An-Najah National University

Faculty of Graduate Studies

VARIATIONS OF THE CHEMICAL COMPONENTS AND BIOLOGICAL ACTIVITIES OF *THYMUS CAPITATUS* ESSENTIAL OIL FROM THREE REGIONS IN PALESTINE

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This Thesis Dissertation was Defended Successfully on 29/5/2022 and approved by

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Dedication

This thesis is dedicated to:

The sake of Allah, my Creator, and my Master

My great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life

My homeland Palestine, the warmest womb

An-Najah National University, my second home

My academic advisers who guided me in this process

My parents, a source of inspiration who gave me strength when I thought of giving up

My husband a strong and gentle soul who always believed in my abilities and me even against better judgment

My sons who are the soul of life

My beloved brothers and sister; Abdullah, Qutaiba and Dana

My friends who encouraged and supported me,

All the people in my life who touched my heart, I dedicate this research

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Finally, I would like to thank my husband and my sons for their patience and encouragement to complete my studies.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

VARIATIONS OF THE CHEMICAL COMPONENTS AND BIOLOGICAL ACTIVITIES OF *THYMUS CAPITATUS* ESSENTIAL OIL FROM THREE REGIONS IN PALESTINE

I declare that 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: _____

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VARIATIONS OF THE CHEMICAL COMPONENTS AND BIOLOGICAL ACTIVITIES OF *THYMUS CAPITATUS* ESSENTIAL OIL FROM THREE REGIONS IN PALESTINE

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ABSTRACT

Introduction: In Palestinian traditional medicine, *Thymus capitatus* is a widely utilized medicinal plant. The main goal of this study was to assess the phytochemical content of *T.capitatus* essential oils (EOs) from three Palestinian regions using hydro distillation and microwave ultrasonic technologies. Also, the essential oil that was taken from the plant was put through some biological tests.

Methodology: identification and quantification of the various elements in the EOs examined were accomplished using GC-MS spectrometry. The DPPH assay and the β-carotene-linoleic acid assay were utilized in order to determine the levels of antioxidant activity. In order to determine whether or not *T.capitatus* possesses anti-lipase activity. α-amylase inhibitory activity of the EOs samples was studied in comparison with the hypoglycemic drug, Acarbose. An anti-microbial assay was conducted against seven different types of the most common bacteria and fungi. Additionally, Hep-G2 cells were used to assess the anticancer activity.

Results: The EOs presented several components, mainly monoterpenes, thymol and carvacroal. Chemical components of the EOs varied between districts (Ramallah: carvacrol (31.25%), γ-terpinene (30.94%), Jenin: γ-terpinene (67%), cis-b-terpineol (12.91%), Hebron: thymol (40.35%), b-Caryophyllene (13.23%) were the main components of the EOs in the districts). According to the results, the antioxidant activity of *T.capitatus* EOs was shown to be high and dose dependent. DPPH assay results showed that the three districts had nearly the same IC50, which was a fourth-fold of gallic acid. On the other hand, β-carotene-linoleic acid assay results showed that all samples had higher antioxidant efficiency than water (control) and the synthetic

antioxidant α-tocopherol, which gave the highest β-carotene degradation. *T.capitatus* EO worked against all bacteria and fungi that were tested in terms of antibacterial activity, with MIC values between 0.1953 and 1.5625 μg/ml. The Hebron sample gave distinguishable results at low concentrations. All samples showed anti-lipase activity even higher than Orlistat at concentrations equal to or higher than 200 g/ml. Furthermore, all three EO samples inhibited α-amylase concentration dependently; statistical analysis revealed a slight difference between the samples, but all of them showed nearly the same percent inhibition at 400 g/ml, which is 50% acarbose.

Finally, according to cytotoxic activity, all samples showed promising results against Hep-G2, with an average percent inhibition of 85% at a concentration of 62.5 μg/ml with slight differences between the districts.

Conclusion: The chemical structure of the EO of *T.capitatus* can be related to the plant's origin, soil components, genetic variables, and climatic conditions, which in turn reflect on the biological activity of it.

Key words: *Thymus capitatus*, hydro distillation, thymol, carvacrol, anti-lipase, DPPH

Chapter One

Introduction

1.1 General project overview

In the past decade, medicinal herbs have been seen as a possible source of therapeutic aids in health care systems around the world, both for people and for animals. Several scientific studies have been undertaken to find natural compounds extracted from herbs that have the potential to cure a variety of health conditions. Among these herbs are *thyme, rosemary,* and *oregano,* which are used mostly for their antioxidant properties [1, 2]. However, it is essential to keep in mind that the potency of herbal extracts is dependent on the substrate they work on and the dose supplied to patients.

Thymus capitatus is known in Palestine as ‘‘Zetmane". It's a common type of thyme that is known to have various biological effects, including antimicrobial and antioxidant activities [3, 4]. *T. capitatu* grows in different regions in Palestine under variable environmental conditions. In our study, the impact of these environmental conditions on the chemical composition of *T. capitatus* after extraction, as well as their influence on the essential oil's biological activity, were evaluated.

1.2 Herbal medicine as a source of bioactive compounds (advantages& disadvantages)

Despite the fact that there is still a gap between what is considered to be "scientific evidence" and what is truly used to treat sickness, herbal treatments are very popular in many parts of the world. Both misleading and factual premises support the medical community's rejection of herbal remedies. It is essential that herbal medicine be taught to all healthcare practitioners. Efforts must be made to conduct high-quality studies on herbal medicines in order to produce an "evidence-based herbal medicine" that will improve and protect human health and well-being. Herbal medications offer the advantage of having a more complex composition, which is comprised of a variety of active ingredients with multiple functions. Several possible theories have been floated for this phenomenon (cumulative effects, enhanced bioavailability, and synergy) [5, 6].

In recent years, in the developed world, people have returned to using herbal medicines and other forms of traditional medicine. Also, the World Health Organization (WHO) says that plants are the source of about a quarter of all medications that are currently prescribed, and that 65 percent of the world's population uses plants to stay healthy [7, 8].

The basic medicinal herb compounds to produce valuable synthetic preparations are secondary metabolites synthesized from these herbs. Newly, secondary metabolites have been revealed as potential new drugs, herbicides, insecticides, antibiotics, and active compounds in cosmetic preparations. Their therapeutic effect is related to their antioxidant, anti-aging, anti-cancer, anti-atherosclerotic, antibacterial and also anti-inflammatory activities [9, 10].

Due to the toxicity and side effects of allopathic medicines, the usage of herbal medicines and the number of their manufacturers have been expanded. Herbal medications have become increasingly popular among non-prescription users in recent decades. These medications have withstood thousands of years of human testing in the real world. Because of their toxicity, some medications have been withdrawn, while others have been changed or combined with additional herbs to counteract negative effects. One of the most significant benefits of these herbal medicines is that they are derived from natural sources. The body has a higher chance of balancing these nutrients in the system because they originate from a variety of foods. As a result, the body absorbs all of the needed nutrients with no side effects, unlike artificial treatments [11, 12].

In addition, there are several advantages of herbal remedies, such as low cost, complete accessibility, few side-effects, efficiency, and potency, which are very high. While their disadvantages are that they are unable to treat acute illnesses and accidents, there is a risk with self-dosing, and standardization is hard [13].

1.3 Essential oils (EOs) and the identification of active therapeutic compounds in T. capitatus EOs

In fact, essential oils of Thymus capitatus are classified as volatile molecules that are the output of specialized secretory structures found in medicinal plants, while in higher

medicinal plants, these EOs are biosynthesized via the secondary metabolism pathway [14].

In general, mainly two chemical groups form the basis of EO constituents, which are terpenoids (sesquiterpenes of low molecular weight and monoterpenes), and less commonly phenylpropanoids [15]. Comparing the EO chemical components' mass spectra to reference spectra in the computer library was also used to identify them [16].

Table 2.a (appendix 2) provides the major chemical composition of *T.capitatus* EOs in different sites around the world according to previous studies

1.4 Description and literature review of T. capitatus EOs

Thymus capitatus belongs to the *Lamiaceae* family, and it's native to the eastern Mediterranean region. As a perennial shrub with fragrant flowers and leaves, it grows in many places in the mountains of Palestine.

Monoterpenoids make up a large portion of its essential oil's composition (around 90 percent). Carvacrol, thymol, and p-cymene/γ-terpinene are all present in large quantities. Linalool, borneol, and 1,8-cineole are other essential components, but they are present in less amounts. Because of their pleasant scent and flavor, they are commonly used as a spice in the preparation of traditional meals in many regions of the world. In addition, *T.capitatus* EO has antibacterial, antifungal, and antioxidant effects. Because of this, it can stop the growth of many germs that cause diseases [22].

Since ancient times, people have turned to *T. capitatus* for a variety of medical purposes, including as an anthelmintic, carminative, antispasmodic, expectorant, sedative, stimulant, tonic, anti-inflammatory, and analgesic. *T. capitatus* essential oil has been certified for use due to the pharmacological effect it possesses, which includes both antioxidant and antibacterial properties [23, 24].

This herb, locally known under the common name (Zetmane), in the past was used to flavor meats, stews, and soups and was also used as raw material without any preliminary preparation [25, 26].

Figure 1.1

Thymus capitatus

The antimicrobial activity of *T.capitatus* EO has been tested against a wide range of bacteria and fungi, including gram-negative bacteria as *Escherchia coli* and *Klebsiella pneumoniae*, gram-positive bacteria as *Salmonella analum and Listeria monocytogenes*, fungi as *Mucor ramamnianus* and *Aspergillus ochraceus*, and yeast species like *Saccharomyces cerevisiae* and *Candida albicans*. The results showed that *E. coli, L. monocytogenes, and K. pneumonia* bacteria were suppressed by the tested *T.capitatus* EOs. Furthermore, considerable efficacy against fungi and yeasts was observed [27]. The radical cation 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate) and the free radical 2,2-diphenyl-1-picrylhydrazyl were examined in antioxidant activity experiments of *T. capitatus* EO, and the findings revealed strong inhibitory concentration values [28,29]. Tepe et al. conducted research on the in vitro antioxidant properties of essential oils of two *Thymus* species (*Thymus sipyleus* subsp. *sipyleus* var. *sipyleus*) extracts, and they presented their findings. About 71 compounds were identified and characterized by the presence of high monoterpene especially phenolic carvacrol, thymol, *p*-cymene, and γ-terpinene. The oils were tested

for their possible antioxidant activity by using (DPPH) and β-carotene/linoleic acid assays, and both of them showed high antioxidant activity in different proportion [28].

1.5 In vitro assessments based on free radical scavenging and enzymatic inhibition protocols for *T. capitatus* EOs

Herbal plants have long been thought to be good antioxidants. Thyme herb, as a potential source of pharmacological aids, has played an important part in global health systems for both humans and animals, not only in diseased conditions but also as a potential means for maintaining proper health. Thyme has been found to be a source of bioactive chemicals in several investigations. Thymol and carvacrol are two phenolic components of thyme oil [29]. An antioxidant's fundamental trait is its capacity to trap free radicals. Antioxidants like phenolic acids, polyphenols, and flavonoids get rid of free radicals like lipid peroxyl, peroxide, and hydroperoxide. This stops the oxidative pathways that lead to diseases that get worse over time [30].

Obesity is a global health problem which since decades had significant attention, one of the used methods to control this disorder was the inhibition of lipase enzyme which is responsible of the transformation of fats to simple fatty acids which are easily absorbed to blood stream, this enzyme secreted from pancreas to begin its function in small intestine [31].

Orlistat is a potent inhibitor of pancreatic lipase and leads to moderately reduced fat absorption by lipase inhibition. However, it can also cause side effects, including diarrhea, steatorrhea, oily stools, and incontinence. Several research groups have focused on screening plant extracts for potential lipase inhibition, since medicinal plants have been used as dietary supplements for weight reduction and management [32].

Diabetes is considered a metabolic disorder from which more than 100 million people worldwide suffer. This condition is caused by an absolute or relative lack of insulin secretion as well as different degrees of insulin resistance. One strategy for the treatment of diabetes is enzymatic inhibition procedure especially pancreatic α- amylase [33].

In recent years, medicinal herbs with a lowering blood glucose effect have piqued the interest of researchers in this field, and they have been studied in diabetes treatments because medicinal herbs contain various chemical constituents that have the potential to inhibit α-amylase and thus delay carbohydrate digestion, lowering the glucose absorption rate and thus lowering the postprandial plasma glucose rise. Several indigenous medicinal plants, including thymus, exhibit strong -amylase inhibition [34, 35].

Because of the limited and ineffective efficacy of available treatments for metastasis, cancer sickness remains a leading cause of death. Cancer is a disease that gets worse over time because cells keep dividing without being told to [36]. Apoptosis, cytotoxicity, and anti-proliferative activity are all processes that can slow the progression of cancerous cells. Anti-proliferative activity slows cell growth, preventing cancer cells from spreading quickly. The cytotoxic and anticancer activities of Thymus essential oils and monoterpenes have been extensively studied. In Thymus essential oils, the key components responsible for their therapeutic effects include carvacrol, thymol, terpinene, and p-cymene [37].

1.6 Hypothesis & significance for this project

T.capitatus dried aerial parts from three regions in Palestine were extracted using the microwave-ultrasonic method and the hydro distillation method to get information about the most efficient method for extraction. *T.capitatus* essential oil chemical active components were characterized by gas chromatography coupled mass spectroscopy. *T.capitatus* essential oil might also provide phytochemicals that have anticancer activities, possibly free of the safety and side-effect concerns of those currently used in chemotherapy, in addition to antioxidant, anti-microbial, anti-obesity, and finally antidiabetic activities.

1.7 Aims of study

- Compare the efficiency between two separation methods: the hydro-distillation method and the microwave ultrasonic method.

- GC-MS was used to identify the phytochemical composition of this extracted EO and try to compare EOs of *T.capitatus* from different regions in Palestine with each other.

- To evaluate *in-vitro T.capitatus* EO antioxidant levels as well as to assess its antimicrobial effects, including several fungal and bacterial strains.

- Enzyme inhibition assessments of *T.capitatus* EOs on lipase and α-amylase as indicators in the treatment of obesity and diabetes.

- To test *T.capitatus* EOs for anticancer efficacy against some available human cancer cell lines.

- Make a comparison between the three regions EO for all the tested biological activities to study the effect of variation in the chemical composition on them.

Chapter Two

Materials and methods

2.1 General methodology

In order to separate essential oils (EOs) from medicinal plants, researchers have access to a wide array of extraction techniques. These odorant chemicals are normally extracted using steam, dry distillation, or another suitable mechanical technique. This extraction does not include heating the odorant compounds. Most of the time, a physical method is used to get the essential oils out of the water phase. . Additionally, they have found that Solvent-Free Microwave Extraction is an effective method for extracting essential oils because traditional extraction methods like solvent and hydro diffusion resulted in the loss of a number of evaporative constituents, toxic solvent residues in the finished product, and poor isolation coherence [39]. In our research, both microwave ultrasonic and hydro-distillation methods were performed for the sake of comparison, and after that, several biological tests were done to calculate the activity of the EOs.

2.2 Listing of materials and chemicals

Several chemical reagents were used in the experimental part of this research, including the following liquids and solids in Table 2.1.

Table 2.1

chemicals used for essential oil (EOs) extraction and laboratory assessments

Reagents	Supplier	Country of Supplier
Dimethyl sulfoxide (DMSO)	Riedel-de Haën	Germany
Methanol, 99.9%	Loba Chemie	India
6-Hydroxy-2,5,7,8 tetramethylchroman-2 carboxylic acid (Trolox)	Sigma-Aldrich	USA
2,2-Diphenyl-1picrylhydrazyl (DPPH)	Sigma-Aldrich	USA
DPPH	Sigma-Aldrich	Denmark
Orlistat	Sigma-Aldrich	Germany
P-nitrophenyl butyrate (PNPB)	Sigma-Aldrich	Germany
Porcine pancreatic lipase, type II	Sigma-Aldrich	USA
Acarbose	Sigma-Aldrich	USA
α-Amylase	Sigma-Aldrich	India
3,5-Dinitrosalicylic acid (DNSA)	Sigma-Aldrich	USA
Chloroform 99.9%	Loba Chemie	India
Tween 40	Loba Chemie	India
Linoleic acid	Sigma-Aldrich	USA
B- carotene	Sigma-Aldrich	USA
Potassium phosphate	Sigma-Aldrich	USA

- Analytical grade RPMI 1640 culture medium, glutamine, Trypsin, amphotericin B, fatal calf serum, *Hank's* balanced solution, Trypan blue solution, penicillin, and gentamicin.

