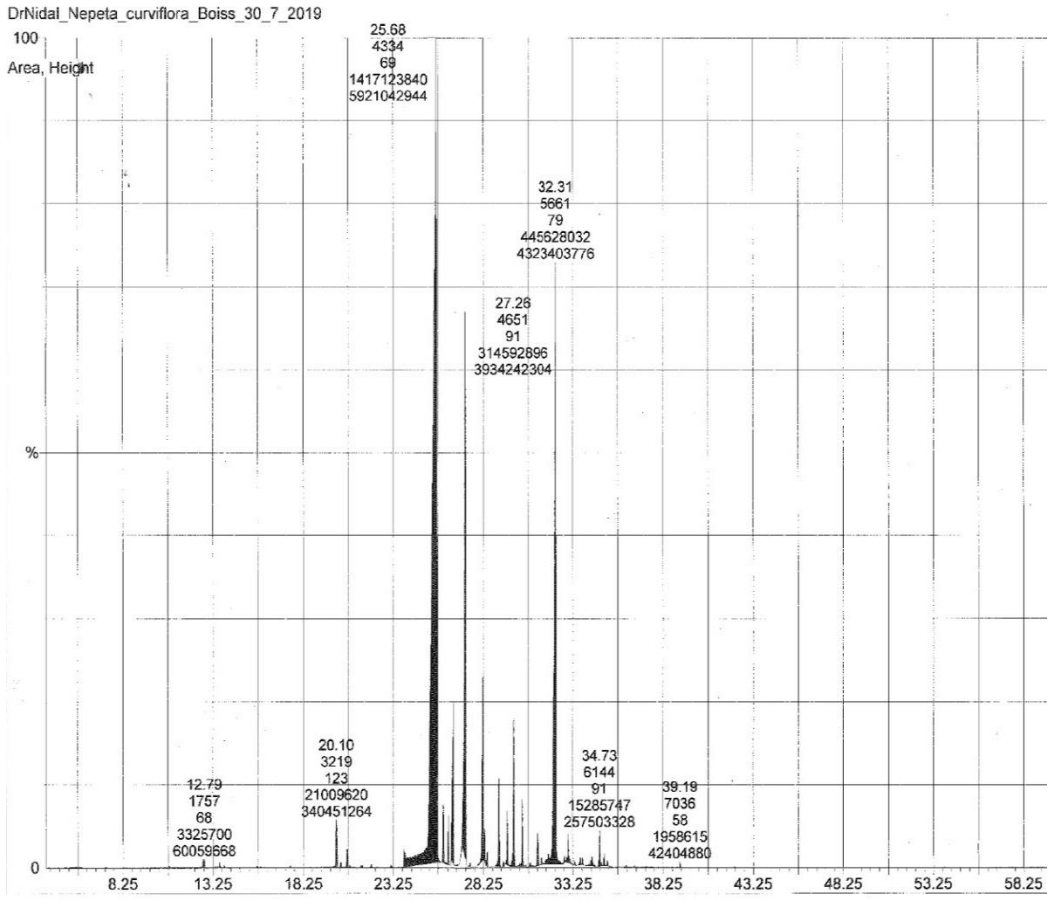


Fig. 3.3. *Nepeta curviflora* essential oil GC-MS chromatogram.

3.2 Antioxidant activity of *N. curviflora* EO

The DPPH assay was used as an *in vitro* approach to determine the free radical-scavenging activity of *N. curviflora* EO and to screen for potential antioxidant activity. IC_{50} values were used to assess the ability of the examined samples to inhibit DPPH. They identify the amount of antioxidant required to inhibit the radical (DPPH) concentration by 50% and they are inversely linked to their antioxidant activities. The radical scavenging activity of *N. curviflora* EO and trolox was articulated as a percentage of DPPH inhibition. The DPPH radical scavenging activity of the EO was determined as 6.3 ± 0.43 μg/mL, two-fold weaker than that displayed by trolox (IC_{50} = 3.1 ± 0.92 μg/mL), as indicated in Table 3.2 and Fig. 3.4.

Table 3.2. The inhibitory activity of *Nepeta curviflora* essential oil and trolox against DPPH. Values represent the mean ± SD (n = 3/group). IC_{50}=half maximal inhibitory concentration, DPPH = 1,1-diphenyl-2-picrylhydrazyl.

Concentrations (µg/mL)	Trolox	*N. curviflora* essential oil
0	0 ± 0.00	0 ± 0.00
2	53.41 ± 2.26	52.24 ± 0.00
5	61.52 ± 0.33	52.24 ± 0.00
10	91.42 ± 0.33	53.46 ± 1.62
20	97.28 ± 0.33	77.02 ± 0.31
50	97.28 ± 0.33	77.02 ± 0.33
100	97.55 ± 0.68	81.58 ± 0.3
IC_{50}	3.1 ± 0.92	6.3 ± 0.43

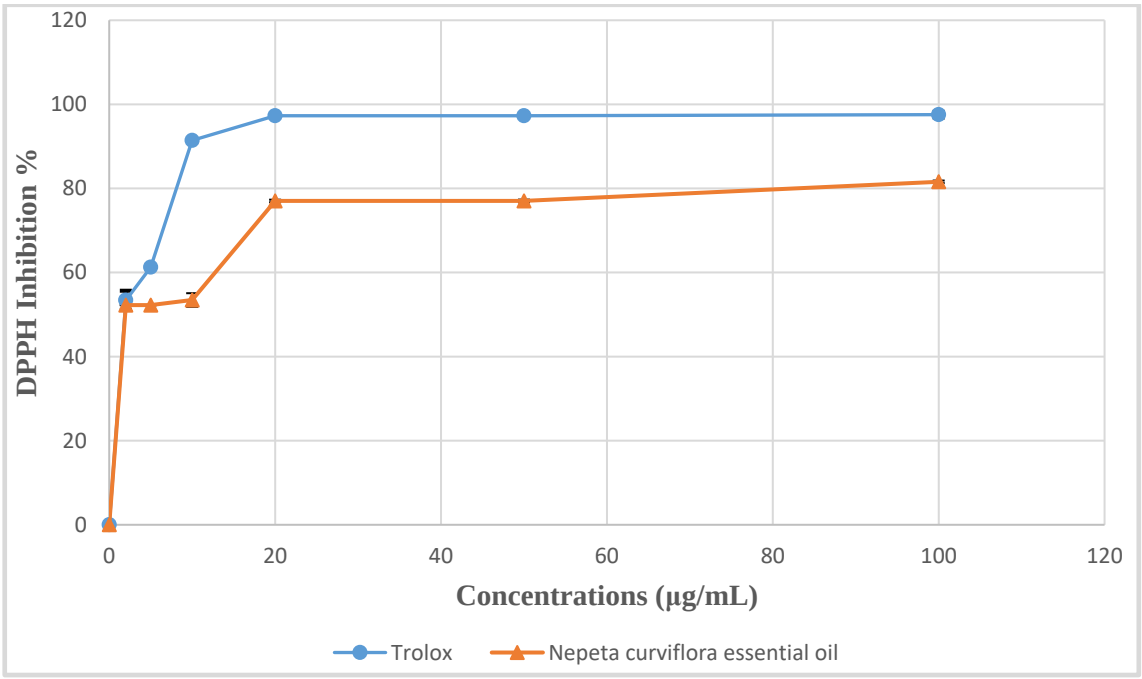


Fig. 3.4. Inhibition% of DPPH (1,1-diphenyl-2-picrylhydrazyl) by trolox and *Nepeta curviflora* essential oil.

3.3 Porcine pancreatic lipase inhibitory activity

In this assay, the anti-obesity activity of *N. curviflora* EO was compared with that of orlistat, a potent lipase inhibitory agent which is responsible for the digestion of dietary fat. The plant EO and orlistat exhibited anti-obesity activity with IC_{50} values of 26.3 ± 0.57 and 12.3 ± 0.33 µg/mL, respectively, as shown in Table 3.3 and Fig. 3.5.

