An-Najah National University

Faculty of Graduate Studies

PHYTOCHEMICAL COMPOSITION, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF *CLINOPODIUM INSULARE* ESSENTIAL OIL FROM PALESTINE

By

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This Thesis was Defended Successfully on 5/2/2023 and approved by

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Dedication

To my wife

To my parents

To my brothers and sisters

To all my friends

This project will be beneficial.

Acknowledgments

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Declaration

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

PHYTOCHEMICAL COMPOSITION, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF *CLINOPODIUM INSULARE* ESSENTIAL OIL FROM 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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PHYTOCHEMICAL COMPOSITION, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF *CLINOPODIUM INSULARE* ESSENTIAL OIL FROM PALESTINE

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ABSTRACT

Clinopodium insulare (*Calamintha incana*) is one of the medicinal aromatic plants that is dominant in the eastern Mediterranean regions, including Palestine, and has a distinctively pleasant mint-like smell. Traditionally, *C. incana* leaves are used as a spice and herbal tea. The current work's goal is to examine the potential pharmacological characteristics of chemical constituents.

The essential oil of the plant was extracted using the hydrodistillation (Clevenger method) technique. The use of gas chromatography–mass spectrometry (GC–MS), the chemical components of the plant's essential oil were determined. The essential oil's antioxidant capacity was evaluated by inhibiting 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The 3,5-dinitrosalicylic (DNSA) assay was used to assess anti-amylase activity. Anti-lipase activity was assessed using the p-nitrophynel butyrate method.

Antimicrobial activity was assessed using the broth microdilution method for antibacterial testing and the poisoned food technique for fungal testing. Six bacterial strains were used: four Gram-negative: *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, and Klebsiella pneumonia;* and one yeast: *Candida albicans*. Two were gram-positive: *MRSA (Methicillin-Resistant Staphylococcus aureus)*; in addition, the oil was tested against three dermatophytes: *Microsporum canis, Trichophyton rubrum, and Trichophyton mentagrophyte*. Anti-cancer activity was evaluated by using the MTS assay on HeLa cells.

Plant essential oil yield was 0.213 percent (w/w). GC-MS analysis detected the presence of sixty compounds, which accounted for 98% of the total oil composition. The major compound was piperitone oxide, representing 41.178%. *C. incana* showed anti-oxidant activity with an IC_{50} of 390 µg/mL.. Essential oil has a moderate amylase inhibitory effect with an IC_{50} value of 120 µg/ml and a moderate anti-lipase inhibitory effect with an IC_{50} value less than 800 µg/ml. The sample showed broad antimicrobial activity, potent against Gram-negative bacteria with MICs ranging between 0.4883 ug/ml and 62.5 ug/ml , between 26.041 ug/ml and 31.25 ug/ml for Gram-positive bacteria, and yeast with a MIC of 0.2441 ug/ml. The antifungal activity against *Microsporum canis, Trichophyton rubrum, and Trichophyton mentagrophyte was observed,* with MICs ranging from 0.15 µl/ml against *Trichophyton mentagrophyte* to 0.37 µl/ml against *Trichophyton rubrum*. The anticancer activity of the oil was tested against HeLa cells and showed promising results with an IC_{50} dose of 50 µg/mL.

The plant's essential oil contained varying percentages of various phytochemicals, which provided various potential biological activities such as antioxidant, anti-lipase, anti-amylase, anti-cancer, and antimicrobial effects.

Keywords: Essential oil; Antibacterial activity; Antifungal activity; *Clinopodium insulare*.

Chapter One

Introduction

1.1 Traditional medicine

Medicinal plants have been used since the Vedic era. They have been used to treat and prevent many types of infections, as well as epidemics, for many centuries. In Indian, Egyptian, Chinese, Greek and Roman cultures, they have been utilised to heal illnesses and revitalise body systems. According to estimates, there are around 300,000 plant species on our planet. A biologically active substance that can be utilised to treat illness can be made from over 135,000 plant species [1].

Herbal medicines are becoming more and more popular in the treatment of illnesses due to their low cost, safety, improved acceptance, compatibility with the human body, few side effects and ease of storage [2-4].

The pharmaceutical and scientific communities have recently focused on traditionally used medicinal plants as a result of the growing interest in herbal medicines among Western countries and the urgent need to create new, efficient medications. Part of this focus includes putting the secondary metabolites of plants into clinical settings in order to establish their biological properties [5].

Secondary metabolites that come from plants are called phytochemicals and have pharmacological properties such as antioxidative, anti-allergic, antibiotic, hypoglycaemic and anticarcinogenic [6].

Some plants' essential oils have potent antioxidants or have a low risk of side effects when used to treat diabetes. Researchers are interested in this and want to study these plants to learn more about how they work biologically [7].

The use of a unique systematic approach for the separation, identification and determination of chemical constituents is combined with various biological activity tests on plant extracts [8].

1.2 Modern phytochemistry

The basis of traditional medical methods that have been practiced for thousands of years by people in China, India and many other nations has been developed from plants. For the development of new therapeutic agents against serious illnesses, a full knowledge of phytochemicals is essential [9].

Plant compounds are thought of as secondary metabolites since the plants that produce them might not have much need for them. They can be found in all plant body parts, including the stems, leaves, bark, seeds, flowers, fruits and roots, confirming that every part of a plant can have active ingredients. The identification of bioactive standards found in medicinal plants is assisted by the screening of phytochemicals, which can also lead to the development of new medications. In the current research, the primary phytoconstituents of 30 traditionally used medicinal herbs were identified, and their existence was connected to the bioactivities of the plants [10].

Standard methods for identifying and analysing chemical constituents, such as LC-MS (liquid chromatography-mass spectrometry), LC-NMR (liquid chromatography-nuclear magnetic resonance), GC-MS (gas chromatography-mass spectrometry) and LC-MS/MS (liquid chromatography-tandem mass spectrometry), were used for the screening of the plants. The results made it possible to determine if the plant had tannins, flavonoids, phenolics, saponins, steroids, cardiac glycosides or alkaloids [11].

1.3 Main chemical constituents in medicinal plants

Through the process of photosynthesis, plants produce carbohydrates and oxygen in their leaves by utilising carbon dioxide from the atmosphere and water taken up through their roots. In all plants, the biosynthetic pathways are the cause of the occurrence of both primary and secondary metabolites. Secondary metabolites are chemicals that have a role in how an organism interacts with its environment but are not essential for an organism to survive. These compounds frequently contribute to a plant's ability to resist biotic or abiotic stresses. Alkaloids, polyphenols, terpenes, flavonoids, sugar alcohols, phenolics, quinines and many other metabolite groups make up secondary metabolites [12].

Some secondary metabolites serve as highly valuable chemicals that are used as drugs, flavours, fragrances, pesticides and dyes. They can also serve as a manufacturer's defence against pathogens, wild animals or competitors; as a resource for pollination or seed dispersal; as a means of preventing or modifying extrinsic abiotic elements; or a combination of these purposes [13]. Flavonoids and phenolic acids have antioxidant and anticarcinogenic properties as well.

1.3.1 Phenolic compounds

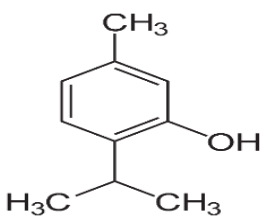
One of the groups that plants may produce during development or as a reaction to stimuli such as pollution, injury and UV radiation are phenolic chemicals. Based on the proportion of phenolic subunits, a basic class attempts to divide the broad category of phenolics into simple phenols and polyphenols. Thus, "plant phenolics" include simple phenols, flavonoids, phenolic acids, stilbenes, hydrolysable and condensed tannins, lignans, lignins and coumarins [14].

Phenolic acids that contain rings in their structure comprise two distinct carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures. The number and location of the hydroxyl groups on the aromatic ring distinguish the different types, even if the basic structure remains the same. Examples include vanillic, caffeic, p-coumaric and ferulic. Certain natural sources contain extra acids (*e.g.*, gentisic, syringic). There are many examples of the main groups of compounds in plants [15].

Thymol: Terpinene, the key element of Lamiaceae essential oils, is anticipated to be used in the synthetic process of this fragrant monoterpene. It is a derivative of hydroxybenzoic acid, which certain plant species create as a chemoprotective mechanism against diseases, parasites, predators or environmental exposure **(**Figure 1.1) [16].

Figure 1.1

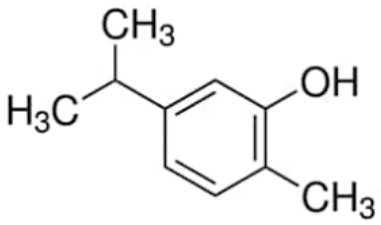
Chemical structure of thymol



Carvacrol: This is a phenolic monoterpene present in the essential oils of several plants, including pepperwort (*Lepidium flavum*), wild bergamot (*Citrus aurantium bergamia*), oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*). Carvacrol is reported to have an extensive variety of bioactivities, including antibacterial, antioxidant and anticancer properties that may be beneficial in therapeutic settings. Carvacrol has more antibacterial action than other volatile substances found in essential oils because it has a free hydroxyl group, is hydrophobic and contains phenol [17].

