An-Najah National University
Faculty of Graduate Studies

Chemical composition and pharmacological screening of *Micromeria fruticosa serpyllifolia* volatile oils collected from West Bank-Palestine

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Dedication

To my parents and sister spirits

To my sisters and brothers

To National University of Pharmacy *represented by* ***Rector;*** *Prof. Chernykh Valentyn Petrovych*

To Zeiad El Bargouthi spirit

To all my loyal friends

I dedicate this work

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الإقرار

أنا الموقعة أدناه، مقدمة الرسالة تحمل العنوان:

Chemical composition and pharmacological screening of *Micromeria fruticosa serpyllifolia* volatile oils collected from West Bank-Palestine

التركيب الكيميائي والفحص الدوائي للزيوت الطيارة لنبتة "زعتر البلاط"، التي تم جمعها من الضفه الغربيه- فلسطين

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، وأن هذه الرسالة كاملة، أو أي جزء منها لم يقدم من قبل لنيل أي درجة علمية أو لقب علمي أو بحث لدى أي مؤسسة تعليمية أو بحثية أخرى.

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 or qualification.

Student's name: **اسم الطالبة:**

Signature: **التوقيع:**

Date: **التاريخ:**

List of abbreviation

Symbol	Abbreviation
Amu	**Atomic mass unit**
ANNU	**An-Najah National University**
ATCC	**American Type Culture Collection**
BC	**Before Christ**
BHA	**Butylaed hydroxyanisole**
BHT	**Butylated hydroxytoluene**
CAM	**Complementary and Alternative Medicine**
CI	**Clinical Isolate**
CNS	**Central nervous system**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
DNSA	**3, 5-dinitrosalicylic acid**
DPN	**Diabetic peripheral neuropathy**
DPPH	**2, 2-diphenyl-1-picrylhydrazyl**
GC-MS	**Gas Chromatography/Mass Spectrometry**
HCT	**Human Colon Tumor cells**
I%	**Percentage inhibition**
IC_{50}	**Inhibition concentration of 50%**
M fruticosa	***Micromeria fruticosa***
MAC	**MacConKey Agar**
MAP	**Medicinal aromatic plants**
MCF7	**Mammary carcinoma F7**
MFH	***Micromeria fruticosa* Hebron**
MFN	***Micromeria fruticosa* Nablus**
MFR	***Micromeria fruticosa* Ramallah**
MHB	**Mueller Hinton Broth**
MIC	**Minimum Inhibitory Concentration**
MRSA	**Methicillin resistant *Staphylococcus aureus***

Symbol	Abbreviation
MSA	**Mannitol Salt Agar**
NA	**Nutrient Agar**
ND	**Diabetic nephropathy**
NIST	**The national institute of standards and technology**
NSAID	**Non-steroidal anti-inflammatory drug**
PDA	**Potato Dextrose Agar**
PL	**Pancreatic Lipase**
PNPB	***p*-nitrophenyl butyrate**
ROS	**Reactive Oxygen Species**
STDV	**Standard deviation**
S.S	**Stock solution**
SEB	**Staphylococcal enterotoxin B**
Subsp.	**Subspecies**
Syn.	**Synonym**
UV-Vis	**Ultraviolet visible**
VO	**Volatile oil**
W/W%	**Weight by weight%**
WB	**West Bank**
WHO	**World Health Organization**

Table of Contents

List of Tables

List of Figures

List of Equations

List of Schemes

Chemical composition and pharmacological screening of *Micromeria fruticosa serpyllifolia* volatile oils collected from West Bank-Palestine

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Abstract

Background and Objectives

Micromeria fruticosa subspecies *serpyllifolia* (M. Bieb.) is one of the Medicinal Aromatic Plants (MAP) which are dominated in the eastern Mediterranean regions including Palestine, has pleasant minty fragrance, in hot summer provide sensation of coolness. The objective of the current work was to screen and compare the chemical constituents and potential pharmacological properties of *Micromeria fruticosa serpyllifolia* volatile oils collected from three different regions in the West Bank -Palestine.

Methods

The volatile oils of three samples of *Micromeria fruticosa serpyllifolia* were extracted using Microwave - ultrasonic apparatus method. The volatile oils samples were analyzed for chemical constituents using GC-MS. The antioxidant activity of the volatile oils of the three samples were screened by the inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The antilipase activity was evaluated using porcine pancreatic lipase (PL) and *p*-nitrophenyl butyrate (PNPB). The anti amylase activity was assessed using porcine pancreatic α- amylase, starch

and 3,5-dinitrosalicylic (DNSA). The antimicrobial activity was examined using broth microdiltution method separate for antibacterial and then for antifungal and agar dilution method for fungal assessment. Nine bacterial strains were used four Gram-positive: *Staphylococcus aureus*, *Staphylococcal enterotoxin* B (SEB), *Enterococcus faecium*, "methicillin"-resistant *Staphylococcus aureus* (MRSA), and five Gram-negative strains; *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnie*, *Klebsilla pneumoniae* and one fungus *Epidermophyton floccosum* and one yeast *Candida albicans.*

Results

Plant extracts yield range was (0.67 to 0.99%) (w/w%). GC-MS analysis showed the high percentages of oxygenetated components with the range of (86.1-89.88%), non oxygenated components in the range of (4.38-4.71%), the total identified compounds range was (90.48-94.44%). Seven components were observed, pulegone was the most abundant components in the three samples in the range of (74.43-86.04%), isomenthone was the second abundant components with the range of (3.16-14.41%).The sample from Ramallah (middle region) showed the potent antioxidant agent with IC_{50} 0.45 µg/mL, the sample from Hebron (southern region) was the potent antilipase agent with IC_{50} 85.00 µg/mL. The sample from Nablus (northern region) was the potent antiamylase agent with IC_{50} 3.00 µg/mL. The three samples exhibited broad antimicrobial activity; the three samples showed potent antifungal activity at minimum inhibitory concentration (MIC) with

the range of (0.206 to 0.781 mg/mL). The sample from Hebron (southern region) showed the highest potency against *Shigella sonnie* with lowest reported MIC value (1.56 mg/mL), the sample of Nablus (northern region) demonstrated the least potency against *Staphylococcal enterotoxin* B (SEB) and 'methicillin" resistance *Staphylococcus Aureus* (MRSA) with highest MIC value (6.250 mg/mL). However, the three samples showed broadspectrum antibactreial activity with MIC value (3.125 mg/mL).

Conclusion

The study showed that *Micromeria fruticosa serpyllifolia* volatile oils samples from different regions in Palestine contained different proportions of phytochemicals which provided different potential biological activities such as: antioxidant, antiobesity, antidiabetes and antimicrobial activities that were in line with traditional uses of the plant extracts. The plant extracts showed higher antioxidant, antilipase and antiamylase potency higher than that of the relative references and there were significant differences in these activities compared to each other. Further *in vivo* studies are required to evaluate the potential pharmacological activities, safety and toxicity of plant extract. Also further studies are needed to isolate, identify and characterize the main components responsible for potential pharmacological activities.

Chapter One

Introduction

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Introduction

1.1 History of Medicinal Plants

Plants have been always used as an important source of food, cosmetics, medicine and widely used as traditional medicine or in drug development of pure active ingredients [1, 2]. The first use of traditional plants for medicinal purposes was as old as 4000-5000 Before Christ (BC) by Chinese, later between 1600-3500 BC by Indian peoples [3]. Medicinal plants are considered to be the principal source of treatment in Chinese traditional medicine (TCM) and are reported to be one fifth of the overall pharmaceutical market in China [4]. Plants are represented by over than 85% of Chinese materia medica [5].

In India medicinal plants have been used by Ayurveda the primarily medical system practiced in India which believed that everything can be a medicine. Recently around 70% of Indians depend on medicinal plants to treat diseases. Nearly 25,000 formulation used in traditional Indian medicines are based on plants [4, 5].

In Japan the traditional medicinal plants were included in their first edition of pharmacopeia in the 19th century [4]. Whereas, herbal medicine in the Western world (Europe and USA) is included into complementary and alternative medicine (CAM) as they believe that those product are natural and more safe than the synthetic one [4].

In the Arab world the first records of using herbs in medicine were 2600 BC in Iraq. Besides, around 700 herbal medicines were documented back to 1500 BC in Egypt. In the time of Arab Islamic Empire (632-1258) over than 1400 of herbal medicine drugs were used by the Arabic physicians. In the Middle East over than 2600 plant species are grown among them over than 700 plant species are considered medicinal plants but only less than 250 plant species are used in Arab traditional medicine [5]. In Mediterranean region 250-290 herbal species are still in use according to ethnopharmacologists, especially in Historical Palestine around 129 herbal species are used in Arabic traditional medicine for treating digestive, liver, cancer, respiratory, skin diseases and for lowering cholesterol and weight loss [5].

1.2 Current use of Medicinal Plants

Traditional herbal medicines have been used by large numbers of populations from the ancient centuries till now to meet their health care requirements; according to World Health Organization (WHO) around 80% of inhabitants around the world use primarily traditional medicines in their health care need such as plant extracts or their active principles [1, 3]. In the developing countries herbal medicines have been used as an alternative source of medication to solve the health problems and the high costs of drugs [6]. In the developed countries the drugs derived from plants are taking an important place in their health care system [1]. In North America the huge attention in nutraceuticals and medicinal herbs products were

augmented in the last years, in which phytochemical compounds can promote medicinal qualities and long-term health [7]. The percentage use of herbal medication in USA are reported to be 25% of the total drug market and the global herbal medicine market is expected to increase from 61 billion US $ to 5 trillion US $ in 2025 [5]. Investigating plants as a source of new drug is interested by the pharmaceutical industries in China and Japan [1, 8]. According to WHO investigations about 62 of verified different therapeutic drug categories used around the world are produced from 119 chemical isolated from 91 plant species [1]. Dissimilar to conventional single drug, plant extracts or raw plant have a range of phytochemicals and bioactive constituents that provide synergistic effects which allow for multi-target effect in curing of diseases [5]. The medicinal plants and their claimed traditional use are considered one of the major approaches in developing new drug from natural products [1].

Plants produce primary and secondary metabolites that are important for plant growth, protection and beneficial for human body [9]. Secondary metabolites include alkaloids, glycosides, flavonoids, phenols, steroids, saponins, tannins, terpenoids and volatile oils, are important for healing diseases and are responsible for the therapeutic effect of plants, for example terpenoids and volatile oils have anti-inflammatory, anticancer, anthelmintic, antimalarial, antiviral, antibacterial, cholesterol inhibition and insecticide [10, 11]. Different drugs used in recent time were derived from medicinal plants such as artemisinin, atropine, quinine, ephedrine, colchicine, aspirin and digoxin [10]. Up to 122 compounds have been

isolated from medicinal plants and used in modern medicine; 80% of these components have traditional use similar or related to the use of currently isolated active compounds [5]. Medicinal plants have also an economic value if they cultivated in appropriate geographical and environmental conditions that give the plants the suitable habitat, harvested in suitable seasonal conditions and collected from the suitable parts of the plant to yield a good and a high quantities of their active principles [1, 12, 13]. The importance of natural products including medicinal plants and the wide use in modern medicine refers to (1) the diversity of chemical structures of their constituents used in semi and total synthesis of new chemical products (2) the large number of diseases treated or prevented by these components and finally (3) the ability of their frequency use in disease treatment [3].

1.3 Volatile oils

Volatile oil also called "ethereal oil" or "essential oil". VOs are extracted from different parts of plant (roots, bark, twigs, buds, leaves, flowers, seeds, fruits, wood and herbs) by several methods depending on the plant origin and the volatility of the oils; simple pressing (orange and lemon oils), fermentation followed by distillation (mustard and bitter almond oils), extraction and the most common commercially method steam distillations [14]. VOs are hydrophobic, and are soluble in alcohol, non-polar or weakly polar solvents, waxes and oil. They are without color or with pale yellow liquid with lower density of water. They are easily oxidized by air, heat, and light and have different actions depending on

their chemical composition [15]. The greatest use of VOs especially in European Union in cosmetics such as perfumes as aftershaves and fragrances, flavoring and preservative agents in food, in alternative medicine and in pharmacy for their therapeutic effects. The investigation of those plants and their VOs used in traditional medicine is important to improve the quality of healthcare [16-18]. The chemical compounds of VOs can be classified into oxygentated (ketones, alcohols, phenols, etc.) and hydrocarbons (limonene, pinene , etc.) or into phenylpropanoids and terpenoids [15, 16]. The active compounds can be divided into four groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and “others”[19].

Terpenes are hydrocarbons produced from binding of several isoprene units (C_5H_8) (Figure 1).The main terpenes are monoterpenes ($C_{10}H_{16}$) and sesquiterpene ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) and triterpenes ($C_{30}H_{48}$), examples of terpenes (limonene, pinene).

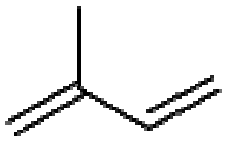


Figure (1): Isoprene [20]

Terpenoids are terpenes that produced by adding oxygen molecules and moving or removing methyl groups under the influence of enzymatic biochemical modifications, terpenoids also called "isoprenoids" organize a large number of natural components obtained from isoprene units C_5

linked head to tail form (Figure 1) [19]. Terpenoids can be sub- divided into aldehydes, alcohols, ethers, esters, epoxides, phenols and ketones [19].

Phenylpropanoids are presented in VOs in few amounts and derived from Shikimic acid (Figure 2) [20]. Phenylpropanoids - contain a large organic products produced by plants to protect themselves from wounds, infections, herbivores and ultraviolet irradiation [21]. Examples of Phenylpropanoid are eugenol, vanillin [19].

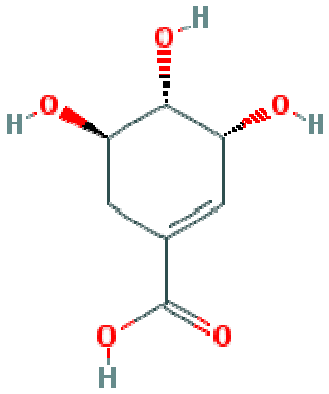


Figure (2): Shikimic acid [22]

Other volatile oil constituents: volatile oils contain a number of different degradation products originating from terpenes, unsaturated fatty acids, glycosides, lactones, and sulfur-and nitrogen-containing compounds (allyl isothiocyanate (AITC) and allicin) [19].

The chemical composition and the aroma of VOs may be different due to growing condition (climate, type of soil and composition, amount of water exist, altitude), plant age, geo-climatic location and environmental conditions of collection time and site [23].

The chemical structures of VOs determine their therapeutic activities; as antiviral, antimicrobial, antiseptic, vasodilators, hypotensive, calming, sedative, spasmolytic, antipyretic, analgesic , anti-inflammatory, expectorant, stimulant, tonic, antitumor, anaesthetic, cell regenerating, and digestive [15].

1.4 Background

1.4.1 *Micromeria fruticosa* (L.) Druce

Micromeria fruticosa (L.) Druce related to *Micromeria* Benth genus and *Lamiaceae* family [24, 25] and has six subspecies: *M fruticosa subsp. fruticosum* (L.), *M fruticosa subspecies barbata* (P. H. Davis), *M fruticosa subspecies brachycalyx* (P. H. Davis), *M fruticosa subspeices giresunica* (P. H. Davis), *Micromeria futicosa* var. *italica* (Huter), *M fruticosa* subsp. *serpyllifolia* (M. Bieb.) P. H. Davis [24].

1.4.2 *M fruticosa* subspecies *serpyllifolia* (M. Bieb.)

1.4.2.1 Scientific name, synonyms and traditional names

M fruticosa subspecies *serpyllifolia* (M. Bieb.) is one of the Medicinal Aromatic Plants (MAP) which are dominated in the eastern Mediterranean regions including Palestine, has pleasant minty fragrance, in hot summer provide sensation of coolness [26, 27]. *Clinopodium serpyllifolium* (M.Bieb) is the accepted scientific name for *M fruticosa* subspecies *serpyllifolia* [28]. The synonyms are: *Micromeria fruticosa* subsp. *serpyllifolia* (M. Bieb.) Boiss, *Nepeta serpyllifolia* (M. Bieb.),

Melissa serpyllifolia (M. Bieb), *Satureja serpyllifolia* (M. Bieb.) [24, 28, 29]. *M fruticosa* subsp. *serpyllifolia* (M. Bieb) known as White *Micromeria* is an aromatic herb with peppermint smell [12]. In Palestinian society known as Duqat ‘Adas (دقــة عــدس), ‘Ishbit esh-shai (عشــبة الشــاي), Qurnya (قرنيــة) and as Thyme-leave savory in English, the aerial parts of plant (flower, leaves, and stalk) are used in folk medicine [30].

1.4.2.2 Description of *M fruticosa serpyllifolia*

M fruticosa serpyllifolia is a perennial Mediterranean plant habitant in rocky areas in Palestine, Lebanon, Syria and Turkey has a height of 20-80 cm. It is short-shrub plant grown in the period of end winter and spring, starts flowering in summer (July) until autumn (November) with white color (Figure 3) [2, 13, 27, 31]. Stems straight, whitish, covered with short, dense and soft hair, thick and solid, simple or paniculated at the end above, branches divaricated. Leaves greyish-white, thyme-leaved, covered with very finely hair (finely puberulent), opposite, oblong ovate (Figure 4). Inflorescence is a cluster of cymes with many branching flowers (Figure 5). Corolla yellow or white, scarcely female and self-pollinated in unopened flowers (Figure 6) [24, 32, 33].