- Among the bacteria examined under the microscope were:

- *Klebsiella pneumoniae* (ATCC 13883)

- *Proteus vulgaris* (ATCC 8427)

- *Staphylococcus aureus* (ATCC 6538)

- *Escherichia coli* (ATCC 25922)

- *Pseudomonas aeruginosa* (ATCC 9027*)*

- *and MRSA* (Clinical sample).

- Candida albicans was the fungus studied in our research.

The college of medical sciences of An-Najah National University in Nablus provided all of the previously examined microorganisms.

2.3 List of instruments used

Table 2.2

List of instruments used for essential oil (EOs) extraction and laboratory assessments

Instrument	Supplier	Country of Supplier
Oven	Ari Levy, Inc.	Israel
Balance - AS 220/C/2	Radwag	Poland
Micropipette	MRC, Ltd.	Israel
Grinder - Uno	Moulinex	China
Micropipettes	Macherey-Nagel	USA
Filter papers – MN 617and Whatman No.1	Macherey-Nagel	USA
Vortex	Heidolph	Germany
Microwave-ultrasonic reactor extractor	LAB-KITS	China
Microplate reader	Unilab	USA
Water bath - BPXOP1001040	Lab Tech	South Korea
GC-MS	Perkin Elmer	UK

2.4 Collection of plant materials

T.capitatus aerial parts were collected in March (2021) from three cities that resembled three regions of Palestine: Jenin (north), Ramallah (middle), and Hebron (south). Areal parts of the plant were carefully separated, washed twice with distilled water, dried for 15 days in the shade, grounded well, and stored in cloth bags until the extraction process began. Dr. Nidal Jaradat identified the plants at the pharmacy department.

2.5 Extraction process from *T.capitatus* aerial parts

2.5.1 Microwave ultrasonic and hydro-distillation methods

Three samples of *T.capitatus* aerial parts were dried powder and extracted using an ultrasonic microwave technique while being exposed to ultrasonic waves to optimize the process of extraction. A device that consists of an oven microwave and an ultrasonic extractor was employed. This apparatus was filled with a 1 L round bottom flask holding 100 g of dried aerial parts powder and 500 mL of deionized water. The power of the microwave-ultrasonic extractor was set at 1000 W during the extraction process. Ultrasonic power was also increased to (50 W and a frequency of 40 kHz). The extraction process lasted for 10 minutes at 100°C. Each plant sample was subjected to three rounds of this procedure. A clean beaker was used to gather the extracted EO, which was then chemically dried and kept cool (between 2 and 8 °C) [40, 41].

The second extraction method for this essential oil was utilized by hydro-distillation used by Jaradat and colleagues [42]. Briefly, the EO was extracted applying a Clevenger apparatus running at atmospheric pressure for 180 minutes at 100 °C with a hydro-distillation rate of 0.54 ml/min after 100g of the dry powder was suspended in 1 L of distilled water. The EO was chemically dried with calcium carbonate and remained in the refrigerator at 4 °C until use. The following formula was used to compute the yield of each sample:

Yield percent = (extract weight/dry weight of *T.capitatus*) *100

2.5.2 Chemical composition by Gas Chromatography/Mass spectrometry

The chemical composition of the three EO samples examined was determined using the GC-MS method. Shimadzu QP-5000 GC-MS with Rtx-5ms column (30m long, 0.25m thickness, 0.250 mm inner diameter) was used to record GC-MS chromatograms. At a flow rate of 1 mL/min, helium was used as the carrier gas. 220°C was the injector temperature. The oven temperature was programmed to rise from 50°C (1 minute hold) to 130°C at 5°C/min, then to 250°C at 10°C/min and kept isothermally for 15 minutes. The temperature on the transfer line was 290°C. An electron ionization system with detector voltages of 1.7 KV was employed for GC-MS detection. The mass range was 38-450M/Z, with a scan rate of 0.5 s and a scan speed of 1000 amu/sec [43].

2.6 Identification of the components for EOs from *T.capitatus*

In mass spectrometry, standard reference libraries are frequently used to identify unknown substances. The library curator's primary responsibility is to guarantee that each entry is correct.

NIST mass spectrometry data center and literature references were used to compare MS retention times and Kovats indices, which allowed us to determine which chemical constituents were present in the EOs. electronically produced quantitative data from integrated peaks [44].

2.7 Antioxidant assay for samples of EOs from *T.capitatus*

2.7.1 DPPH assay

In methanol, a 100 µg/ml stock solution for the three *T.capitatus* EO samples was produced. In addition, a Trolox and galic acid solution of 100 µg/ml were produced (the reference standard). For each sample, serial dilutions were made from the stock solutions, yielding (5,10,20,30,40,50,80, and 100 µg/ml) using the (C1V1=C2V2) equation. One milliliter of each sample dilution was combined with one milliliter of 0.002 g/ml DPPH in methanol. To make a final working volume of 3 ml, 1 ml of methanol was added. The DPPH solution had to be made fresh because it was light-sensitive. The series concentrations were blank controlled with DPPH in methanol at a 1:2 ratio, without the addition of an extract. For around 30 minutes, all working solutions were incubated at room temperature (25 C) in the dark. A spectrophotometer was used to detect optical densities at a wavelength of 517 nm. The following equation was used to compute percent DPPH inhibition for three samples of EO from *T.capitatus.*

with trolox or gallic acid as the standard compound:

$$\text{DPPH inhibition \%} = (ABl - Ats)/ABl \times 100\%$$

ABl: the absorbance measured for the blank solution,

Ats: the absorbance measured of the tested sample of *T.capitatus* solution.

2.7.2 assay of β carotene- linoleic acid

The antioxidant activity is measured by the β-carotene-linoleic acid system model for the three EO samples from *T.capitatus* by Miller [45]. Based on oxidative breakdown products of the linoleic acid, this assay measures the oxidation of β-carotene-linoleic acid. 1 mg of carotene was dissolved in 2 ml of chloroform with 20 mg of linoleic acid and 200 mg of Tween 40, and then the chloroform was completely evaporated with a rotary evaporator at a low temperature and lower pressure. Then we added 200ml of distilled water that had been saturated with oxygen, shaking vigorously for 30 minutes. (0.1 mL) of the EO samples from *T.capitatus* aerial parts and the positive control (α-tocopherol) were combined with aliquots (5 ml) of these prepared solutions. A sample without antioxidants was also made as a control sample.

The mixture was maintained in a thermostatic bath at 50 °C after an initial absorbance reading at 470 nm, and absorbance was recorded at intervals of 15 minutes to 120 minutes.

2.8 Pancreatic lipase inhibition assay for *T.capitatus* EOs

The steps of the protocol for the porcine pancreatic lipase inhibitory assay were mostly the same as those described by Bustanji et al. (2010) [46, 47], with a few changes.

The three samples of EOs from *T.capitatus* were used to make stock solutions of 500 µg/ml in 10% DMSO. From the stock solution, serial dilutions of five concentrations (50, 100, 200, 300, and 400 µg/ml) were made. Just before usage, a 1 mg/ml stock solution of porcine pancreatic lipase in Tris-HCl buffer was produced fresh. 20.9 mg of p-nitrophenyl butyrate (PNPB) was dissolved in 2 mL of acetonitrile to make the substrate.

Each working solution contained 0.1 ml of 1 mg/ml porcine pancreatic lipase and 0.2 ml of each dilution series member of the EO. Tris-HCL was added to produce the working solutions' final volume of 1 mL, and they were incubated for 15 minutes at 37 °C. After incubation, each test tube received a 0.1 mL p-nitrophenyl butyrate solution. The mixture was then incubated at 37°C for another 30 minutes. A UV spectrophotometer was used to measure the hydrolysis of PNPB into p-nitrophenolate at 410 nm, which was used to estimate pancreatic lipase activity. Using Orlistat as a standard reference

chemical, the same technique was done. The following equation was used to compute the percentage lipase inhibition by EOs in the three regions:

Lipase inhibition % = (ABl ─ Ats)/ABl × 100%

A_{Bl} is the obtained absorbance of the blank solution, and A_{ts} is the obtained absorbance of the tested sample solution.

2.9 *In-vitro* evaluation of α-amylase inhibition

α-Amylase inhibitory activity of the three samples was assessed by the standard method of Wickramaratne, M.N., et al. (2015) with minor modifications [48-50].

Each *T.capitatus* EO sample was diluted in a few milliliters of 10% DMSO, then further dissolved in (Na 2 HPO 4 /NaH 2 PO 4(1:1) (0.02 M), NaCl (0.006 M) at pH 6.9) to yield stock solutions with 1000 µg/ml concentrations. The following dilutions were made from these: 50, 100, 200, 300, 400, and 500 µg/ml, using 10% DMSO as the diluent. A 0.2 ml amount of 2 units/ml porcine pancreatic amylase was mixed with 0.2 ml of EO prepared solutions and incubated for 10 minutes at 30 °C. a Following incubation, the tubes were given 0.2 mL of a freshly prepared 1 percent starch solution in water and incubated for at least three minutes more.The reaction was then paused by adding 0.2mL (3,5-dinitro salicylic acid (DNSA) reagent, which was diluted with 5 mL distilled water before being heated in a water bath at 90 °C for 10 minutes. The combination was then allowed to cool to the ambient temperature before being measured at 540 nm. The blank control was made using the identical ingredients as the above but with 0.2ml buffer instead of EOs. Following the process outlined above, acarbose was utilized as a standard reference. The -amylase inhibitory activity was estimated using the following equation for the three EO samples from *T.capitatus:*

% Of α-amylase inhibition = (ABl – ATs)/ ABl × 100%

ABl: the absorbance of the blank sample

ATs: the absorbance of the test sample.

2.10 Antimicrobial activity of *T.capitatus* EOs

2.10.1 Microorganisms and condition for cultivation

EOs samples of *T,capitatus* were tested against the following bacteria strains:

- *Klebsiella pneumoniae* (ATCC 13883)
- *Pseudomonas aeruginosa* (ATCC 9027)
- *MRSA (Clinical sample)*
- *Proteus vulgaris* (ATCC 8427)
- *Staphylococcus aureus* (ATCC 6538)
- *Escherichia coli (ATCC 25922)*
- Antifungal activity of the EOs was examined against the growth of a diagnostically confirmed *Candida albicans* clinical isolate.

2.10.2 Antimicrobial assays for *T.capitatus* EOs for the three samples

The antimicrobial activity of the EOs samples from three regions of Palestine was assessed by using the broth micro-dilution method according to the previous protocols by Balouiri, M., et al. (2016) and Bariş, Ö., et al. (2006) with some modifications [51, 52].

For 18 hours, bacteria stains were developed in cultured broth. Ten percent DMSO was used to dissolve each of the isolated *T.capitatus* EOs. After filter sterilization, the *T.capitatus* EOs solutions were serially micro-diluted ten times in sterile nutritional broth before use. In 96-well plates, the dilution procedures were carried out in an aseptic environment.

2.10.3 Antibacterial and anti-fungal assay for *T.capitatus* EOs for three samples

For the antibacterial assay, each 200 µL of the isolated EOs samples was dissolved in 150 µL of 10% DMSO and then diluted with 150 µL distilled water and left on UV for 15 min.

Bacterial suspensions were made; a swab was collected from the different types of bacteria and then placed in normal saline. Turbidity was measured using the UV at λ = 620. It should be between (0.08 and 0.12). If it was less than that, bacteria were introduced, and if it was larger than this value, normal saline was added. Then 50µL of the bacterial suspension was combined with 5mL of the media to obtain the final bacterial suspension. The prepared *T.capitatus* EOs solutions were filtered, sterilized and then micro-diluted serially 10 times, starting with 50 µL of the EOs solutions that were added to sterile nutrient broth containing 50L of the media. The initial obtained concentration was 20% v/v of the extracted essential oil at the first line of the 96 well plates. This process was repeated until plate 10, at which 50 µL taken from it and removed. Then after, 50µL of the prepared bacterial or fungal suspensions were added (one type of the mentioned bacterial strains for each line) to each plate, except the 12^{th} vertical line of the 96 well plates, so that the final concentration obtained at the first vertical line of the essential oil became 10% v/v, equal to 100 µg of the EO per ml. The EOs-free nutrient broth on vertical line 11 was used as a positive control for bacterial growth. Vertical line number 12, on the other hand, contained EOs-free nutrient broth that had not been inoculated with any of the tested bacterial cells and served as a negative control for the media. We also had a compound control (compound + media) to ensure that there was no contamination or turbidity, and that the change at the last horizontal line of the 96 well plates was not due to the compound itself.

The *T.capitatus* EO samples were tested in triplicate on each of the bacterial cells that were included in this investigation. At 35°C, all of the inoculation plates were incubated. The incubation period was around 18 hours long. The minimum inhibitory concentration of *T.capitatus* EOs was defined as the lowest concentration at which no observable bacterial growth in that micro-well was observed.

2.11 Anticancer activity

2.11.1 Types of cancer cell used

There are numerous distinct forms of cancer, which are classified according to the site of infection or the underlying biological process [53]. In our study, we used hepatic G2 cancer cells to study the anticancer activity of the *T capitatus* EOs.

2.11.2 Cytotoxicity assay

RPMI 1640 media supplemented with 10% heated fetal bovine serum, 1% of 2 mM l-glutamine, 50 IU/ml penicillin, and 50 µg/ml amphotericin B was used to culture hepatic G2 carcinoma cells. Once mycoplasma and bacteria were ruled out, cells were cultured in RPMI 1640 media with 10% calf serum as a monolayer confluent at 35°C. As a precaution, antibiotics were not administered as a precaution to avoid sensitizing the cell membranes. Phosphate buffer saline (PBS) was used to wash cells three times for the experiment. PBS was decanted, cells detached with 0.025% Trypsin–EDTA and RPMI 1640 medium was added to make up a volume of 10 ml. To make a single cell suspension, the cell suspension was centrifuged at 1000 xg for 10 minutes, and the pellet was resuspended in 10 ml of media. Trypan blue exclusion was used to measure cell viability, which was found to be greater than 96 percent in a hemocytometer. After inoculation, stock cultures were duplicated weekly. The cell line was grown in 6-well tissue culture plates (9.8 cm2) at 35 degrees Celsius in a humidified atmosphere containing 5% CO2. The cells were treated with the isolated EOs after 24 hours. 0.1 ml of each EOs extract from the three districts was serially diluted to 1000, 500, 250, 125, and 62.5 µg/ml.

Chapter Three

Results and Discussion

3.1 Extraction yield of the collected EOs from *T. capitatus*

In our study, hundreds of dried aerial parts of *T. capitatus* from three districts in Palestine were grinded and subjected to two methods of extraction: hydro distillation and microwave ultrasonic. Microwave ultrasonic extraction is a sophisticated technique that employs the use of a microwave oven in the extraction process. This method was studied and compared with conventional hydro distillation. Table 3.1 shows the percent of oil from each district by the two methods.

Results from table 3.1 showed that, according to the percentage yield, the hydro distillation method was more efficient with nearly a double fold than the microwave ultrasonic method, which can be explained by the longest time consumed by hydro distillation that may have led to complete extraction or the volatility nature of the EO. In addition, there were noticeable differences in mass values between the districts.

As shown in Ramallah, which has an average elevation of 880 meters above sea level and an average humidity and average temperature of (47%), (40°F to 84°F) respectively, gave the highest yield in both methods (2.8% hydro distillation and 1.39% microwave ultrasonic), followed by Hebron, which has an average elevation of 930 meters above sea level and an average humidity and average temperature of (62%), (27°F to 78°F) respectively, gave a yield of (1.26% hydro distillation and 0.4% Microwave ultrasonic). Finely, the Jenin sample that is located at 250 meters above sea level with an average temperature of (52°F to 89°F) and an average humidity of 69% gave 1.15% by the hydro distillation method and 0.89 by the microwave ultrasonic method. In addition to the varied geographical locations and environmental conditions, the three districts also have different soil types, soil components, and soil pH. All these factors worked together, not separately, to cause the changes in quantity that were seen between the three samples.