Figure 1.2

Chemical structure of carvacrol



1.3.2 Flavonoids

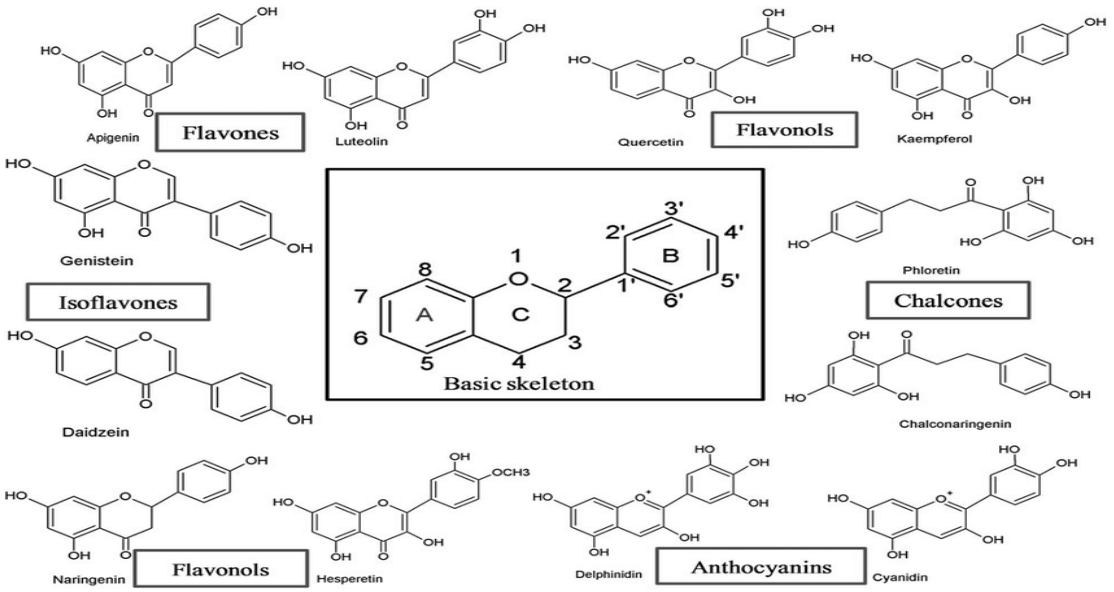
The polyphenolic chemical family known as flavonoids, which has over 2500 distinct forms, is widely cultivated across the plant world. These substances can be found in fruits, vegetables, cereals, tea, wine, and the bark, roots and stems of plants [18].

It is becoming more and more significant that flavonoids are involved in biological processes in plants, animals and microbes. Flavonoids give flowers their colour and fragrance and have long been recognised to be generated in certain places in plants. Because of this, they attract pollinators, which help spread the fruit, germinate the seeds and spores, and help young plants grow and develop [19].

Flavonoids are divided into subgroups depending on the carbon of the c-ring to which the b-ring is bound, as well as the level of unsaturation and oxidation of the c-ring. Isoflavones are flavonoids that bind the b-ring to the c-ring at position 3. Neo-flavonoids are b-rings coupled in position 4 of the c-ring, whereas b-rings connected in position 2 can be further differentiated based on the structural functions of the c-ring. These compounds are categorised as chalcones, anthocyanins, flavones, flavonols, flavanones and flavanonols. (Figure 1.2) [20].

Figure 1.3

Flavonoids subgroups



Due to their anticarcinogenic, antimutagenic, anti-inflammatory and antioxidant characteristics, as well as their ability to alter important cellular enzyme processes, flavonoids are linked to a wide range of health benefits. They are also believed to function as potent inhibitors of a number of enzymes, including cyclo-oxygenase (COX), lipoxygenase and phosphoinositide 3-kinase [21]. Almost all flavonoid families have antioxidant properties. According to some research, the most potent flavonoids for protecting the body from oxygen radicals are flavonoids and catechins [22].

1.3.3 Flavonoid and phenolic biosynthesis pathway

1.3.3.1 Flavonoid

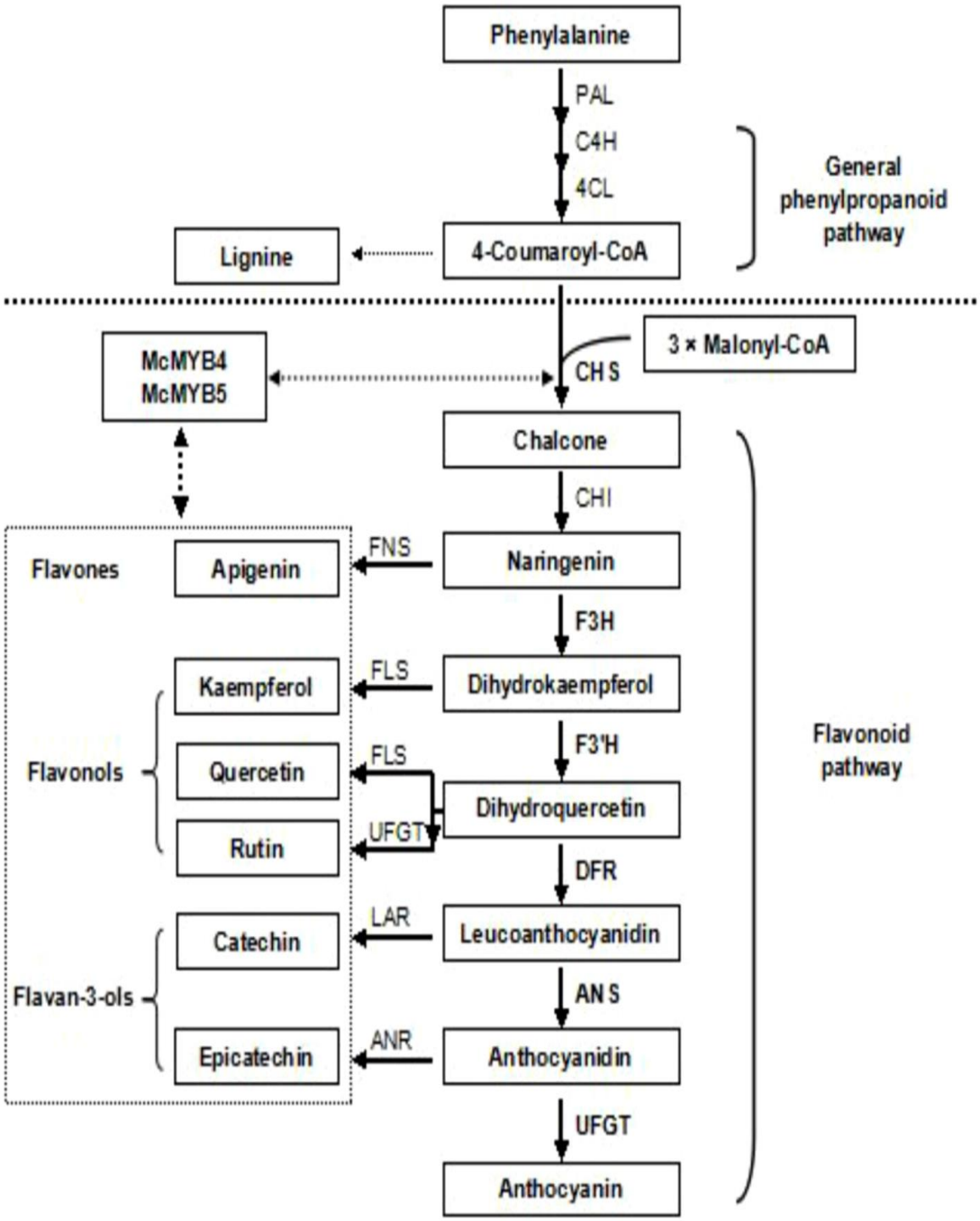
Robert Boyle discovered flavonoids for the first time in 1665 as components of plant colour. There are around 6350 different types of flavonoid chemicals. They are highly abundant in the kingdom of plants and are low-molecular-weight polyphenolic chemicals. Flavonoids are concerned with unique survival and adaptive strategies. These include producing pigment for UV defence, promoting pollen germination and fertility, providing defence against harmful microbes, and acting as signal molecules in plant-microbe interactions [23].

The condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA results in a tetra-ketide, which is the first step in the biosynthesis of flavonoids. To create naringenin-chalcone, the resultant tetra-ketide intermediate proceeds through intramolecular cyclisation. Chalcone synthase (CHS) catalyses this reaction. Malonyl-CoA is decarboxylated into an acetyl-CoA carbanion during the condensation process. However, since iso-flavonoids are generated from 2,4,4-trihydroxychalcone (iso-liquiritigenin), which lacks a hydroxyl group, there may be a slight alteration in the reaction catalysed by CHS for iso-flavonoid production. Prior to cyclisation, the hydroxyl group is probably reduced by the polyketide . Chalcone ketide reductase, a NADPH-structured enzyme that may interact with CHS, catalyses this process [24].

From this point on, the flavonoid biosynthesis route is divided into multiple aspect-branches, which together generate various types of flavonoids, including flavonols, pro-anthocyanidins, flavones, isoflavones, flavanones and anthocyanins. Naringenin is a key intermediate in this process [25-27].

Scheme 1.1

Enzyme entry into the flavonoid biosynthesis pathway



1.3.3.2 Phenolic acid

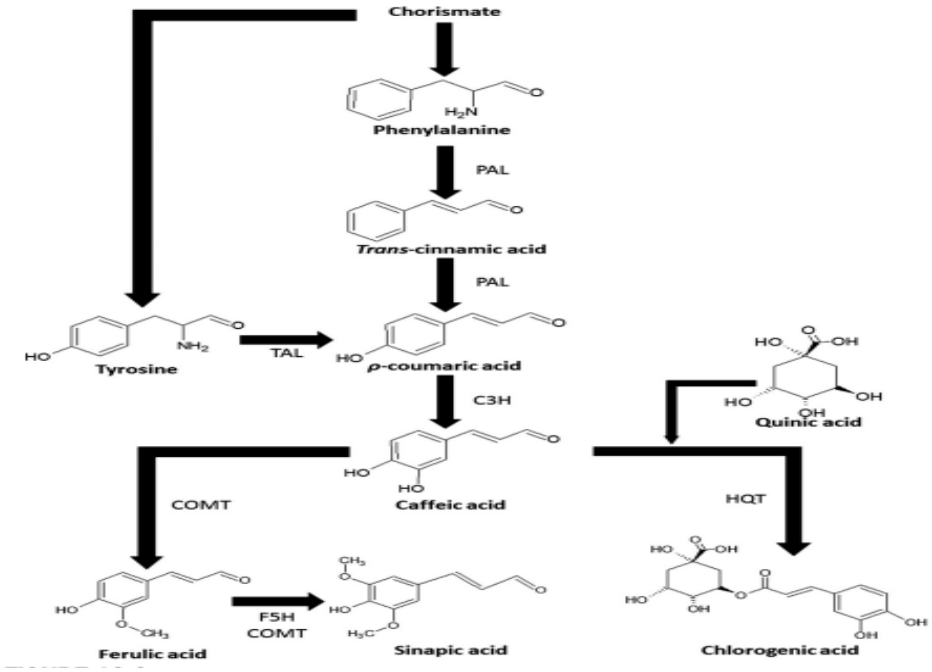
Tyrosine is required in smaller amounts by some plant species, while phenylalanine is often the main substrate for phenolic compounds synthesis through the phenylpropanoid pathway in vegetation. There are various classes for phenolic compounds [28].