Figure (3): ***M fruticosa serpyllifolia*** **[29]**

Figure (4): Leaves of ***M fruticosa serpyllifolia*** **[34]**

Figure (5): Inflorescence of *M fruticosa* (L.) Druce subsp. *serpyllifolia* (Bieb.) Davis [35]

Figure (6): *Corolla* of *M fruticosa* (L.) Druce subsp. *serpyllifolia* (Bieb.) Davis [34]

1.4.2.3 Folk uses

Micromeria fruticosa L Druce subsp. *serpyllifolia* is a medicinal herb is widely spread in eastern regions of Mediterranean coast Palestine, Lebanon, Syria and Turkey. In those regions *M fruticosa* is used as: a replacement for mint, herbal tea, spice, flavoring agent in food and soups, in addition it has different uses in traditional medicine such as: treatment of hypertension, heart disorders, diarrhea, abdominal pains, colds, headache, wounds, infections such as skin and eye infections and anti-inflammatory, exhaustion, weariness [27, 36-40].

Palestine: In Palestinian society *M fruticosa* is considered one of the most wild edible plant in Palestine [26]. In Palestinian society the leaves are prepared as tea for colds and relieve intestine and stomach pain in addition to exhaustion and weariness [27]. In addition to that the extracts of leaves have been used for relief chest, respiratory system, asthma, fever, for skin infections, wounds and eye inflammation [2, 31]. Drinking an infusion of *M fruticosa* stalks and leaves in Palestinian society is used for calming and strengthening the nerves, and in treatment of diabetes, cough, respiratory system disorders, headaches, fever and urinary diseases [2, 30].

Jordan: In Jordan *M fruticosa* is imported, used for colds and opening of respiratory ducts [41]. The species recoded in Jordan was *Micromeria nervosa* [42].

Lebanon: In Lebanon *M fruticosa* has been used widely for curing from different ailment such as colds, skin infections, wounds, diarrhea and abdominal pains [43]. Fluid extracts or volatile oils of aromatic plants have been used for a long period of time for producing soaps which were used for regular washing and as a disinfectant for wounds and in aromatherapy such as massage or baths [18].

Greece: In Greece and Asia minor *M fruticosa* was used in detoxification of metal salts poisoning like sulfate salts of zinc, copper and iron (Zn/Cu/$FeSO_4$) [2].

Turkey: *M fruticosa* subspecies rich in pulegone have been used as herbal teas for stomach troubles among Turkeys communities [44].

Iran: The mainly traditional use of *Micromeria* species in Iran is for treating cold. They use the aerial part of plants as herbal teas because of their pleasant smell and medical advantages. They are also used for headache, tooth pain, skin infections, wounds, eye inflammations, cardiac disorders and chest pain [45].

1.4.2.4 Chemical composition

The chemical composition and the level of the constituents of the VOs of *Micromeria* species may different depending on the variation in cultivation, growing season, origin, vegetative stage of the plants; from younger and older branches and from the leaf pairs in the same plant [13, 40]. The major constituents of VOs and aqueous extract of the plant were,

monoterpens (pulegone, menthol, isomenthol, isomenthon, limonene, α-pinene, β-pinene, piperitone, piperitenone oxide) and sesquiterpenes (β-caryophyllene and germacrene) [13, 27, 36].

1.4.2.5 Evidence based uses

The oil and the aqueous extract of *M fruticosa* showed remarkable antitumor activities against Human Colon Tumor cells (HCT) and Mammary Carcinoma F7 (MCF 7) [36]. The aqueous extract of *M fruticosa serpyllifolia* can be used as anti-inflammatory and as a protection against gastric ulcer so can be used as supplement or alternative herbal therapy for NSAIDs which can cause gastric ulcers [31, 37]. VOs of *M fruticosa serpyllifolia* also exhibit antibacterial, antifungal, antioxidants, insecticide, analgesic, anticonvulsants and CNS depressant [12, 27, 38]. The methanolic and aqueous extracts of *M fruticosa serpyllifolia* have antioxidant activities and thus may be used as palliative in liver injuries, and have a remarkable effect in inhibition inflammatory pain [36, 46]. According to Yaniv *et al*. the excessive use of *M fruticosa serpyllifolia* may be associated with neuro- and hepatic toxicities due to pulegone [27]. Essential oil of *M fruticosa serpyllifolia* can be used as a natural substance for replacement of synthetic herbicides due to the presence of pulegone which consisted of 70% pulegone and 30% other substances [47].

1.5 Problem statement

Dangerous health problems causing major load to the global health sector. Oxidative stress caused by disrupting the balance between the

oxidants and antioxidants affect the cell activity, viability, lead to cell damage and consequence affect the organ jobs and participate in many diseases such as diabetes, hypertension, atherosclerosis, asthma, pulmonary fibrosis, ischemia, neurological dysfunction, immune system diseases, cancer and promote aging [48, 49]. The highly active oxidants either stay at the site of production or transfer to other sites destroying the structure of the cells like nucleic acids, proteins, carbohydrates and lipids by changing their role in the cells [48, 49]. The accumulation of oxidants and depletion of antioxidants induce the cell to return to normal balance by activation or paralysis genes responsible for production protective enzymes, structural proteins, and reproduction factors. The increase of production of oxidants modify DNA structure causing change in lipids and proteins, induce cytokines anti-inflammatory and proinflammatory, activate transcription factors induced by stress [48]. Antioxidants are considered the first choice of cell defense system for protection against oxidants or reactive oxygen species (ROS) by preventing or delaying the process of oxidation [49]. Recently there is a big attention towards natural antioxidant from plants. Antioxidant can act as radical scavenger, promote health and produce anticancer activity [50].

Obesity and overweight are defined as a condition where the fat is accumulated in the body and negatively affect the health [51]. Obesity is a global health danger. About 52% of adult European people are overweight or obese which negatively affect personal professional quality of life, morbidity and mortality [52]. Several studies in Europe indicated that

obesity causes emotional disease and depression [53]. According to World Health Organization (WHO) in 2016, the numbers of adult obese was 650 million represented 13% of adults [51]. Obesity is considered one of the key causes of the death in USA, approximately 300,000 deaths take place each year due to nutrition and physical inactivity [54]. Obesity and overweight and their related health problems have considerable economic outcomes. The growing expansion of obesity and overweight is correlated with diagnostic, preventive and medical services like nursing home care, hospital care and physician visits costs. The greatest cost expended for obesity is due to coronary heart disease, diabetes type 2, and hypertension [55]. It was estimated that the total treatment cost for obesity per year was increased to $147 billion in 2008 [56]. Different natural components have been investigated to have good activities for preventing or treating obesity and related metabolic and cardiovascular disease [52]. Orlistat is the most common drug for obesity treatment, but there is still deficiency of safe medicine for treating obesity [52].

Diabetes is produced when the body does not generate sufficiently enough insulin or can't be used effectively, this can cause hyperglycemia and with long time it causes dangerous impairment in the different systems in the body, particularly in blood vessels and nerves [57]. Diabetes has caused a major load to the global health sector [58]. Diabetes is a dangerous disease and has serious complications; WHO reported that 8.5% of adults around the world have diabetes, and 1.6 million deaths occurred in 2015 [57]. Patient with diabetes are susceptible to vascular diseases;

stroke and heart attack are two to three times more than none diabetes people [59]. Diabetic peripheral neuropathy (DPN) causes different diseases like neuropathic pain, foot sores and amputation, and combined with cardiovascular, hypertension, nerve ischemia and elevated mortality. DPN affected 15-20% of diabetes [60]. Diabetes causes retinopathy which after long period of time produces blindness. According to WHO 2.6% of the worldwide blindness can be referred to diabetes [57]. Diabetic nephropathy (DN) is considered a leading cause for kidney failure [61]. The percentage annual rate of deaths resulted from DN reaches 20% of deaths [62]. DN appears in 25-40% of diabetic patient after 25 years of treated and uncontrolled diabetes, in US, DN appears in 44% of diabetic patients and the expenses on treatment have been raised 11 times in last ten years [63]. Diabetes type 2 previously known adult onset or insulin independent, mostly happened as a result of physical inactivity, overweight and obesity, depending on that, WHO has recommended that diabetes type II can be controlled by exercise, healthy food and medication [57]. Thus encouraging patients with diabetes type II to follow healthy life style and consuming greens that produce antidiabetic functions would be healthier and more economic ways for controlling diabetes [58]. Therefore the investigating for antidiabetic agents from plant extract has increased, as discovering new effective drugs is important for controlling the disease [58].

Antimicrobial resistance occurs when microscopic organisms like bacteria, viruses, parasites and fungus modify when they exposed to

antimicrobial medicine like antibiotics, antivirals, anthelmintics, antimalarials and antifungal. As a consequence, the drugs become powerless and infections continue in the body creating a danger of prevalence to others [64]. Annually, not less than two million persons in United State are infected with bacteria that resist antibiotics and not less than twenty three thousands persons die as a direct consequence of bacterial infections each year [65]. Over time the antimicrobial resistance develops naturally out of changing in genetic deformation [64]. The overuse and misuse of antibiotics in animals, human being and environment such as using antibiotic for viral infection or using antibiotic for promoting animal growth without official prescription speeds the antimicrobial resistance. Inappropriate sanitary conditions, inadequate infection control and in infection control and food handling are factors that promote the prevalence of antimicrobial resistance [64]. The microorganisms continuously develop novel resistance mechanism and spread worldwide, threat the human ability to cure common infectious illness, producing prolonged diseases, failure and death [64]. Medical procedures such as cancer chemotherapy, organ transplantation, management of diabetes and surgery processes, in absent of antimicrobial medicines for sake of protection and treatment turn into huge risk [64].The expense on health care, hospitalization are increased with antimicrobial resistance and needed for intense care [64]. Sustainable development goals will be delayed and be at risk due to the spread of antimicrobial resistance [64]. The complications of multidrug resistance enforced the scientists to

search for new antimicrobial agents from various sources such as medicinal plants [66]. Aromatic plants have been applied since old times for their preservative and medicinal advantages. In recent time there has been considerable interest in essential oils and extracts of medicinal plants for the development of alternatives to prevent or to delay the growth of pathogens [67].

Depending on the aforementioned problem statements; oxidative stress, obesity, diabetic diseases and antimicrobial resistance and their consequences of developing serious diseases in human being resulting in elevated levels of mortality worldwide. The global trend toward the natural products is growing due to their wide range of chemical products that can produce a synergistic effect in treating diseases with lower toxicity effects. The variety of chemical composition of VOs of aromatic plants related to different variables such as: the soil, the part of plant, the seasons and climate. It was encouraging to study the national and traditional herbs that historically used to treat different diseases. Thus, the aim of the study was to define the chemical compositions and their characteristics of VOs of *Micromeria fruticosa serpyllifolia*.

1.6 Objectives of the Study

1.6.1 General objective

The main purpose of the study was to compare the chemical composition of *M fruticosa serpyllifolia* Volatile oils collected from three geographical regions in Palestine (i.e., northern, middle, and southern

areas), and to screen the potential biological activities and pharmacological properties of VOs of *M fruticosa serpyllifolia*.

1.6.2 Specific objectives

In the current study *in vitro* screening of the potential antimicrobial, antioxidant and enzymatic activity (antilipase and antiamylase) of the VOs were addressed and compared. However specific objectives of the current thesis were:

a. To analyse the chemical composition of *M fruticosa serpyllifolia* VOs using GM/MS.

b. To test the antioxidant activities of *M fruticosa serpyllifolia* VOs

c. To assess the enzymatic properties of *M fruticosa serpyllifolia* VOs such as antilipase and antiamylase.

d. To investigate the antibacterial, antifungal activities of *M fruticosa serpyllifolia* VOs.

e. To conduct a comparative study of the findings of the aforementioned tests among three geographical regions in Palestine.

1.7 Significance of the study

Previous studies suggested that *M fruticosa serpyllifolia* VOs have potential to act as antioxidants, antibacterial,anti-fungal and other biological properties. However, to the best of our knowledge there has been

no research that explores the potential changes in these activities regarding to the differences in geographical conditions and subsequently the chemical constituents. This is the first study conducted on the chemical compositions and biological activities of *M fruticosa serpyllifolia* VOs relative to different geographical conditions in three regions in Palestine. Consequently, the study may be of a valuable tool to:

a. Explore the chemical constituents of *M fruticosa serpyllifolia* VOs.

b. Illustrate if there are differences in the chemical constituents of *M fruticosa serpyllifolia* VOs in the different regions of Palestine.

c. Investigate the biological activities, the antioxidant and the enzymatic properties of *M fruticosa serpyllifolia* VOs in the different regions of Palestine.

d. Serve as a tool to choose the suitable oil to be used for traditional use in a more effective & efficient manner in treating related diseases depending on the results of the chemical composition and concentration of the important constituents of *M fruticosa serpyllifolia* VOs from different regions in Palestine.

e. Give good information about the suitable environmental and geographical conditions for commercial agricultural cultivation.

f. Add economical value to *M fruticosa serpyllifolia* VOs produced in Palestine.

Chapter Two

Materials and Methods

Chapter Two
Materials and Methods

2.1 Materials

The materials used in the study were of analytical grade and used without further purification. DMSO 100% (Dimethylsulphoxide) was purchased from (CARLO ERBA, France), DMSO 100% was used in antimicrobial screening and the dilution 10% was used in the enzymatic screening.

2.1.1 Material used in producing VOs

Calcium Chloride which was used for drying of VOs was purchased from (Sigma-Aldrich, USA).

2.1.2 Material used for antioxidant screening

DPPH (2,2-Diphenyl-1-picrylhydrazyl) was purchased from (Sigma-Aldrich, Germany), Trolox (6 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from (Sigma-Aldrich, China) and Methanol was (self backing Haifa).

2.1.3 Material used for antilipase screening

Porcine pancreatic lipase, Tris-HCl, and PNPB (*p*-nitrophenyl butyrate) were purchased from (Sigma-Aldrich, USA), Orlistat was purchased from (Sigma-Aldrich, China) and Acetonitrile was purchased from (CARLO ERBA, France).

2.1.4 Material used for antiamylase screening

Amylase type VI -B, ≥ 10 unit/mg and Acarbose were purchased from (Sigma-Aldrich, USA), 3-5 dinitrosalysylic acid (DNSA) was purchased from (Sigma-Aldrich, India), sodium potassium tartrate tetrahydrate was purchased from (MERCK, Germany), sodium hydroxide (NaOH) was purchased from (Sun Pharm.drug stars, Nabluse-Palestine), Disodium hydrophosphate/dihydrosodium phosphate (Na_2HPO_4/NaH_2PO_4) was purchased from (Alfa Aesar, USA), Sodium chloride (NaCl) was self backing (Haifa) and Starch (Alzahra company, Nablus-Palestine).

2.1.5 Material used for antimicrobial screening

2.1.5.1 Antibacterial

Methanol (self backing, Haifa), Nutrient Agar 28.0 g/L, Mannitol 111 g/L, MacConKey Agar 49.53g/L and Mueler Hinton Broth 21.9 g/L were purchased from (HiMedia Laboratories, Mumbai-India), Cefuroxime 250 mg (as axetil) tablet, Doxycycline 100 mg (Hyclate) tablet (Jerusalem Pharmaceutical Company, Albiereh-Ramallah-Palestine), Levofloxacin 500 mg tablet (Birzeit Pharmaceutical Company, Birzeit-Ramalla- Palestine), and Azithromycin 250 mg capsules (Pharmacare company, Birzeit-Ramalla- Palestine), all these drugs were donation from Military Medical Services Ramallah Palestine.

2.1.5.2 Antifungal

Ethyl alcohol 99.9% and Sodium Hydroxide 1 M, were purchased from (Sun Pharm.drug stars, Nabluse-Palestine), Sodium Chloride and

Sodium potassium tartrate were (self backing, Haifa), Sabouraud Dextrose Agar 65g/L was purchased from (oxoid, UK), 3-[N-morpholino] propanesulfonic acid (MOPS) buffer 34.53 g/L and RPMI-1640-medium (with L-glutamine, without sodium bicarbonate) 1.165 mol/L (was developed at Roswell Park Memorial Institute) were purchased from (Sigma-Aldrich, UK), 0.05% Tween 80 were purchased from (ACROS Organics, Belgium), Tinidazole 500 mg tablet (Jerusalem Pharmaceutical Company, Albiereh-Ramallah-Palestine), Terbinafine hydrochloride 250 mg tablet (Birzeit Pharmaceutical Company, Birzeit-Ramalla- Palestine), all these drugs were donation from Military Medical Services Ramallah Palestine.