Table 3.1

The percentage yield EOs from T. capitates

District	Mass of sample (g)	% Eos	
		Hydro distillation	Microwave ultrasonic
Ramallah	100	2.8	1.39
Jenin	100	1.15	0.86
Hebron	100	1.26	0.4
S.D		0.922	0.495

Our results are consistent with the results held on different types of plants which emphasizes the effect of environmental factors on the chemical composition and amount of the essential oils [54, 55]. Also, it emphasized the study done by Elyemni et al. 2019 [56] that the microwave ultrasonic method was shown to be superior in terms of both the amount of energy saved and the amount of time required for extraction. Also, gas chromatography–mass spectrometry analysis of the extracted essential oils indicated that the use of microwave irradiation had no negative impact on the essential oil content [56, 57]**.**

3.2 components of the essential oils

GC/MS was used to identify all the EO components of *T.capitatus*, their concentrations and output orders that reveal information about the volatile nature of the EOs.

An essential oil chromatogram and the reference substances in a spectrum library with a computerized data bank were compared to each other. The GC/MS method was used to get the different mass spectra and retention indices of compounds that might be in this extract [52,53].

Today, *T.capitatus* EO has been meticulously investigated, and the compositional diversity of plants growing in different nations and even in different regions of the same country has produced numerous chemotypes [58].

Table 3.2.1 showed the GC-MS analysis of EO components from three districts in Palestine (Ramallah, Jenin, Hebron). It showed more than (21) compounds were separated and identified, the EOs percentages yield of *T.capitatus* were (98.96%, 97.47% ,92.61%) for (Ramallah, Jenin, Hebron) respectively. Components identified included sesquiterpenes, monoterpenes, and other compounds like, alcohols, phenols, and organic acids.

Variation of geographical origin of the EOs leads to variation in the chemical composition of it, results are described in (tables 3.2.1 and 3.2.2). Starting with Ramallah, carvacrol (31.25%), γ-terpinene (30.94%), o-cymene (16.84%) and linalool (6.19%) were the main components. Looking at the other samples, γ -terpinene (67%), cis-b-terpineol (12.91%), carvacrol (6.44%) and thymol (5.51%) were the main components in Jenin. On the other hand, thymol (40.35%), b-Caryophyllene (13.23%), (carvacro, methyl ether) (10.7%), p-cymene (8.41%) and camphene (5.56%) were the main components appeared in Hebron sample. According to the results, there were many clearly noticeable differences in three main components including (γ-Terpinene, carvacrol and thymol) as their percentages differed clearly between the three districts, so we concluded that the influence of variation of the geographical origin over them was evident.

The variation of chemical components of the EO samples for the chosen districts can be explained by results of previous studies that emphasized the effects of plant's origin, harvest period, soil components, genetic variables, and climatic conditions on the chemical structure of essential oils. This study's findings are compatible with these findings [59, 60].

For example, Vaičiulytė, V., et al. in their research (*Variation of essential oil composition of Thymus pulegioides in relation to soil chemistry),* studied the effects of soil PH and 14 chemical components of the soil on the chemical composition of *Thymus pulegioides* that represents important genus of Thymus (*Lamiaceae*) and results obtained showed that the amount of aluminium, copper, iron, potassium and manganese in soil when increased led to decreasing in amount of essential oils in raw material of *T. pulegioides.* Also, abundance of higher amount of phosphorus in the soil led to increased biosynthesis of α-terpinyl acetate. Moreover, higher amount of sulphur in soil was a reason to increase in percentages of carvacrol and linalool but decreasing the percentage of *p*-cymene in *T. pulegioides* EOs. Additionally, high amounts of manganese in soil affected the biosynthesis of main compounds in EOs of *T. pulegioides* [54]. So, we concluded there were important effects of the soil chemical compositions on the composition of the essential oil of the plants, and furthermore research needed to study the influence of each factor separately on the chemical composition of the EOs.

Table 3.2.1

GC-MS analysis results

Ramalla	Rt	RI	%	Jenin	Rt	RI	%	Hebron	Rt	RI	%
α-Thujene	8.403	925	0.65	α-Thujene	8.415	926	0.31	********	***	***	***
a-Pinene	8.71	933	0.7	a-Pinene	8.695	933	0.49	a-Pinene	8.7	933	2.52
Camphene	9.37	949	0.15	Camphene	9.35	949	0.9	Camphene	9.351	949	5.56
Sabinene	10.501	976	0.17	b-pinene	10.486	976	0.49	b-pinene	10.501	976	0.23
Myrcene	11.07	990	0.73	Myrcene	11.06	990	0.33	Myrcene	11.071	990	0.17
a-Phellandrene	11.71	1006	0.08	α-Terpinene	12.157	1017	1.41	α-Terpinene	12.162	1017	0.35
α-Terpinene	12.167	1017	2.25	p-Cymene	12.49	1025	1.41	p-Cymene	12.487	1025	8.41
o-Cymene	12.507	1025	16.84	γ-Terpinene	13.887	1059	67	Limonene	12.672	1029	0.47
Sylvestrene	12.687	1029	0.4	cis-4-thujanol	15.608	1101	0.19	γ-Terpinene	13.887	1060	3.61
γ-Terpinene	13.91	1059	30.94	cis-b-Terpineol	18.698	1181	12.91	cis-4-thujanol	15.603	1101	2.98
linalool	15.903	1109	6.19	carvacrol methyl ether	20.86	1240	0.07	Camphor	17.389	1147	0.004
cis-b-Terpineol	18.674	1181	0.02	2-Isopropyl-4-methylphenol	21.78	1266		2-Methyl isoborneol	18.389	1173	2.24
thymol methy ether	20.825	1239	0.24	o-cymenol	21.996	1278	0.14	Terpinene-4-ol	18.689	1181	0.02
thymol	22.131	1276	3	thymol	22.776	1293	5.51	a-Terpineol	19.286	1196	0.23
carvacrol	23.136	1304	31.25	carvacrol	23.056	1301	6.44	**********	20.465	1229	0.58
b-Caryophellene	26.988	1420	4.61	b-Caryophellene	26.993	1419	1.65	Carvacrol, methyl eth	20.855	1240	10.7
	27.608	1440	0.28	trans-a-Bergamotene	27.618	1440	0.1	Isobornyl acetate	22.461	1285	0.38
α-Caryophyllene	28.143	1457	0.19	α-Caryophyllene	28.14	1457		thymol	22.771	1293	40.35
Viridiflorene	29.284	1493	0.27	Caryophellene oxide	32.03	1585	0.11	carvacrol	23.056	1301	0.58
Caryophellene oxide	32.035	1586	o.77					b-Caryophellene	26.993	1420	13.23
								Caryophellene oxide	32.02	1585	1.8
Sum			98.96				99.35				92.61

Table 3.2.2

Major components of the EOs in their districts.

Ramalla	Rt	RI	%	Jenin	Rt	RI	%	Hebron	Rt	RI	%
o-Cymene	12.507	1025	16.84	γ-Terpinene	13.887	1059	67	Camphene	9.351	949	5.56
γ-Terpinene	13.91	1059	30.94	cis-b-Terpineol	18.698	1181	12.91	p-Cymene	12.487	1025	8.41
linalool	15.903	1109	6.19	thymol	22.776	1293	5.51	Carvacrol, methyl ether	20.855	1240	10.7
carvacrol	23.136	1304	31.25	carvacrol	23.056	1301	6.44	thymol	22.771	1293	40.35
								b-Caryophellene	26.993	1420	13.23
								Caryophellene oxide	32.02	1585	1.8
Sum			85.22				91.86				78.25

3.3 Antioxidant activity

3.3.1 DPPH assay

Antioxidant activity was assessed using DPPH (2,2-diphenyl-1-picrylhydrazil), one of the earliest free radicals utilized in research of antioxidant activity [61]. DPPH is a radical that is stable and has a solution that is purple. Its distinctive absorption maximum occurs at 517 nm. The application of the regular protocol relies on the disappearance of the maximum that occurs when DPPH is decreased by a chemical with anti-radical properties, which causes the discoloration to become yellow.

Table 3.3.1 showed percent inhibition of EO's of the districts at different concentrations ranging from (0-100g/ml), in addition to the values of IC_{50}.

Analysis of table 3.3.1 and figure 3.3.1 showed that the three districts had nearly the same IC50 which was four-fold of gallic acid. On the other hand, they revealed a 50% inhibition of gallic acid at 50 μg/ml

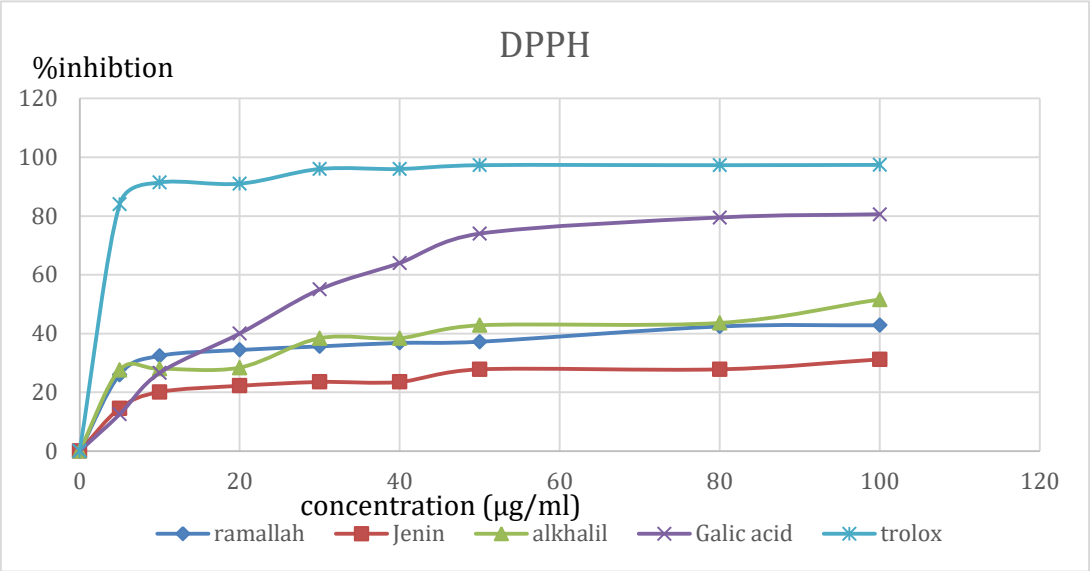
Table 3.3.1

DPPH assay for the EO of three the three districts

Conc.(μg/ml)	Ramallah	Jenin	Hebron	Gallic acid	Trolox
0	0	0	0	0	0
5	26	14.5	27.6	12.5	84
10	32.4	20.1	28	26.6	91.4
20	34.4	22.2	28.4	40	91
30	35.6	23.5	38.4	55	96
40	36.8	23.5	38.4	64	96
50	37.2	27.8	42.8	74	97.3
80	42.4	27.8	43.6	79.5	97.3
100	42.8	31.2	51.6	80.6	97.4
IC50 (μg/ml)	>100	>100	95	25	5

Figure 3.3.1

DPPH assay of T.capitatus EOs in three districts



Statistical analysis of the results was done by SPSS software using the *Kruskal-Wallis Test*. The results obtained are detailed in table B.2 (appendix B) Asymp. Sig. value obtained according to DPPH assay was equal to (0.000) and was less than (0.05), so we concluded that there are significant differences between districts according to DPPH assay.

3.3.2 β -Carotene assay

The β -carotene linoleic acid test is another antioxidant test that may be performed on *T.capitatus* table 3.3.2. In order to evaluate the antioxidant activity of the EOs, an emulsion system consisting of β -carotene and linoleic acid was used. This was done on the basis of the hypothesis that β -carotene loses its color in the absence of antioxidants. table 3.3.2 and figure 3.3.2 showed the nature of the EOs in three districts within two hours. All of them showed higher antioxidant efficiency than water (control) and the synthetic antioxidant α-tocopherol which gave the highest β-carotene degradation. After two hours, the oil of the three districts showed some variances in the absorbance. The values of absorbance for Ramallah, Jenin, and Hebron were (0.79, 0.744, and 0.723) respectively.

Numerous studies have found a correlation between monoterpenes and their oxygenated monoterpenes, which include phenols and alcohols, and the presence of antioxidant

activity. It was discovered that carvacrol (main component in Ramallah EO sample) possessed the greatest antioxidant activity [62, 63], and as results of β -Carotene assay showed, the Ramallah sample gave the highest antioxidant activity. Several additional components of essential oils, such as α-terpinene and γ-Terpinene, may possibly be responsible for the found antioxidant activity [64] and these results go along with our results as the second sample that followed the Ramallah sample in terms of anti-oxidant activity was the Jenin sample that was rich in γ-Terpinene (67%). Sabinene and other non-phenolic terpenoids have been shown to have significant antioxidant properties [65].

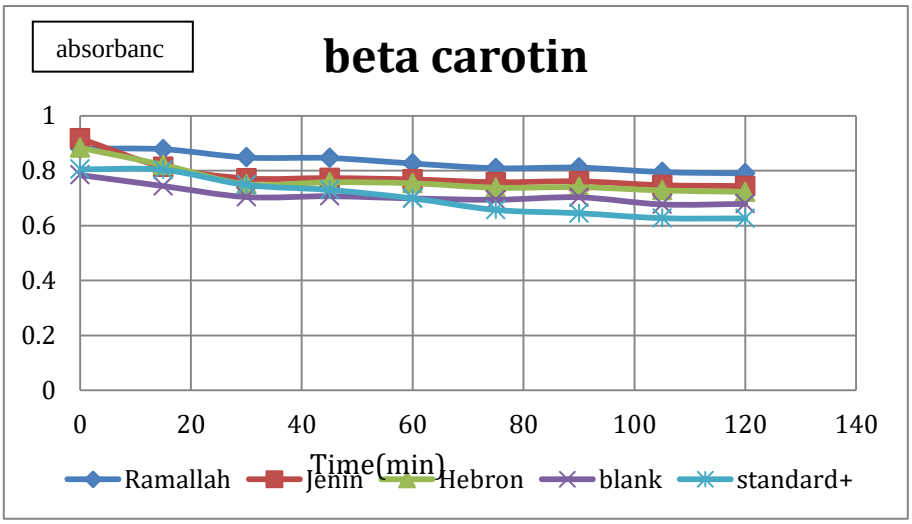
Table 3.3.2

β -Carotene assay of T.capitatus EOs for three districts

Time (min)	Ramallah	Jenin	Hebron	Blank	α-tocopherol
0	0.88	0.917	0.883	0.784	0.805
15	0.878	0.814	0.821	0.744	0.803
30	0.848	0.772	0.751	0.704	0.748
45	0.846	0.773	0.758	0.707	0.73
60	0.826	0.768	0.754	0.699	0.699
75	0.809	0.758	0.738	0.694	0.657
90	0.811	0.761	0.74	0.703	0.645
105	0.795	0.747	0.728	0.677	0.627
120	0.79	0.744	0.723	0.679	0.626

Figure 3.3.2

β -Carotene assay of T.capitatus EOs in three districts



3.4 Anti-lipase activity

In terms of metabolic illnesses, obesity is one of the fastest growing. Some ways to treat obesity are to limit the number of calories you eat and take medicines that stop your body from absorbing fat from food, speed up your metabolism, and stop it from storing fat [66].

Lipases cannot hydrolyze dietary fat while orlistat is present because it forms a covalent bond with the active serine site, rendering them ineffective. Flatulence, fecal urgency, deficiency in fat-soluble vitamins, steatorrhea, and abdominal cramping are all unwelcome side effects of the drug [67].

The anti-lipase activity of *T.capitatus* against the positive control anti-lipase commercial medication Orlistat was evaluated using the porcine pancreatic lipase inhibitory assay.

With Orlistat as an active positive control, the results shown in table 3.4 and figure 3.4 reveal that all tested extracts have anti-lipase action. At concentrations of (200 μg/ml) and above, the three district EOs demonstrated anti-lipase activity even greater than Orlistat.

Table B.3 (appendix B) showed the results of the Kruskal-Wallis Test using SPSS software for anti-lipase activity of the EOs for the three districts. Asymp. Sig. value was 0.946, which is higher than (0.05), so we considered that the differences between the districts were not significant.