A. Hydroxycinnamic acids

The first step in the production of phenolic compounds from l-phenylalanine is the removal of an amino group via the enzyme phenylalanine ammonia lyase. Finally, p-coumaric acid is created by adding a hydroxyl group to position 4 of trans-cinnamic acid by the action of the enzyme cinnamate-4-hydroxylase (CH4). Tyrosine ammonia lyase (TAL), a tyrosine deaminase, may also be used to produce p-coumaric acid from tyrosine [29]. The addition of a hydroxyl group at position 3 by the enzyme coumaryl 3-hydroxylase (CH3), which yields caffeine, is a significant step in the production of hydroxycinnamic acids. Caffeic acid o-methyltransferase is an enzyme that turns caffeic acid into ferulic acid via 3-O-methylation (COMT). When the enzyme hydroxycinnamoyl-coenzyme quinate transferase (HCQT) esterifies caffeoyl-CoA with quinic acid from the conjunction of caffeine and one coenzyme (CoA) via the enzyme 4-coumarate CoA ligase, chlorogenic acid is produced. This is the final stage in the biosynthesis of hydroxycinnamic acid, and sinapic acid is obtained from ferulic acid by means of its hydroxylation at position 5 and subsequent O-methylation by the action of ferulic 5-hydroxylase (F5H) and COMT, respectively. It is possible to use coumaric, ferulic and sinapic acids to produce lignin [25, 30].

Scheme 1.2

Hydroxycinnamic acid pathway of synthesis

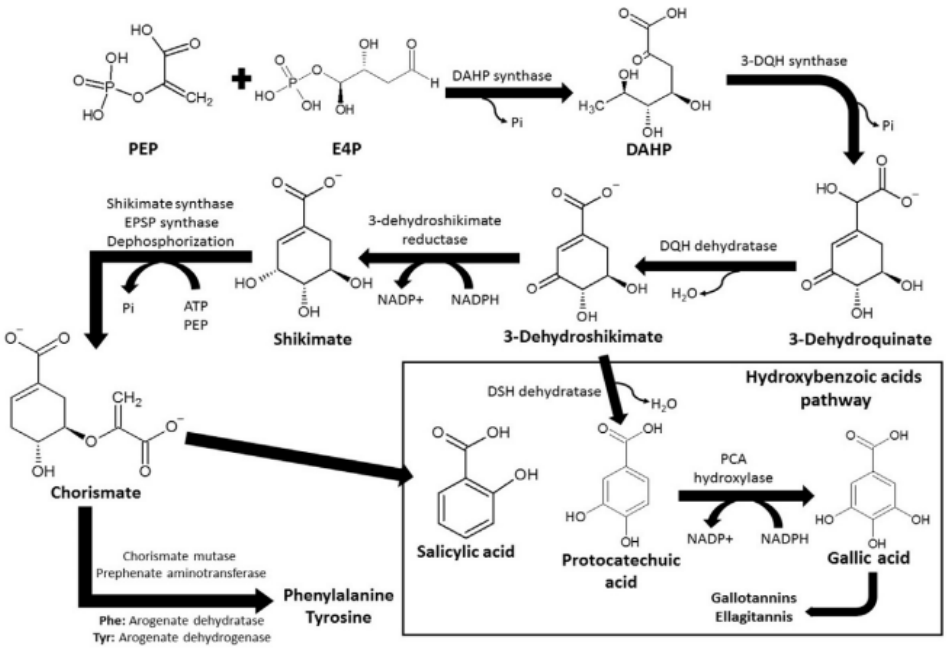


B. Hydroxybenzoic acids

Since hydroxybenzoic acids are no longer phenylpropanoids, they can be synthesised even if Buddy isn't constantly active. They can be made directly from the shikimic acid pathway. The most useful hydroxybenzoic acids are gallic and salicylic acids [31]. To produce gallic acid, the enzyme shikimate dehydrogenase generates 3,5-di-dehydro shikimate from three dehydro-shikimate (DHS) via the shikimic route [32]. Gallic acid may be used to create polymeric substances like gallo and ellagi-tannis. The shikimic acid route can be used to generate salicylic acid in plastids [25, 33].

Scheme 1.3

Shikimic acid and hydroxybenzoic acid biosynthesis



1.4 Medicinal plants among the past and the present

Important aromatic plants utilised in both conventional and complementary medicine, as well as the food and pharmaceutical industries, may be found in the *Lamiaceae* family, which includes species of thyme, sage, *Calamintha* and rosemary [34].

The *Lamiaceae* plant family, which includes over 3000 species of plants found in both tropical and subtropical climates worldwide, is a particularly significant one for medicinal purposes. They can resist the hot weather because they generate a lot of essential oil. This family includes spearmint, *Coleus*, *Tulsi*, mint and thyme. It is grown mainly for therapeutic, fragrant, nutritional and ornamental purposes. The potent essential oil, tannins, saponins and organic acids are all therapeutic components [35].

The oil is generated using steam distillation and is used in aromatherapy for its ability to improve mood and for its sedative, diuretic, tonic and antibacterial characteristics. Important plant species used in studies on antibacterial activity include those in the *Lamiaceae* family [36].

The essential oils from more than half of the plants in the *Lamiaceae* family have effective antifungal properties minimum inhibitory concentration (MIC) 1000 µg/ml. In the treatment of fungal infections, particularly those of the skin and mucous membranes, the microbiological findings suggest that they may be used either alone or in conjunction with antifungal medications. Several essential oils from *Lamiaceae* plants and the parts that contain them are extracted and used in both cosmetics and medications. Future therapy of multi-drug resistant fungus may involve the use of essential oils [37].

The amount and quality of phenolic chemicals found in the *Lamiaceae* family contribute significantly to their strong antioxidant and antibacterial properties. Eugenol, carvacrol and thymol, three of the substances that make up the majority of essential oils, are principally responsible for their bactericidal and bacteriostatic effects [38].

The most widely used plants in the *Lamiaceae* family are the *Calamintha* species, which are used to treat inflammation, dermatitis, infections, gastritis and other illnesses.

Calamintha incana is a herbaceous perennial plant found in Europe, the Eastern Mediterranean, Central Asia, America and North Africa that belongs to the Lamiaceae family. This genus has fragrant characteristics that give its leaves and aerial parts a unique, pleasantly mint-like odour. *C. incana* leaves are usually used to make herbal tea and as a spice. It is a diaphoretic, expectorant and antispasmodic in traditional medicine and is used to treat general weakness and gastrointestinal distress. The biological effects of *C. incana* have been studied in a few studies. *C. incana* essential oil is especially remarkable given its potential cytotoxic effects [39].

Antimicrobial, antioxidant and enzyme inhibitory activity (α-amylase) have also been demonstrated for *C. incana*. Its essential oil extract is characterised by its non-terpenoid aromatic compounds, with more phenols than flavonoids, and showed non-selective cytotoxic action and a strong antioxidant capability [40].

Figure 1.4

Calamentha incana aerial part

a :*calamentha incana* flowers b : *calamentha incana* leaves

1.5 Methods of isolation and quantitative determination

1.5.1 Extraction

Following collection and drying, extraction is the next stage in the investigation of medicinal plants. Prior to analysis, the major stage of the process is the recovery and isolation of bioactive phytochemicals from plant sources. Liquid-liquid and solid-liquid extraction are the methods most often employed and remain the most widely utilised techniques particularly due to their simplicity, effectiveness and broad application. The extraction process is heavily influenced by variables including the pattern-to-solvent quantity ratio, pH, the number and length of male or female extraction steps, and temperature [14].

The following are the most popular methods for extraction:

- Hydro-distillation
- Decoction
- Infusion
- Soaking
- Soxhelt extraction
- Steam distillation

Hydro-distillation is a method used to separate water-insoluble, mildly volatile chemicals from plants. The plant material is crushed and boiled in water. A separatory funnel is used to catch and separate the distillate [41].

1.5.2 Chromatographic and detection techniques

1. Gas chromatography/mass spectrometry

GC was developed in 1953, with the help of Martin and James, and has since become one of the most important and widely used analytical techniques in analytical chemistry [42]. Compounds are divided into two different phases, one mobile and one stationary, in GC. As they are moved side by side over a bed of the stationary phase, each chemical in a mixture creates separate barriers between these two phases, causing separation to occur. A high-molecular-weight liquid is deposited on the surface of finely split particles or the walls of a long capillary tube as the stationary phase in GC. The mobile phase is an inert CF_4 gas [43]. The amount of separation carried out before the additives emerge one by one from the bed into a detector increases with the length of time this approach is utilised. With its high pattern throughput and extraordinarily high resolution, capillary gas chromatography (CGC) can identify hundreds of different chemicals [44].

Mass specrtrometry: The prepared sample is introduced into a high vacuum, allowing the molecules to move freely before being destroyed into individual ions by an electron stream. A clear pattern of the quantity of ions present at each mass will be found if the ions are separated based on their mass. The mass spectral range is a pattern that is as specific to a chemical as a fingerprint is to a person. We can recognise a chemical by looking at its mass spectra [43, 45].

(GC/MS): MS is the preferred detection method for GC because it provides an excellent mix of sensitivity and dependability. It is also significantly more robust than LC-MS and far more sensitive than NMR [46].

2. UV spectroscopy

In UV-visible absorption spectrometry, before reaching the detector, light from a suitable source passes through the sample and a prism or grating monochromator (filter instruments are still often employed). This setup, which is similar to that used in infrared spectrometry, reduces the sample's exposure to light, which may lead to photo-decomposition [47].

1.6 Importance of essential oils

Essential oils are herbal oils produced by distillation that have a distinctive smell specific to the plant or sources from which they were obtained. Terpenoids, which are produced from isoprene, are the main components of these oils, followed by diterpenes and aromatic chemicals [48]. The genetic makeup or developmental stages of plants have absolutely no bearing on the synthesis of essential oils. The environment and its changes can have a significant influence on the physiological processes and biochemical pathways that control plant metabolism and, consequently, oil generation. Food and pharmaceutical employers use them because of their therapeutic, antibacterial and antioxidant properties. Essential oils have biological properties that make them useful as insecticides, herbicides, and chemicals that fight cancer. However, they also have ecological functions, such as protecting plants and attracting pollinators [49].