2.2 Instruments

2.2.1 Volatile oils (VOs) extraction and chemical screening

Grinder (Moulinex model, Uno. China) was used to fracture the dried herbs. Balance max 220 g (Radway, Poland) was used to weigh the plant material, Microwave-ultrasonic cooperative extractor/reactor (CW-2000, China) was utilized for extraction volatile oil, GC-MS (Gas Chromatography Mass Spectrometry (QP-5000 Shimadzu GC-MS, Japan) was utilized for chemical screening of VO.

2.2.2 Antioxidant and enzymatic screening

Balance maximum capacity 4500 g (boeco, Germany), UV-Vis (Ultraviolet-Visible) Spectrophotometer (Jen WAY 7315, UK) was utilized

for assessment the antioxidant and enzymatic activities of VOs. Water bath (Memert, Germany), water bath sonicator (MRC, Haifa), Heater (Lab-Tech, Korea), Refrigerator (beko, UK), Single micropipette 100-5000 µL (Nichiryo Nichipet, Japan), single micropipette 100 -1000 µL and 20-200 µL (Huma pette, Germany), large glass test tubes, volumetric flasks 10, 50, 100 mL, plastic cuvettes.

2.2.3 Antimicrobial screening

Balance max 300 g, d=0.001g was purchased from (Sartorius AY 303, Canada), Heater (Lab-Tech, Korea), Autoclave used for sterilization media, water and disposed materials was purchased from (MRC, Haifa), Bunsen Burner (Ningbo I.G.I Gas Industry, China) and Hood (BIOBASE, China) were used for working under aseptic conditions. Refrigerator (Ariston, USA), Water bath and Incubator (Ari j Levy, Haifa), pH meter was used to adjust pH of RPMI solution was purchased from (Jen WAY, UK), Multichannel micropipette 30-300µL (MRC, Haifa), Multichannel micropipette 1-10 µL (Eppendorf research, Germany), Single micropipette 100-1000 µL (Microliter, BRAND, Germany), Single micropipette 20-200 µL (Huma pette, Germany), tips white, yellow and blue were used for measuring the minute volumes of plant extract, nutrient broth and bacterial solutions were purchased from (Labcon, USA). Microplates 96 Well Cell Culture (Greiner bio-one CELLSTAR, Austria). Aluminum foil (Reynold consumer products company, USA) used for enveloping the material for autoclaving. Disposable sterile syringe 5, 10 mL (Changzhou Heany,

Jiangsu-China), sterile syringe filter 0.25 µm (KDL, China), in addition to other equipments were used like large and small glass test tubes, large and small plates, Loops, disposable sterile pipette volumes 1, 5, 10 mL and Eppendorf tubes (Nichipet EX, Japan), autoclave sterilization tapes and parafilm M (Bemis, USA)

2.3 Methods

2.3.1 Plant materials collection and preparation

The aerial parts of *M fruticosa serpyllifolia* were collected in April 2017, before flowering period from three cities resembling three regions in the West Bank (WB) in Palestine: Nablus, Ramallah and Hebron represented North, Middle and South of the WB in Palestine respectively. The samples were botanically identified and coded by Dr. Nidal Jaradat the Pharmacognosist at An-Najah National University (ANNU). The extraction of VOs was followed the procedure in reference [68]. The fresh aerial parts of *M fruticosa serpyllifolia* were separated carefully, washed two times with distilled water, dried for two weeks in the shade at room temperature. The dried specimens were fractured, and stored in well closed plastic bags for future use in the Laboratory of Pharmacognosy at ANNU Faculty of Medicine and Health Sciences.

2.3.2 Volatile oil extraction

The VOs of the three specimens of *M fruticosa serpyllifolia* plant were extracted utilizing the apparatus Microwave-ultrasonic method which

was examined by Jaradat et al, 2016 by which the suspension of plant fractures were exposed to ultrasonic waves to improve the extraction process [69]. The apparatus was consisting of a microwave oven combined with an ultrasonic extractor (Figure 7). Approximately 100 g of the fractioned dried aerial parts of each plant sample were placed in an one litter round-bottom flask, about 300 mL deionized water was added, the flask was placed in the apparatus and then connected with Clevenger apparatus, which placed in the same apparatus. The power of the microwave-ultrasonic extractor apparatus was fixed at 1000 W. The ultrasonic power of the apparatus was fixed at 50 W and the frequency of 40 kHz at its maximum power. The extraction process was prolonged for 10 min at 100 ˚C. This was repeated three times for each plant sample. The resulted VOs were collected into a separate clean, well closed small glass bottle, chemically dried over calcium chloride and the dried oil was transferred into small clean, well closed glass bottle, labeled with the name of plant, date, number of the sample, covered with aluminum foil and stored in the refrigerator at 2-8˚C until use [69, 70].The average percentage yield (w/w%) of VOs were: Nablus was 0.67 ± 0.29% Ramallah was 0.99 ± 0.55% and Hebron was 0.70 ± 0.17%.

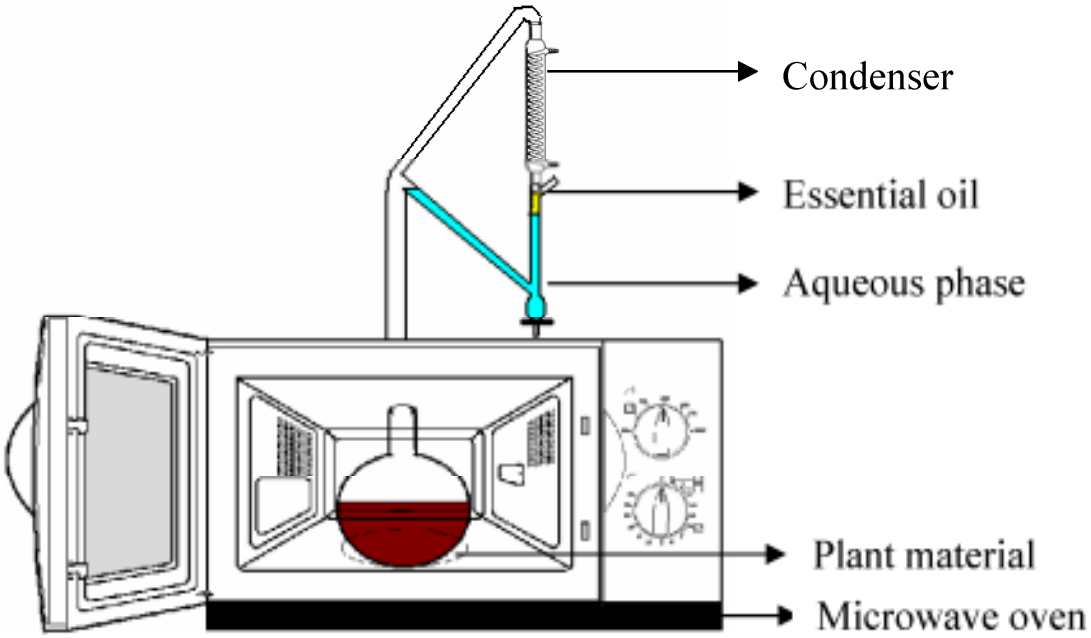


Figure (7): Microwave - ultrasonic apparatus [71]

2.3.3 Gas Chromatography/Mass Spectrometry (GC-MS)

The chemical composition of the three samples of *M fruticosa serpyllifolia* VOs was detected using GC-MS technique. The method used was described by Al-Hamwi *et al*.[12] and Jaradat *et al* [70] with some modifications. GC-MS chromatograms were displayed using (Shimadzu QP-5000 GC-MS), equipped with column Rtx-5ms (0.250 mm inner diameter, 0.25µm thickness and 30 m long). A carrier gas was Helium at a flow rate of 1 mL/min. The temperature of the Injector was 220°C. The temperature of the Oven was programmed from 50°C (1min hold) at 5°C/min to 130°C, then at 10°C/min to 250°C and kept at constant temperature for 15 min. The temperature transfer line was 290°C. An electron ionization method was used for detection of GC-MS, with detector volts of 1.7 KV. A scan speed 1000 amu/sec and scan rate of 0.5 s, and were used, covering a mass range from 38-450 M/Z [12, 70]. The mass

spectrometry data center of the national institute of standards and technology (NIST) was used as a reference to identify the chemical components of the VOs by comparing their MS spectra with data of NIST in addition of using Kovats index in the literature to compare their retention times. The quantitative data were obtained electronically from integrated peaks, area percentages without the use of correction factor [12, 70].

2.3.4 Antioxidant activity DPPH assay

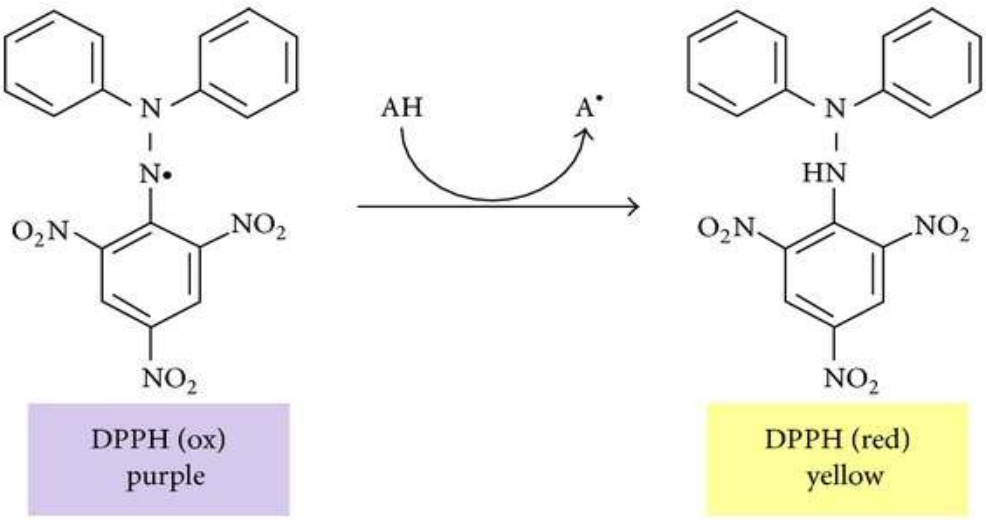
Scavenging activity of *M fruticosa serpyllifolia* VOs of the three samples from three regions of West Bank in Palestine was assessed using the method of Sonboli and Jaradat et al [45, 70]. Stock solutions of VOs of the three samples and Trolox (the reference product) were prepared in methanol at a concentration of 0.1 mg/mL(10 mg of sample in 100 mL methanol). Working solutions with the following concentrations: 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µg/mL was prepared by taking volumes of (0.1, 0.2, 0.3, 0.5, 0.7, 1, 2, 3, 4, 5, 8 and 10 mL) respectively of stock solution and bringing them up to 10 mL with methanol, using 10 mL volumetric flask (VF). The DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared freshly at concentration (0.002% w/v), 2 mg of DPPH was dissolved in 100 mL of methanol using 100 mL VF. A mixture of DPPH, methanol and VO of each of the above mentioned working solutions was prepared at 1:1:1 ratio. A blank solution was prepared by mixing the DPPH solution with methanol at 1:1 ratio. After that, all of those solutions were incubated at room temperature in a dark cabinet for 30 minutes. The

absorbance of those solutions was measured after the incubation period by UV-Vis spectrophotometer at 517 nm wavelength, and methanol was used to zero the spectrophotometer. The antioxidant activities of *M fruticosa serpyllifolia* VOs and Trolox was assessed by their ability to donate a hydrogen atom or electron and was identified from converting the deep violet color of methanol solution of DPPH to colorless or pale yellow as shown in Scheme 1, for that the inhibition percentage of DPPH activity was used to determine the antioxidant activity of *M fruticosa serpyllifolia* VOs and Trolox using the following equation:

$$\boldsymbol{In\%} = \frac{\boldsymbol{A\ blank - A\ sample}}{\boldsymbol{A\ blank}} \times \mathbf{100} \qquad \boldsymbol{Eq.1}$$

Equation (1): Inhibition% of antioxidant activity [40]

Where A_{blank} represented the absorption of the control reaction (all reagent without the sample) and A_{sample} represented the absorbance of the sample.



Scheme (1): Principle of DPPH radical scavenging capacity assay [72].

The antioxidant half-maximal inhibitory concentration (providing 50% inhibition, IC_{50}) for each of the studied *M fruticosa serpyllifolia* VOs and Trolox standard solution as well as their standard deviations, was calculated from the graph plotted of inhibition percentage against extract concentration. using Microsoft Office Excel 2007.

2.3.5 Pancreatic Lipase (PL) Inhibition

The porcine pancreatic lipase (PPL) inhibitory assay was conducted using the methods from Jaradat et al. [73], Bustanji et al. [74] and Siew-Ling et al. [75] with some modifications.

2.3.5.1 Preparation stock and working solutions

a. Volatile oil and Orlistat stock and working solutions

VOs stock solution of 1mg/mL was prepared in 10% Dimethyl sulfoxide (DMSO), and diluted with 10% DMSO to produce five different concentrations (200, 400, 600, 800, and 1000 μg/mL). Orlistat was used as a reference for pancreatic lipase inhibition assay and was prepared by the same procedure of plant extract.

b. Pancreatic lipase enzyme

Pancreatic lipase enzyme stock solution was prepared immediately before use by suspending in 10% DMSO at concentration 1 mg/mL. By weighing 25 mg of lipase suspending in small amount of 10% DMSO, bringing up the volume up to 25 mL in VF (25 mL), putting in water bath sonicator at 37 ºC for 15 minutes.

c. *p*-nitrophenyl butyrate (PNPB) lipase substrate

The stock solution of *p*-nitrophenyl butyrate (PNPB) was prepared according to manufacture structures (20.9 mg of PNPB in 2 mL of acetonitrile) by dissolving 104.5 mg of PNPB in acetonitrile bringing up the volume to 10 mL in V.F (10 mL).

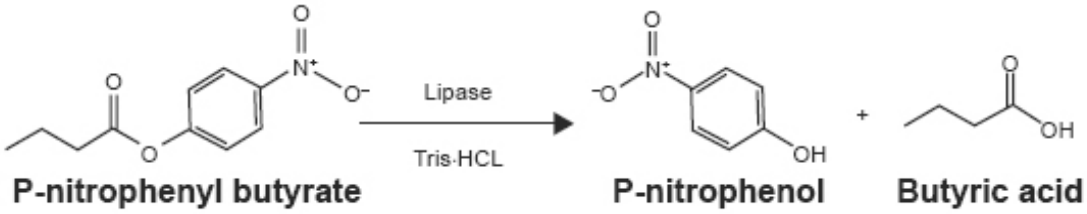
2.3.5.2 Assay of Pancreatic Lipase (PL) Inhibition

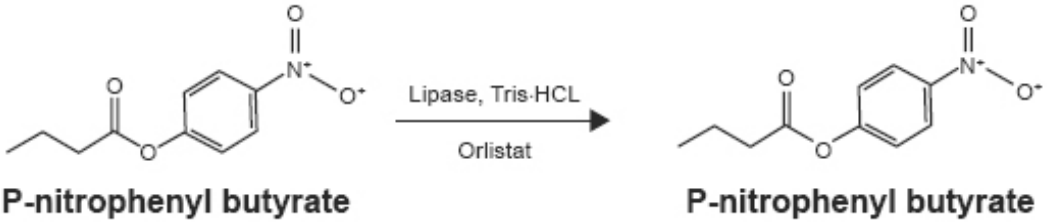
The Pancreatic Lipase (PL) Inhibition Assay was conducting using the procedure in the references [73-75] with some modifications. From each working solution of plant extract prepared above, 200 µL plant extract was taken in a separate test tube, 100 µL of porcine pancreatic lipase (1 mg/mL) was added to it. The resulting mixture was then made up to 1000 µL by adding 700 µL of Tris– HCl solution and incubated at 37 °C in water bath for 15 min. After the incubation time, 100 µL of PNPB (*p*-nitrophenyl butyrate) solution was added to each test tube. The mixture was again incubated in water bath for 30 min at 37 °C. A negative control solution was prepared without plant extract, by mixing 100 µL of porcine pancreatic lipase (1 mg/mL) solution with Tris– HCl solution made up to 1mL by adding 900 µL. The same procedure was followed for Orlistat used as positive control. Tris-HCl buffer was used to zero UV-Vis spectrophotometer at 405 nm. Pancreatic lipase activity was determined by measuring the hydrolysis of *p*-nitrophenolate to *p*-nitrophenol (Scheme 2) at 405 nm using UV-Vis spectrophotometer. The lipase inhibition activity of *M fruticosa serpyllifolia* VOs or Orlistat as a reference was identified by

measuring the effect on the enzyme reaction rate after adding extracts, compared with the control. I% was calculated by the using equation 2 [76].

$$\mathbf{I\% = [(Absorbance_{Control} - Absorbance_{Test})/Absorbance_{Control}]*100}$$

Equation (2): Pancreatic lipase inhibition [76]





Scheme (2): Hydrolysis of *p*-nitrophenyl butyrate (PNPB) with and without Orlistat [77]

2.3.6 α-amylase inhibitory screening

The α-amylase inhibition assay was done according to procedure conducted by Wickramaratne et al. [58] with some modifications. The assay was performed using the 3,5-dinitrosalicylic acid (DNSA) method.