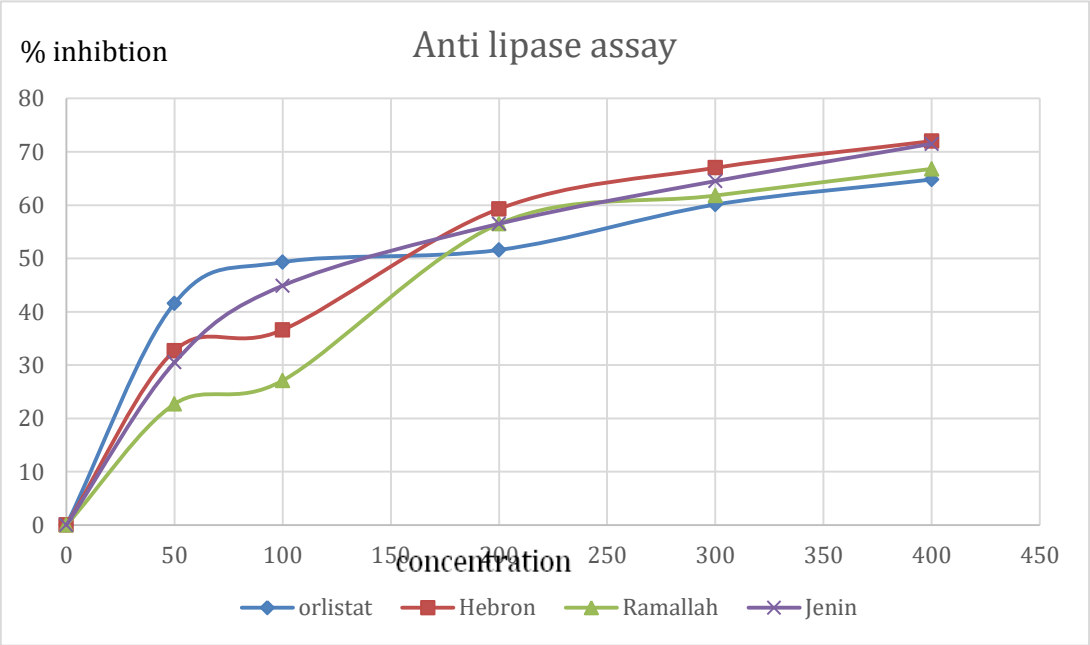
Table 3.4

Anti-lipase activity of the EOs for the three districts

Conc.	District			Standard
% Inhibition (μg/ml)	Hebron	Ramallah	Jenin	Orlistat
0	0	0	0	0
50	32.7	22.7	30.5	41.6
100	36.6	27.1	44.9	49.3
200	59.3	56.5	56.5	51.6
300	67	61.8	64.5	60.1
400	72	66.8	71.5	64.8

Figure 3.4

Anti-lipase activity of EOs in three districts



3.5 α-Amylase

A diabetic is a metabolic condition due to insulin resistance or insulin shortage. Chronic hyperglycemia can create a wide range of problems that affect numerous cells and organs, resulting in several fatal disorders [68].

Oral anti-diabetic medicines and/or parental insulin are used to treat diabetes [69]. A significant number of these pharmaceuticals are associated with a variety of major adverse effects and potentially hazardous contraindications. Patients have shown a strong preference in recent years for herbal supplements and pharmaceuticals that combine high therapeutic efficacy with low incidences of adverse effects. Anti-diabetic medicines derived from herbs show a great deal of promise, and the anti-diabetic potential of plants that have been used in traditional medicine for a long time is currently the subject of research [70]. Numerous plants have been confirmed to exhibit hypoglycemic capabilities, as evidenced by literature sources from multiple databases; these planets tend to reduce blood glucose either as an insulinomimetic or through insulin secretory activity. The majority of hypoglycemic plants are found in the following families: (Leguminoseae, *Lamiaceae, Liliaceae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae*, and *Araliaceae).* Anti-diabetic properties are attributed to

polyphenols, flavonoids, terpenoids, and coumarins as well as other components in these medicinal plants [71].

Table 3.5 and figure 3.5 showed the α-amylase inhibitory activity for the EOs of the three districts contrasted with Acarbose, which is utilized therapeutically in a concentration-dependent manner for its inhibitory action. Statistical analysis reveals a slight difference between them. All of them showed nearly the same percent inhibition at a concentration of 400 µg/ml which is 50% of Acarbose.

Table 3.5

α- Amylase percent inhibition of EOs for three districts

% Inhibition				
conc.(µg/ml)	Jenin	Ramallah	Hebron	Acarbose
0	0	0	0	0
50	10.2	7.3	5.3	54.9
100	11.8	12.6	17.5	66.1
200	15	19.1	19.5	68
300	19.5	27.6	30.5	69
400	41.9	42.7	38.6	70.1
1000	46.7	47.6	43.5	71

Figure 3.5

Alfa amylase percent inhibition of EOs in three districts

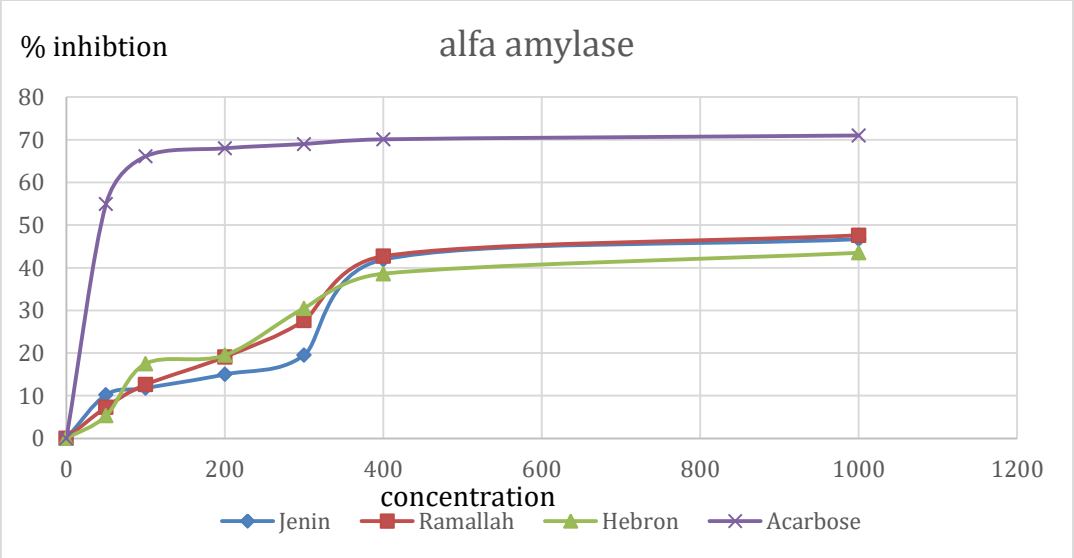


Table B.4 (appendix B) statistical analysis of the results of α-amylase percent inhibition for the three districts that make up the sample of study using chi-square test showed that there is an Asm. Sig. difference of value (8.272) and sig vale (0.041). The ranking of the effect is according to the following sequence: Jenine 11.3, Ramallah 12.21, Hebron 12.14, Acarbose 22.21). Those results confirmed that there are differences in the EO activities between the districts.

3.6 Antimicrobial assay

Microbial infections are a global concern that poses a life-threatening threat to humanity. Antibacterial and antifungal resistance has emerged as an urgent global health issue, owing to the abuse of antibiotics. With an estimated 2 million patients afflicted with medication-resistant germs each year, an alternate strategy to combat drug resistance is required. As a result, there is a proclivity to employ traditional or unusual approaches to solve the problem and prevent the spread of infectious diseases [72].

Gram-negative and gram-positive bacteria were both shown to be highly susceptible to

T.capitatus EO, despite the fact that it was previously stated that essential oils are less effective against gram-negative bacteria [73]. A possible explanation for this is that the EOs contain hydrophobic components, which may damage the cell membranes of bacteria and hence impede their ability to operate [64].

Moreover, further research has shown and explained that *T.capitatus* essential oil's effectiveness against fungus may be due to the quantity of phenolic chemicals (carvacrol and thymol), which may interfere with enzymes involved in cell wall synthesis, such as chitin synthase and chitinase, and with α and ß-glucanases. The antifungal impact of *T.capitatus* was previously shown to involve telomerase suppression as it increased the rate of cell senescence and apoptosis, as previously reported. (γ-terpinène and p-cymene) have also been implicated in antifungal action [73].

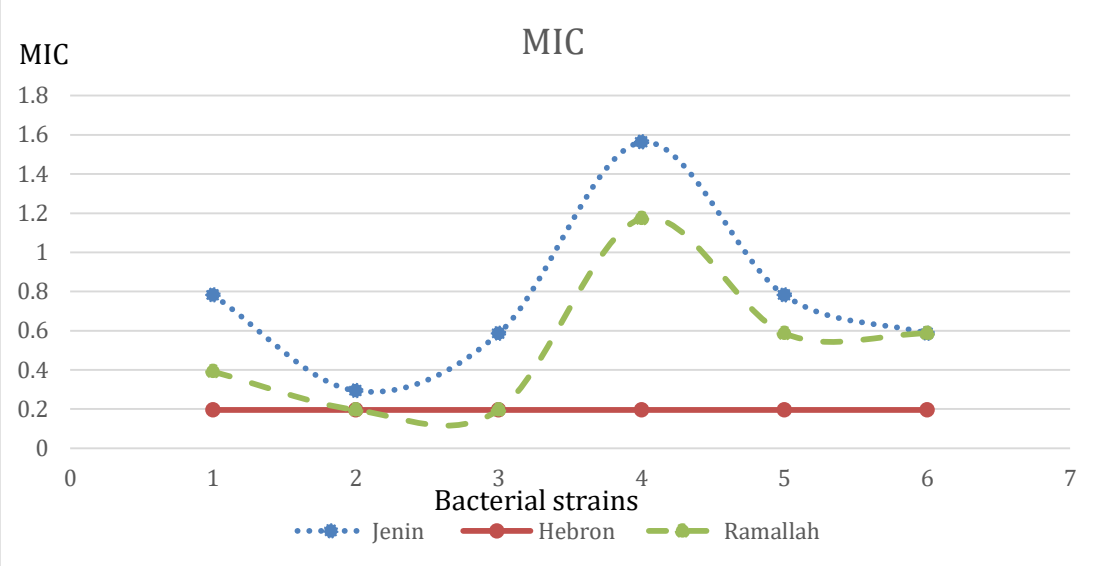
In our research, antimicrobial activity of EOs samples from three regions of Palestine was assayed by using the broth micro-dilution method according to a previous protocol (Balouiri, M., et al.2016; Bariş, Ö., et al.2006) with some modifications.

Table B.5 (appendix B) and Figure 3.6 reveal the activities of the EOs on different types of bacteria. EOs from all districts showed MIC values between (0.1953 and 1.5625 µg/ml) except for *pseudomonas*, which showed from 9.375µg/ml in Jenin to 37.5µg/ml in Hebron. Compared to the results of the three districts, the Hebron EO sample had the highest activity of 0.1953g/ml against all types of bacteria, and this was explained by the high percentage of thymol (40.35%) in it. Another important result is the efficiency of the EOs against the MRSA strain, which is resistant to most antibiotics.

Those results may be explained by the differences in types and concentrations of components in the districts. (Table : 3.2.1)

Figure 3.6

MIC values of T.capitatus EOs in three districts



(1-proteus, 2-Candida, 3-MRSA, 4-S.areus, 5-Klebseiela, 6-E.coli)

Table B.6 (appendix B) showed the results of the Kruskal-Wallis Test for anti-microbial assay of the EOs for the three districts. Asymp. Sig. value was 0.053, which is slightly higher than (0.05), so we considered that there are slight differences between the districts according to anti-microbial activity.

3.7 Anticancer activity

Cytotoxic chemotherapy refers to a group of chemicals and medicinal plants that are used to kill various kinds of cancer cells. Cytotoxic medications include both plants and pharmaceuticals. They do this by preventing the division of cells, which ultimately results in the death of cancer cells. Additionally, they can improve the results of radiotherapy and surgery as well as decrease the number of metastases [74].

One of the vital essential oil bearing families is *Lamiaceae*, *Thymus* (thyme) genus is a member of this family, and *T.capitatus* is one of its species.

The cytotoxic and anticancer activities of Thymus essential oils and monoterpenes have been extensively studied. Carvacrol, thymol, terpinene, and p-cymene are the main parts of Thymus essential oils that give them their healing effects [75].

In this test, Hep-G2 cells were used to predict the anticancer assay of *T.capitatus* EO.

The Hep-G2 cells were subjected to increasing quantities of the investigated samples from three districts (1000, 500, 250, 125, and 62.5 µg/ml) for a day. The MTS test was used to get a quantitative reading on the cell viability.

Table 3.7 shows the percent inhibition of thymus EOs from three sites in Palestine on Hep G2 cells. They showed nearly the same results with more than 85% inhibition at concentrations greater than 62.5µg/ml, except Ramallah EO showed 82.5% inhibition at 62.5µg/ml. This means that the active substance that affects Hep G2 cells is different in the districts and more concentrated in Hebron and Jenin. More phytochemical and in vivo pharmacological research are required to validate these exceptional results.

Table 3.7

% inhibition of T.capitatus EOs in three districts against hepatic G2 cancer cells

Conc.	% Inhibition		
µg/ml	Hebron	Ramallah	Jenin
Control	0	0	0
1000	87.8	88	88.3
500	86.5	88.7	89
250	87.9	88.7	88.4
125	85.6	84.4	88.5
62.5	85.9	82.5	87.1

Chapter Four

Conclusion

Tested samples of *T.capitatus* were collected from three regions of Palestine (Hebron, Jenin, and Ramallah). Selected districts have different altitudes above sea level, resulting in differences in weather, humidity, and the nature of soil and its components. The results of *T.capitatus* extraction using two methods to study the influences of these factors on the percentage of yield, essential oil composition, and the consequent effects on biological activity revealed that, in terms of percentage of yield, the hydro distillation method was twice as efficient as the microwave ultrasonic method, with significant differences in mass values between the districts. About 21 compounds were separated and identified by GC-MS analysis of EO components from the three samples, with clear variations in the percentage of them between districts, mainly (γ-Terpinene, carvacrol and thymol), as the influence of environmental factors over them was evident.

In vitro assessment of the antioxidant activity of the EOs was carried out by using the *Beta*-Carotene assay and DPPH assay. The samples showed good antioxidant activity. Similar results of Alfa amylase and Anti-lipase showed activity of EOs against both enzymes when compared with the positive control. Those results were approved by SPSS analysis. Regarding antimicrobial activity, *T.capitatus EO* was effective against all of the bacteria and fungi that were tested, with the exception of *pseudomonas. The* Hebron sample gave distinguishable results at low concentrations against all bacterial and fungal strains put to the test. Finally, regarding cytotoxic activity against Hep G2 cells, they showed nearly the same results (more than 85% inhibition at concentrations greater than 62.5μg/ml, except in Ramallah, where the oil showed 82.5% inhibition at 62.5μg/ml) which means that the active consistent is different in the districts and more concentrated in Hebron and Jenin.