Table 3.3. The inhibitory activity of *Nepeta curviflora* essential oil and orlistat against porcine pancreatic lipase.Values represent the mean ± SD (n = 3/group). IC_{50}=half maximal inhibitory concentration

Concentrations (µg/mL)	Orlistat	*N. curviflora* essential oil
0	0 ± 0.00	0 ± 0.00
50	91.05 ± 0.77	64.52 ± 0.47
100	93.1 ± 0.42	64.69 ± 0.72
200	94.3 ± 0.42	64.69 ± 0.72
300	97.4 ± 0.12	97.96 ± 0.47
400	97.5 ± 0.00	98.95 ± 0.49
IC_{50}	12.3 ± 0.33	26.3 ± 0.57

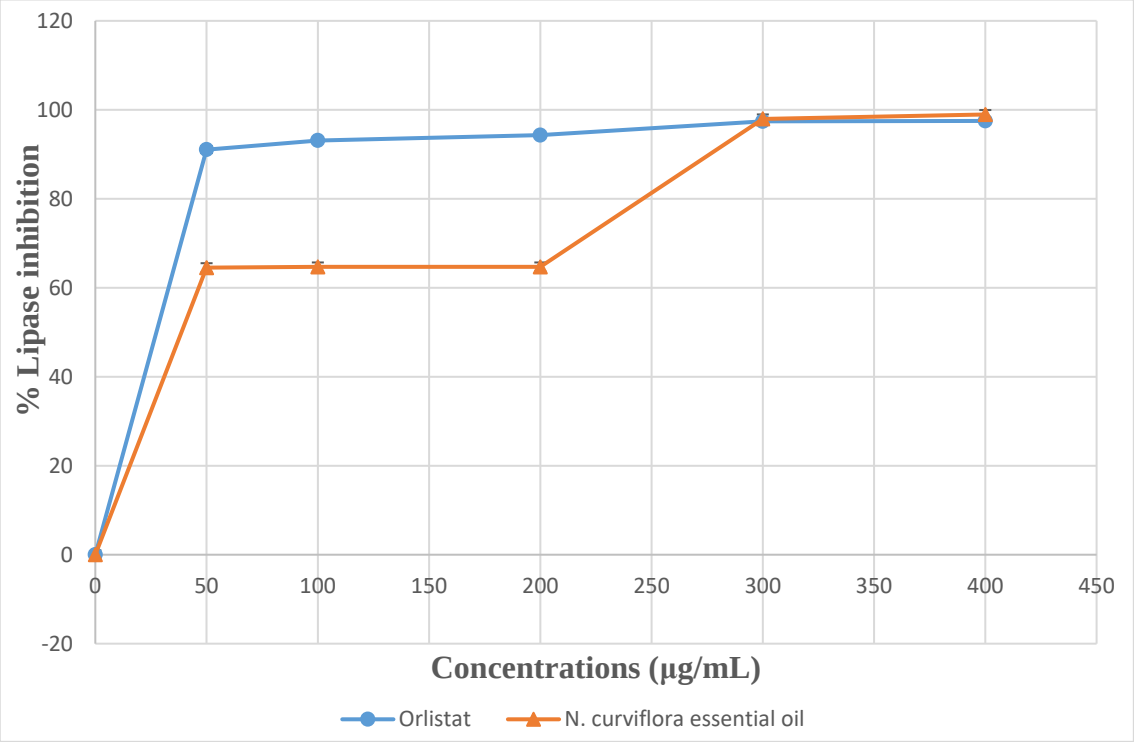


Fig. 3.5. Inhibition percentage of lipase by *Nepeta curviflora* essential oil and orlistat**.**

3.4 *α*-Amylase activity

The lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate to the chromophore (*p*-nitrophenol) assay was used to determine the inhibitory activity of *N. curviflora* EO on the porcine pancreatic α-amylase enzyme. The assay was compared with acarbose (positive control), a potent *α*-amylase inhibitory agent, and the IC_{50} values were calculated for *N. curviflora* EO and acarbose (Table 3.4 and Fig. 3.6). The IC_{50} of the EO and the positive control were measured as 45.7 ± 0.26 and 28.84 ± 1.22 µg/ mL, respectively.

Table 3.4. α-Amylase inhibition of Nepeta curviflora essential oil compared with acarbose.Values represent the mean ± SD (n = 3/group). IC50=half maximal inhibitory concentration.

Concentrations (µg/mL)	Acarbose	*N. curviflora* essential oil
0	0 ± 0.00	0 ± 0.00
10	53.22 ± 1.2	20.3 ± 0.007
50	54.91 ± 0.58	63.53 ± 0.21
70	66.1 ± 1.34	63.53 ± 0.21
100	66.1 ± 1.62	65.21 ± 0.41
500	72.54 ± 1.37	65.8 ± 0.45
IC_{50}	28.84 ± 1.22	45.7 ± 0.26

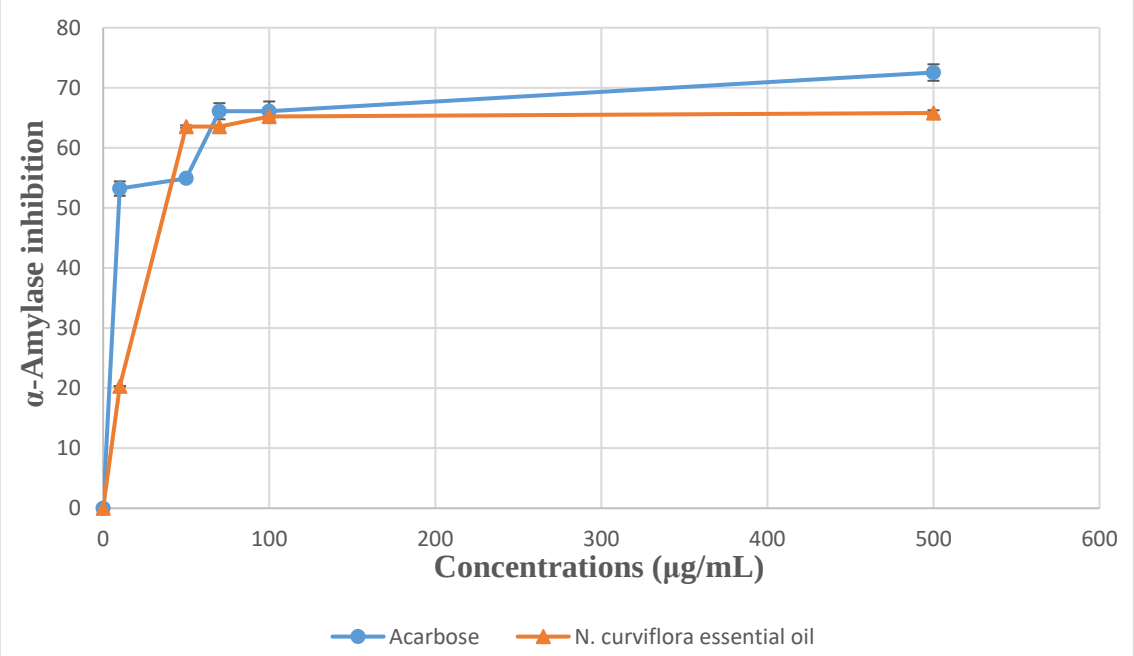


Fig. 3.6. *α*-Amylase inhibition of *Nepeta curviflora* essential oil compared with acarbose.

3.5 *α*-Glucosidase inhibitory activity

Results for *α*-glucosidase were compared with those for acarbose, a strong *α*-glucosidase enzyme inhibitory drug, and the IC_{50} doses were calculated for *N. curviflora* EO (Table 3.5 and Fig. 3.7). The IC_{50} values for the EO and the

positive control were found to be 54.9 ± 0.34 and 37.15 ± 0.32 µg/mL, respectively.

Table 3.5. *α*-Glucosidase inhibition of *Nepeta curviflora* essential oil compared with acarbose. Values represent the mean ± SD (n = 3/group). IC_{50}=half maximal inhibitory concentration.