2.3.6.1 Preparation of stock and working solutions

a. Sodium phosphate buffer (20 mM) with sodium chloride (6.7mM), pH 6.9.

Solutions of 20 mM sodium phosphate monobasic and sodium phosphate dibasic buffer containing 6.7 mM sodium chloride (NaH_2PO_4 and Na_2HPO_4 both containing 6.7 mM NaCl, pH 6.9), (sodium chloride

(NaCl) was used for ionic strength and osmolarity purposes). The solutions were prepared by partially filling a beaker with the NaH_2PO_4 and NaCl solution, the mixture was subjected to magnetic stirrer, while adjusting the pH by inserting a calibrated pH electrode in the solution. Then gradually adding the Na_2HPO_4 and NaCl solution until the pH reached 6.9. A weight of 5.36 g of 20 mM $Na_2HPO_4.7H_2O$ and 0.39 g of 6.7 mM NaCl were dissolved in distilled water to make 1 liter and a weight of 2.76 g of $NaH_2PO_4.H_2O$ and 0.39 g of NaCl were dissolved in distilled water to make 1 liter.

b. Volatile oil stock and working solutions

M fruticosa serpyllifolia VOs stock solution (S.S) of 1mg/mL concentration, was prepared in a minimum amount of 10% DMSO (1:100 dilution) and was further dissolved in buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M), NaCl (0.006 M) at pH 6.9). Working solution of concentrations (10, 50, 100, 500 and 1000 µg/mL) were prepared by mixing (0.1 mL, 0.5 mL, 1 mL, 5mL, 10 mL) respectively of *M fruticosa serpyllifolia* VOs S.S and further diluting with buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M)), NaCl (0.006 M) at pH 6.9) and bring up to 10 mL using VF (10 mL).

c. Acarbose stock solutions

Acarbose was used as a reference. The stock and working solutions of Acarbose were prepared using the same procedure of *M fruticosa serpyllifolia* VOs.

d. α-amylase solution

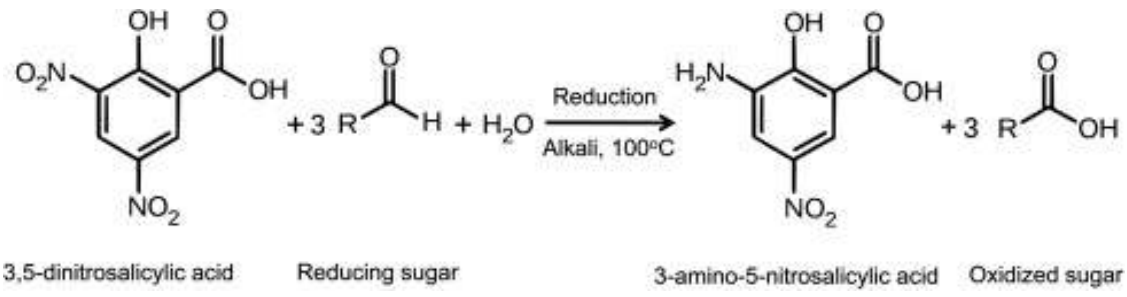
α-amylase solution (2 unit/mL) was prepared by dissolving 12.5 mg of amylase enzyme in a minimum amount of DMSO10 %, then bringing up to 100 mL with buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M)), NaCl (0.006 M) at pH 6.9) in VF (100 mL).

e. Starch stock solution

Starch solution was prepared in concentration of 1% (w/v) by suspending 1 g of starch in 100 mL distilled water using V.F (100 mL), and kept in water bath at 37ºC until use with slightly mixing to prevent starch precipitation.

f. 3.5-dinitrosalicylic acid stock solution (DNSA)

DNSA was used as reactive reagent which reacts with reducing sugars to form 3-animo-5- nitrosalicylic acid (Scheme 3) which strongly absorbs light at 540 nm. It was prepared by dissolving 12 g of sodium potassium tartratetetrahydrate in 8.0 mL of 2 M NaOH (8 g in 100 mL distilled water) and further dissolved in 20 mL of 96 mM of 3.5-dinitrosalicylic acid solution.



Scheme (3): DNSA reaction with reducing sugars to form 3-animo-5- nitrosalicylic [78].

2.3.6.2 Assay of α-amylase inhibition

A volume of 200 µL of α-amylase solution (2 unit/mL) was mixed with 200 µL of each VOs working solutions and incubated for 10 min at 37°C. Then 200 µL of the starch solution was added to each tube and incubated for 3 min at 37°C. The reaction was terminated by the addition of 200 µL DNSA reagent and boiled for 10 min in a water bath at 85–90 °C. The mixture was cooled to ambient temperature and diluted with 5 mL of distilled water, and the absorbance was measured at 540 nm using a UV-Vis. spectrophotometer. The blank with 100% enzyme activity was prepared by replacing the plant extract with 200 µL of buffer. Acarbose was used as a positive control sample. The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using equation 4. The % α-amylase inhibition was plotted against the extract concentration and the IC_{50}values was obtained from the graph [58].

$$\%\ \alpha\ amylase\ inhibition = \frac{Abs\,100\%\ control - Abs\,Sample}{Abs\,100\%\ Control} \times 100$$

Equation (3): % α-amylase inhibition [58]

2.3.7 Antimicrobial screening

2.3.7.1 Microorganisms and condition for cultivation

a. Bacterial strains

The VOs samples of *M fruticosa serpyllifolia* were studied for their antimicrobial activities. The antibacterial activities of *M fruticosa*

serpyllifolia VOs were investigated against the growth of nine references bacterial strains obtained from the American Type Culture Collection (ATCC): *Escherichia coli* (ATCC 25922), *Enterococcus faecium* (ATCC 700221, USA), *Klebsiella pneumonia* (ATCC 13883,UK), *Pseudomonas aeruginosa* (ATCC 27853, USA), *Shigella sonnie* (ATCC 25931,USA), *Staphylococcus aureus* (ATCC 25923, USA). In addition to diagnostically proven clinical isolates *Proteus mirabilis*, *Staphylococcal enterotoxin* B (SEB) and MRSA (methicillin resistant *Staphylococcus aureus*).

b. Fungal strains

The antifungal activity of VOs was examined against the growth of two fungal strains acquired, from the American Type Culture Collection (ATCC) *Candida albicans* (ATCC 90028, USA) and *Epidermophyton floccosum* (ATCC 52066, UK).

2.3.7.2 Preparation of growth media

a. Bacterial growth media

Nutrient Agar (NA) was prepared by dissolving 5.6 g of NA powder in 200 mL autoclaved distilled water. The mixture was heated to boiling point with stirring with magnetic stirrer, then autoclaved for 15 min at 121ºC, the sterilization process was confirmed by using sterilization indicator tapes. After autoclaving and under aseptic condition, the sterilized solution of NA was poured into small and large plates (around 15 mL and 20 mL respectively), allowed the media to solidify and condensate to dry,

the plates were labeled, incubated in invert position to prevent condensation of vapor on agar, and to reduce contamination, enveloped with plastic bag, labeled and kept in refrigerator at 4-8 ºC until use.

MacConKey Agar (MAC) was prepared by dissolving 5.15 g of MAC powder in 100 mL autoclaved distilled. The same procedure was implemented as NA steps. However, small plates were used in the current case.

Mannitol Salt Agar (MSA) was prepared by dissolving 11.1 g of MSA powder in 100 mL autoclaved distilled water in 100 mL size flask as 111.1g/l and followed the procedure with NA using small plates.

Mueller Hinton Broth (MHB) was prepared by dissolving 8.4 g of MHB powder in 400 mL autoclaved distilled water. The solution was heated to boiling point with stirring with magnetic stirrer, then after reaching a touchable temperature, the solution was poured into 10 small test tubes with 5 mL in each, and into another 10 small test tubes with 4 mL in each and finally the rest amount of MHB was poured in 10 mL in large test tubes. All the tubes were autoclaved for 15 min at 121ºC, the sterilization was confirmed using sterilization indicator tape then the tubes were kept in refrigerator at 4-8 ºC, until use.

Sabouraud Dextrose Agar (SDA) was used to culture fungal strains *Candida albicans* and *Epidermophyton floccosum*. Following the manufacturer's instructions, SDA was prepared in by dissolving 6.5 g of

SDA powder in 100 mL autoclaved distilled water, then followed the procedure with NA using large plates

McFarland 0.5 standard was utilized to visually compare the turbidity of bacterial and fungal solutions, to standardize the estimate number of bacteria and fungus in a liquid suspension. Generally McFarland 0.5 standard represents 1.5 x 10^8 CFU/mL. It was prepared in 100 mL VF, by mixing approximately 85 mL of 1% H_2SO_4 with 0.5 mL of 1.175% $BaCl_2$. $2H_2O$ and gradually adding 1% H_2SO_4 to bring the mixture to 100 mL. The flask was sealed with Parafilm, stored in dark at room temperature until use [79].

b. Fungal growth media

Potato Dextrose Agar (PDA) was used for fungal culture and was prepared by dissolving 39.0 g of PDA powder in 1000 mL autoclaved distilled water in 1000 mL size flask as 39.0 g/l, followed the procedure with NA.

SDA for slant tube was prepared in 50 mL for slant tube for *Epederophyton floccosum*, by dissolving 3.25 g of SDA powder in 50 sterilized distilled water, the medium was dispensed 1 mL in small tubes, the tubes were sterilized for 15 min at 121ºC, the sterilization was confirmed using sterilization indicator tapes. Then after, the tubes were directly placed in water at 40 ºC for further use in agar dilution method.

Preparing normal saline with tween 80 was use to prepare fungal suspension of *Epidermophyton floccosum*, and was prepared in 100 mL of

distilled water (sodium chloride 0.9% with 0.05% tween 80), was stirred, heated for 5 mint, with sterile disposable pipette size 10 mL, a volume of 10 mL of solution was discharged in large tubes to prepare 10 tubes, the tubes were sterilized for 15 min. at 121ºC the sterilization was confirmed using sterilization indicator tapes and were kept in refrigerator 4-8 ºC until use.

RPMI 1640 Medium preparation was used to culture *Candida albicans*. Followed the NCCLS (The National Committee for Clinical Laboratory Standards) Guidelines, RPMI 1640 medium with 0.165 M MOPS (3-[N-morpholino] propanesulfonic acid) buffer was prepared in 50 mL by dissolving 0.52 g of RPMI 1640 powder (with glutamine, without bicarbonate) in 40 mL autoclaved distilled water and adding 1.7265 g MOPS. The pH of the solution was adjusted to 7.0 at 25 ºC using NaOH (1 M). Sterilized distilled water was added to bring volume to 50 mL. The solution was sterilized using syringe filter with 0.25 µm micropores, RPMI solution was tested for turbidity by disposing 2 mL in tube with cover and let in incubator for 24 hours and it was clear [80].

2.3.7.3 Preparation of microorganism strains

a. Bacterial strains

Freshly prepared bacterial strains were used; the strains were cultured before 24 hours of use. Initially the bacterial strains were cultured each on its specific growth medium; *Enterococcus faecium* on Nutrient

Agar, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Shigella sonnie* on MacConKey Agar, *Staphylococcus aureus*as, *Staphylococcal enterotoxin* B (SEB) and MRSA on Mannitol Salt Agar. Stock solution of bacterial strains suspensions were prepared in 5 mL MHB by gently swabbing the colony surface of overnight bacterial stains subcultured onto specific Agar with a sterile swab, adjusting the turbidity to 0.5 McFarland solution with concentration of $1.5*10^8$ CFU/mL, then working solutions were prepared by mixing 2 mL of stock solutions with 4 mL MHB in a large plate to produce a concentration of 0.5 $*10^8$ CFU ($5*10^7$) which was further used in the experiment of microdilution method, the concentration achieved in the wells was $2.5*10^5$ CFU.

b. Fungal strains

The fungal strains were cultured freshly using Potato Dextrose Agar and Sabouraud Dectrose Agar; *Candida albicans* before 24 hours and *Epidermophyton floccosum* before 48 hours of using:

Candida albicans stock solution in sterile MHB was prepared by gently swabbing the colony surface of overnight *Canida albicans* subcultured onto SDA with a sterile swab, adjusting the turbidity to 0.5 McFarland solution with concentration of $1*10^6$ to $5*10^6$ CFU/mL, then working solution was made to be used for inoculation in microdilution method with final concentration of $1*10^3$ to $5*10^3$ CFU/mL by 1: 1000 dilution in two times; first dilution was 1:50 in MHB (100 µL to 4900),

second dilution was 1:20 in RPMI µL (500 µL to 9500 µL), and finally 100 µL was used in microdilution method [81], the final concentration in wells was $0.333*10^3$ to $1.666*10^3$ CFU/mL.

Epedermophyton floccosum fungus had originally been subcultured onto potato dextrose agar (PDA) plate, incubated at 30°C until plentiful growth from which fresh isolate was subcultured onto Sabouraud Dextrose Potato Agar (SDA) before 48 hours of the experiment. A suspension of *Epedermophyton floccosum* was prepared by adding Sterile normal saline containing 0.05% Tween 80 to the surface growth and spores and hyphae were scraped off using sterile scalpel. The turbidity of resulting suspension was adjusted to be equivalent to 0.5 McFarland (absorption 0.8 to 1 at 600 nm), and finally 20 µL of the solution was used for inoculation using agar dilution method.

2.3.7.4 Preparation of plant VOs solutions

The initial concentrations used of *M fruticosa serpyllifolia* VOs for bacterial and *Candida albicans* assays were 50 mg/mL and for *Epidermophyton floccosum* were 25mg/mL. The concentration of 50 mg /mL was obtained by initially mixing 50 mg of VOs with 0.5 mL of DMSO 100% then bringing up the volume to 1 mL with DMSO 100% using eppendorf tubes size 2 mL. The same procedure was used to prepare the concentration of 25 mg /mL, except the weight of VOs was 25 mg.

2.3.7.5 Preparation of antimicrobial agents

a. Antibacterial agents

The antibacterial agents used for susceptibility tests on the nine bacterial strains that were used with *M fruticosa serpyllifolia* VOs, were dissolved in a certain volume of relevant solvents according to solubility test to obtain stock solution:

Azithromycin was dissolved in Ethanol 95% to get stock solution of concentration (4.5 mg/mL), two dilutions of (1:10) were made to get concentration (45µg/mL) carried out on all bacterial strains.

Levofloxacillin was dissolved in sterile distilled water to acquire stock solution of concentration (3.28 mg/mL), three dilutions of (1:10) were made to get concentration (3.28 µg/mL) was performed on *Staphylococcal enterotoxin* B (SEB), MRSA, *Enterococcus faecium* and *Klebsilla pneumoniae*, and a fourth dilution was made of (1:10) to get concentration (0.328 µg/mL) was performed on *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella sonnie*.

Cefuroxime was dissolved in Dimethyl sulfoxide (DMSO) 100% to obtain a stock solution of concentration (3.77 mg/mL), two dilutions of (1:10) were made to get concentration (37.7 µg/mL), was tested on all bacterial strains. **Doxycycline** was dissolved in DMSO 100% to get a stock solution of concentration (6.20 mg/mL), three dilutions were made of

(1:10) to get concentration (6.20 µg/mL), was carried out on all bacterial strains [82-85].

b. Antifungal agents

The antifungal agents used for susceptibility tests on *Candida albicans*, used with *M fruticosa serpyllifolia* VOs, were dissolved in a certain volume of relevant solvents according to solubility test to obtain stock solution:

Terbinafine was dissolved in equal portion of sterile distilled water and methanol (1:1) to get a stock solution of concentration (50 mg/mL), two dilutions were made of (1:10) to obtain concentration (500 µg/mL).

Tinidazole was dissolved sterile distilled water to get a stock solution of concentration (50 mg/mL), two dilutions were made of (1:10) to obtain concentration (500 µg/mL) [86].

2.3.7.6 Antimicrobial assays

The susceptibility tests carried out on microorganism were determined using broth microdilution method and agar dilution method as defined by procedure in the following references with some modifications [40, 70, 87, 88].

a. Antibacterial assay

Broth microdilution method was used to evaluate the Minimum inhibitory concentration (MIC) values to estimate the antibacterial activity.

Overnight cultures of four Gram-positive bacteria i.e. *Staphylococcus aureus*, *Staphylococcal enteotoxin* B (SEB), *Enterococcus faecium* "methicillin"-resistant *Staphylococcus aureus* (MRSA) and five Gram-negative strains; *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnie*, *Klebsilla pneumoniae* were adjusted to 0.5 McFarland standard turbidity. Sterile Mueller Hinton broth (100 µL) for bacterial investigation was added to all microplate wells using multichannel pipette (30-300 µL) . The stock solutions of samples (100 µL) were added to microplate wells in duplicate; at row A and B for first sample, C and D for the second sample, E and F for the third sample, G and H for DMSO 100 % to detect the presence of antibacterial activity for DMSO in broth microdilution method conditions. Then, using multichannel pipettes (30 - 300 µL) 100 µL of the mixture (the samples or DMSO 100% and the sterile broth) from wells number one was transferred to wells number two in order to obtain (2-fold) serial dilution and so on till row wells number 11, wells number 12 served as positive control without plant extracts or DMSO 100%. The inoculate (1µL) from bacterial suspension was added to each well using multichannel pipettes (1-10 µL). The entire process was conducted under aseptic conditions. The final concentrations of bacteria were 2.5 $*10^5$ CFU/mL. The concentrations of *M fruticosa serpyllifolia* VOs were ranged from 0.049 to 25 mg/mL and for DMSO 100% from 0.098% to 50%. Wells numbers 11 were served as a negative (free of inoculate) controls. Plates were incubated for 16-20 hours at 35°C. Bacterial growth was indicated by the presence of turbidity in the wells.