List of Abbreviations

Abbreviation	Meaning
ATCC	American Type Culture Collection
°C	Degree Celsius
DMSO	Dimethyl sulfoxide
DNSA	3,5-Dinitrosalicylic acid
E. coli	*Escherichia coli*
EO	Essential oil
GC-MS	Gas Chromatography Mass Spectrometry
Hep-G2	Hepatic G2
K. pneumonia	*Klebsiella pneumoniae*
L	Letter
L. monocytogenes	*Listeria monocytogenes*
MIC	Minimum inhibitory concentration
NIST	National institute of standards and technology
PBS	Phosphate buffer saline
PNPB	P-nitrophenyl butyrate
RBMI	Roswell park Memorial Institute Medium
ROS	Reactive Oxygen Species
T. capitatus	*Thymus capitatus*
UV	Ultraviolet
W	Watt
WHO	World Health Organization

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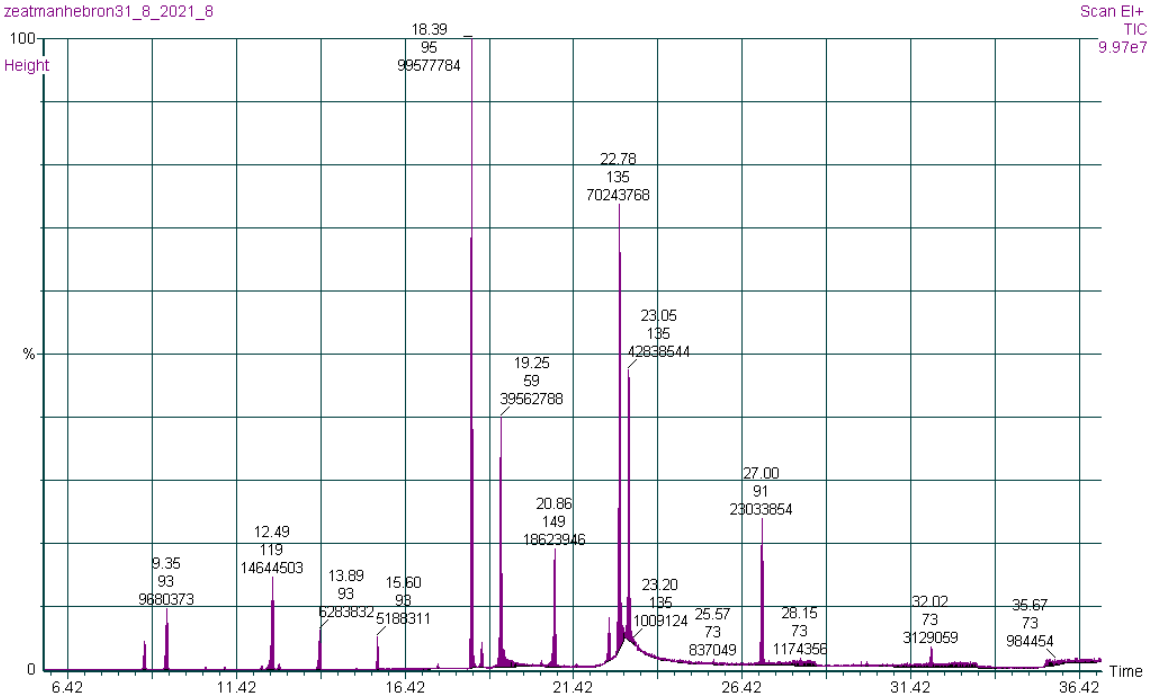
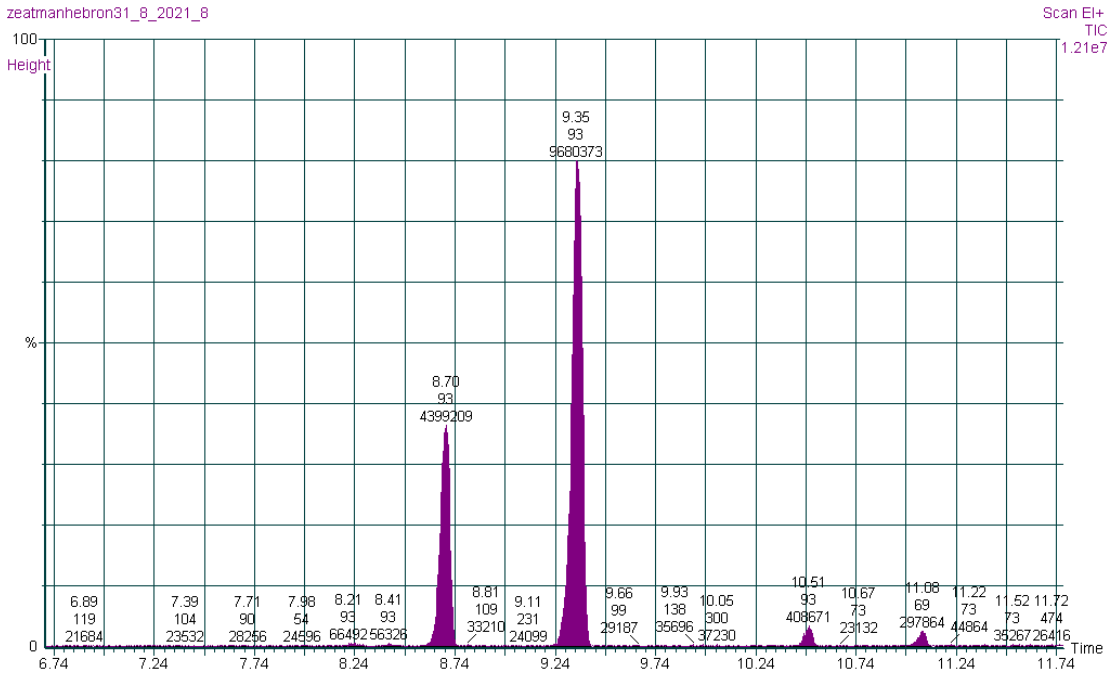
Appendices

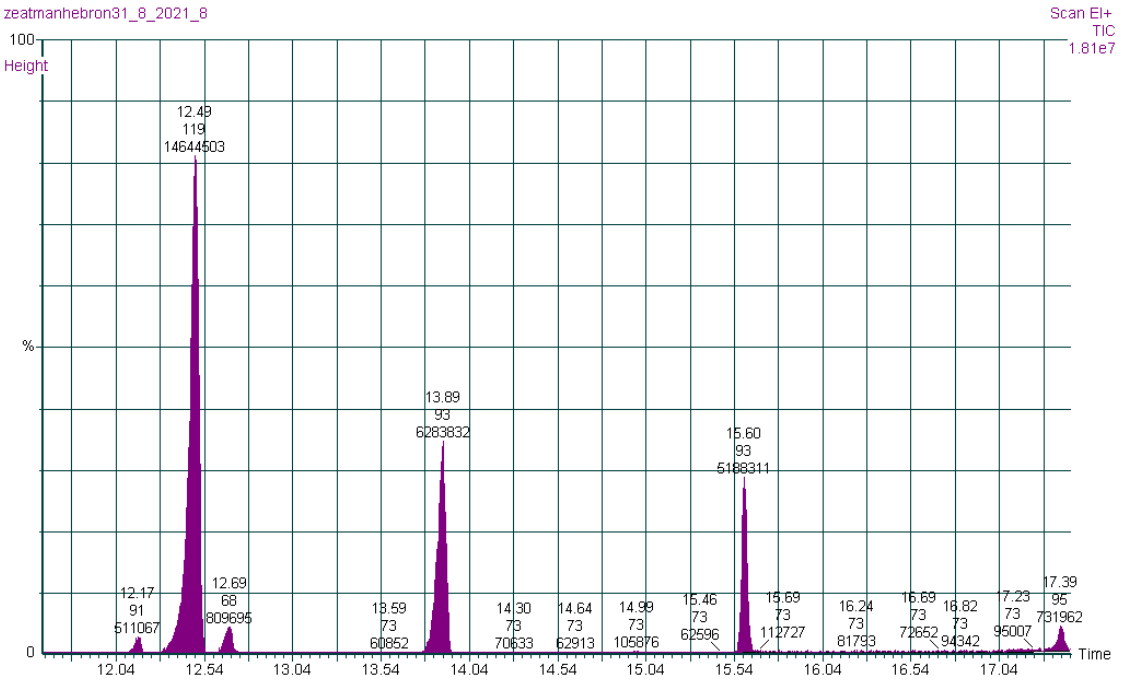
Appendix A

GC-MS analysis results

1.a- GC-MS analysis results for Hebron sample:



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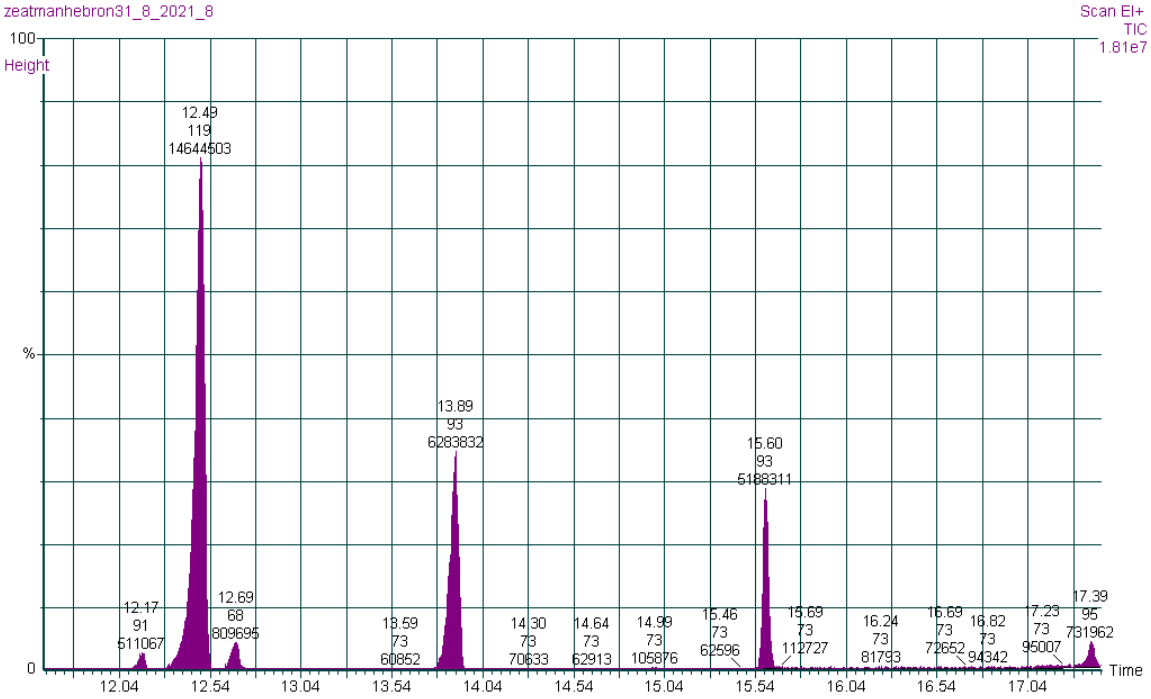
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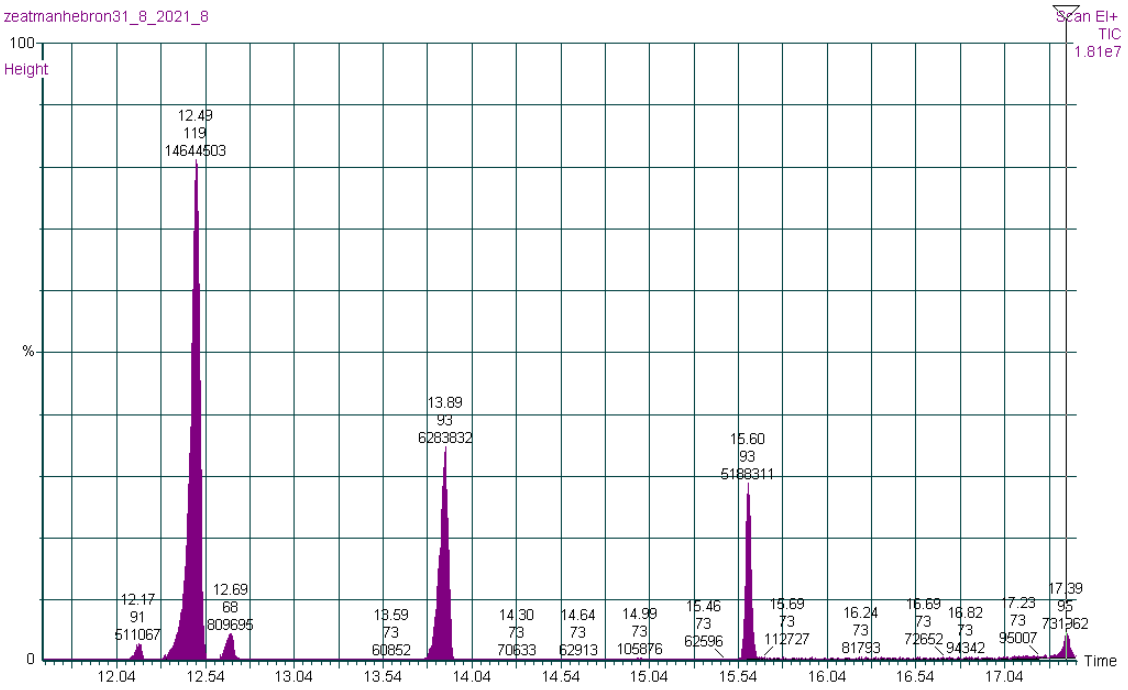
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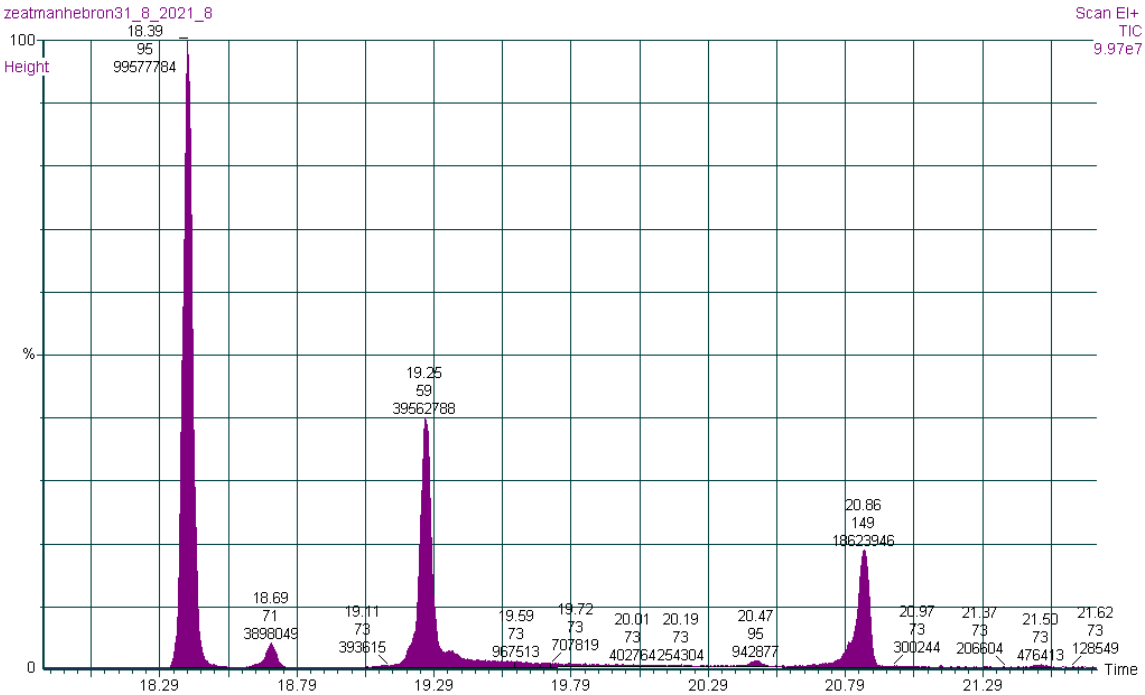
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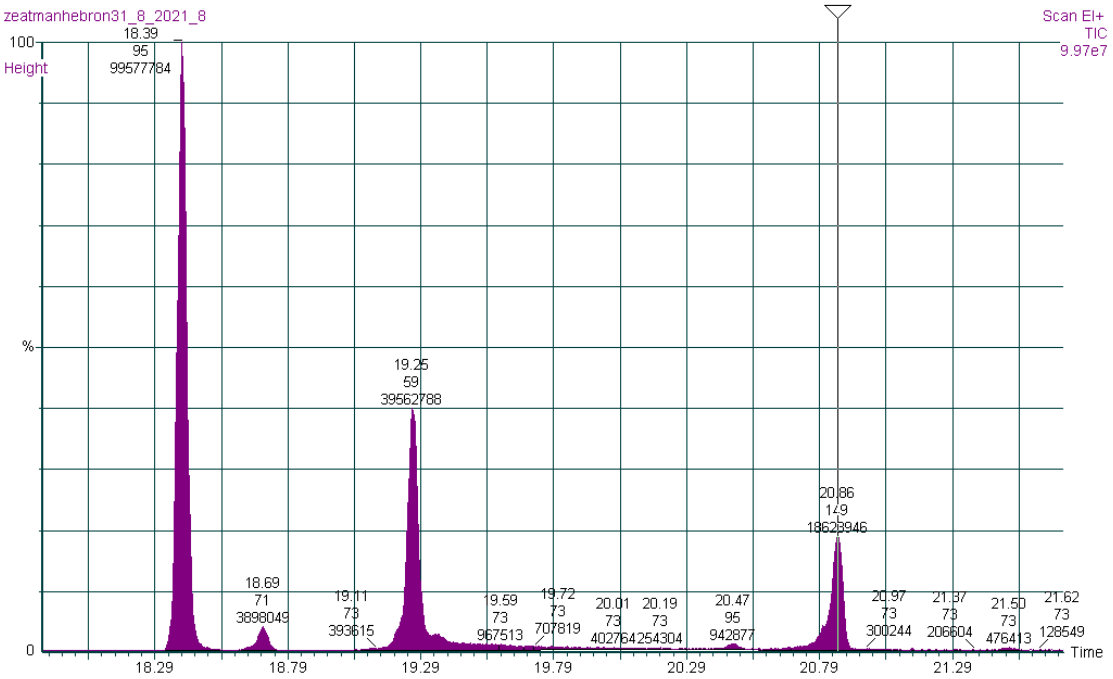
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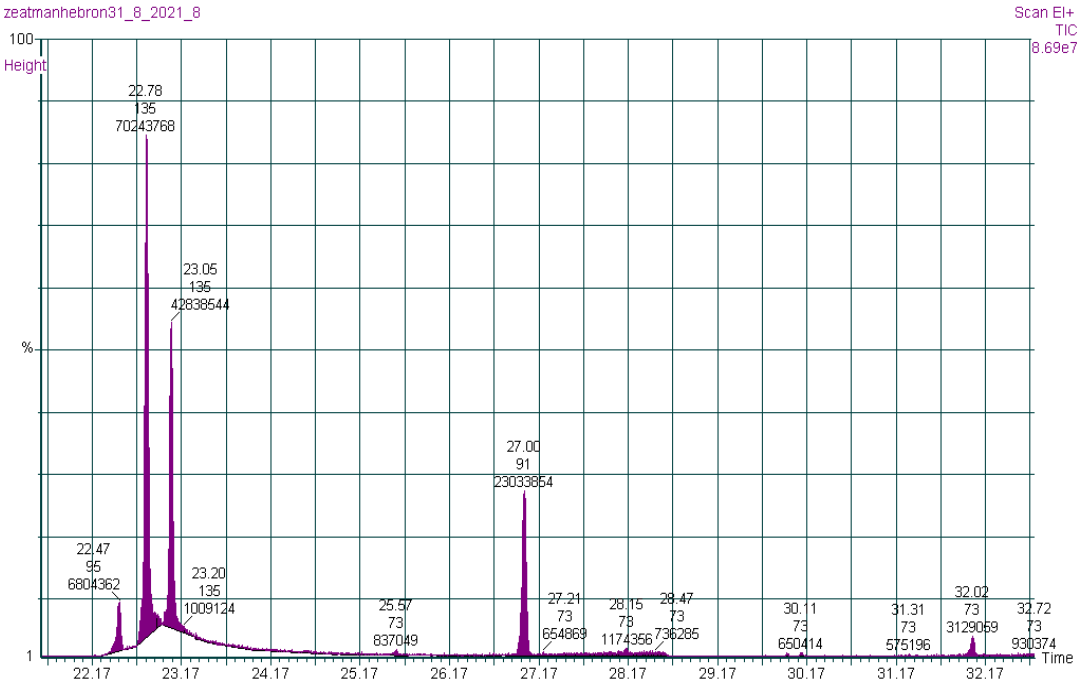
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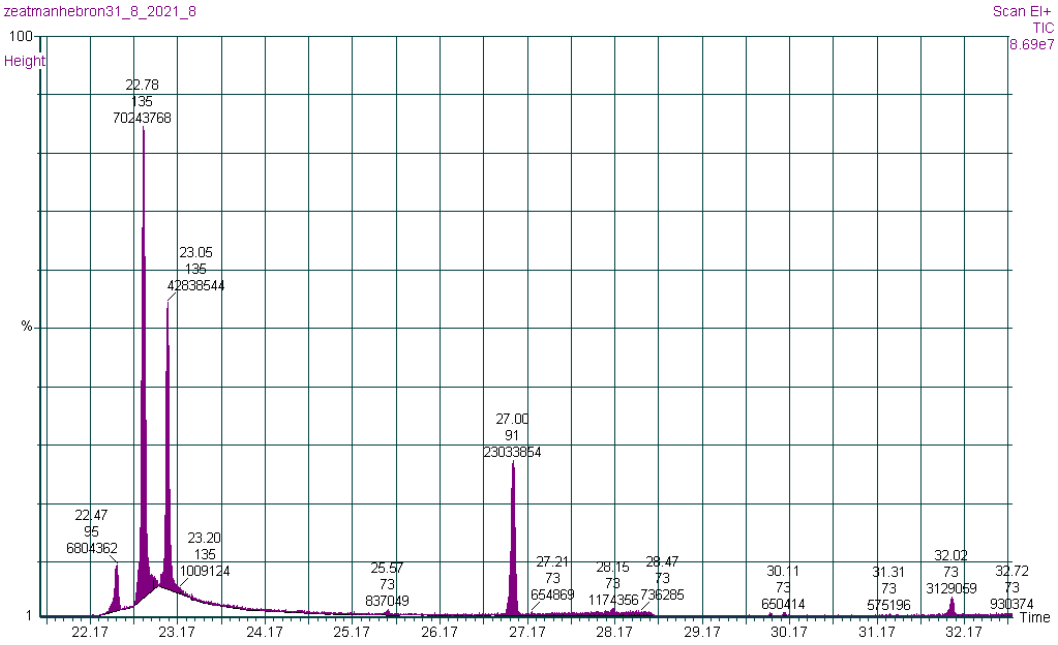
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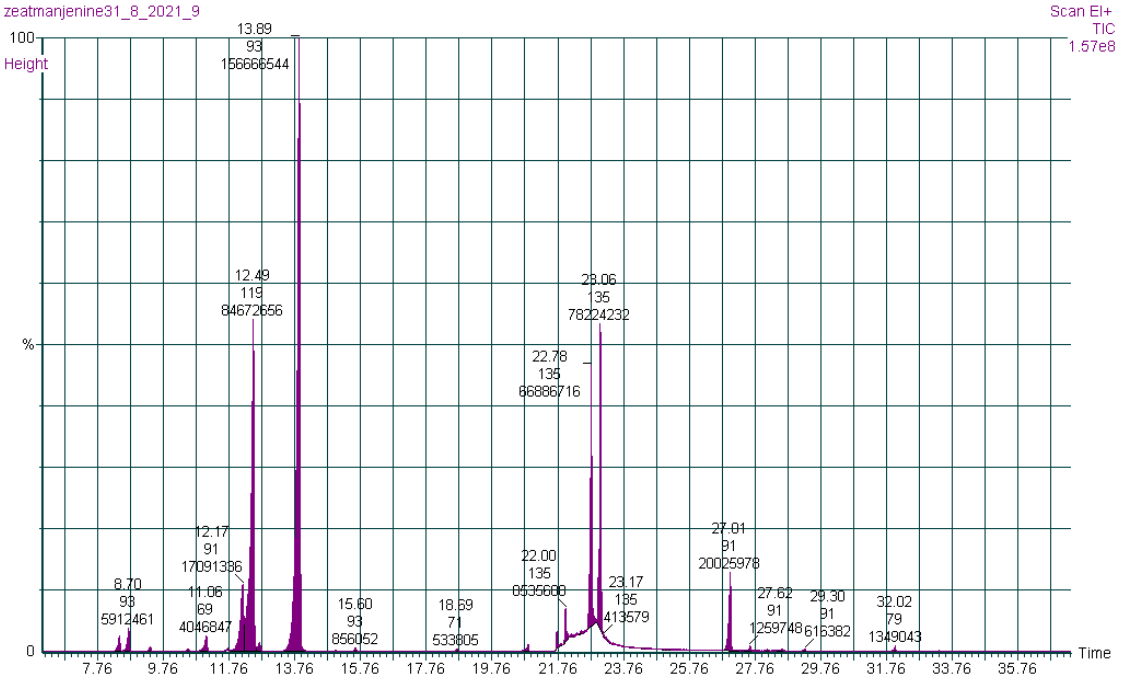