Concentrations (µg/mL)	Acarbose	*N. curviflora* essential oil
0	0 ± 0.00	0 ± 0.00
100	65.81 ± 0.46	38.33 ± 0.73
200	67.76 ± 0.34	55.51 ± 0.22
300	73.34 ± 0.45	81.76 ± 0.11
400	85.39 ± 0.36	81.76 ± 0.01
500	92.28 ± 0.11	92.72 ± 0.72
IC_{50}	37.15 ± 0.32	54.9 ± 0.34

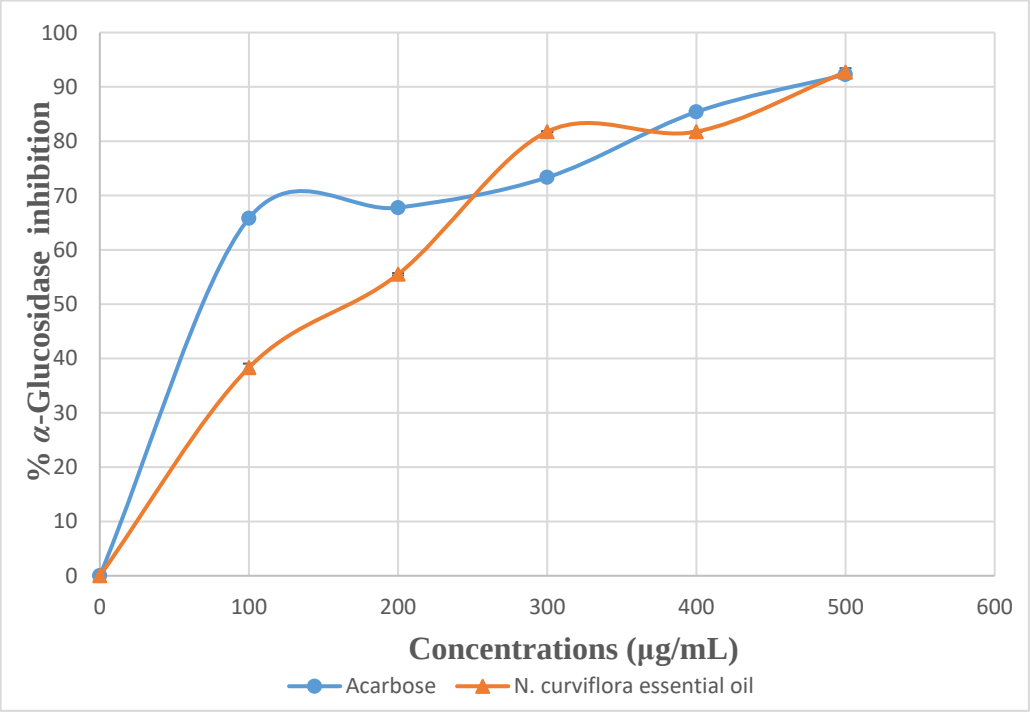


Fig. 3.7. *α*-Glucosidase inhibition of *Nepeta curviflora* essential oil and acarbose.

3.6 Phytochemical content of *N. curviflora* stem extracts

The isolation procedure was carried out using silica gel chromatography. Three extracts were obtained. Out of the three extracts, a crude extract was labeled as A, and the other two were labeled as B and C. The purity of the obtained compounds was checked by TLC plates and HPLC analysis. There was one concern raised in this study about the HPLC findings (fig.a.3 – fig.a.9, Appendix), since there were problems with calibration that were detected late in the process. A calibration curve should have been generated for each analyte to assay samples in each analytical run, but this was not done for each run. As a result, the data quality has declined, and the HPLC data in this study has been overlooked. Another limitation of this study is the lack of NMR (Nuclear magnetic resonance) techniques at An Najah National University, along with the restrictions due to COVID-19. Because of this, we were not able to send the compounds abroad to run ^{1}H- and C-NMR, which will enable us to identify the purified compounds.

IR analysis of the compound B (fig.a.1, Appendix) has shown that it has a hydroxyl (OH) group (3395 cm^{-1}) indicated by the broadband in this zone, an aromatic ring (2931 cm^{-1}), a carbonyl group (1735 cm^{-1}), the peak at 1707 cm^{-1} corresponds to C=O stretching of a ketone, and the peak at 1616 cm^{-1} corresponds to C=C stretching of an aromatic ring.

On the other hand, IR analysis of compound C (fig.a.2, Appendix) is not informative. It is aromatic without carbonyl groups.

3.7 Anti-oxidant inhibitory activity of *N. curviflora* extracts

Antioxidant activities of the obtained extracts of *N. curviflora*'s stems were investigated using the DPPH assay as an *in vitro* approach and were expressed as DPPH % inhibition activity and IC_{50} values. Table 3.6 and figure 3.8 present the DPPH inhibitory activity and the IC_{50} values of extract A, compounds B, and C, and Trolox. The higher % inhibition and lower IC_{50} values reflect better anti-oxidant action of the extracts. Extract B is the most potent, with an IC_{50} = 43.365 µg/mL.

Table 3.6: IC_{50} (µg/ml) values for different extracts. Values represent the mean ± SD (n = 3/group). IC_{50}=half maximal inhibitory concentration.

	Trolox	A	B	C
IC_{50} (µg/mL) ± Standard deviation	2.7 ± 0.095	78.53 ±1.09	43.365 ± 0.165	171.48 ± 1.57

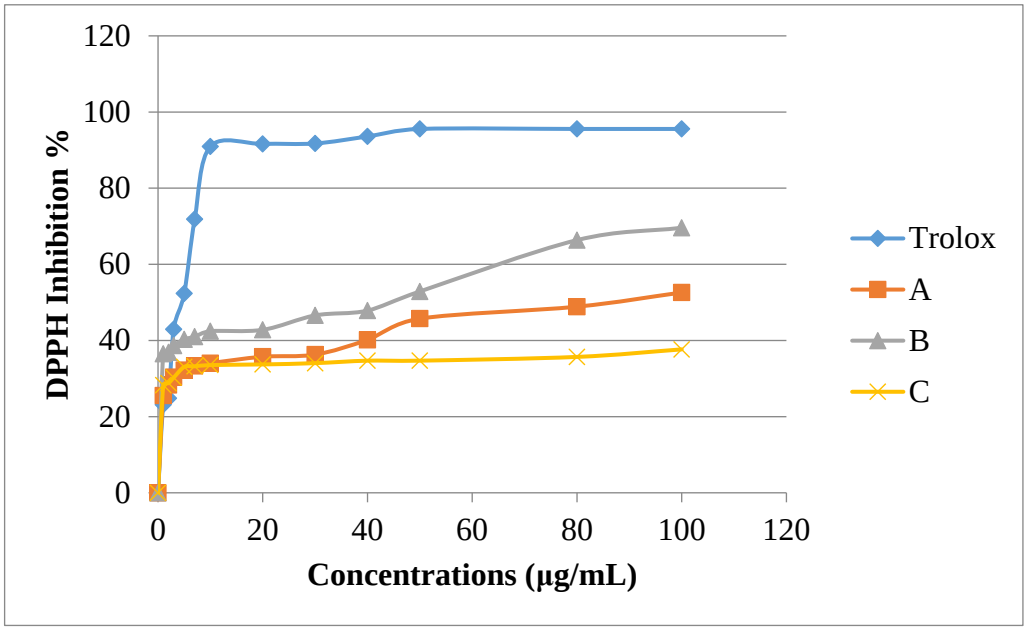


Fig. 3.8 Anti-oxidant % Inhibition concentration (µg/ml) of different extracts from N. curviflora stem extracts**.**

3.8 Anti-microbial activity

The micro-dilution assay was used to evaluate the antimicrobial activity of A, B, and C extracts against six bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *klebsiella pneumonia*, *staphylococcus aureus,* and MRSA, in addition to the yeast *C. albicans*. The lower MIC value indicates the higher antibacterial activity of the extracts on the tested bacterial strains. These results (Table 3.7) indicated that extract B had a moderate inhibitory effect against MRSA and *S.aureus.* On the other hand, both A and C extracts showed a weak or negligible inhibitory effect against all the microorganisms tested. The findings also revealed that none of the extracts inhibited *C. albicans* growth.