The lowest concentrations where did not show any bacterial growth were estimated as the MIC value. In order to control the sensitivity of the tested microorganisms, the MIC of antibacerial agents; Azithromycin, Levofloxacillin, Doxycycline, Cefuroxime were also evaluated in parallel experiments.

b. Antifungal assay

Broth microdilution method was used to evaluate the Minimum inhibitory concentration (MIC) values to estimate the activity against *C. albicans*. The same procedure of bacterial strains was used for *C. albicans* with some modifications. Sterile RPMI 1640 medium (100 µL) was used for *C. albicans* investigation, the inoculate of *C. albicans* was (100 µL) was added to each well using multichannel pipettes (30-300 µL). Thus the final concentration of *C. albicans* was in the range of ($0.333*10^3$-$1.666*10^3$ CFU/mL); the concentrations of *M fruticosa serpyllifolia* VOs were ranged from $8.467*10^{-4}$ to 16.666 mg/mL and for DMSO 100% from $1.69*10^{-3}$ to 33.33%. Plates were incubated for 48 hours at 35°C. The growth of *C. albicans* was indicated by the presence of pellets at the bottom of the wells. The lowest concentrations which did not show any *Candidal* growth were estimated as the MIC value. In order to control the sensitivity of the tested *C. albicans*, the MIC of antifungal agents; Terbinafine and Tinidazole were also evaluated in parallel experiments.

Agar dilution method was used to evaluate the Minimum inhibitory concentration (MIC) values to estimate the activity against

Epidermophyton floccosum. The sterilized SDA slant tubes with one mL medium were directly placed in water bath at 40ºC and in a quick manner and under aseptic conditions, one mL of *M fruticosa ssp. serpyllifolia* VOs samples or of DMSO 100% (to insure the antifungal activity of DMSO) was added to the first tube, quickly mixed and one mL was transferred to the second tube and so on (serial dilution), the tubes number six were served as positive control (no plant extract or DMSO), the tubes were directly slanted after transferring 1 mL. The concentrations of *M fruticosa serpyllifolia* VOs samples were ranged from 0.78 to 12.5 mg/mL and for DMSO were from 3.125% to 50%. Each sample of VOs or DMSO has 6 tubes and the assay was done in duplicate to insure antifungal properties. All slanted tubes were tightly closed and incubated at room temperature for 24 hours. Then after, all the tubes were inoculated with 20 µL of *Epidermophyton floccosum* suspension which was prepared previously above. The tubes were closed tightly with cotton and incubated for two weeks at room temperature, after two days of incubation the tubes were sealed with parafilm M till two days before the end of incubation time and then the parafilm M was removed. The plates were evaluated for the presence or absence of white fungal growth at the end of the incubation period. The lowest concentration at which the absence of growth was registered and identified as the MIC value was identified.

2.3.8 Statistical Analysis

Statistical analysis was conducted using One-way ANOVA with post-hoc Tukey-Kramer HSD multiple comparison calculation, *p* values of 0.05 or less were considered statistically significant [89].

Chapter Three
Results

Chapter Three
Results

The main goal of our study was to screen the chemical constituent and the potential pharmacological activities of three samples of *Micromeria fruticosa serpyllifolia* volatile oils representing three regions in West Bank of Palestine and finally to make comparison between the findings.

3.1 Volatile oils analysis

Volatile oils of the three samples of *M fruticosa serpyllifolia* were extracted using Microwave-Ultrasonic Apparatus method, the produced oils were viscous, colorless and with a nice peppermint smell. Studying the VOs yields of the aerial parts of *M fruticosa serpyllifolia* samples, indicated variations between the three samples: 0.67 ± 0.29%, 0.99 ± 0.55% and 0.70 ± 0.17% (w/w%) from Nablus, Ramallah and Hebron respectively (Table 1). The data were expressed as mean ± STDV (n=3). The chemical analysis conducted using GC-MS, characterized the VOs with seven components classified into oxygenated ingredients mainly ketones and non-oxygenated ingredients mainly hydrocarbons in all three samples with different proportions (Table 1and Figure 8). The most abundant components in all of three samples were pulegone and isomenthone. The total identified components in the three samples were almost consistent in which 90.48, 94.44 and 93.55% of the constituents were identified in Nablus, Ramallah and Hebron districts respectively. Detailed results are represented in Table 1 and Figure 8. Other five common compounds identified in all of three samples with total

percentage less than 2% were: D- Limonene, β-Pinene, Isocaryophyllene, α-Pinene and β-Myrcene (Table 1). Referring to the Appendices components 1, 2 and 3 the basic chemical structure chromatograms of *M fruticosa serpyllifolia* VOs from Nablus, Ramallah and Hebron were shown in chromatograms. The results of GC-MS analysis were shown in Appendices 4, 5 and 6. The chemical analysis of *M fruticosa serpyllifolia* VOs from three cities in Palestine was illustrated in Figure 8.

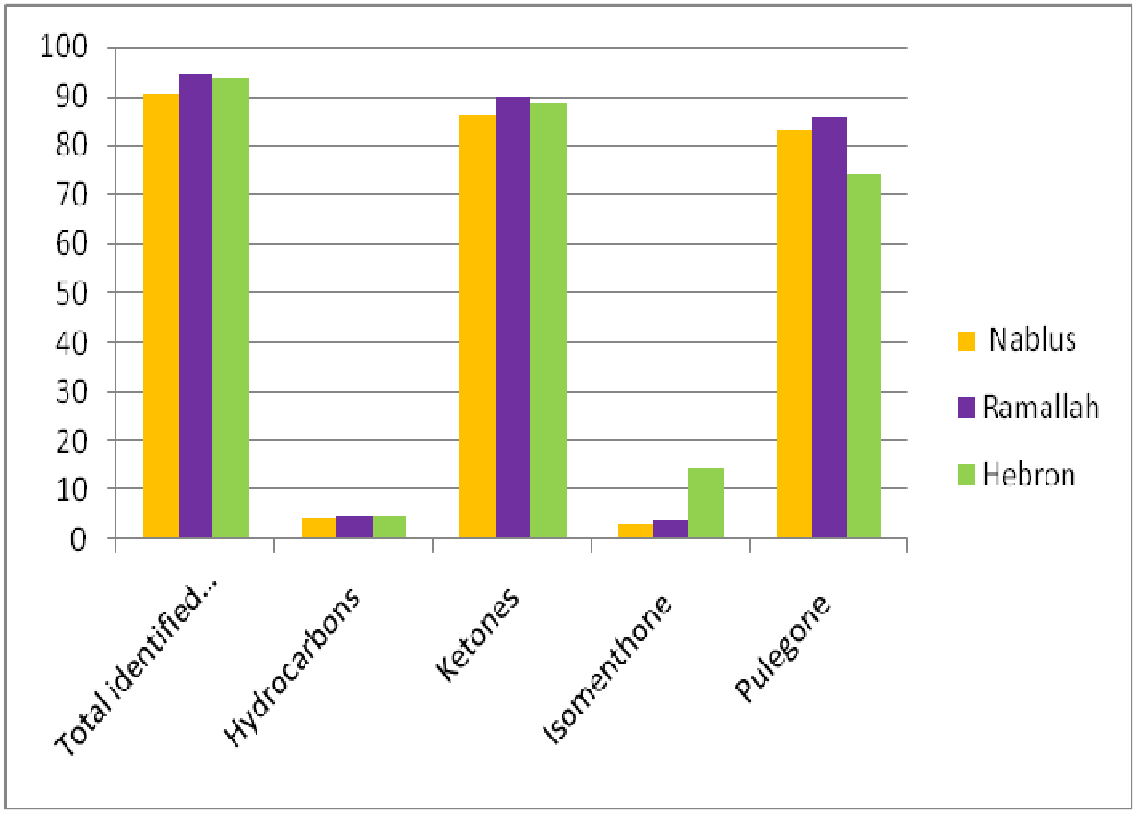


Figure (8): Chemical analysis of three samples *M fruticosa serpyllifolia* VOs

Table (1): The total % of yields, chemical compounds, total identified compounds, and chemical groups of tree samples of *M fruticosa serpyllifolia* VOs

	% total VO Nablus	% total VO Ramallah	% total VO Hebron
(w/w) % yield	0.67% ± 0.29	0.99% ± 0.55	0.70% ± 0.17
α-Pinene	0.91	0.71	0.83
β-Pinene	1.48	0.94	1.08
β-Myrcene	< 0.04	0.26	0.35
D- Limonene	1.73	1.65	1.26
Isocaryophyllene	0.26	1	1.19
Isomenthone	3.16	3.84	14.41
Pulegone	82.94	86.04	74.43
Total non-oxygenated constituents	4.38	4.56	4.71
Total oxygenated constituents	86.1	89.88	88.84
Total identified components %	90.48	94.44	93.55

3.2 Antioxidant

DPPH assay was used as *in vitro* approach to determine the free radical-scavenging activity and to screen for the possible antioxidant activity of the *M fruticosa serpyllifolia* VOs from different regions in Palestine. IC_{50} values were used to assess the ability of the examined samples to inhibit DPPH, they identified the amount of antioxidant required to inhibit the radical (DPPH) concentration by 50% and they inversely linked to their antioxidant activities. The assay revealed that the three samples of VOs exhibited DPPH free radical less than 50%, except for Ramallah VO sample that showed the highest scavenging effect of (50.19 ± 0.65%) at concentration 100 µg/mL, and highest potency with IC_{50} (0.45 ± 0.01 µg/mL). The VOs samples from Nablus and Hebron showed lowest

potency and scavenging effect. Trolox owned scavenging effect (96.80 ± 2.83) and potency at IC_{50} value (0.64 ± 0.12 µg/mL). The results of IC_{50} and the DPPH radical scavenging effect of the three samples and Trolox were shown in (Table 2 and Figure 9). Statistical analysis using one-way ANOVA was performed to compare the antioxidant potency (IC_{50}) and efficacy among samples. The three VOs samples showed higher antioxidant potency compared to Trolox. There were significant differences in antioxidant potency and efficacy of VOs compared to Trolox $p < 0.05$ or < 0.01. There were significant differences in antioxidant efficacy of VOs compared to each other $p < 0.05$ or < 0.01, but there were no significant differences in antioxidant potency of VOs compared to each other $p > 0.05$.

Table (2): IC_{50} of DPPH radical scavenging activity of *M fruticosa serpyllifolia* VOs from different regions of Palestine and Trolox

	Trolox	**Nablus**	**Ramallah**	**Hebron**
IC_{50} µg/mL	$0.64 \pm 0.12^{*}$	0.47 ± 0.02^{a}	0.45 ± 0.01^{a}	0.47 ± 0.01^{d}
% DPPH radical scavenging activity	96.80 ± 2.83	49.25 ± 0.33^{d}	50.19 ± 0.65^{bd}	45.29 ± 0.43^{cde}

$^{a}p < 0.05$ compared to trolox, $^{b}p < 0.05$ compared to Nablus, $^{c}p < 0.01$ compared to Ramallah, $^{d}p < 0.01$ compared to trolox, $^{e}p < 0.01$ compared to Nablus, *mean ± STDV, n=3

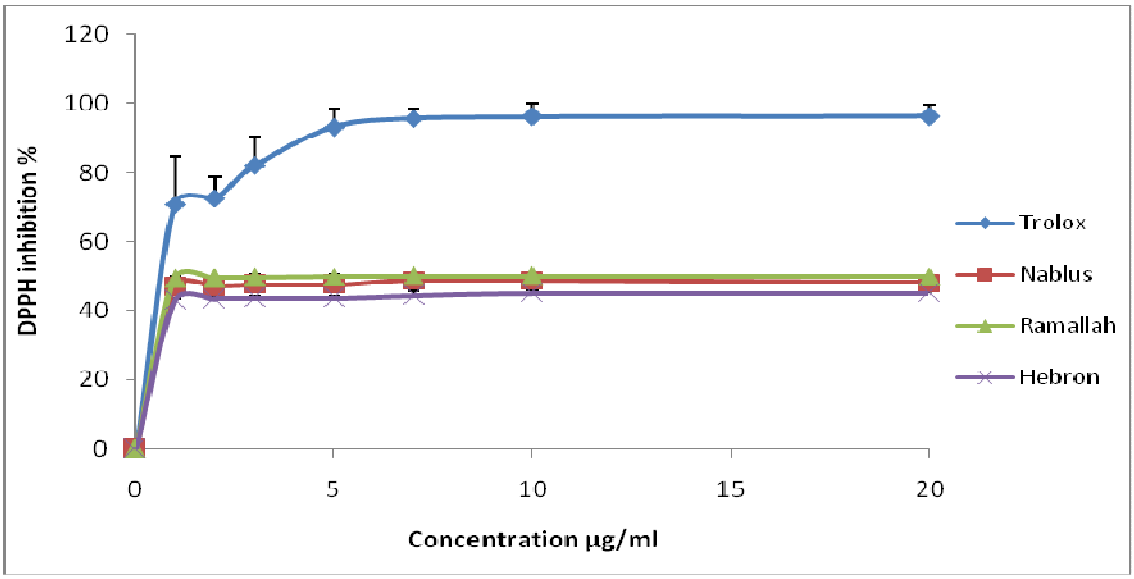


Figure (9): DPPH radical scavenging activity of the three samples *M fruticosa serpyllifolia* VOs and Trolox

3.3 Lipase inhibition assay

The hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol was used to measure the influence of *M fruticosa serpyllifolia* VOs of three samples on pancreatic lipase enzyme. The assay detected by comparing to Orlistat a strong lipase inhibitory agent, the three VOs samples showed nearly the same activity but the VO sample from Hebron showed the highest potency with IC_{50} value of (85.00 µg/mL) whilst the VOs sample obtained from Nablus showed the highest I% effect (65.40%). However, Orlisat owned potency at IC_{50} of 91.00 µg/mL with antilipase inhibition of (99.13%). The results of IC_{50} values and the antilipase activity of the three samples and Orlistat were shown in (Table 3and Figure 10). Comparative statistical analysis of the findings of the three samples of VOs showed that there were significant differences in antilipase potency and efficacy of VOs compared to Orlistat ($p < 0.01$). In addition, there were significant differences in antilipase potency and efficacy of VOs compared to each other $p < 0.01$,

but there were no significant differences in antilipase potency between the samples of Nablus and Ramallah $p > 0.05$.

Table (3): Lipase inhibition assay of the three samples of *M fruticosa serpyllifolia* VOs and Orlistat

	Orlistat	Nablus	Ramallah	Hebron
IC_{50} µg/mL	91.00	87.00[a]	87.00[a]	85.00[abc]
Antilipase activity	99.13%	65.41%[a]	54.94%[ab]	36.92%[abc]

[a] $p < 0.01$ compared to Orlistat, [b] $p < 0.01$compared to Nablus sample, [c] $p < 0.01$ compared to Ramallah

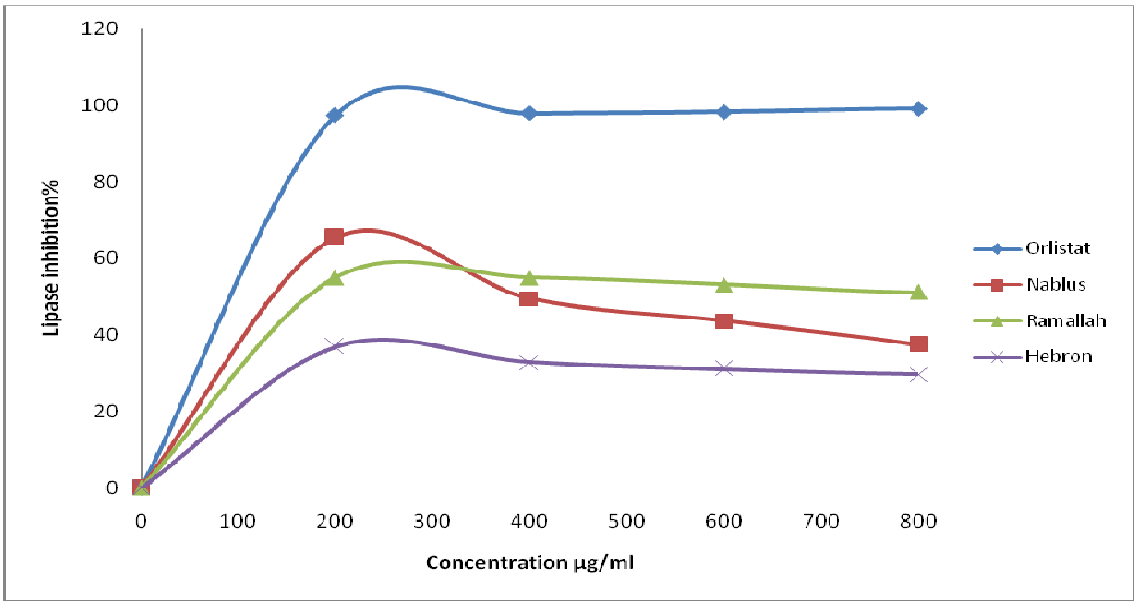


Figure (10): Lipase inhibition assay of the three samples of *M fruticosa serpyllifolia* VOs and Orlistat

3.4 α-Amylase inhibition assay

In vitro assay of alpha amylase inhibitory activities by using starch as a substrate and Acarbose as a positive control was conducted on *M fruticosa serpyllifolia* VOs of three samples. The outcomes revealed that the three samples of VOs showed different degree of inhibition. Nablus VO sample showed highest potency with IC_{50} value of (3.00 µg/mL) and

highest antiamylase activity (64.34%), the VOs samples from Ramallah and Hebron showed nearly the similar potencies and activities, while Acarbose showed potency at IC_{50} value (9.00 µg/mL) and I% effect (91.39%). The results of IC_{50} values and the antiamylase activity of the three samples and Acarbose were illustrated in (Table 4 and Figure 11). The three VOs samples showed higher potency in α-Amylase inhibition compared to Acarbose. There were significant differences in antiamylase potency and efficacy of VOs compared to Acarbose and compared to each other $p < 0.01$.