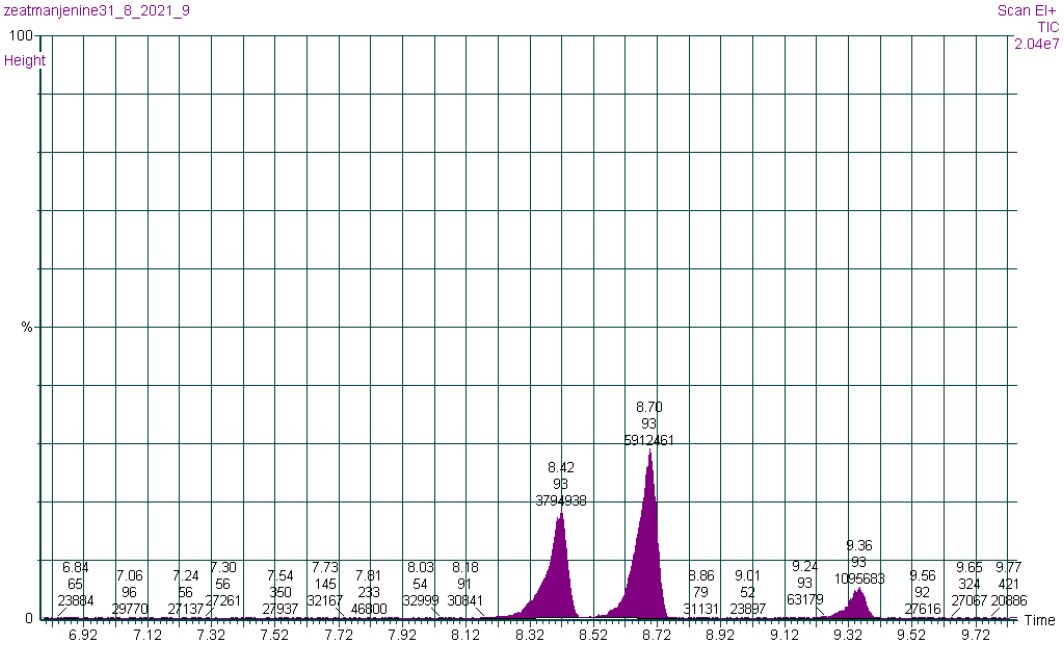

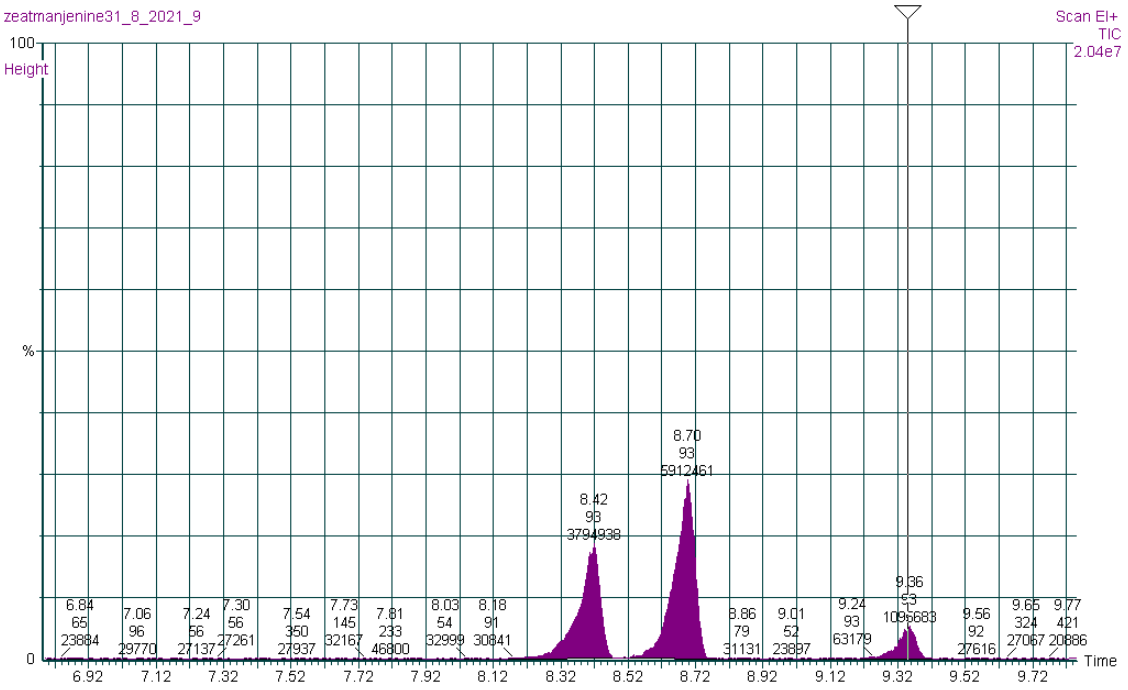
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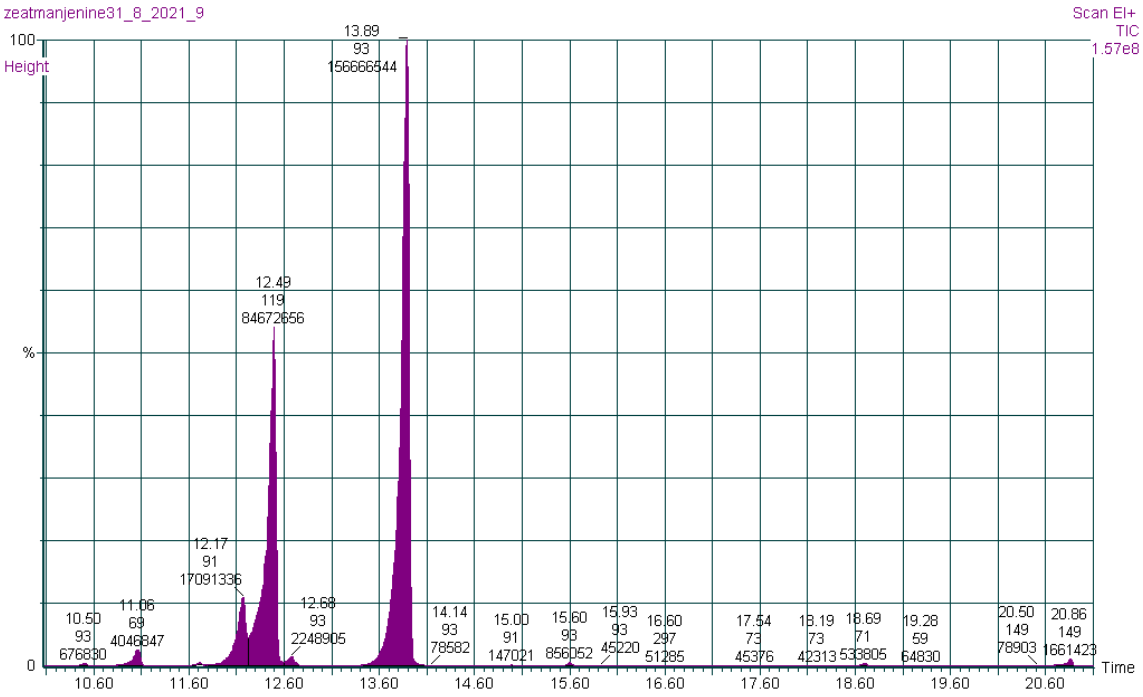
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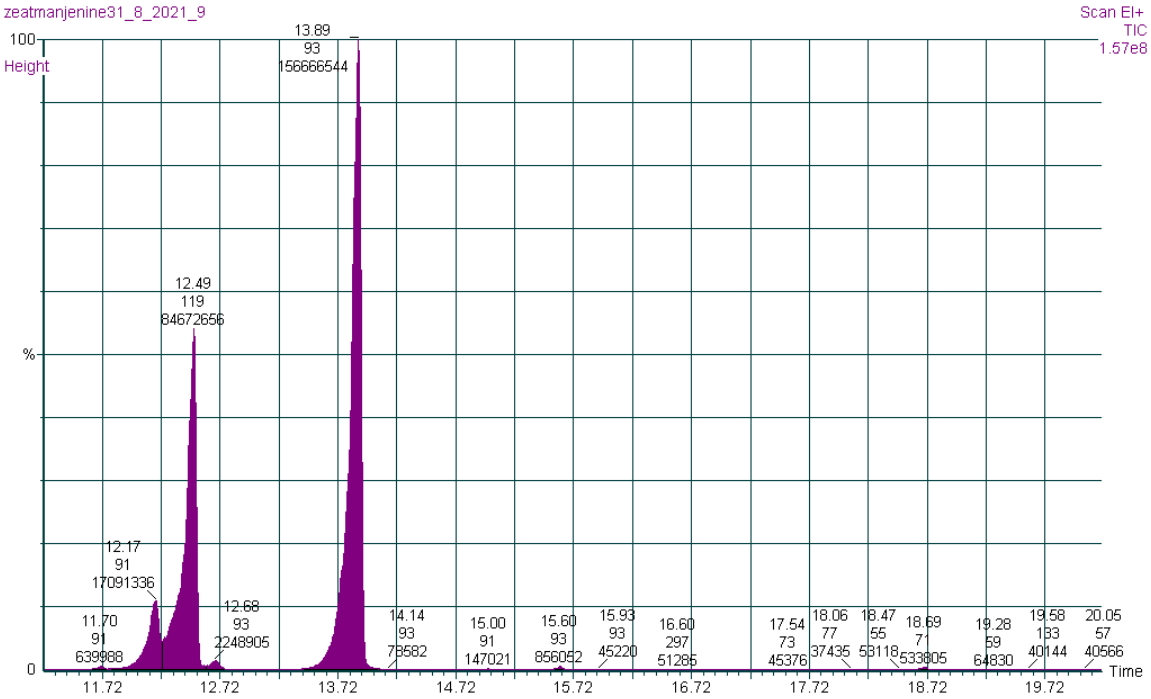
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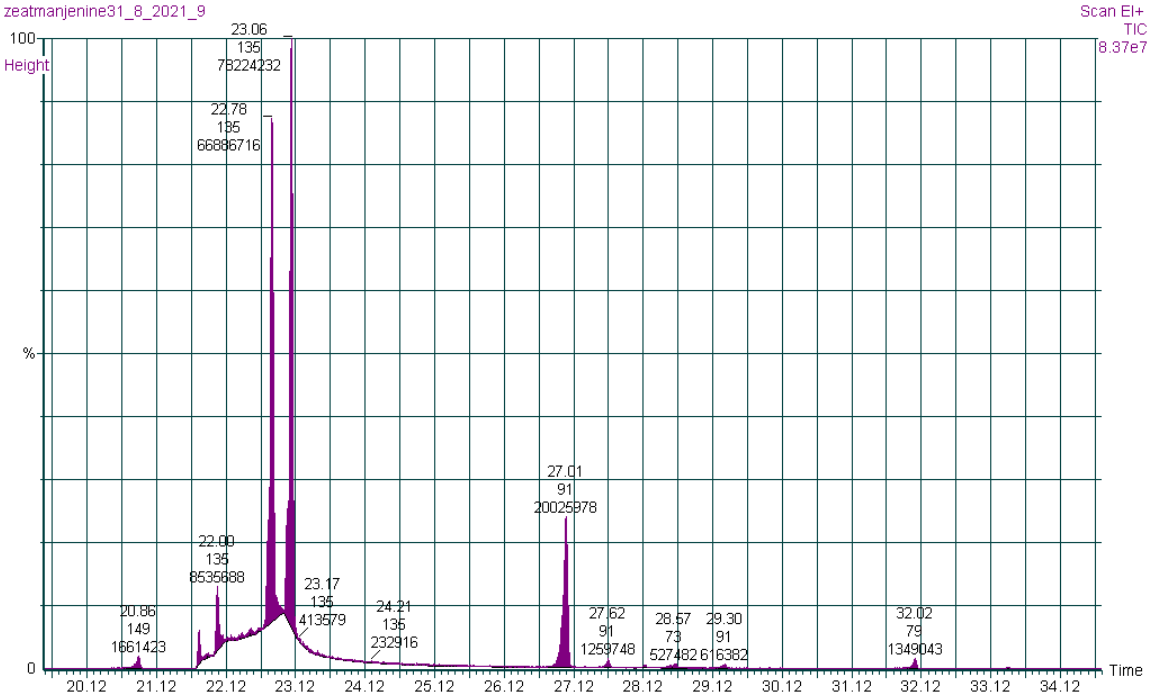
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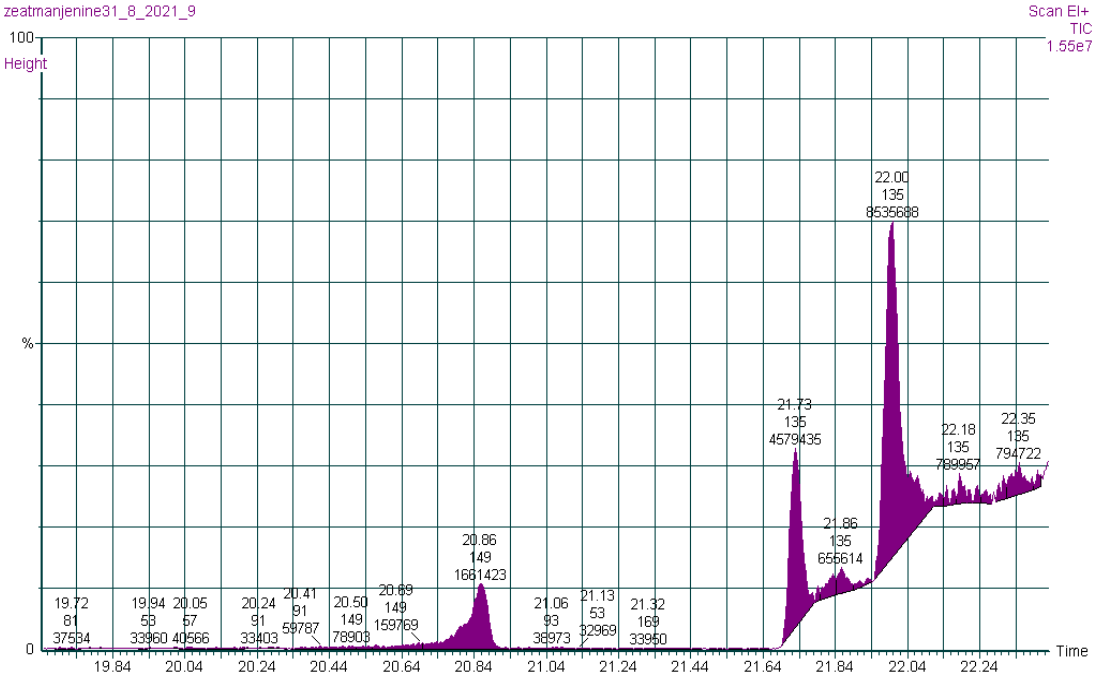