Under various environmental circumstances, these substances, like other secondary metabolites, are crucial for maintaining plant health. Because of this, the quantitative and qualitative reaction to the environment is a typical issue in the production of aromatic flowers [50].

1.7 Essential oil as a potential anti-obesity agent

Around 20 chronic illnesses and health issues, including hypertension, type 2 diabetes, osteoarthritis, dyslipidaemia and high cholesterol, are all made more likely by obesity. The adverse effects of many antiobesity medications, such as mood swings, suicidal behaviour, and digestive or cardiovascular problems, have restricted their use [51].

The antiobesity effects of essential oils are thought to be mediated by a number of different mechanisms, including anti-lipase activity, antihyperlipidaemia through the downregulation of adipogenic transcription factors, such as PPARγ and CEBPα at both the protein and mRNA levels, raising the plasma glycerol concentration (a marker of lipolysis), and suppressing fat accumulation and intracellular triglyceride levels [52].

1.8 Global disease, diabetes and cancer

“Diabetes mellitus is a serious and growing health problem worldwide and is associated with severe acute and chronic complications that negatively influence both the quality of life and survival of affected individuals” [53].

“The association between cancer and diabetes has been investigated extensively and most, but not all studies, found that diabetes mellitus is associated with an increased risk of several types of cancer. If diabetes is associated with even a small increase in the risk of cancer, this may have important consequences at the population level” [54].

Diabetes mellitus is the fourth most common cause of mortality among Palestinians, with an incidence of 10.5% in patients between the ages of 18 and 80. An increased risk of type 2 diabetes and its consequences is linked to lifestyle modifications and uncontrolled glycaemic levels [55].

In 2021, around 7 million people died from cancer, and 15 million new cancer cases were reported by the WHO and the American Cancer Society [56]. Breast, lung and colorectal malignancies were seen in the greatest number of individuals, sequentially. The most popular kind of cancer treatment is chemotherapy, although it has considerable toxicity and side effects. Due to the high mortality rate among cancer patients , patients frequently begin looking for alternative treatments, such as herbal medicine, in addition to or in instead of traditional therapy [57].

1.9 Anti-bacterial activity of essential oil

There are now several antibiotics available to treat different bacterial diseases. However, the severity of bacterial pathogen-related disorders has worsened as a result of growing multidrug resistance. The prevalence of life-threatening bacterial infections in humans has also grown as a result of the host cells' inadequate immunity and the bacteria's capacity to acquire biofilm-associated medication resistance. Thus, even today, bacterial infections continue to be a key factor in human mortality. Moreover, the use of a number of antibacterial drugs at higher doses may cause toxicity in humans. Researchers are now investigating new critical chemicals that may be effective in combating bacterial strains [58]. The antibacterial effects of essential oils may prevent bacterial development (bacteriostatic) or kill bacterial cells (bactericidal). Even so, it might be difficult to distinguish these activities separately. In consideration of this, antibacterial activity is typically assessed using the least bactericidal concentration or the lowest inhibitory concentration (MIC). The primary components of essential oils are monoterpenes, which have been demonstrated to have a strong antibacterial effect against associated microbes [59].

Strong antibacterial activity against *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* has been demonstrated by the essential oils of cinnamon, clove, pimento, thyme, oregano and rosemary [60].

1.10 Anti-fungal activity of essential oils

Essential oil components may act as antifungal agents due to their accumulation in the lipophilic hydrocarbon molecules of the cell lipid bilayer; such action also allows the easier transfer of other essential oil constituents to the inner cell. “The different activity may be explained by the variety of water solubility and lipophilic properties of the essential oils” [61].

“Many studies have reported that the constituents of essential oils could act synergistically or antagonistically. The combination of some particular oils can increase fungistatic activity. The antifungal activity of terpenoids is generally related to their functional groups, *i.e.*, to the hydroxyl group of phenolic terpenoids. Carvacrol and thymol, produced from *p*-cymene, exhibit an important antifungal effect; they cause damage in the cell membrane by interacting with sterols and with ergosterol in particular” [62].

Finding a "lead compound" that acts as the "active component" of potential drugs that cause fungal death is the primary goal of drug discovery. If our "lead" oil kills some fungi, this might unlock the gates for the creation of novel chemical entities (NCEs) that have a significant impact on eradicating these fungi [63].

1.11 Aims of the study

The aims of our study are as follows:

1. The separation, recognition and identification of the active ingredients of *C. incana.*
2. The evaluation of the following biological activities of *C. incana:*

a. Antioxidant
b. Antimicrobial
c. Antifungal
d. Anti-lipase
e. α-Amylase

3. The examination of *C. incana's* cytotoxic effect on a cancer cell line.

Chapter Two

Materials and Methods

2.1 Materials

As illustrated in Tables 1, 2, 3, 4, 5 and 6, all of the chemicals and reagents used in this study were purchased from reliable suppliers and were received from the Pharmacy Department at An-Najah National University.

Table 2.1

Chemicals and reagents used for antioxidant evaluation

Chemicals and reagents	Manufacturer	Country
(DPPH) 2, 2-Diphenyl-1-picrylhydrazyl	Sigma-Aldrich	Germany
Methanol	Lobachemie	India
Trolox (6-hydroxy- 2, 5, 7, 8-tetramethychroman-2 carboxylic acid)	Sigma-Aldrich	Denmark

Table 2.2

Chemicals and reagents used for -α-amylase inhibitory evaluation

Chemicals and reagents	Manufacturer	Country
Sodium chloride (NaCI)	Self backing	Haifa
3-5 dinitrosalysylic acid (DNSA)	Sigma-Aldrich	USA
Acarbose	Sigma-Aldrich	USA
sodium potassium tetrahydrate	MERCK	Germany
Starch	Alzahra company	Nablus-Palestine
Amylase type VI -B, >10 unit/mg	Sigma-Aldrich	USA
Di-sodium hydrophosphate/dihydrosodiun phosphate (Na,HPO./NaH,PO4)	Alfa Aesar	USA

Table 2.3

Chemicals and reagents used for anti-lipase evaluation

Chemicals and reagents	Manufacturer	Country
PNPB (p-nitropheny! Butyrate)	Sigma-Aldrich	USA
Tris-HC	Sigma-Aldrich	USA
Acetonitrile	CARLO ERBA	France
Orlistat	Sigma-Aldrich	China
Porcine pancreatic lipase	Sigma-Aldrich	USA

2.2. Instrumentation

All of the instruments used in this study, with the exception of the microbiological tests, were obtained from the Pharmacy Department at An-Najah National University. The microbiological tests were carried out at the Faculty of Science, Microbiology Department, An-Najah National University.

The glassware included calibrated cylinders (1000 mL, 100 mL and 50 mL), glass rods, separatory funnels and simple funnels. Volumetric flasks (50 mL, 150 mL, 100 mL and 25 mL) were also employed.

2.3 Plant collection

The plant species used in this study was obtained from Beit Lead Village in Tulkarm (32° 15' 38" N, 35° 06' 53" E) in May and June of 2021. The aerial portion of *C. incana* was dried in the shade, at 30 °C. It was classified by Prof. Nidal Jaradat at the Pharmacognosy and Herbal Products Laboratory at An-Najah National University, Nablus, Palestine (Faculty of Medical Sciences). A mechanical blender was used to blend the dried leaves into a fine powder after they had been well cleaned and totally dried. The resulting powders were kept separate and in specific containers until they were used, at which point they were properly stored.

2.4 Oil separation

A Clevenger apparatus was used to carry out standard hydrodistillation; 500 mL of deionised water and 100 g of the dried aerial parts of the *C. incana* plant were added to a flask with a cylindrical bottom. The volatile oil was then collected and weighed. The following equation was used to calculate the essential oil yield [64].

$$the\ yeild\ of\ oil = \frac{net\ weight\ of\ extracted\ oil(g)}{weight\ of\ dry\ matter\ (g)} \times 100 \qquad (2.1)$$

After collecting the volatile oil yield in a beaker and adding the drying agent $CaCl_2$, the volatile oil was weighed and put in an amber-coloured bottle [65].

2.5 Identification of some constituents from the plant oil by GC-MS

Sampling:

First of all, 5 mg of the oil was dissolved in 5 mL of ethanol. A sample of the ethanolic solution (10 µL) was then injected into the GC-MS spectrophotometer.

Conditions of GC-MS:

GC-MS chromatograms were captured using a Shimadzu QP-5000 GC-MS with a Rtx-5ms column that was 30 m long, 0.25 µm thick and 0.250 mm in diameter. Helium was utilised as the carrier gas at a flow rate of 1 mL/min. The injector temperature was 220 °C. The oven temperature was set to increase from 50 °C for one minute to 130 °C at 5 °C/min, then to 250 °C at 10 °C/min, and maintained at this temperature for 15 minutes. The temperature of the transfer line was 290 °C. For GC-MS detection, an electron ionisation system with 1.7 kV detector was used. The mass range was 38-450 m/z, with a scan rate of 0.5 s and a scan speed of 1000 A/sec., A small number of compounds were identified by GC-MS depending on the total ionic concentration (TIC) values and mass spectra for each, in addition to several more compounds that were found but not recognised.

2.6 Antioxidant assays

2.6.1 DPPH assay

This technique is used to assess the ability of the plant's essential oil to scavenge DPPH radicals [66]. The ability of natural compounds to get rid of free radicals can be judged by how much the absorbance of the stable free radical DPPH goes down at 519nm. A yellow, colourless substance results from the interaction between the scavenger and the purple free radical 1,1-diphenyl-2-picrylhydrazyl [67]. The oil's capacity to scavenge free radicals was examined in comparison to Trolox, which was used as the reference.

In summary, a stock solution was generated for each oil and for Trolox at a concentration of 10 mg/100 mL of methanol, and subsequent serial dilutions were produced at varying concentrations (10, 20, 30, 40, 50, 100, 200, 300, 500, 700 and 1000 µg/mL). The 2,2-diphenyl-2-picrylhydrazyl (DPPH) reagent was then dissolved in 0.002% w/v methanol and combined in a 1:1:1 ratio with the working quantities that had previously been generated. A pure methanol solution was employed as a control.