Table (4): α-Amylase inhibition assay of the three samples of *M fruticosa serpyllifolia* VOs and Acarbose

	Acarbose	Nablus	Ramallah	Hebron
IC_{50} µg/mL	9.00	3.00^{a}	3.50^{ab}	3.60^{abc}
antiamylase activity	91.39%	$64.34\%^{a}$	$23.77\%^{ab}$	$25.00\%^{abc}$

$^{a}p < 0.01$ compared to Acarbose, $^{b}p < 0.01$ compared to Nablus, $^{c}p < 0.01$ compared to Ramallah

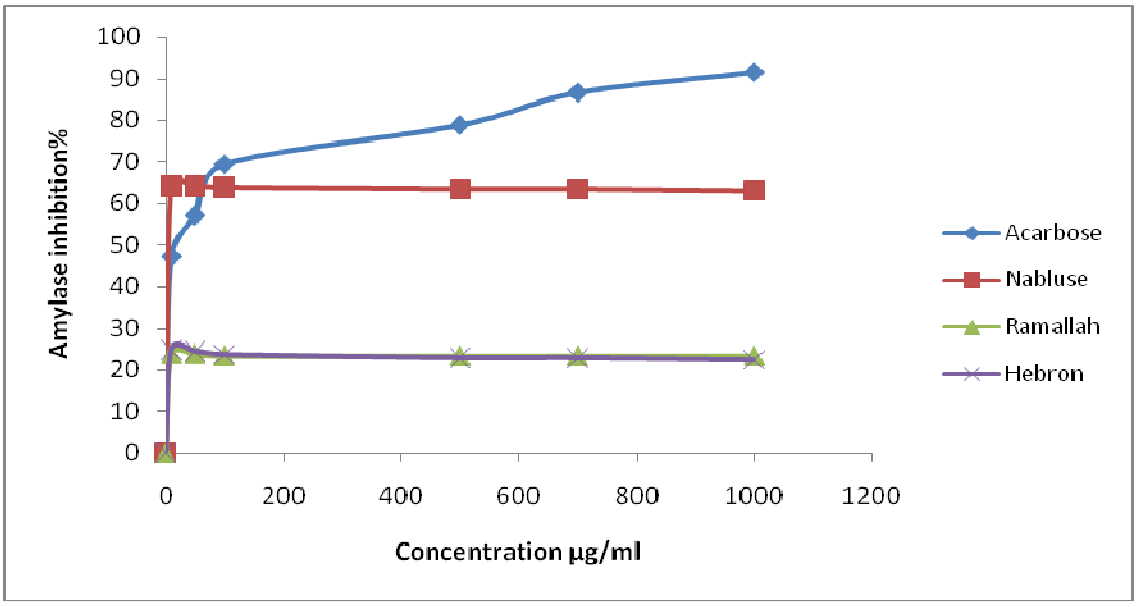


Figure (11): Amylase inhibition assay of *M fruticosa serpyllifolia* VOs from different regions of Palestine

3.5 Antimicrobial activity

3.5.1 Antibacterial activity

The minimum inhibitory concentrations (MIC) of *M fruticosa serpyllifolia* VOs from different regions of Palestine were reported in Table 5. The majority of Gram (+) and Gram (-) bacterial strains were sensitive to *M fruticosa serpyllifolia* VOs at MIC of 3.13 mg/mL. There were no statistically significant differences in activity against nine microbial strains between *M fruticosa serpyllifolia* VOs from the three regions in Palestine. There were significant differences of Hebron VO sample compared to Nablus and Ramallah VOs samples against the American Type Culture Collection *Shigella sonnie* (ATCC 25931), $p < 0.01$, the VOs sample from Hebron had the highest potency at MIC value of 1.56 mg/mL. There were significant differences of Hebron and Ramallah VOs samples compared to Nablus VOs samples against two clinical isolate (CI) of Gram (+) bacterial strains; *Staphylococcal enterotoxin* B (SEB) and MRSA, $p < 0.01$, the VOs sample from Nablus provided the lowest potency at MIC value of 6.250 mg/mL. To evaluate the sensitivity of bacterial strains, four antibacterial drugs were used: Azithromycin 250 mg, Levofloxacin 500 mg, Doxycyclin 100 mg, Cefuroxime 250 mg. The MICs values of the drugs were in the range ($1.28125*10^{-6}$ mg/mL-$22.5*10^{-3}$ mg/mL), Table 6 listed the MICs for drugs.

3.5.2 Antifungal activity

Among the fungal strains were tested for sensitivity to *M fruticosa serpyllifolia* VOs, the yeast was the most sensitive followed by the fungus;

the American Type Culture Collection *C. albicans* (ATCC 90028) yeast was found to be the most sensitive to *M fruticosa serpyllifolia* VOs samples at MIC value of (0.206 mg/mL) followed by the fungus *Epederophyton floccosum* (ATCC 52066) at VOs MIC value of (0.78 mg/mL) (Table 5 and Appendices 7, 8). To evaluate the sensitivity of fungal strains, two antifungal dugs were used: Terbenafine 250 mg and Tenidazole 500 mg and the MIC value of antifungal drugs was (18.52 µg/mL) Table 6.

Table (5): Antimicrobial activity (MIC in mg/mL) of *M fruticosa serpyllifolia* VOs from different regions of Palestine based on broth microdilution method and agar dilution method

	MIC MFN	**MIC MFR**	**MIC MFH**	**DMSO 100%**
Yeast				
***C. albicans* (ATCC 90028)**	0.206	0.206	0.206	3.70%
Fungus				
***Epederophyton floccosum* (ATCC 52066)**	0.781	0.781	0.781	6.25%
Bacterial strains				
***Staphylococcus aureus* (ATCC 25923)**	3.125	3.125	3.125	12.50%
***Staphylococcal enterotoxin* B (SEB) CI**	6.250	3.125[a]	3.125[a]	12.50%
MRSA (CI)	6.250	3.125[a]	3.125[a]	12.50%
***Enterococcus faecium* (ATCC 700221)**	3.125	3.125	3.125	6.25%
***Escherichia coli* (ATCC 25922)**	3.125	3.125	3.125	12.50%
***Pseudomonas aeruginosa* (ATCC 27853)**	3.125	3.125	3.125	12.50%
***Shigella sonnie* (ATCC 25931)**	3.125	3.125	1.5625[ab]	12.50%
***Proteus mirabilis* (CI)**	3.125	3.125	3.125	12.50%
***Klebsiella pneumoniae* (ATCC 13883)**	3.125	3.125	3.125	12.50%

[a]$p < 0.01$compared to Nablus, [b]$p < 0.01$ compared to Ramallah

Table (6): MICs (µg/mL) of Antimicrobial drugs

	Azithromycin	**Levofloxacin**	**Doxycycline**	**Cefuroxime**
Bacterial strains				
***Staphylococcus aureus* (ATCC 25923)**	0.352	$5.125*10^{-3}$	0.012	2.356
***Staphylococcal enterotoxin* B (SEB) CI**	0.352	$6.4*10^{-3}$	0.097	4.713
MRSA CI	0.176	$6.4*10^{-3}$	0.097	4.713
***Proteus mirabilis* CI**	5.625	$1.28*10^{-3}$	0.387	4.713
***Pseudomonas aeruginosa* (ATCC 27853)**	0.703	$1.28*10^{-3}$	0.387	2.356
***Escherichia coli* (ATCC 25922)**	0.703	$1.28*10^{-3}$	0.012	2.356
***Klebsiella pneumoniae* (ATCC 13883)**	1.406	0.012	0.387	4.713
***Shigella sonnie* (ATCC 25931)**	0.703	-	0.387	2.356
***Enterococcus faecium* (ATCC 700221)**	22.5	1.64	0.0.097	4.713
	Ternenafine	**Tinidazole**		
***Candida albicans* (ATCC 90028)**	18.5185	-		

Chapter Four

Discussion

Chapter Four
Discussion

4.1 Chemical analysis

The yields of *M fruticosa serpyllifolia* VOs in the current study were lower than the findings of a study conducted in Palestine by Shehab *et al*., [36] which reported a yield of VOs of (2.2%). Also our data were lower in yield than that of *M fruticosa serpyllifolia* growing in Turkey examined by Gulluce *et al*., [40] who reported a yield of (1.85%) of VOs of the plant collected in the flowering period.

The GC-MS analysis under the conditions mentioned above, identified seven compounds listed in Table 1. *M fruticosa serpyllifolia* VOs chromatographic profiles of the three samples and the analysis the results (Appendices 1,2,3,4,5 and 6) were dominated by oxygenated ingredients presented by ketones with the range of (86.1 - 89.88%) and hydrocarbons ranging from (4.38 -4.71%). Among the oxygenated compounds pulegone (Appendix 9) was the most distinguished component with a range of (74.43 - 86.04%) and isomenthone (Appendix 9) was detected to be the second most abundant component with a range of (3.16 - 14.41%). The non-oxygenated components detected were less than 2%: Isocaryophyllene, D-limonene, β-pinene, α-pinene and β- myrcene (Appendix 9). Studies conducted previously on *M fruticosa serpyllifolia* VOs growing in Palestine reported that pulegone, neo-Menthol and Isomenthone (Table 7) were the dominant compounds (Shehab *et al*., 2012) [36], for that growing in Lebanon Pulegone and D-limonene were the prevalent components (Table

7) (Al-Hamawi *et al*., 2011) [12] and for that growing in Turkey piperitenone, pulegone and Isomenthone were the most abundant components (Table 7) (Gulluce *et al*., 2004) [40]. Isa Telci1 and Mustafa Ceylan (2007) [38] reported that the VOs of subspecies of *Micromeria fruticosa* belong to different chemotypes: a) pulegone, linalool and *p*-menthone, b) piperitenone and linalool type, and revealed that pulegone was the most prominent compound in *Micromeria* species mainly in *M fruticosa*. In the current study Isocaryophyllene was lower than that identified in a previously published article conducted in Palestine (Table 7) [36]. The rest components in the current study such as D-limonene, β-pinene and α-pinene were presented in higher levels than that of the Palestinian sample and of the Turkey sample. β-myrcene was not identified in the Turkey sample [36, 40]. Pulegone, limonene, α-pinene, β-pinene, β-myrcene were also being identified in *M Barbata* growing in Lebanon but in different proportions [18, 90]. The differences in the total percentages of; yields, identified components and chemical compounds may be explained by the variations in environmental conditions including location, climate, seasonal factors and geographical factors [36]; the part of the plant studied and the growing period of leaves, the younger leaves were investigated to be rich mainly in pulegone accounted for 70% of the VOs [13], it was accounted for 29.19% of VOs in the flowering stage [40] and 58.5% before flowering period [36].

Table (7): Main components and their structures of *M fruticosa serpyllifolia* VOs from different origin

Origin	Sample period	Extraction method	Compound and concentration	Reference
Palestine	before flowering stage (March)	hydrodistillation	pulegone 58.5% neo-Menthol 8.7% Isomenthone 3.9% Isocaryophyllene 3.9%	Shehab *et al*., (2012) [36]
Turkey	flowering stage	hydrodistillation	piperitenone 50.61% pulegone 29.19% Isomenthone 3.92%	Gulluce *et al*., (2004) [40]
Lebanon	full flowering stage in July	hydrodistillation	Pulegone 30.41% D-limonene 15.64% Menthalactone10. 28%	Al-Hamawi *et al*., (2011) [12]

4.2 Antioxidant

Natural antioxidant have been widely investigated to find protective compounds against damages and diseases developed from free radicals and oxidative stress. *Micromeria* species were identified to be as a rich source of antioxidant agents [91]. DPPH assay was used as *in vitro* approach to investigate the free radical-scavenging function and to screen for the potential antioxidant activity of the three plant VOs samples. Different results were reported by Gulluce and coauthors in Turkey in which antioxidant activity of the VOs of *M fruticosa serpyllifolia* was observed with IC_{50} value of 98.2 µg/mL [40]. The study showed that VOs abundant with oxygenated monoterpene such as pulegone have antioxidant activity

[92, 93]. However, this result may be supported the antioxidant potency of Ramallah sample VOs, as owned the highest amount of total oxygenated compounds and pulegone (89.88 and 86.04%) respectively among the three samples of VOs.

4.3 Lipase inhibition assay

Overweight and obesity produced by increased caloric absorption, which may develop chronic diseases such as: arteriosclerosis, hyperlipidaemia, hypertension, coronary heart diseases, diabetes mellitus etc., can be ameliorated by suppressing or retarding lipid absorption. Pancreatic lipase (PL) is the key enzyme for breaking down triglycerides and controlling the hydrolysis of dietary fat [94]. Traditional medicinal plants are widely investigated for their phytochemicals to produce new lipase inhibitory agents for preventing or curing chronic diseases [74]. The anti-lipase properties of *M fruticosa serpyllifolia* VOs samples from different regions of Palestine were investigated by the inhibition of pancreatic lipase (PL). To the best of our knowledge, there were no previous studies conducted to explore the activity of *M fruticosa serpyllifolia* VOs against PL enzyme. The results of the current study showed that VO sample from Hebron owned the highest potency with IC_{50} value 85.00 µg/mL but with the lowest inhibition effect with 36.92%, (Table 3, Figure 10). However, through an *in vitro* screening of the phytochemical properties of 30 plants growing in Mexico, Villa-Ruano *et al*., (2013) [95], concluded that plant rich with sesquiterpenes and other

phytochemical (steroids, tannins, glycoside) showed very strong antilipase activity. Other studies reported that plant extracts rich in terpenes showed antilipase activity [96, 97]. Investigating *Pinus massoniana* L. volatile oil growing in China by Wang M *et al*.,(2017) [98], indicated that the dominant components were related to monoterpene and sesqueterpene (α-pinene, β-pinene, D-limonene and caryophyllene) and were responsible for antilipase activity of the oil at IC_{50} 25.10 ±0.49 µM .The phytochemical screening of the three samples of *M fruticosa serpyllifolia* VOs support the existence of monoterpenes and sesquiterpenes in all of three samples of VOs. Hebron sample owned the highest percentages of monoterpenes and sesqueterpines, while Nablus sample owned the highest percentage of (α-pinene, β-pinene, D-limonene), which support the highest antilipase potency and efficacy of Hebron and Nablus samples respectively.

4.4 Amylase inhibition assay

Diabetes mellitus is a leading endocrine disease, characterized by irregularity in lipid, carbohydrate and lipoprotein metabolism, causes many health complications such as hypertension, hyperlipidemia, atherosclerosis, hyperglycaemia and hyperinsulinemia, annually resulting in three million mortality. Some edible and traditional plants have been used to reduce diabetic symptoms. α-amylase is essential enzymes in metabolizing and absorption of starch, reduction of the enzyme can actively control blood glucose levels in diabetes mellitus patients. Therefore, plants extracts showing inhibition of α-amylase enzyme properties are useful in

controlling diabetes [99]. The anti-diabetic properties of *M fruticosa serpyllifolia* VOs samples from different regions in Palestine were investigated by the inhibition of α-amylase activity. According to our knowledge, there was no previous studies conducted for the purpose of assessing the activity of *M fruticosa serpyllifolia* VOs against α-amylase enzyme. The inhibition of α-amylase activity of *Sideritis galatica Bornm* VOs sample growing in Turkey studied by Zengin *et al*.(2016) [100], was related to abundant of monoterpene hydrocarbons ingredients mainly α-pinene and β-pinene. In screening the α-amylase inhibitory activity of J. *phoenicea* volatile oil growing in Tunisia, the results showed a powerful α-amylase inhibition properties due to presence of terpenes like α-pinene [76]. The *M fruticosa serpyllifolia* VOs sample from Nablus owned the highest amount of α-pinene and β-pinene components (0.91and 1.48%) respectively in comparing with samples of Ramallah and Hebron which may explained its highest potency against α-amylase enzyme.