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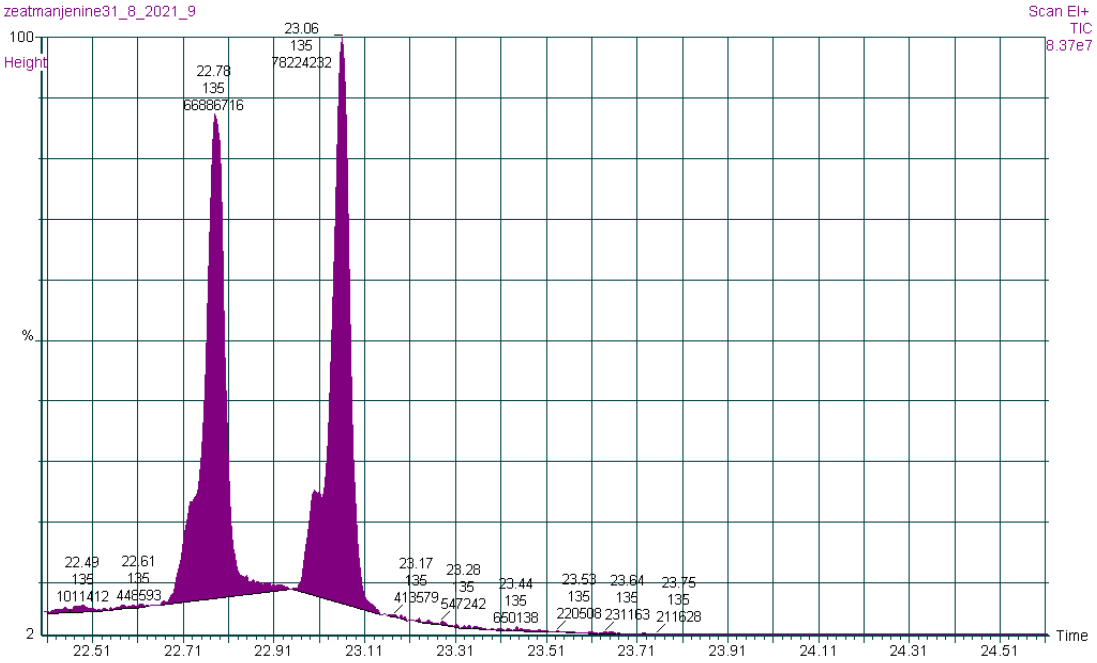
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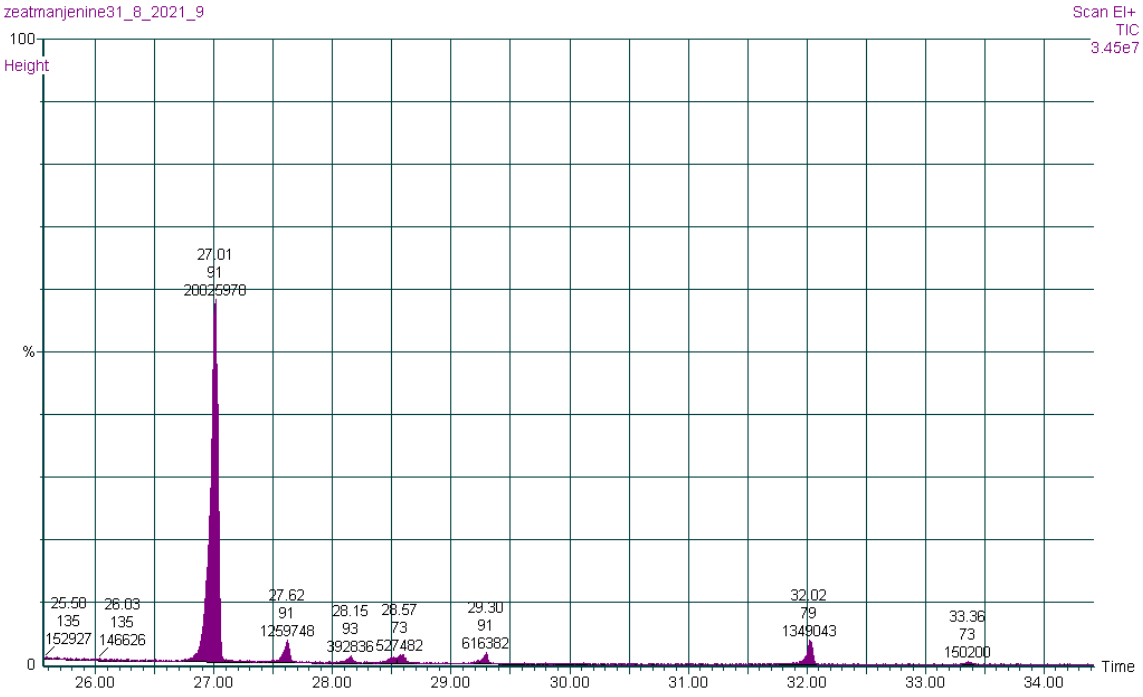
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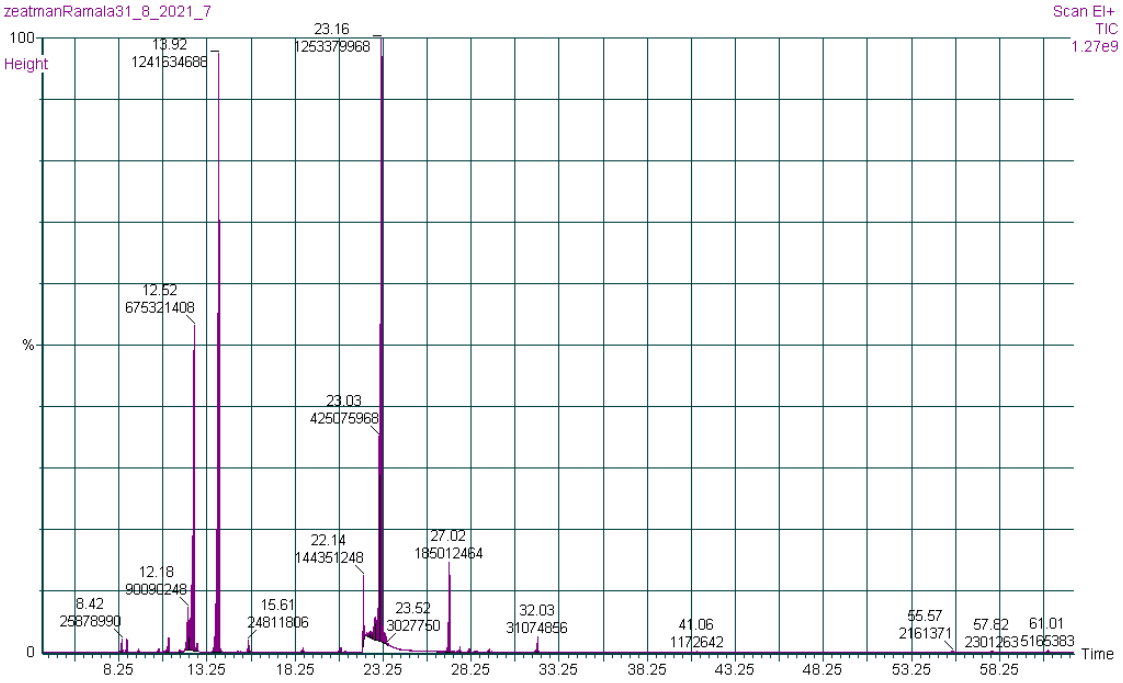
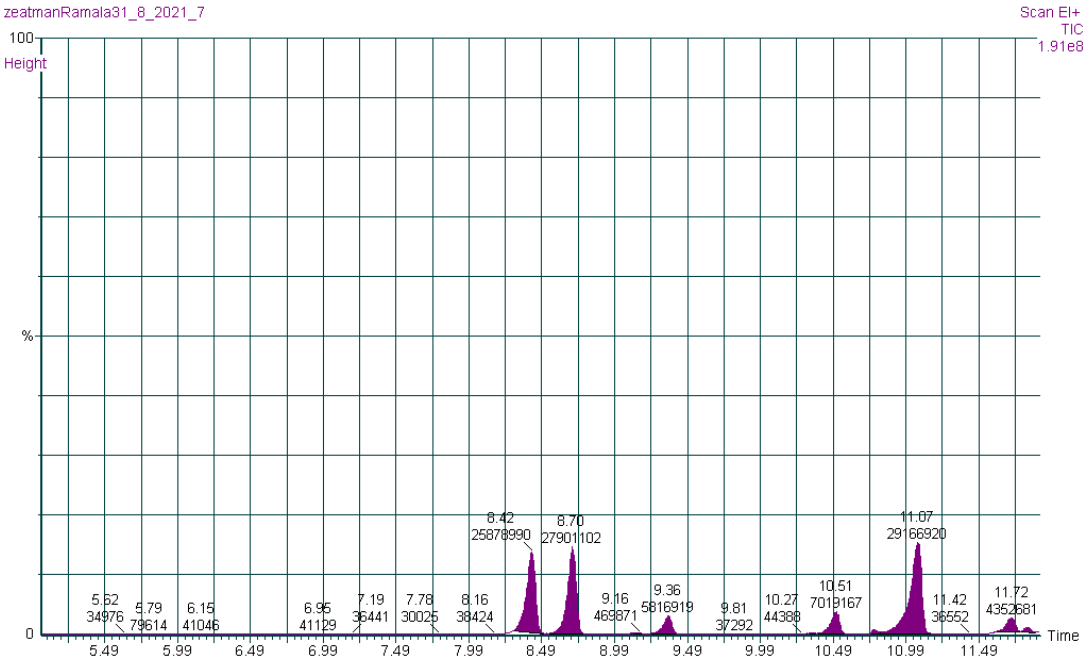
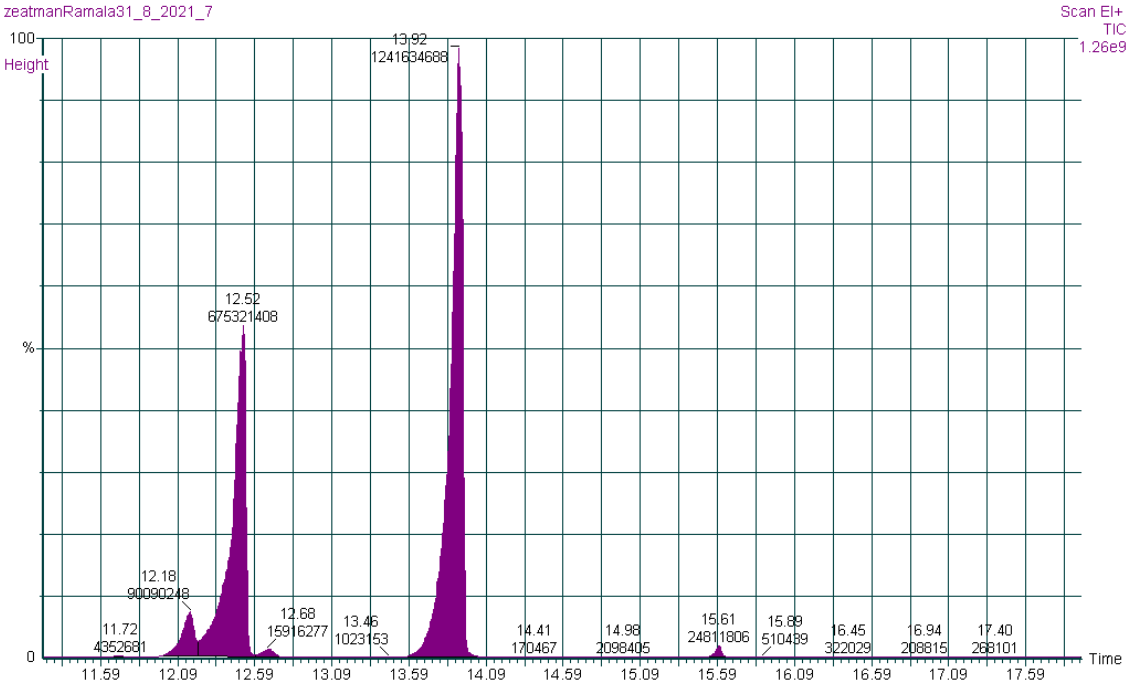
1.c- GC-MS analysis results for Ramallah sample:



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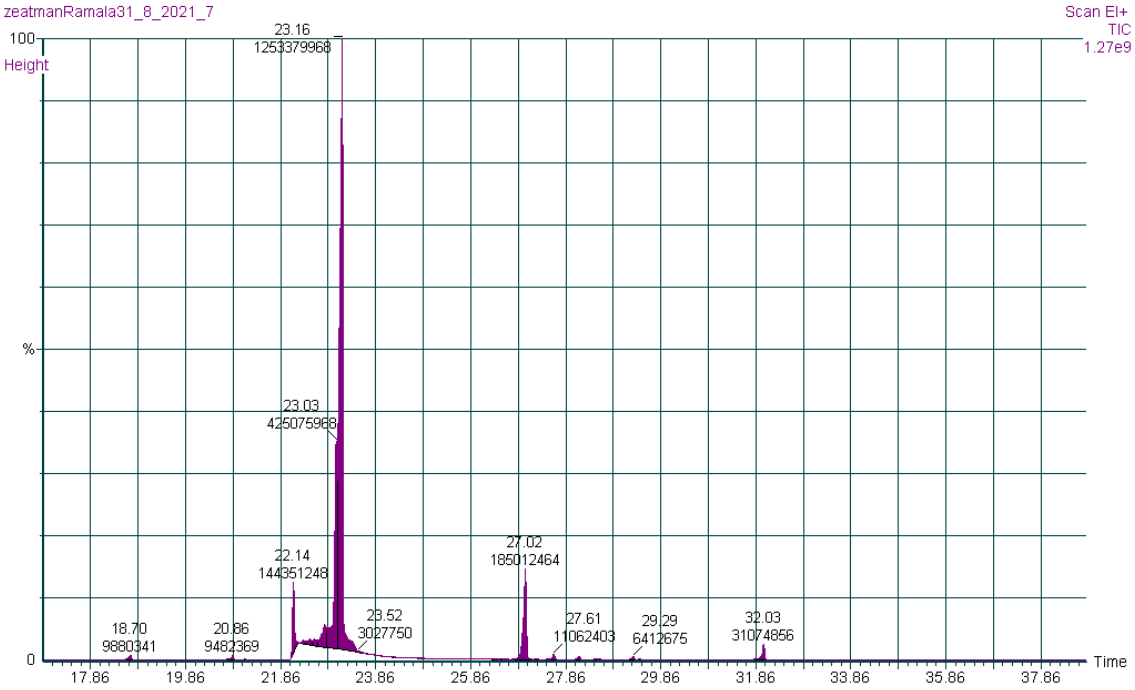
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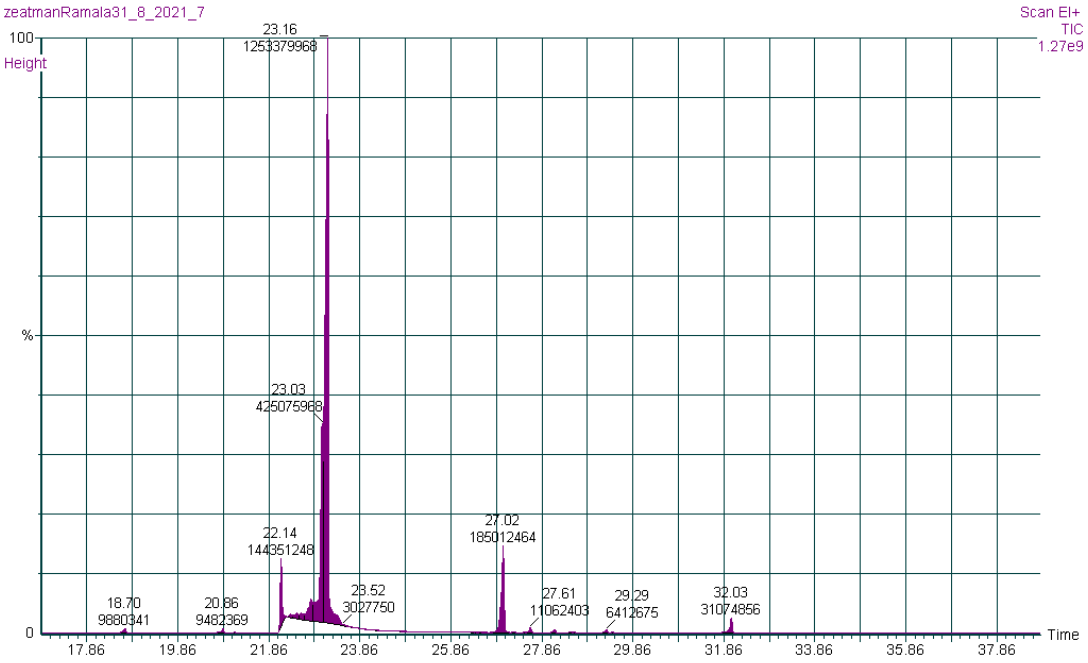
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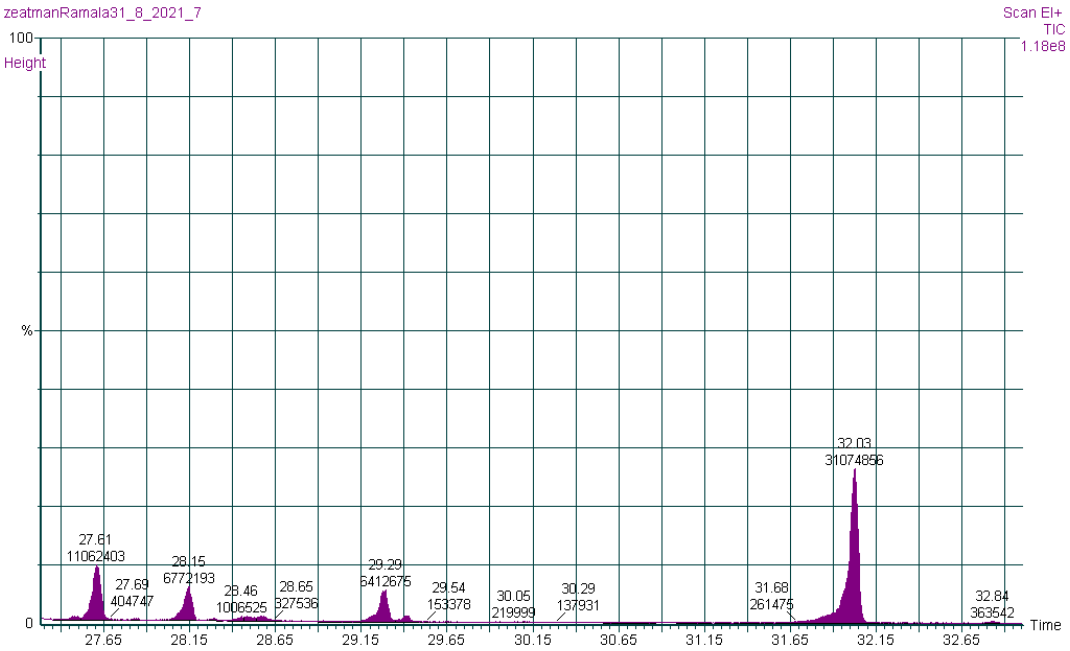
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Time

Appendix B

Tables of study

Table B.1

Chemical composition of T.capitatus EOs according to previous studies

Occurrence	**Main components**	References
Algeria, Balearic Islands, Corsica,	Carvacrol 6-86%. *P*-Cymene 2-26%. Γ-Terpinene t-17%. Thymol t-72%. B-Caryophyllene t-9%. Borneol t-10%. Myrcene t-9%.	[17, 18]
Italy, Morocco, Spain	Carvacrol 6-86%. Thymol t-72%. *P*-Cymene 2-26%. Γ-Terpinene t- 17%. Borneol t-10%. Myrcene t-9%. B-Caryophyllene t-9%. Linalool t-7%. Camphene t-6%. Terpinen-4ol t-6%. *Trans*-β- Ocimene t-5%	[19]
Matmata, Tunisia	Thymol (89.06%), p-Cimene (5.04%), g- Terpinene (3.19%).	[20]
Sicilian	Carvacrol (86.3%), β-Caryophyllene (2.8%), α-Elemol (1.4%)	[21]

Table B.2

SPSS analysis for DPPH results.

DPPH

Kruskal-Wallis Test

Ranks

Mean Rank	N	district number	
17.92	13	1.00	inhibition_1
30.73	13	2.00	
32.50	13	3.00	
54.62	13	4.00	
29.23	13	5.00	
	65	Total	

Test Statistics

inhibition_1	
25.985	Chi-Square
4	Df
.000	Asymp. Sig.

Table B.3

SPSS analysis for anti-lipase activity of the EOs for the three districts

Kruskal-Wallis Test

Ranks

Mean Rank	N	district_number	
12.83	6	1	Inhibition
11.17	6	2	
13.58	6	3	
12.42	6	4	
	24	Total	

Test Statistics[a,b]

Inhibition	
0.373	Chi-Square
3	Df
0.946	Asymp. Sig.

a. Kruskal Wallis Test

b. Grouping Variable: district_number

Table B.4

SPSS analysis results for Alfa amylase percent inhibition of EOs for three districts

Ranks			
Mean Rank	N	district number	
11.43	7	1.00	inhibition_1
12.21	7	2.00	
12.14	7	3.00	
22.21	7	4.00	
	28	Total	

Test Statisticsa,b	
inhibition_1	
8.272	Chi-Square
3	Df
0.041	Asymp. Sig.

Table B.5

Microbial growth MIC values of T.capitatus EOs for three districts

Bacteria	**MIC(µg/ml)**		
	Jenin	**Hebron**	**Ramallah**
1-proteus	0.78125	0.1953	0.3906
2-Candida	0.29295	0.1953	0.1953
3-MRSA	0.585925	0.1953	0.1953
4-S.areus	1.5625	0.1953	1.1718
5-Klebsiela	0.78125	0.1953	0.585925
6-E.coli	0.585925	0.1953	0.585925
S .D	0.4297	0.0	0.3636

Table B.6

statistical tests of antimicrobial assay for the three districts

Test Statisticsa,b	
	inhibition_1
Chi-Square	5.861
Df	2
Asymp. Sig.	.053

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

تباينات المكونات الكيميائية والأنشطة الحيوية للزيت العطري لنبتة الزعيتمان من ثلاث مناطق في فلسطين

إعداد

ألاء بركات

إشراف

د. نضال جرادات

د. أحمد خساتي

قدمت هذه الرسالة استكمالا لمتطلبات الحصول علي درجه الماجستير العلوم الصيدلانية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

2022

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الملخص

المقدمة: يعتبر نبات الزعيتمان من العقاقير الطبية التقليدية واسعة الانتشار في فلسطين، لذا هدفت هذه الدراسة إلى تقييم المكون العلاجي الكيميائي لزيت هذا النبات المستخرج من ثلاث مناطق فلسطينية من خلال استخدام أسلوب التقطير المائي وتقنيات الميكروويف الفرط صوتية، كذلك خضوع الزيت المستخرج من النبات لبعض الاختبارات البيولوجية.

المنهجية: تم تحديد العناصر المختلفة في زيت الزعيتمان وحصرها واختبارها من خلال استخدام مقياس الطيف GC- MS. وقد تم استخدام مقايسة ال DPPH ومقايسة حمض كاروتين ب (اللابنوليك) من أجل تحديد مستويات نشاط الأكسدة وذلك من أجل تحديد إذا ما كان مركب (ت كابيتاتس) بمتلك نشاطاً مضاداً (لليباز) أم لا، وتمت دراسة النشاط التثبيطي (ألفا أميليز) لعينات زيت الزعيتمان التي تم دراستها مفارنةً مع عقار سكر الدم (Acrabose). وكذلك تم إجراء اختبار مضاد للميكروبات ضد سبعة أنواع مختلفة من البكتيريا والفطريات الأكثر انتشاراً، وبالإضافة لذلك تم استخدام خلايا (Hep-G2) لتقييم النشاط المضاد للسرطان.

النتائج: أظهر زيت الزعيتمان عدة مكونات أساسية هي: أحادي التربيت، والثيمول، والكرافاكورال، واختلفت المكونات الكيميائية لزيت الزعيتمان باختلاف المناطق حيث أن (رام اللة كرافاكورال بنسبة 31.25%،

وبيتا تريبنين بنسبة (30.94%)، وجنين بيتا تريبنين بنسبة (67.0%) وبيتا تريبنول بنسبة (12.91%)، والخليل ثيمول بنسبة (40.35%)، و وبيتا كاريوفيلين بنسبة (13.23%) التي كانت المكونات الرئيسة لزيت الزعيتمان في هذه المناطق. ووفقاً لهذه النتائج، تبين أن النشاط المضاد للأكسدة مرتفع ويعتمد على الجرعة. كذلك اظهرت نتائج فحص ال(DPPH) ان المناطق الثلاث لديها نفس النسبة من IC50 تقريباً والتي كانت في المرتبة الرابعة تقريباً من احتوائها على حمض الغاليك. في المقابل، أظهرت نتائج فحص حمض (بيتا كاروتين لينوليك) أن جميع العينات لديها مضادات للأكسدة أعلى من الماء (الضابط) ومضاد الأكسدة الصناعي ألفا توكوفيرول والذي أعطى أعلى تحلل للبيتا كاروتين. عمل النبات ضد جميع البكتيريا والفطريات التي تم اختبارها من حيث فعاليتها في هذا المجال حيث تراوحت قيم (MIC) بين 0.1953 و 1.5625 ميكروغرام /مل، وأعطت عينة الخليل نتائج مميزة بتراكيز منخفضة. أظهرت جميع العينات نشاطاً مضاداً لليباز أعلى من (Orlistat) بتركيزات تساوي أو تزيد عن 200جم/مل. وأكثر من ذلك، عملت عينات النبات الثلاث على تثبيط تركيز ألفا أميليس بشكل معتمد وكشفت نتائج التحليل الإحصائي عن اختلاف طفيف بين العينات الثلاث لكن جميعها أظهرت نفس النسبة المئوية للتثبيط عند 400جم/ مل وهو 50.0% أكاربوز. وأخيرا، ووفقاً للنشاط السام للخلايا، أظهرت جميع العينات نتائج واعدة ضد (Hep-G2) بمتوسط تثبيط بنسبة 85.05 وبتركيز 62.5 ميكروغرام/ مل مع وجود اختلافات طفيفة تعود للمناطق.

الخاتمة: يمكن أن يرتبط التركيب الكيميائي لزيت الزعتر بأصل النبات، ومكونات التربة، والمتغيرات الوراثية، والطروف المناخية المحيطة، والتي بدورها تنعكس على النشاط البيولوجي لها.

الكلمات الرئيسة: الزعيتمان، والتقطير المائي، والثيمول، والكارفاكورال، ومضادات الليباز، وفحص ال(DPPH).