One mL of each concentration of oil or Trolox was combined with 1 mL of methanol and 1 mL of 0.002% DPPH The absorbance was measured at 519 nm after each sample was incubated at room temperature for 30 minutes in the dark. After that, all samples were incubated at room temperature for 30 minutes in a dark environment.

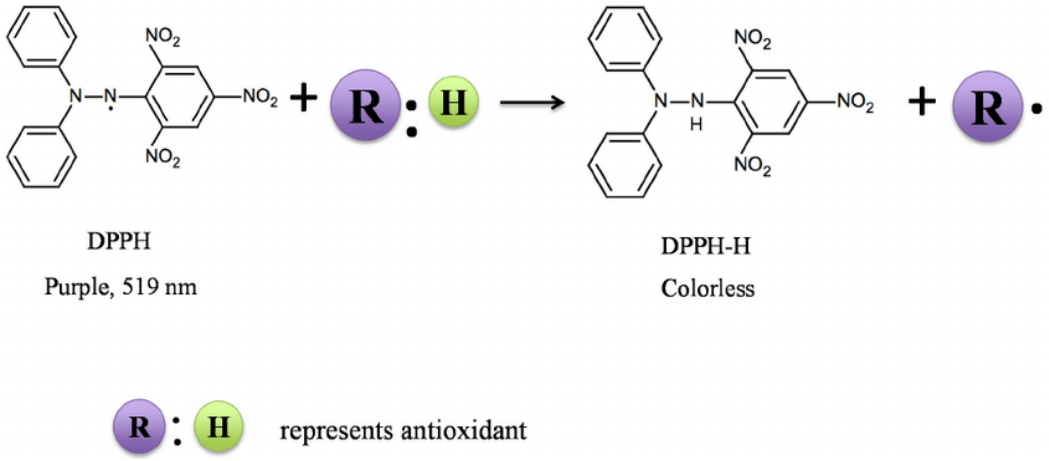
DPPH solution and methanol were combined in a 1:1 ratio to generate a blank solution, and the absorbance of this mixture was used to determine the percentage of inhibition:

$$\% \text{ of Inhibition} = [(A_C - A_T) / A_C]*100 \quad (2.2)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample. The reaction between DPPH (oxidising agent) and Trolox (antioxidant) is shown in Scheme 2.1 [68].

Scheme 2.1

The DPPH Radical Scavenging Capacity Assay's basic principles



Using Microsoft Office Excel 2012, a graph plotting the percentage of inhibition against extract concentration was used to calculate the antioxidant half-maximal inhibitory concentration (IC_{50}) for each of the tested *C. incana* essential oil and Trolox standard solutions.

2.7 Enzymatic screening

2.7.1 α-amylase inhibitory screening

Wickramaratne's method was used to conduct the α-amylase inhibition technique, with minor changes [69]. The experiment was carried out using the 3,5-dinitrosalicylic acid (DNSA) technique.

2.7.1.1 Preparation of stock and working solution

A. Sodium phosphate buffer (20 mM) with sodium chloride (6.7 mM),, ph 6.9.

NaH_2PO_4 and Na_2HPO_4, both of which contained 6.7 mM sodium chloride and had a pH of 6.9, were made by partially filling a beaker with the sodium phosphate and sodium chloride solution, applying the mixture to a magnetic stirrer, and trying to adjust the pH by inserting a calibrated pH electrode into the solution. When the pH hit 6.9, the Na_2HPO_4 and NaCl solution were gradually added. A mass of 5.36 g of 20 mM $Na_2HPO_4.7.H_2O$ and 0.39 g of 6.7 mM NaCl was used to create 1 litre, and 2.76 g of NaH_2PO_4 and 0.39 g of NaCl was dissolved in distilled water to create 1 litre.

B. Essential oil stock and working solution

A buffer solution containing Na_2HPO_4/NaH_2PO_4 (0.02 M) and NaCl (0.006 M) at pH 6.9 was used to further dissolve a *C. incana* essential oil stock solution of 1 mg/mL concentration. This buffer solution was produced at a minimum concentration of 10% dimethyl sulphoxide (DMSO) (1:100 dilution). By combining the synthesised molecules in volumes of 0.1, 0.5, 0.7, 1 and 5 mL, respectively, further diluting with buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M) and NaCl (0.006 M) at pH 6.9), and then increasing the volume to 10 mL using volumetric flask , working solutions with concentrations of 10, 50, 70, 100 and 500 µg/mL were created in 10 mL VF.

C. Acarbose stock solution

Acarbose was utilised as a basis for comparison. The acarbose stock and working solutions were made in a similar way to that used for the *C. incana* essential oil.

D. α-amylase solution

To create the α-amylase solution, 12.5 mg of the α-amylase enzyme was dissolved in at least 10% DMSO, and up to 100 mL of the buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M) and NaCl (0.006 M) at pH 6.9) in VF was added.

E. Starch stock solution

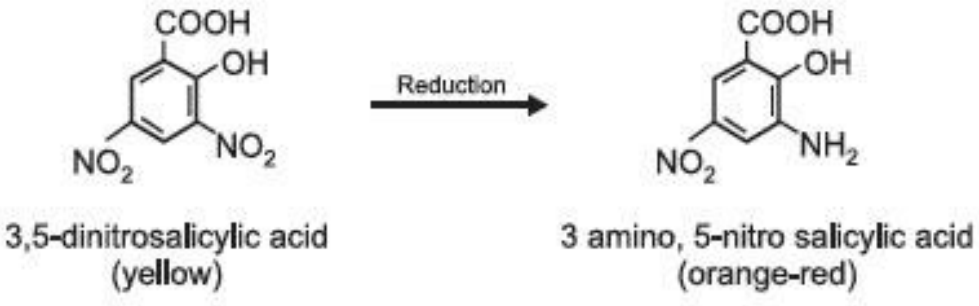
To prevent precipitation, the starch solution was created at a concentration of 1% (w/v) by dissolving 1 g of starch in 100 mL of distilled water using VF (100 mL). The solution was maintained in a water bath at 37 °C until use, with little mixing.

F. 3,5-dinitrosalicylic acid stock solution

As a reactive agent, DNSA reacts with reducing sugars according to Scheme 2.2 to produce 3-amino-5-nitro-salicylic acid, which is highly absorbent of light between 538 and 542 nm [70]. To make this solution, 12 g of sodium potassium tetrahydrate were first dissolved in 8.0 mL of 2 M NaOH (8 g in 100 mL of distilled water), and then 20 mL of a solution containing 96 mM DNSA were added.

Scheme 2.2

DNSA with reducing suger reaction



G. Assay of a-amylase inhibition

First of all, 200 µL of α-amylase solution (2 units/mL) were added to each essential oil working solution, and the mixture was incubated at 30 °C for 10 minutes. Each tube was then filled with 200 µL of the starch solution before being incubated at 30 °C for 3 minutes. Then, 200 µL of DNSA reagent were added to the reaction to stop it, and the solution was then boiled for 10 minutes in a water bath at 85 to 90 °C. A UV-Vis spectrophotometer was used to measure the absorbance at 540 nm after the mixture had

been cooled to room temperature and diluted with 5 mL of distilled water. A blank with 100% enzyme activity was created using 200 µL of buffer in place of the compounds. The positive control sample was acarbose. The equation below expresses the α-amylase inhibitory activity as a percentage of inhibition. The concentration of the compounds was plotted against the percentage of α-amylase inhibition, and the IC_{50} values were determined from the graph [71].

$$\alpha\text{-amylase inhibition} = [(A_C - A_T)/ A_C]*100 \quad (2.3)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample.

2.7.2 Pancreatic anti-lipase inhibitory screening

The porcine pancreatic lipase (PPL) inhibitory test was carried out from Jaradat *et al*. and Bustanji *et al*., with a little modification [72, 73].

2.7.2.1 Preparation of stock and working solution

A. Essential oil and orlistat stock and working solution

The following five concentrations was created from a stock solution of 1 mg/mL of essential oil in 10% DMSO: 50, 100, 200, 300 and 400 µg/mL. Orlistat was produced using the same method as the *C. incana* extract and used as a reference standard for the pancreatic lipase inhibition experiment.

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

The stock solution of PNPB was created by dissolving 104.5 mg of p-nitrophenyl butyrate (PNPB) in acetonitrile and increasing the volume up to 10 mL in VF, in accordance with the manufacturer's instructions (20.9 mg of PNPB in 2 mL of acetonitrile).

C. Pancreatic lipase enzyme

Just prior to use, 1 mg/mL of pancreatic lipase enzyme was suspended in 10% DMSO to create the stock solution. For this, 25 mL of VF was added to 25 mg of lipase that has been suspended in a small quantity of 10% DMSO and the mixture was sonicated at 37 °C for 15 minutes.