4.5 Antimicrobial activity

Multidrug resistance of bacterial species causes health difficulties. Extracts of volatile oils have been investigated as new potential antimicrobial agents, biopreservative products and promising antiseptic enhancer for topical uses [101]. *Micromeria* species VOs were considered strong broad spectrum antimicrobial activity [102]. The antimicrobial properties of *M fruticosa serpyllifolia* VOs of the three samples from different regions of Palestine were examined against nine bacterial strains

(4 Gram (+) and 5 Gram (-)), one yeast and one fungal stains in recent study and were quantitatively determined by MIC values using microdilution and agar dilution methods. The results listed in Tables 5 showed that the VOs of the three samples exhibited considerable antifungal potency but little antibacterial potency. The results of antimicrobial activity of the VOs of three samples revealed that this activity was specific against *Shigella sonnie*, *Staphylococcal enterotoxin* B (SEB) and MRSA and non specific against the rest of microbial organisms. Screening for the potential antimicrobial activity of *M fruticosa serpyllifolia* VOs and methanolic extract, growing in Turkey conducted by Gulluce *et al*. (2004) [40] concluded that the VO provided broader spectrum and stronger antimicrobial properties than that of methanolic extract (methanolic extract did not show any antimicrobial activity), the MIC values of 0.5 mg/mL volatile oil stock solution (for bacterial species which were susceptible to the oil ranged from 31.25 to 125 µg/mL and for fungus which were susceptible to VO ranged from 31.25 to 62.50 µg/mL. A study conducted by Omari *et al*., (2016) [103] evaluating the antifungal activity of *M barbata* growing in Lebanon, using different fungal strains and yeasts, including *Epidermophyton floccosum* and *Candida albicans* concluded that the *M barbata* VOs showed a high fungistatic activity. Investigating the antimicrobial activity of *Micromria cilicica* VOs growing in Turkey resulted that the *Micromria cilicica* VOs and pulegone crude compound (the main component) showed a significant antifungal and antibacterial activity, the activities increased relying on the amount of pulegone and

VOs and *Candida albicans* was the most sensitive to pulegone [104]. *Micromeria congesta* VOs were considered as a significant antibacterial due to abundant components such as pulegone, isomenthone [105]. Studying the chemical ingredients, antibacterial and antifungal activity of the volatile oils of four plants including *Mentha spicata* growing in Iran by (Kasime *et al*.,2012) [106], and crude menthone (the dominant component) for antimicrobial activity, reported that VOs showed very strong antimicrobial properties against *Staphylococcus aureus*, all of *shigella species*, *Escherichia coli*, *Klebsiells sp*, *Pseudomonas aeruginosa*, *Proteus sp*, *Candida albicans* and other strains and concluded that menthone (isomenthone) exhibited strong antibacterial properties with MIC 1.5-3.5 µg/mL. These findings could be linked with our results obtained from Hebron in which the VOs of *M fruticosa serpyllifolia* owned the highest quantity of isomenthone (14.41%) (Table 1) which is thought to be effective against *Shigella sonnie*. On the contrary, isomenthone represented the lowest amount of constituents (3.16%) in Nablus (Table 1) and therefore, exhibited the lowest potency against *Staphylococcal enterotoxin* B (SEB) and MRSA.

Chapter Five

Conclusion and Recommendations

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5.1 Conclusion

M fruticosa serpyllifolia VOs from different regions in Palestine represented by three cities showed variable antioxidant, anilipase, antiamylae and antimicrobial activities depending on the phytochemical constituents of the volatile oils. *M fruticosa serpyllifolia* VOs of three regions owned the same chemical components but in difference proportions. The sample from middle Palestine (Ramallah) showed highest total percentages of yield, identified compounds and oxygenated compounds which may produce the antioxidant potency. The sample of south Palestine (Hebron) exhibited antlipase potency due to the presence of higher amounts isocaryophyllene and hydrocarbons. The sample from north Palestine (Nablus) exhibited highest anti-amylase activity due to higher amount of α-pinene and β-pinene. The plant extract exhibited strong antifungal activities and lower antibacterial activities. The sample of the south region showed higher potency against *Shigella sonnie* while the sample of the northern region showed lower potency against *Staphylococcal enterotoxin* B and MRSA. These findings enable *M fruticosa serpyllifolia* VOs to be a good agents in curing or preventing diabetes, hypercholesterolemia, healing wounds, skin dermatitis and good food preservative agent.

5.2 Recommendations

Future work may include:

1. Further *in vivo* studies are needed to evaluate the potential pharmacological activities.

2. Further studies are required to isolate the basic components responsible for potential pharmacological activities.

3. Further studies are required to evaluate the safety and toxicity of plant extract.

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109. National Center for Biotechnology Information. PubChem Compound Database; CID=440917, ***D-Limonene***. (accessed Nov. 16, 2017).

110. National Center for Biotechnology Information. PubChem Compound Database; CID=6654, ***Alpha-pinen***. (accessed Nov. 16, 2017).

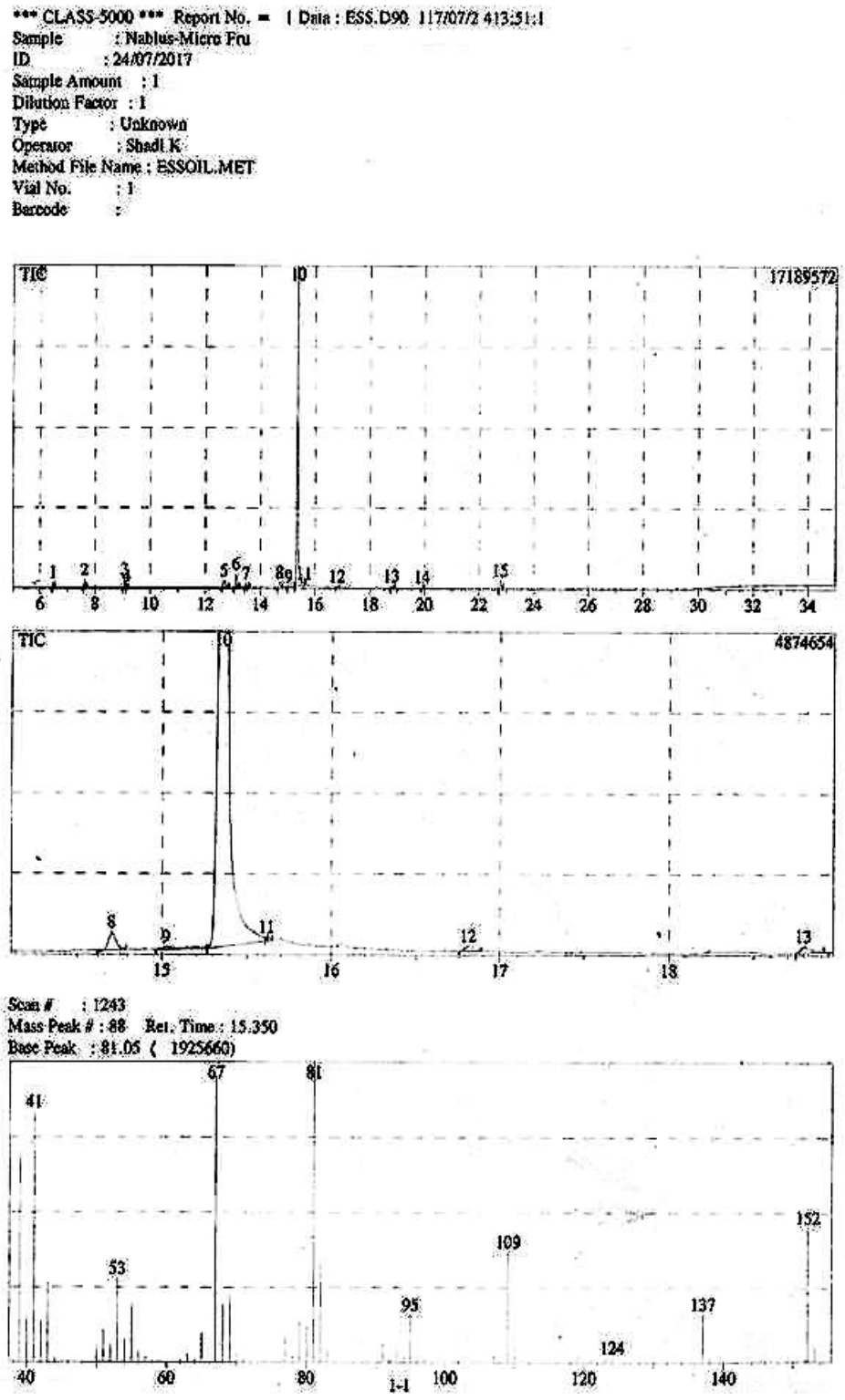
111. National Center for Biotechnology Information. PubChem Compound Database; CID=5281522, ***Isocaryophyllene*** (accessed Nov. 15, 2017).

112. National Center for Biotechnology Information. PubChem Compound Database; CID=14896, ***Beta-Pinene***. (accessed Nov. 16, 2017).

113. National Center for Biotechnology Information. PubChem Compound Database; CID=31253, ***β- myrecene*** (accessed Nov. 16, 2017).

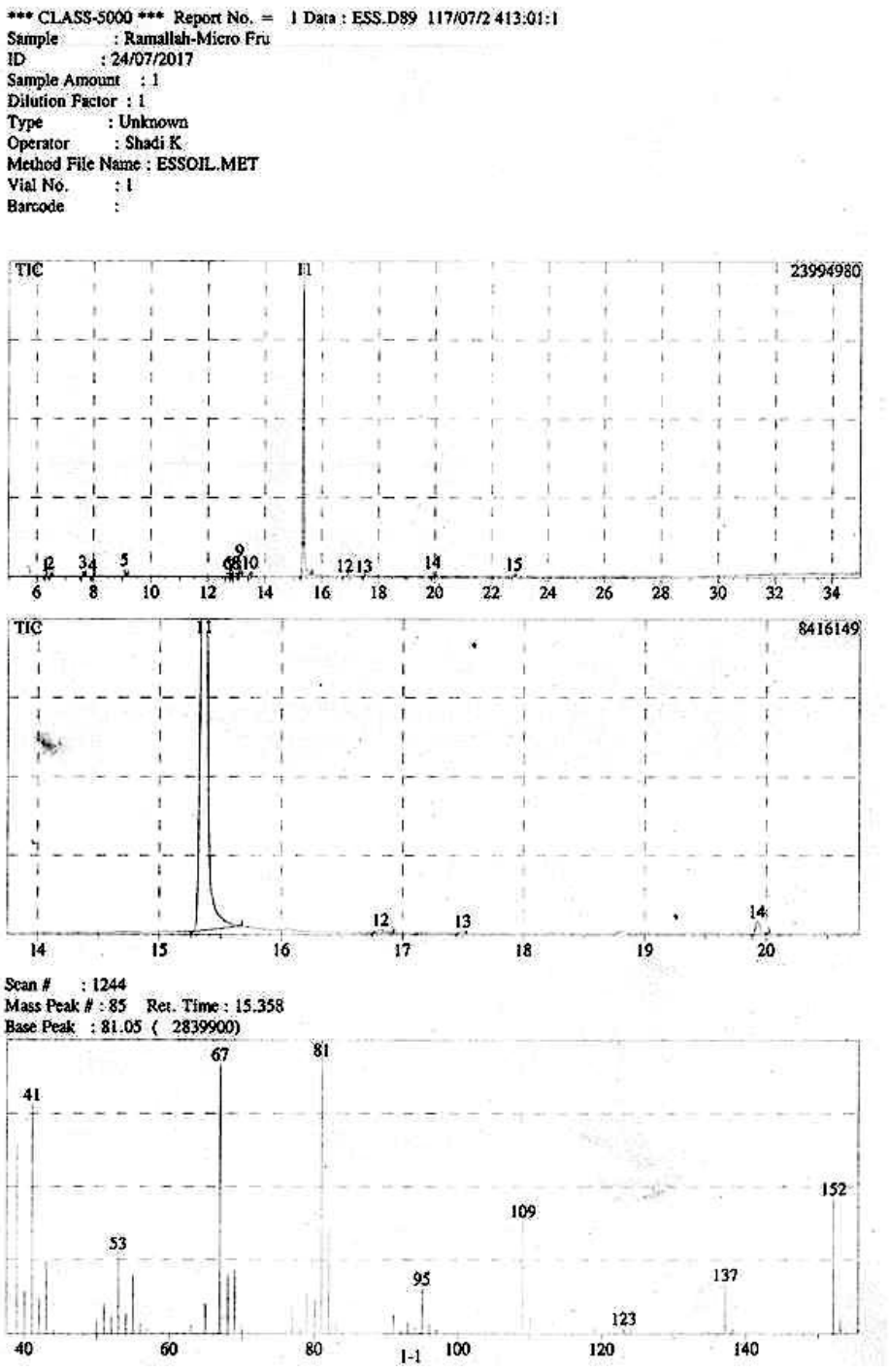
Appendix

Appendix 1



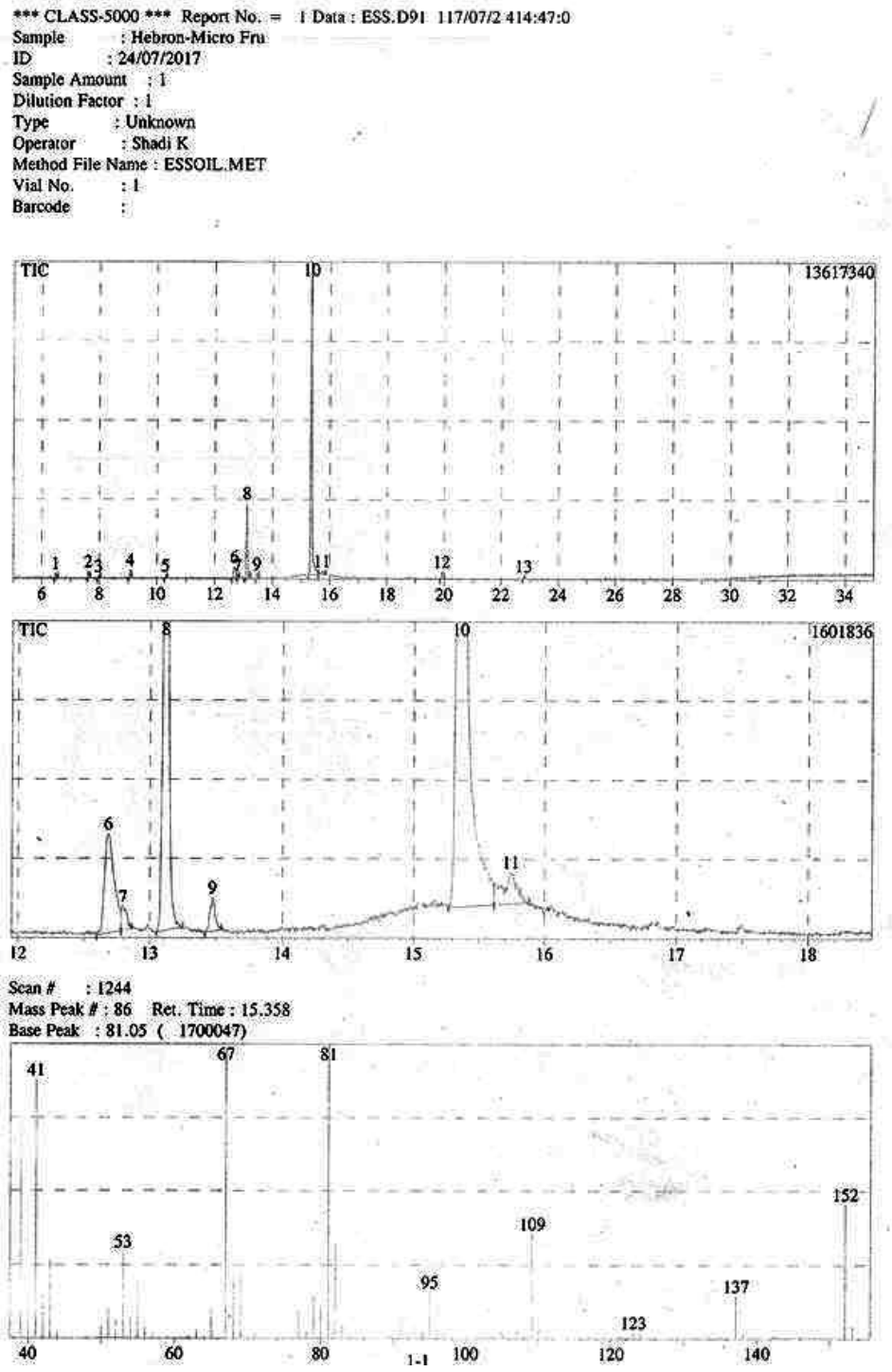
Chromatogram *of M fruticosa serpyllifolia* VO of Nablus