Table 3.7. Minimum inhibitory concentration values (µg/ml) for different *N. curviflora* extracts against selected pathogens (bacteria and fungi). R = Resistant

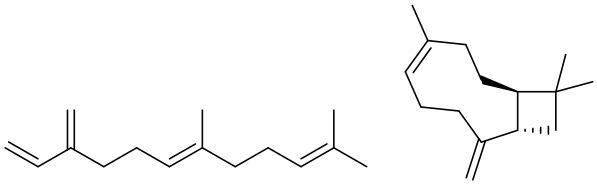
	Bacteria						**Fungus**
ATCC Number	**Clinical strain**	**ATCC 25923**	**ATCC 25922**	**ATCC 13883**	**ATCC 8427**	**ATCC 9027**	**ATCC 90028**
Microbe /extract MIC (µg/ml)	**MRSA**	***S.aureus***	***E. coli***	***Klebsiella pneumonia***	***Proteus vulgaris***	***Pseudomonas aeruginosa***	***Candida albicans***
A	1250	1250	R	R	1250	1250	R
B	312.5	156.3	1250	1250	1250	R	R
C	R	1250	R	R	R	R	R

Chapter Four

Discussion and Conclusions

4.1 The chemical composition of *N. curviflora* EO

Twenty compounds were identified, accounting for 100% of the total EO of *N .currvifloa*. The most abundant components of the EO were found to be: 1,6-dimethyl spiro[4.5]decane **55** (27.51%), caryophyllene oxide **56** (20.08%), and *β*-caryophyllene **38** (18.28%). Sesquiterpenes were discovered to be the main group of the eight groups of identified compounds, accounting for 38.49% of the total EO composition. The main major compounds in this group are *β*-caryophyllene **38** (18.28%), *β*-farnesene **58** (6.2%), and *γ*-caryophyllene **59** (4.95%). Another major sesquiterpene group is the oxygenated sesquiterpene group, which is represented by only caryophyllene oxide **56**, which accounts for 20.08% of total EO. The spiroalkane group is the second most prevalent group of compounds, accounting for 29.14% of the total EO, with 1,6-dimethylspiro[4.5] decane **55** (27.51%) being the most abundant. Hydrocarbon monoterpenes and oxygenated monoterpenoids (aldehyde, ketone, and lactone) made up 9.62% of the total oil, of which nepetalactone was the major, which accounted for 5.38% of the total EO.



58 59

As stated previously, even the same plant species can have a different composition of EO, depending on different aspects such as climate, age, vegetative cycle stage, plant organ, location, and extraction technique [127-129]. Thus, comparing the EO composition of *N. curviflora* from the current study with the findings from the previous studies from the Lebanese and Jordanian mountains shows quantitative and qualitative variations between them. These differences are shown in table 4.1.

Table 4.1 Chemical compositions of *Nepeta curviflora* essential oil growing in Lebanon, Jordan, and Palestine.

Location	The used parts	Technique	EOs chemical compositions	Reference
Lebanon	Aerial parts included flowering tops, seeds, and leaves	Hydro-distillation for 3.5 h	2-Isopropyl-5-methyl3-cyclohexen-1-one, (-)-spathulenol, cis-Zα-bisabolene epoxide, widdrol, (E,Z)-5,7-dodecadiene, dihydronepetalactone and 4-propyl-cyclohexene with 12.51, 11.73, 8.07, 7.0, 6.93, 5.57 and 5.43%, respectively.	[188]
Lebanon	Aerial parts included stems, leaves, and flowers	Hydro-distillation for 3 h	β-Caryophyllene, caryophyllene oxide, (E)-β-farnesene and (Z)-β-farnesene (41.6, 9.5, 6.2 and 4.8%, respectively)	[190]
Lebanon	Aerial parts included stems, leaves, and flowers	Hydro-distillation for 3 h	β-Caryophyllene, caryophyllene oxide, and (E)-β-farnesene (50.2, 6.4, and 5.3%, respectively).	[191]

Jordan	Aerial parts included stems, leaves, and flowers	Hydro-distillation for 3 h	The EO isolated from the fresh aerial parts consisted mainly of hydrocarbon sesquiterpenes (55.27%). In addition, the dried aerial parts were mainly consisting of oxygenated monoterpene (50.31%). However, the main ingredients characterized in the EOs of dried and fresh plant aerial parts were 4aα,7α,7aαNepetalactone (43.8, 17.7%, respectively), E-caryophyllene (11.5, 16.3%, respectively) and γ-murolene (10.4 and 18.54%, respectively).	[189]
Palestine	Arial parts included leaves and flowers	Mild microwave ultrasonic apparatus	The major compounds are 1,6-Dimethyl spiro[4.5]decane (27.5%), caryophyllene oxide (20.08%), and β-caryophyllene (18.28%)	

The variations between the four studies from Lebanon, Jordan and the current investigation could be partially related to the extraction techniques used. The current study used the mild microwave ultrasonic apparatus for the extraction of the EO, whereas the previously conducted studies used the hydro-distillation technique that requires reflux for a long time ($\geq$ 3 h), which can affect the chemical composition of the EO. Moreover, the parts used, location, and collection period greatly affect the qualitative and quantitative plant chemical composition. Leaves and flowers of *N. curviflora* were used in the current study, while the previously conducted studies used either the leaves, flowers, and stems or the flowering tops, leaves, and seeds. As for the location and the collection period, the *N. curviflora* used in prior experiments was harvested in April, when the seeds were still developing.

Whereas the plant in this study was gathered on May 22nd, when the seeds were completely developed.

4.2 Antioxidant activity of *N. curviflora* EO

The current investigation showed that *N. curviflora* EO had strong antioxidant properties with an IC_{50} value of 6.3 ± 0.43 μg/mL, which is half of that of the standard antioxidant compound trolox (IC_{50} = 3.1 ± 0.92 μg/mL). Al-Qudah has reported that the *N. curviflora* EO from Jordan exhibited significant antioxidant potential with an IC_{50} dose of 0.30 ± 7.07 μg/mL compared with that of ascorbic acid ($IC_{50} = 7.22 \times 10^{-5} \pm 2.30 \times 10^{-6}$ μg/mL) [208]. This significant antioxidant activity may be correlated to the high occurrence of antioxidative compounds in the EO, such as caryophyllene, caryophyllene oxide **56**, nepetalactone, and *β*-farnesene **58** [205,206].

4.3 Porcine pancreatic lipase inhibitory activity

Obesity has recently been identified as a major global issue. It is a long-term metabolic disorder that is associated with many chronic diseases such as hypercholesteremia, hypertension, pulmonary hypertension, sleep apnea, type 2 diabetes, and cardiovascular disease. One of the methods to treat obesity is by inhibiting the absorption of dietary fat using many drugs. Orlistat is an anti-obesity medication that contains lipase inhibitors, which are responsible for lowering fat levels by reducing dietary fat absorption [207,208]. The EO of *N. curviflora* has a strong antilipase inhibition activity,

with an IC_{50} value of 26.3 ±0.57 g/mL when the enzyme activity was measured using PNPB as a substrate. The obtained IC_{50} values were compared with those of orlistat (IC_{50} = 12.3 ± 0.33 μg/mL). This result ties in well with previous studies on other *Nepeta* species, Roh and Jung reported that the IC_{50} of porcine pancreatic lipase was 37.3 ± 2.5% for a hydroethanolic extract of *Nepeta japonica* [209]. Also, Salameh et al. reported that the EO of *Nepeta serpyllifolia* (*Micromeria fruticosa serpyllifolia*) displayed antilipase activity with an IC_{50} value of 39.81 μg/mL when compared with that of orlistat (IC_{50} = 43.64 μg/mL) [210].