D. Assay of pancreatic enzyme

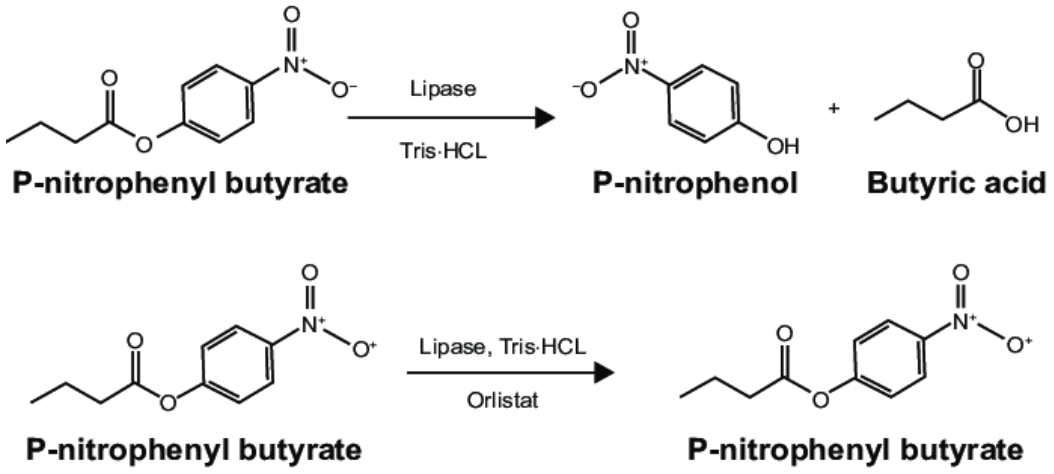
For this assay, 200 µL of the synthesis compounds were collected from each working solution of the plant extract detailed above and added to 100 µL of PPL (1 mg/mL) in separate test tubes. The mixture was made up to 1000 µL by adding 700 µL of Tris-HCl solution and then incubated for 15 minutes in a water bath at 37 °C. Each test tube received 100 µL of PNPB solution following the incubation period. The mixture was incubated once again for 30 minutes at 37 °C in a water bath. By combining 100 µL of PPL (1 mg/mL) solution with 900 µL of Tris-HCl solution, 1 mL of the mixture was generated without the synthetic chemicals. A similar process was followed for orlistat, which was used as a positive control. The UV-Vis spectrophotometer was set to zero at 405 nm using a Tris-HCl buffer and the breakdown of p-nitro-phenyl butyrate to p-nitro-phenol was evaluated at 405 nm to estimate the pancreatic lipase activity. Compared to the control, the effect of the compounds on the enzyme reaction rate was used to determine the lipase inhibitory activity of *C. incana* [74, 75]. Scheme 2.3 shows the effect of lipase enzyme on PNPB (lipase sub) with the presence and absence of orlistat. The percentage inhibition of the synthesised compounds was calculated using the following equation [76] :

$$\% \text{ of Inhibition} = [(A_C - A_T)/ A_C]*100 \quad (2.4)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample.

Schem 2.3

Without and with Orlistat, PNPB degradation



2.8 Anti-microbial screening

2.8.1 Micro-organisms and conditions for cultivation

A. Bacterial strains

The antibacterial activity of *C. incana* was determined using six strains of bacteria brought from the American Type Culture Collection (ATCC): *Escherichia coli* (ATCC # 25922), *Pseudomonas aeruginosa* (ATCC # 9027), *Proteus vulgaris* (ATCC # 8427), *Klebsiella pneumoniae* (ATCC # 13883) and *Staphylococcus aureus* (ATCC # 6538P). Each culture's extracts were taken and gently dissolved in a separate sterile tube with 4-5 mL of normal saline. MRSA (methicillin-resistant *Staphylococcus aureus)* clinical isolates with proven diagnostics are growing less and being inhibited in their proliferation.

B. Fungal strains

The antifungal activity of *C. incana* samples was evaluated against the growth of *Candida albicans* (ATTC # 90028), *Microsporum canis* (CBS 132.88), *Trichophyton rubrum* (CBS 392.58) and *Trichophyton mentagrophyte* (CBS 106.67). Dematophytes are fungi that cause dermatophytoses in humans. The isolates were from the BERC Centre and were maintained on SDA media at room temperature. Experimental cultures were kept on SDA media and subcultured.

2.8.2 Preparation of growth media

A. Bacterial growth media

Nutrient Agar (NA) was used as a subculture for the six bacterial strains.

The McFarland 0.5 standard was utilised to visually compare the turbidity of bacterial and fungal solutions and standardise the estimated number of bacteria and fungus in a liquid suspension. Generally, McFarland's 0.5 standard represents 1.5×10^8 CFU/mL [77]. Mueller-Hinton Broth (MHB) medium, which was used for the antibacterial microdilution assays of bacteria, was prepared by dissolving 8.4 g of MHP powder in 400 mL distilled water. This solution was heated while stirring with a magnetic stirrer until it had completely dissolved, then it was autoclaved for 20 minutes to ensure its sterilisation.

B. Fungal growth media

The subculture medium for fungi was Sabouraud Dextrose Agar (SDA). The MHB media was prepared as described above.

2.8.3 Preparation of micro-organism strains

A. Bacterial strains

Standard solutions of bacterial strain suspensions were made in 5 mL of MHB by gently erasing the colony surface of bacterial strains that had been subcultured overnight onto specific agar with a sterile swab. Once the turbidity was set to 0.5 McFarland solution, which has a concentration of 1.5×10^8 CFU/mL, operating solutions were created by combining 100 µL of the stock solutions with 10 mL MHB on a large plate to produce the concentration attained in the wells, which was 1.5×10^6 CFU. Once the micro-dilution procedure was applied to these solutions, a concentration of 7.5×10^5 CFU was obtained in the wells.

B. Fungal strains

A *C. albicans* stock solution in sterile MHB was carefully made by rubbing the colony face of overnight *C. albicans* subcultured onto SDA with a cotton swab. Working solutions were created by combining 100 µL of the stock solutions with 10 mL MHB on a large plate to achieve a concentration of 2.5×10^4 CFU once the turbidity was established at 0.5 McFarland solution, which has a concentration of 2.5×10^6 CFU/mL. Once the micro-dilution procedure was applied to these solutions, a concentration of 1.25×10^4 CFU was obtained in the wells.

The fungi used in this study were *M. canis, T. rubrum* and *T. mentagrophyte*. The isolates were maintained on SDA media at room temperature, in accordance with Murray *et al*. (1995) and Yaghmour (1997), with some modifications [78].

2.8.4. Preparation of plant Essential oil

The initial concentration of *C. incana* essential oil used for the bacterial and *C. albicans* assays was 250 mg/mL. This was mixed with 250 mg/mL (0.25 mL) of DMSO at 100% to obtain 0.5 mL of the mixture, which was placed under UV rays for 15 minutes for sterilisation purposes.

2.8.5 Antimicrobial assay

The susceptibility tests on the micro-organisms used the broth microdilution method with some modifications [79].

A. Anti-bacterial assay

For the bacteria well microplate, 50 µL of the prepared MHB was filled in columns 1–12 of the colutus (excluding the last row H of the well), and the first column was then filled with 50 µL of *C. incana* extract. A serial microdilution was carried out using a multichannel pipette, starting from the first column, taking 50 µL of this extract and mixing it, then diluting until column 10. Then, 50 µL of each type of bacterial suspension was filled in its specific row in columns 1–11. The microplate was then placed inside the incubator until the next day. Well number 11 was used as a positive control, containing media and bacteria, to compare the effect of the essential oil as an antibacterial, depending on the turbidity. Well number 12 was used as a negative control, containing media alone to make sure that the media was sterile. The MIC of the investigated oil was determined as the lowest concentration of *C. incana* at which no detectable microbial growth was observed in the micro-well.

B. Anti-fungal assay

The MIC values were assessed using the broth microdilution method to evaluate the efficacy against *C. albicans*. The procedure followed that used for the other bacterial species. Using the poisoned food approach as described by Adjou *et al*., the antifungal activity of the essential oil was evaluated against *M. canis, T. rubrum and T. mentagrophyte* [80, 81]. In order to obtain a series of oil concentrations (1 µL/mL, 0.5 µL/mL and 0.25 µL/mL), different volumes of oil were added to pre-sterilised (SDA) media. The solution was then stirred for 5 minutes to homogenise the SDA medium with the essential oil.

A 5 mm-diameter mycelial agar disk was cut from a test fungal culture that was 10 days old and this disk was then inoculated into newly-made agar plates. In the control group, sterile distilled water was used instead of the tested sample. For each treatment, three replicates were employed. After 8 days of dark incubation at 24 °C with the infected plates, observations were made and recorded, measuring the diameter of several fungal colonies. The following formula was used to calculate the % of mycelial inhibition:

$$\% \text{ mycelial inhibition} = (dc\text{-}ds/dc) \times 100\% \quad (2.5)$$

where dc is the colony diameter of the control and ds is the colony diameter of the sample.

2.9 Anticancer test

2.9.1 Preparation of extracts

The extracts were prepared by dissolving 0.1 g of the crude extract or pure compound in 1 mL of DMSO and passing through a 0.2 µm filter to give a final stock solution concentration of 10 mg/mL. All solutions were kept at -20 °C until the cytotoxicity tests were carried out.

2.9.2 Cytotoxicity method

The cytotoxicity was tested on a cervical adenocarcinoma cell line (HeLa). The first step in the cell culture was the culturing of the cell line in T-175 cell culture flasks containing RPMI 1641 basal medium supplemented with L-glutamine (1%), FBS (10%) and penicillin/streptomycin (1%). The cells were kept in a standard cell culture incubator at 5% CO_2, 37 °C and 99% humidity. The medium was suctioned and washed with Ca^{2+}-free PBS before subculturing. Cells were then incubated with 0.025% trypsin for up to 5 minutes until sufficient cells detached. CGM was then used to inactivate the trypsin and the cell suspension was collected. A trypan blue stain was used to determine the viable cell count before adjusting the cell count to 82,000 for HeLa cells. The cells were seeded at 5000 cells per well in a 96-well plate. The cells were then allowed to adhere and accommodate overnight prior to the tests being conducted. After 24 hours, different concentrations for the product (15.6, 31.3, 62.5, 125 and 500 µg/mL) were prepared under pH 7.4 conditions and 100 µL of each concentration was added per well.

After 48 hours, 20 µL of MTS reagent was added to each well and the plates incubated for 2 hours. The absorbance was measured at 490 nm using a plate reader.