Appendix 2



Chromatogram of *M fruticosa serpyllifolia* VO of Ramallah

Appendix 3



Chromatogram of *M fruticosa serpyllifolia* VO of Hebron

Appendix 4

مـركز مختبـرات الفحـوص
Testing Laboratories Center
ISO/IEC 17025:2005

جامعة بيرزيت
BIRZEIT UNIVERSITY

Date: August 7, 2017

Sample Name: Micromeria Fruticosa (Nablus)

The sample was analyzed using GC/MS for essential oil composition, the results are presented in the following table:

NO.	Essential oil Name	% of Total Ess. Oil	Calculated Retention Index (Kovats)
1	Alpha - Pinene	0.91	941
2	Beta - Pinene	1.48	981
3	Beta - Myrcene	<0.04	993
4	D-Limonene	1.73	1031
5	Isomenthone	3.16	1170
6	Pulegone	82.94	1247
7	Isocaryophyllene	0.26	1405

Belal Amous
BZUTL Director

Shadi Kiabni
Technical Director

P.O.Box 14, Birzeit, Palestine, Tel +970 2 298 2102 - 298 2010, Fax +970 2 298 2166 ص ب ١٤، بيرزيت، فلسطين، هاتف ٢٩٨ ٢١٠٢ - ٢٩٨ ٢٠١٠ ٢ ٩٧٠+، فاكس ٢٩٨ ٢١٦٦ ٢ ٩٧٠+
BZUTL@birzeit.edu http://www.birzeit.edu/institutes/inv_occ/

Appendix 5

مـركز مختبـرات الفحـوص
Testing Laboratories Center
ISO/IEC 17025:2005

جامعة بيرزيت
BIRZEIT UNIVERSITY

Date: August 7, 2017

Sample Name: Micromeria Fruticosa (Ramallah)

The sample was analyzed using GC/MS for essential oil composition, the results are presented in the following table:

NO.	Essential oil Name	% of Total Ess. Oil	Calculated Retention Index (Kovats)
1	Alpha - Pinene	0.71	941
2	Beta - Pinene	0.94	981
3	Beta - Myrcene	0.26	993
4	D-Limonene	1.65	1031
5	Isomenthone	3.84	1170
6	Pulegone	86.04	1247
7	Isocaryophyllene	1.00	1405

Belal Amous
BZUTL Director

Shadi Kiabni
Technical Director

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Appendix 6

مـركز مختبـرات الفحـوص
Testing Laboratories Center
ISO/IEC 17025:2005

جامعة بيرزيت
BIRZEIT UNIVERSITY

Date: August 7, 2017

Sample Name: Micromeria Fruticosa (Hebron)

The sample was analyzed using GC/MS for essential oil composition, the results are presented in the following table:

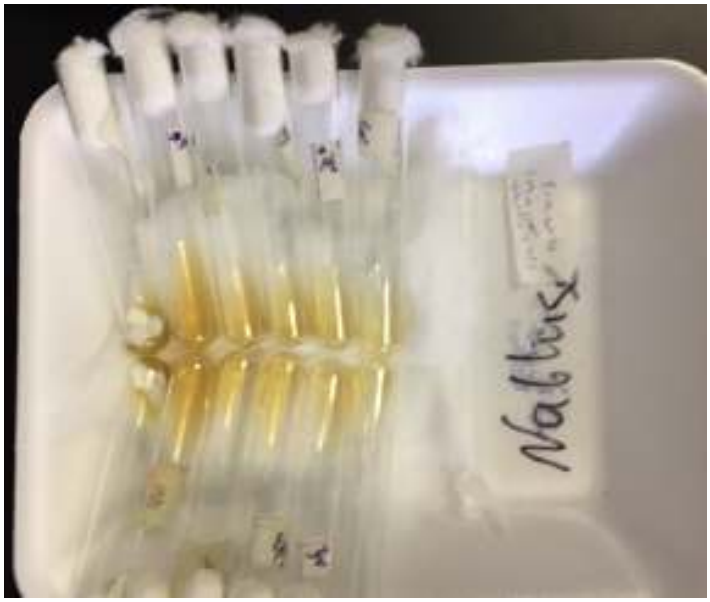
NO.	Essential oil Name	% of Total Ess. Oil	Calculated Retention Index (Kovats)
1	Alpha - Pinene	0.83	941
2	Beta - Pinene	1.08	981
3	Beta - Myrcene	0.35	993
4	D-Limonene	1.26	1031
5	Isomenthone	14.41	1170
6	Pulegone	74.43	1247
7	Isocaryophyllene	1.19	1405

Belal Amous
BZUTL Director

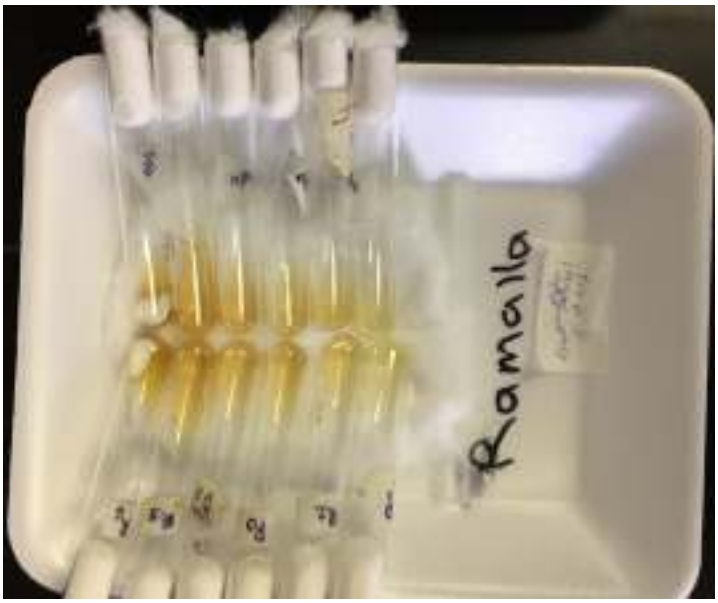
Testing Laboratories Center BIRZEIT UNIVERSITY

Shadi Kiabni
Technical Director

Appendix 7

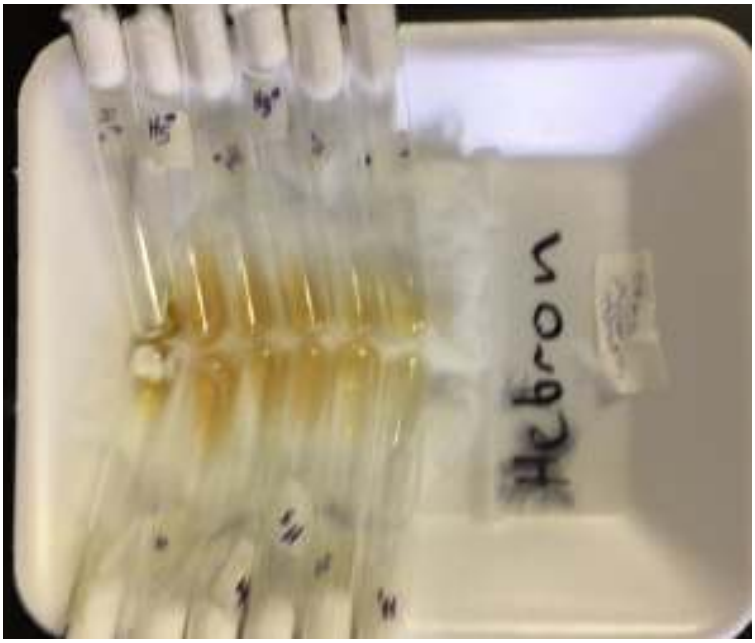


Effect of *M fruticosa serpyllifolia* VOs from Nablus on *Epedermophyton floccosum* using Agar dilution method

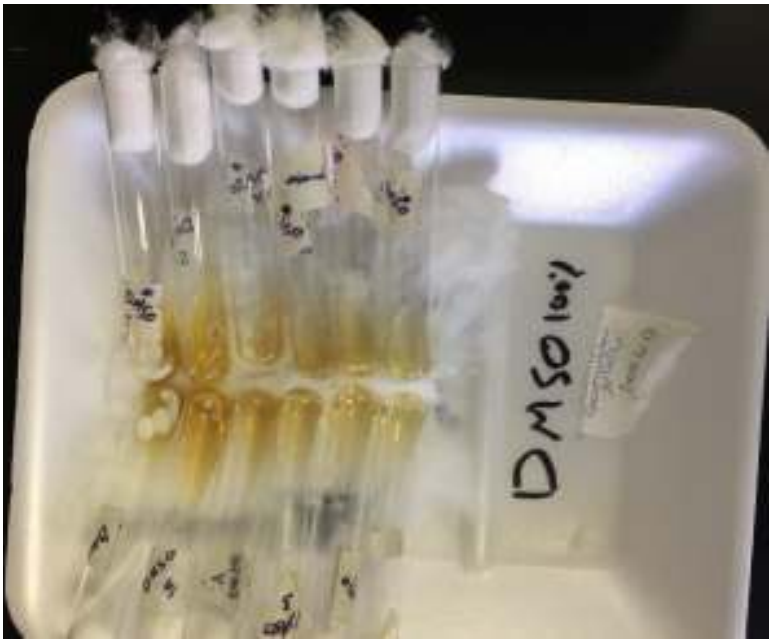


Effect of *M fruticosa serpyllifolia* VOs from Ramallah on *Epedermophyton floccosum* using Agar dilution method

Appendix 8

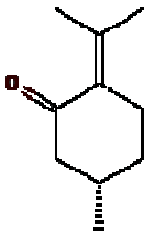


Effect of *M fruticosa serpyllifolia* VOs from Hebron on *Epedermophyton floccosum* using Agar dilution method

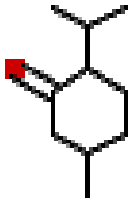


Effect of *M fruticosa serpyllifolia* VOs from DMSO 100% on *Epedermophyton floccosum* using Agar dilution method

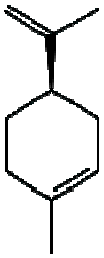
Appendix 9



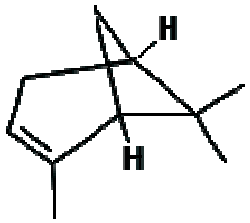
Pulegone [107]



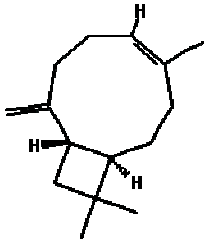
Isomenthone [108]



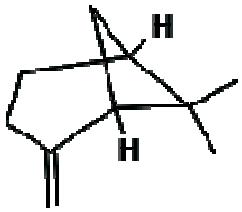
D-Limonene [109]



Alpha-Pinene [110]



Isocaryophyllene [111]



Beta-Pinene [112]

β-Myrcene [113]

Chemical structure of the main components of *M fruticosa serpyllifolia* VOs

كلية الدراسات العليا
جامعة النجاح الوطنية

التركيب الكيميائي والفحص الدوائي للزيوت الطيارة لنبتة "زعتر البلاط"، التي تم جمعها من الضفه الغربيه- فلسطين

إعداد
نهاية محمد يوسف سلامه

إشراف
د.نصر شريم
د. نضال جردات

قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.
2018م

التركيب الكيميائي والفحص الدوائي للزيوت الطيارة لنبتة "زعتر البلاط"،
التي تم جمعها من الضفه الغربيه– فلسطين

إعداد
نهاية محمد يوسف سلامه
إشراف
د.نصر شريم
د. نضال جردات

الملخص

الخلفية والاهداف:

(*Micromeria fruticosa serpyllifolia* (M.Bieb)) هي نبتة طبية عطرية، تنتشر في مناطق شرق البحر الابيض المتوسط بما في ذلك فلسطين. الرائحة العطرية التي تنتجها النبتة تكون برائحة النعنع الحار تعطي احساس بالبرودة في الصيف الحار. كان الهدف من هذه الرسالة هو فحص المكونات الكيميائية والخصائص الدوائية المحتملة للزيوت الطيارة لنبتة (*Micromeria fruticosa serpyllifolia*) من ثلاث مناطق مختلفة من فلسطين

الاساليب:

تم استخراج الزيوت المتطايرة من ثلاث عينات من (*Micromeria fruticosa serpyllifolia)* باستخدام جهاز (Microwave - ultrasonic). وتم تحليل المكونات الكيميائية لعينات الزيوت الطيارة باستخدام (GC-MS). تم فحص فاعلية الزيوت الطيارة من العينات الثلاثة كمضاد للأكسدة من خلال تثبيط (2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical ولفحص الفعالية كمثبط لانزيم (lipase) فقد تم استخدام (porcine pancreatic lipase (PL)) وايضا (*p*-nitrophenyl butyrate (PNPB). تم تقييم الفعالية كمضاد لانزيم (amylase) باستخدام (porcine pancreatic α-amylase)، والنشاء وايضا (3.5-dinitrosalicylic, DNSA). ولفحص الفعالية المضادة للميكروبات فقد تم استخدام طريقة (broth microdiltution) بشكل منفصل كمضاد للبكتيريا وايضا كمضاد للفطر.

وطريقة (agar dilution) للتقييم الفطري. تم اختبار تسعة سلالات بكتيرية أربعة موجبة غرام: *Staphylococcus aureus*, *Staphylococcal enterotoxin* B (SEB), *Enterococcus faecium*, "methicillin"-resistant *Staphylococcus aureus* (MRSA)، وخمسة سلالات سالبة غرام وكانت:

Proteus mirabilis, *Pseudomonas aeruginosa*, *Escherichia coli*,

Shigella sonnie and Klebsilla pneumonia.

وقد استخدام فطر واحد: (*Epidermophyton floccosum*)

وخميرة واحدة: (*Candida albicans*)

النتائج

كان معدل الناتج من الزيوت الطيارة في المدى (0.65 – 0.99٪) (w/w%). وأظهر تحليل GC-MS النسب المئوية من المكونات المؤكسدة كانت في المدى (86.1–89.88٪)، والمكونات غير المؤكسدة في نطاق (4.38–7.71٪)، وكان مجموع المركبات الموجودة في الزيوت الطيارة هو (90.48–94.44٪). وقد تم التعرف على سبعة مركبات، وكان (pulegone) من المكونات الأكثر وفرة في العينات الثلاث في نطاق (74.43–86.04٪)، وكان المكون (isomenthone) في الدرجة الثانية وكان في نطاق (16,16–41,14٪). العينة من رام الله (المنطقة الوسطى) كانت اكثر فعالية كمضاد للأكسدة بتركيز IC_{50} 0.45 µg/mL، واظهرت العينة من الخليل (المنطقة الجنوبية) اكثر فعالية كمثبط لانزيم (lipase) بتركيز IC_{50} 85.00 µg/mL. كما واظهرت العينة من نابلس (المنطقة الشمالية) اكثر فعالية كمثبط لانزيم (amylase) بتركيز IC_{50} 3.00 µg/mL. واظهرت العينات الثلاث نشاطا واسع النطاق كمضاد للميكروبات كما أظهرت العينات الثلاث نشاط غير محددة كمضاد للفطريات في الحد الأدنى من التركيز المثبط (MIC) في نطاق (0.206 to 0.781 mg/mL). كما وأظهرت العينة من محافظة الخليل (المنطقة الجنوبية) نشاطا محددا ضد (*Shigella sonnie*)

في الحد الأدنى من التركيز المثبط (MIC) (1.56 mg/mL) وكانت عينة نـــابلس (المنطقـــة الشمالية) أقل نشاطا ضد "*Staphylococcal enterotoxin* B (SEB) and 'methicillin resistance *Staphylococcus Aureus* (MRSA في الحد الأدنى مـــن التركيـــز المثـــبط (MIC) (6.25 mg/mL)، في حين أظهرت العينات الثلاثة نشاطا غير محدد ضـــد البكتريـــا التي تم اختبارها في الحد الأدنى من التركيز المثبط (MIC) (3.13 mg/mL).

استنتاج

أظهرت الدراسة أن عينات الزيـــوت المتطـــايرة لنيتـــة(*Micromeria fruticosa serpyllifolia*) من مناطق مختلفة من فلسطين تحتوي على نسب مختلفة من المواد الكيميائيـــة النباتية التي وفرت أنشطة بيولوجية محتملة مختلفة مثل: مضادات الأكسدة، والسمنة، ومضادات السكري، وأنشطة مضادات الميكروبات التي تتماشى مع الاستخدامات التقليديـــة للمستخلصـــات النباتية. بالاضافة الى ذلك فان المستخلصات النباتية اظهرت قوة اكبر من المراجع المســـتخدمة لتقييم الفعالية. وايضا اظهرت تفاوت كبير في النشاطات كمضـــاد للاكســـدة، ومثـــبط لانـــزيم (lipase) وايضا انزيم (amylase). انه من الضروري إجراء دراسات في الجسم الحي لتقيـــيم الأنشطة البيولوجية المحتملة وايضا تقييم الامان والســـمية للمســـتخلص النبـــاتي. وانـــه مـــن الضروري إجراء مزيد من التحقيقات لعزل العناصر الرئيسية المسؤولة عن الأنشطة البيولوجية وتحديدها وتوصيفها، وكذلك.