4.4 *α*-Amylase and *α*-glucosidase inhibitory activities

One of the most common chronic diseases in the world is diabetes mellitus. It is characterized by hyperglycemia, which can harm a variety of body parts, such as the eyes, nerves, kidneys, and blood vessels [211]. One of the most effective ways to manage diabetes is to reduce postprandial hyperglycemia (high blood glucose levels after a meal) by inhibiting the carbohydrate hydrolyzing enzymes, especially *α*-glucosidase and *α*-amylase. Acarbose is a synthetic pharmacological inhibitor that is effective against the postprandial hyperglycemia. However, this medication has many undesirable gastrointestinal side effects, like bloating and diarrhea [211]. Thus, research for natural alternatives that are both cost-effective and have fewer side effects is being conducted by many researchers.

The EO of *N. curviflora*, isolated from leaves and flowers, showed strong inhibition activity against porcine pancreatic *α*-amylase enzyme with an IC_{50}

value of 45.7 ± 0.26 μg/mL while acarbose had an IC_{50} value of 28.84 ± 1.22 μg/mL. Zarrabi et al. found that *N. racemosa* aqueous decoction inhabited *α*-amylase and *α*-glucosidase with an IC_{50} value of 9.45, and 33.57 μmol of acarbose equivalent per gram of dried extract, respectively [212].

The activity of *N. curviflora* EO as an inhibitor of *α* -glucosidase was also investigated. The EO had high inhibitory activity against the *α* -glucosidase enzyme, with an IC_{50} of 54.9 0.34 μg/mL, while acarbose had an IC_{50} of 28.84 1.22 μg/mL. Sarikurkcua et al. has reported that the EOs of *N. cadmea* and *N. nuda* subsp. *glandulifera* showed a strong *α*-amylase inhibitory activity with IC_{50} values of 1.35 ± 0.02 and 1.15 ± 0.05 mg/ mL, respectively, which is very close to that of acarbose (positive control, IC_{50} = 1.33 ± 0.01 mg/mL). Furthermore, the same study reported that these EOs have also strong inhibition actions against *α*-glucosidase with IC_{50} values of 5.93 ± 0.18 and 0.99 ± 0.01 mg/mL, respectively, whereas that of acarbose was 0.99 ± 0.01 mg/mL [213]. To the best of our knowledge, the *α*-amylase and *α*-glucosidase inhibitory actions of *N. curviflora* EO have never been reported in the literature.

4.5 The chemical composition of *N. curviflora* stem extracts

Three extracts of the stem of *N. curviflora* were obtained in this investigation. One of these extracts is the crude extract (A). The other extracts were labeled as (B) and (C).

4.6 The antioxidant activity of the stem extracts

The antioxidant activity of the three extracts from the stem of *N. curviflora* was measured using the DPPH assay. The results showed that among the three extracts, extract B showed the most significant antioxidant activity with an IC_{50} of 43.365 µg/mL, less than that of the positive control (Trolox), which has an IC_{50} value of 2.70 µg/mL. A previous study on the methanol and aqueous stem extracts of *Nepeta nepetella* by Seladji M. et al. revealed that the methanol stem extracts exhibited significant antioxidant activity with an IC_{50} value of 7.37±0.17 mg/ml, which is nearly threefold higher than the aqueous stem extracts, which had an IC_{50} of 20.16±0.23 mg/ml [214].

4.7 The antimicrobial activity of the stem extracts

The antimicrobial activity of the three extracts from the stem of *N. curviflora* was assessed using broth microdilution assay against four gram-negative bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *klebsiella pneumonia*, and two gram-positive bacteria, including *Staphylococcus aureus*, and MRSA, also against the yeast *C. albicans*. The results revealed that, out of the three extracts, extract B had the best inhibitory effect against almost all the screened bacterial strains, especially against *S. aureus*, with a moderate MIC value of 156.3 µg/ml. However, all three extracts didn't have any inhibition activity against the fungal strain *C. albicans*. A previous study on the methanolic extracts of *N. juncea* by Sharifi et al revealed significant antibacterial activity against all tested bacterial strains with MIC values ranging from 25 to 100 µg/mL, with *S. aureus* being

the most sensitive bacteria with MIC values of 25, 25, and 50 µg/mL for the methanolic extracts of the leaves, flowers, and roots, respectively [215]. Contrary to our results, *N. juncea* methanolic extracts have also shown antifungal activity against *C. albicans* with MIC values of 25, 50, and 50 µg/mL for the methanolic extracts of the leaves, flowers, and roots, respectively [215].

4.8 Conclusions

In conclusion, the current study investigated the chemical composition of the EO from the leaves and flowers of *N. curviflora*, in which the presence of twenty phytochemicals was identified, with 1,6-dimethyl spiro[4.5]decane 55, caryophyllene oxide 56, and *β*-caryophyllene 38 being the major compounds. Overall, the EO of *N. curviflora* displayed potential antioxidant, antilipase, *α*-amylase, and *α*-glucosidase enzymes inhibition activities compared with the positive controls (trolox, orlistat, and acarbose, respectively). In addition, three methanol extracts from the stem of *N. curviflora* showed DPPH scavenging activity in the following order: B > A > C. Extract B has shown a moderate antibacterial effect against *S. aureus* with a MIC value of 156.3 µg/ml. However, none of the extracts were found to have antifungal activity against the yeast *C. albicans*. These findings provide the potential use of *N. curviflora* as a natural source of antioxidant, anti-obesity, and antidiabetic therapeutic agents, boosting the species' production and consumption.