Chapter Three

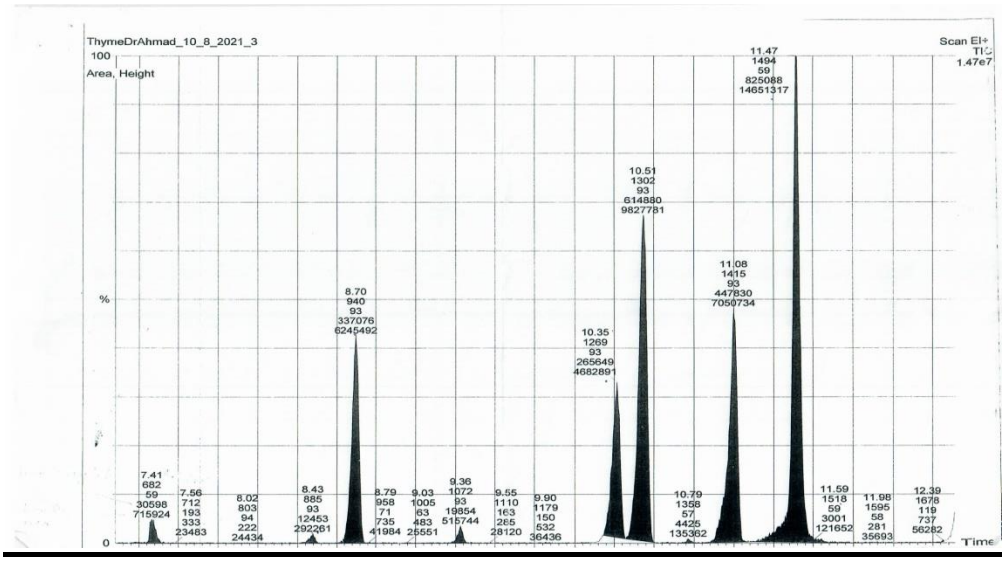
Result

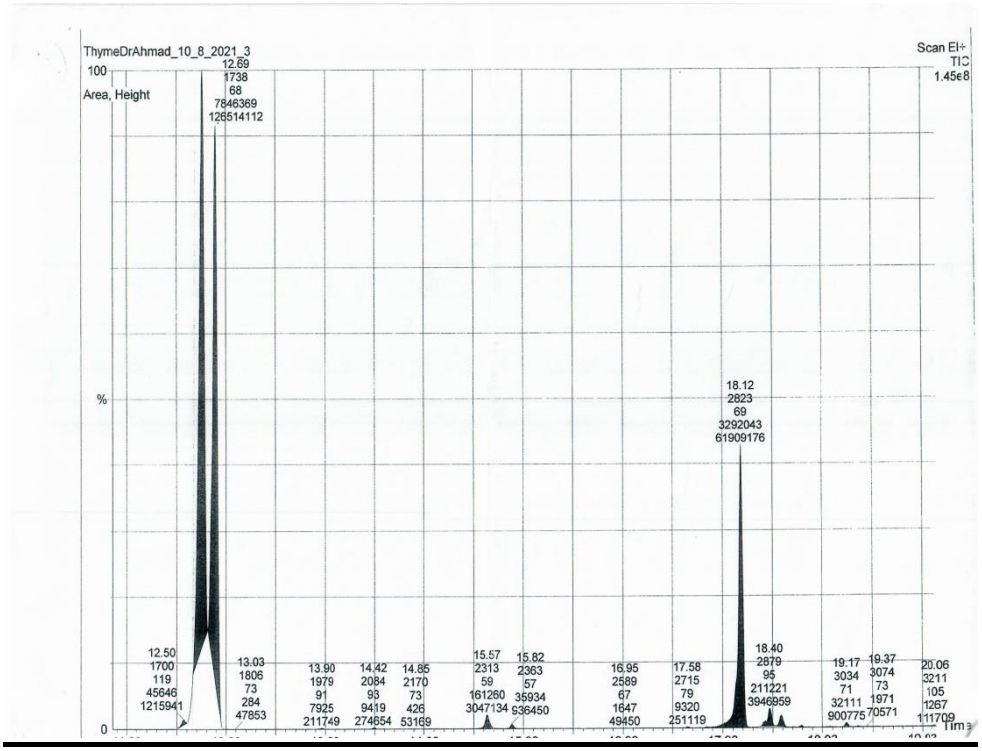
3.1 Chemical composition

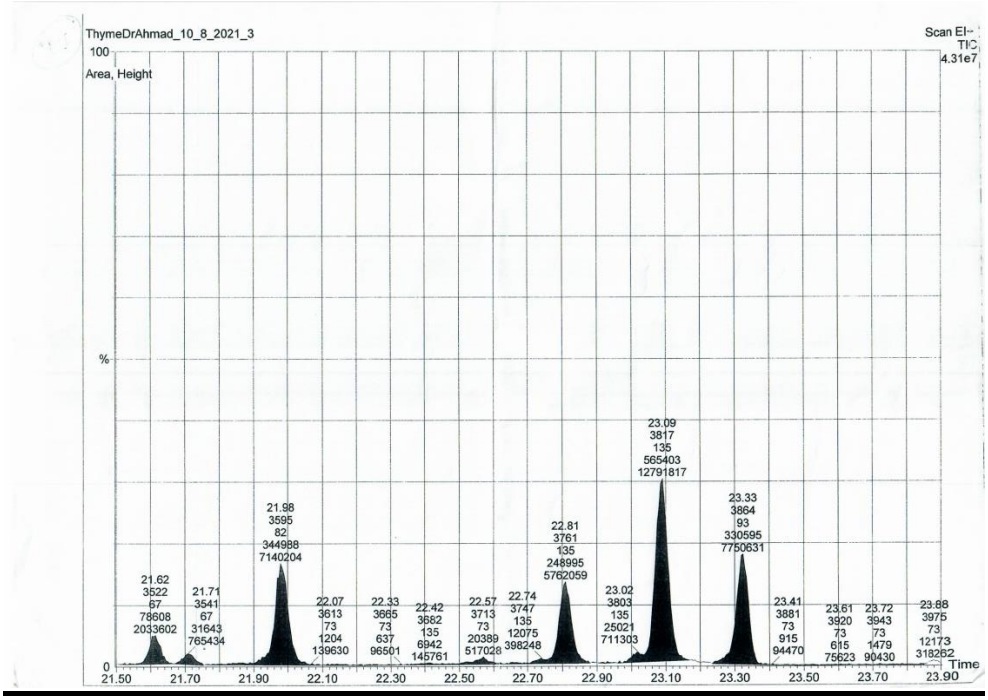
The chemical composition of the EO in the C. incana collected from the Beit Lead forest area revealed sixty compounds, accounting for 98% of the total composition.

Figure 3.1

show GC-MS analysis



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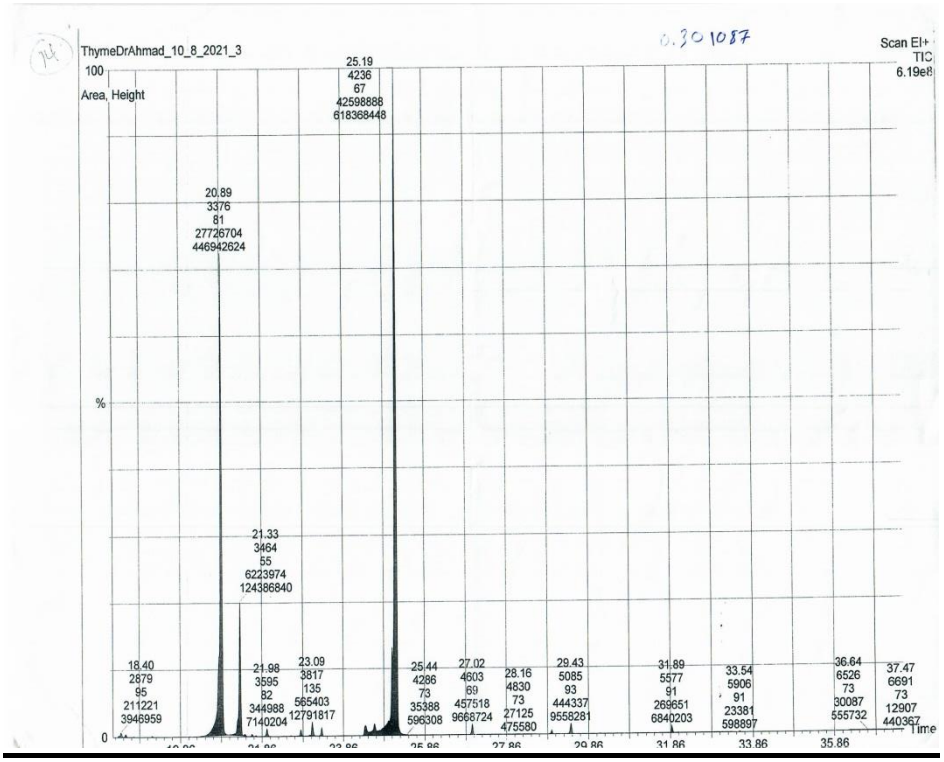
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21.98 3595 82 344988 7140204
23.09 3817 135 565403 12791817
25.19 4236 67 42598888 618368448
25.44 4286 73 35388 596308
27.02 4603 69 457518 9668724
28.16 4830 73 27125 475580
29.43 5085 93 444337 9558281
31.89 5577 91 269651 6840203
33.54 5906 91 23381 598897
36.64 6526 73 30087 555732
37.47 6691 73 12907 440367
25.86
27.86
29.86
31.86
33.86
35.86
Time

Appendix A1 show the yield of the essential oil content, which was calculated using the following equation

$$the\ yeild\ of\ oil = \frac{net\ weight\ of\ extracted\ oil(g)}{weight\ of\ dry\ matter\ (g)} \times 100 = \frac{0.213}{100} * 100\% = 0.213\% \quad (3.1)$$

3.2 Anti-oxidant activity

A yellow, colorless substance results from the interaction between the scavenger and the purple free radical. 1,1-diphenyl-2-picrylhydrazyl . The oil's capacity to scavenge free radicals was examined in comparison to Trolox, as a reference.

$$\% \text{ of Inhibition} = [(A_C - A_T) / A_C] * 100 \quad (3.2)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample

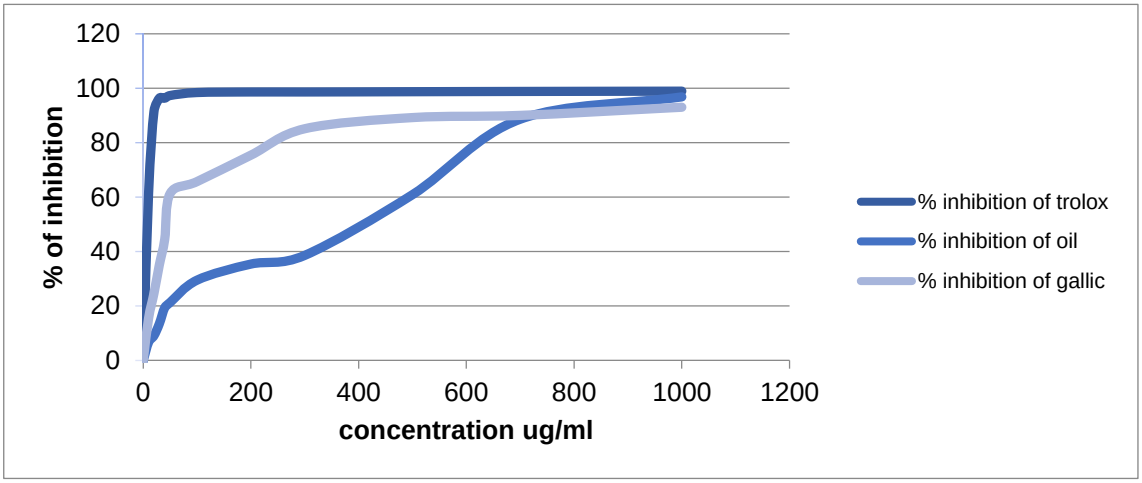
Table 3.1

DPPH and standard were plotted against the concentration of the oil and IC_{50}value