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Appendix

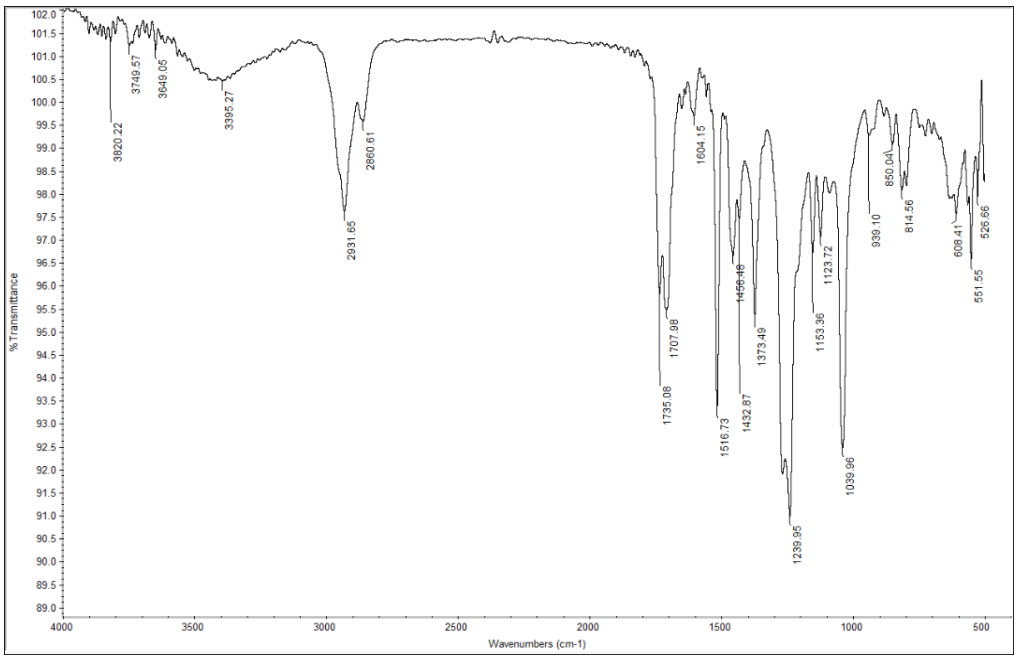

Fig.a.1. IR spectra for compound B

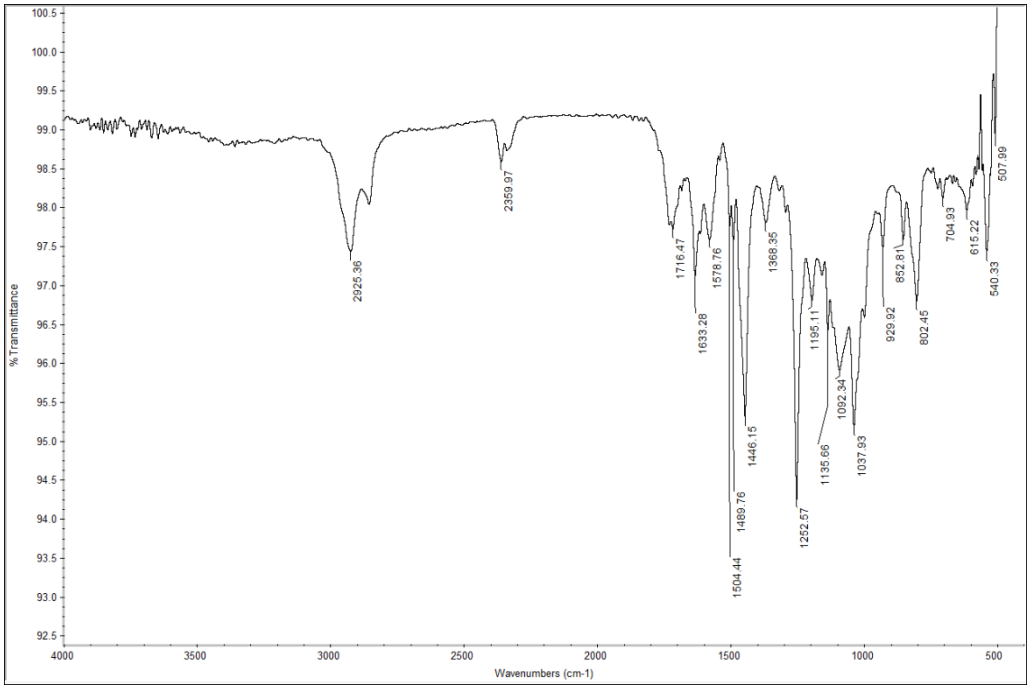

Fig.a.2. IR spectra for compound C

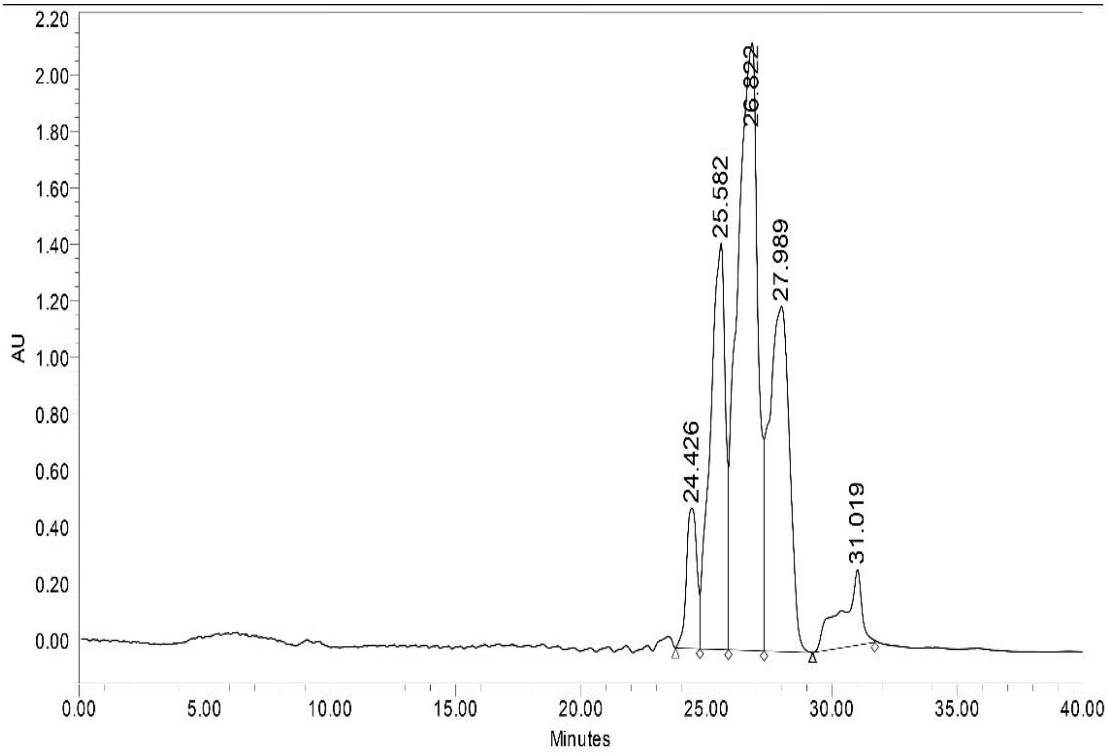

Fig.a.3. HPLC analysis for F1: The first fraction from the first column.

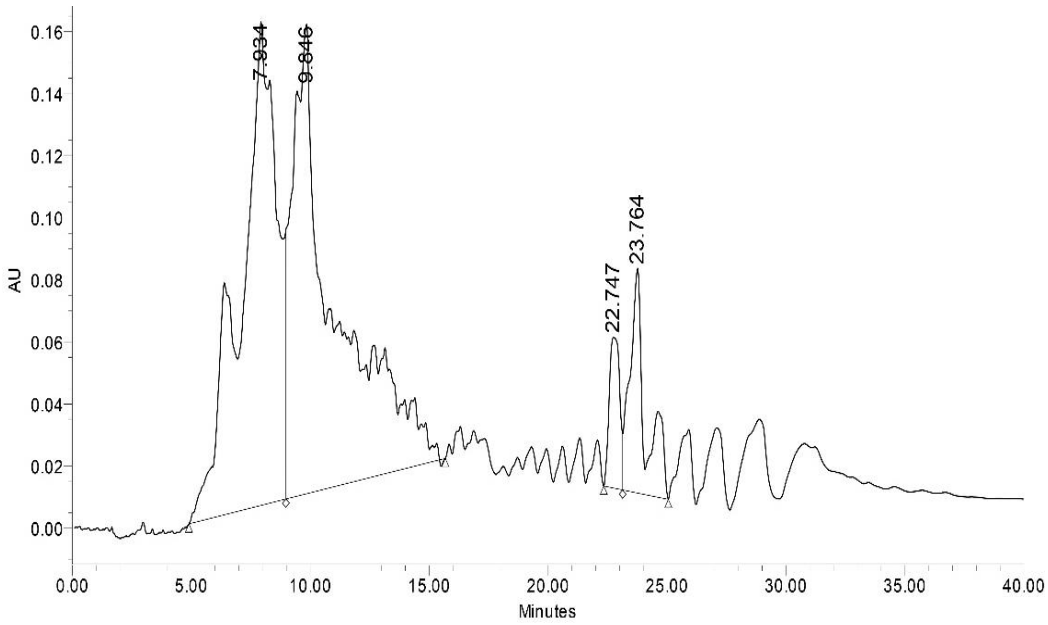

Fig.a.4. HPLC analysis for F2: The second fraction from the first column.

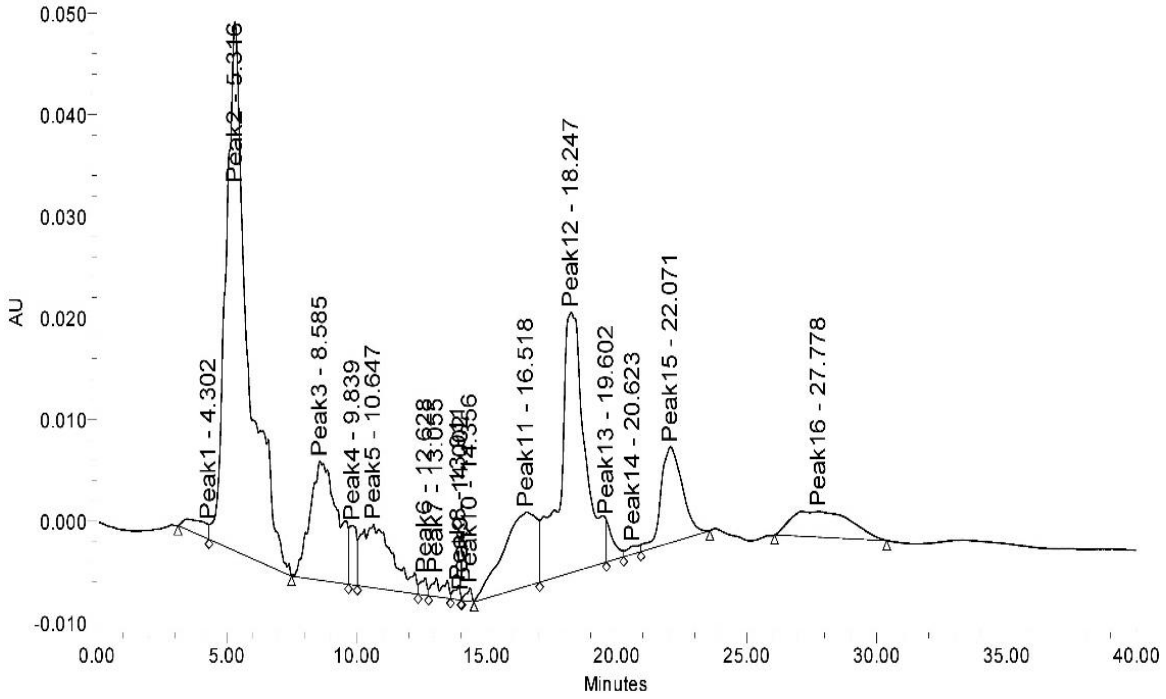

Fig.a.5. HPLC analysis for F3: The third fraction from the first column.

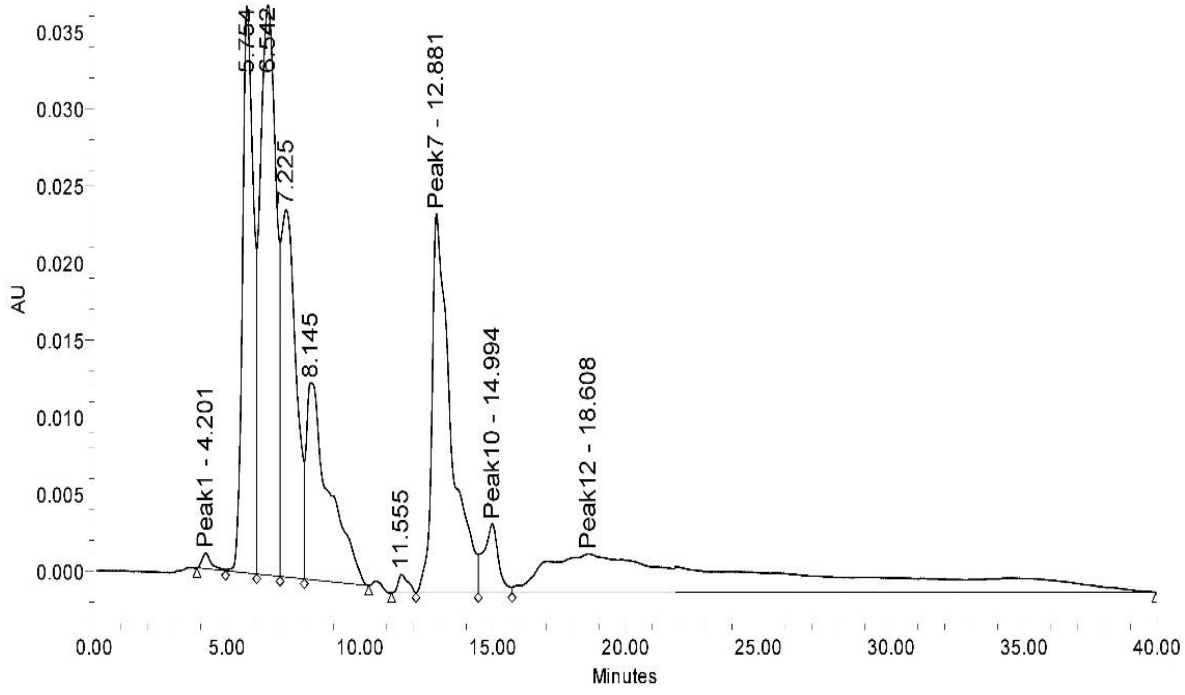

Fig.a.6. HPLC analysis for F4: The fourth fraction from the first column.

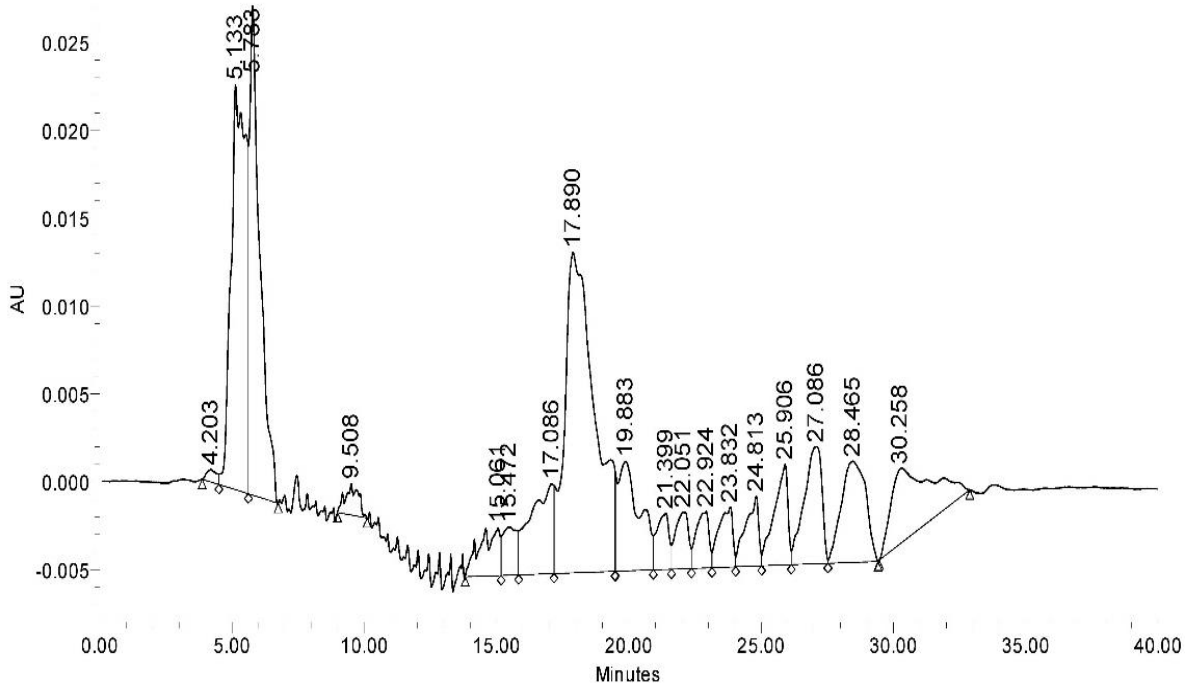

Fig.a.7. HPLC analysis for F5: The fifth fraction from the first column.

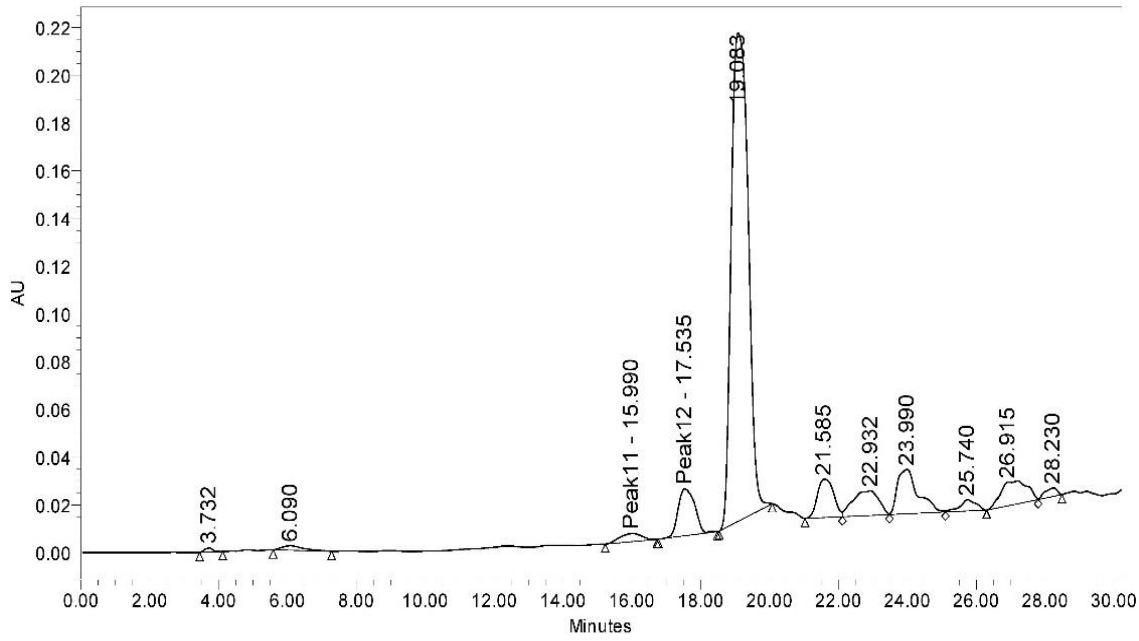

Fig.a.8. HPLC analysis for compound B.

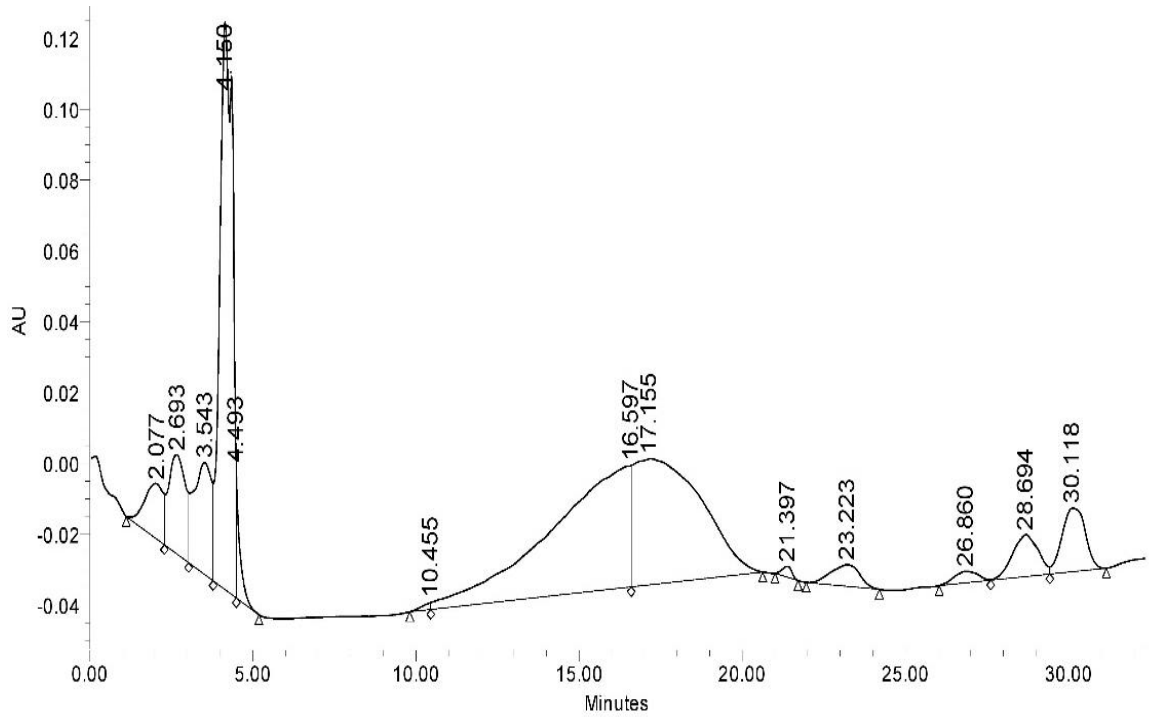

Fig.a.9. HPLC analysis for compound C.

جامعة النّجاح الوطنية

كليّة الدّراسات العليا

الكشف عن الزيوت الطيارة الموجودة في نبتة نعناع القطط وتقييم فعاليتها الحيوية

إعداد

عبير يوسف محمد القطيشات

إشراف

د. نواف المحاريق

د. نضال جرادات

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

2021

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الملخص

منذ آلاف السنين، تم استخدام الأعشاب والنباتات الطبية في علاج أنواع مختلفة من الأمراض ولتحسين الرعاية الصحية للأفراد. أثبتت العديد من الدراسات أن الزيوت الطيارة لها فوائد مختلفة خاصة في مجال الطب والصناعات ومستحضرات التجميل. نبتة نعناع القطط تنمو بشكل طبيعي في الأجزاء الشرقية من منطقة البحر الأبيض المتوسط ، بما في ذلك فلسطين. لها رائحة النعناع اللطيفة وقد استخدمت في الطب الشعبي لعلاج العديد من الأمراض. صممت هذه الدراسة للتحقيق في التركيب الكيميائي للزيت الطيار من أوراق وأزهار نبتة نعناع القطط ومستخلصات جذوعها، بالاضافة الى الكشف عن خصائصها الدوائية.

تم استخراج الزيوت الطيارة لنبتة نعناع القطط باستخدام جهاز(Microwave ultrasonic)، وتم تحليل المكونات الكيميائية باستخدام جهاز (GC-MS)، وتم اختبار فاعلية الزيوت الطيارة والمستخلصات من جذوع هذه النبتة كمضادات للأكسدة من خلال تثبيط الشقائق الحرة لمركب (DPPH). تم فحص فاعلية الزيت الطيار كمثبط ضد انزيم (الليباز) باستخدام (PNPB) ، ضد انزيم الأميليز باستخدام (DNSA) ، وضد انزيم (الغلوكوسيداز) باستخدام (PNPG). تم عزل ثلاثة مستخلصات من جذوع هذه النبتة باستخدام تقنية العمود الكروماتوغرافي ، وتم التحقق من نقاوة المركبات باستخدام تحليل كروماتوغرافيا الطبقة الرقيقة. وتم الكشف عن المجموعات الوظيفية في المستخلصات باستخدام تقنية مطيافية الأشعة تحت الحمراء. ولفحص فعالية المستخلصات من جذوع هذه النبتة المضادة للميكروبات فقد تم استخدام طريقة (broth microdiltution) بشكل منفصل كمضاد للبكتيريا وايضا كمضاد للفطر.

تم تحديد عشرين مركبًا من الزيت الطيار لنبتة نعناع القطط السوري ، بحيث كانت المكونات الاساسية لهذا الزيت هي: 1,6-dimethyl spiro[4.5]decane **55** (27.51%), caryophyllene oxide **56** (20.08%), *β*-caryophyllene **38** (18.28%) .

أظهرت النتائج ان فعالية الزيت كمضاد للأكسدة كانت بتركيز IC_{50} = 6.3 ± 0.43 µg/mL . وفعالية هذا الزيت كمثبط لانزيمات الليباز، الأميليز، و الغلوكوسيدازكانت بتراكيز IC_{50}= 26.3 µg/mL 0.34 ± 54.9, 0.26 ± 45.7, 0.57 ± على التوالي. علاوة على ذلك، تم عزل ثلاثة مستخلصات من جذوع هذه النبتة وسميت كالتالي (A ,B, C). اظهرت هذه المستخلصات فعاليتها كمضادات للأكسدة بتراكيز IC_{50} كالتالي : 171.48 ± 1.57, 43.365 ± 0.165, 78.53±1.09 µg/mL بالترتيب. بالاضافة الى فعاليتها كمضادات للميكروبات، بحيث أظهر المركب (B) أعلى نشاط بحد متوسط من التركيز(MIC) يساوي 156.3 µg/mL وذلك ضد بكتيريا *S. aureus*. لكن جميع هذه المستخلصات لم تظهر أي نشاط ضد سلالة فطريات *Candida albicans*.