	% inhibition		
con. µg/ml	Trolox	E.O	Gallic acid
0	0	0	0
10	61.5	6.7	15.5
20	91.4	9.1	24.6
30	96.3	13.5	35.1
40	96.3	19.4	44.5
50	97.3	21.3	61.3
100	98.4	29.5	65.7
200	98.6	35.4	75.4
300	98.6	38.6	85.1
500	98.7	60.9	89.2
700	98.8	88.6	90
1000	98.9	96.8	93
IC_{50}	7.5	390	46

Figure 3.2

Antioxidant activity of C. incana compared to trolox and gallic acid



3.3 α-amylase inhibitory screening

Using the equation below, the percentage of α-amylase inhibitory activity that was inhibited was calculated.

$$\alpha\text{-amylase inhibition} = [(A_C - A_T)/ A_C]*100 \quad (3.3)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample.

Plotting the % inhibition of α -amylase and the standard versus the oil concentration allowed us to determine the IC_{50} values from the graph. Tables 3.3 and Figure 3.4 demonstrate the essential oil's percentage of inhibition in contrast to acarbose.

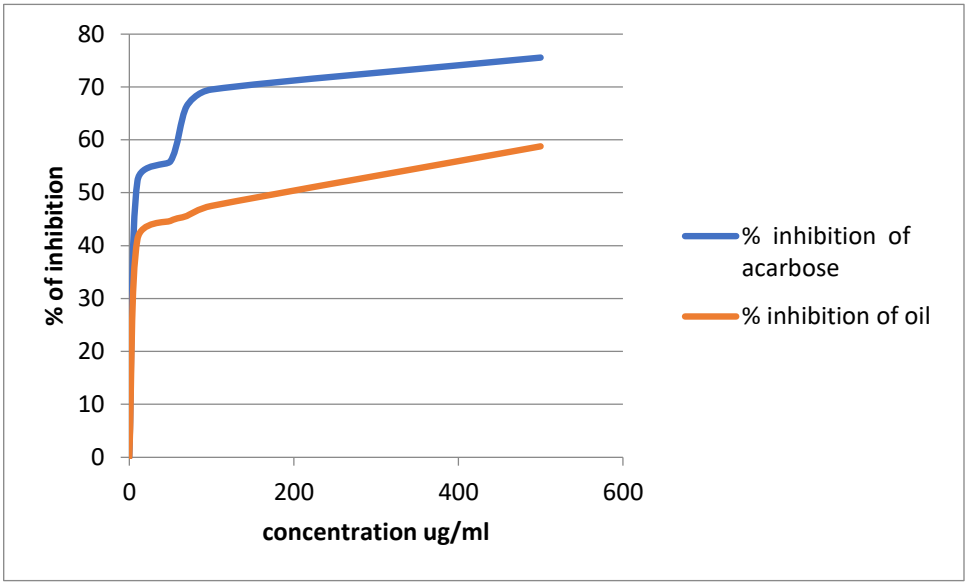
Table 3.2

The percent inhibition of α-amylase and standard were plotted against the concentration of the oil and IC_{50} values

Concentration ug/ml	% of inhibition Acarbose ±SD	*C. incna* ±SD
0	0	0
10	52.22 ±1.2	41.23 ±0.188
50	55.91 ±0.58	44.66 ±0.047
70	66.4 ±1.34	45.6 ±0.081
100	69.5 ±1.62	47.5 ±0.188
500	75.54 ±1.37	58.76 ±0.124
IC_{50}	7.5	120

Figure 3.3

alpha-amylase inhibition of C. incana compared with Acarbose



3.4 Pancreatic anti-lipase inhibitory screening

The ability of *C. incana* to inhibit lipases was assessed by comparing the impact on enzyme reaction rate following the addition of the produced compounds to the control. The following is the equation used to calculate the *C. incana* inhibition percentage.

$$\% \text{ of Inhibition} = [(A_C - A_T)/ A_C]*100 \quad (3.4)$$

where A_C is the absorbance of the blank (all reagents without the sample) and A_T is the absorbance of the sample.

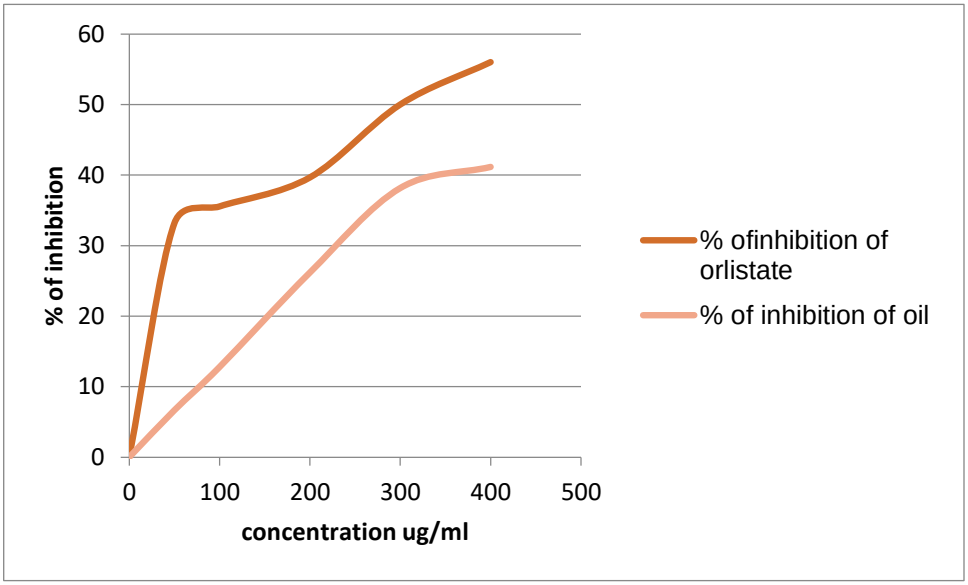
Table 3.3

Percent inhibition of pancreatic lipase and standard were plotted against the concentration of the oil and IC_{50} values

Concentration µg/ml	% of inhibition Orlistat± SD	*C. incana* ± SD
0	0 ±0.0	0 ±0.0
50	33.181 ±0.77	6.66 ±0.54
100	35.55 ±0.42	12.79 ±0.45
200	39.66 ±0.42	26.18 ±0.49
300	50.0 ±0.12	38.16 ±0.72
400	56.01 ±0.38	41.15 ±0.50
IC_{50}	300µg/ml	>800µg/ml

Figure 3.4

anti-lipase activity of C. incana compared with Orlistat



3.5 Anti-microbial screening

3.5.1 Anti-bacterial and selected anti-fungal activity

The results of MIC values and positive standards are recorded. All tests were done in triplicate.

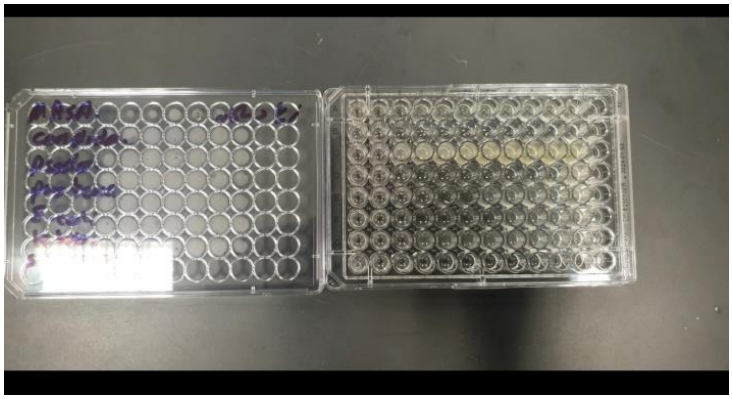
Table 3.4

MIC values (ug/mL) for C. incana oil

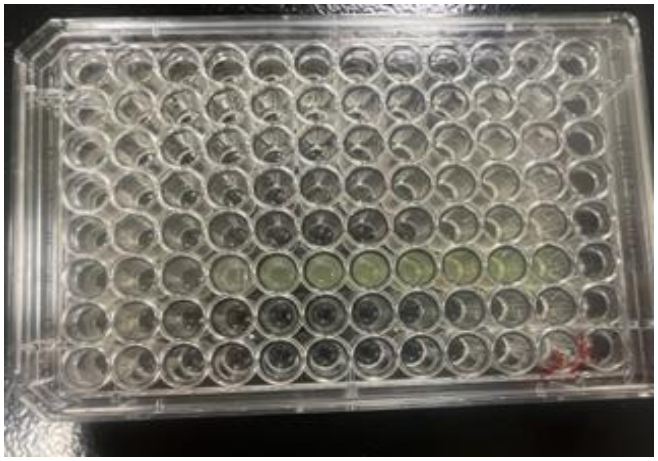
Microorganism	Trial 1 250 mg in 250 µL DMSO	Trial 2 250 mg in 250µL DMSO	Trial 3 250 mg in 250 µL DMSO	Final result MIC (ug/mL) ±SD
MRSA	3	3	4	26.041 ±0.0073
S. aureus	3	3	3	31.25 ±0
K. pneumonia	8	7	8	1.302 ±0.00046
E. coli	7	6	7	2.604 ±0.00092
P. vulgaris	9	9	9	0.48825 ±0.0
P. aeruginosa	2	2	2	62.5 ±0.0
C. albicans	10	10	10	0.2441 ±0.0

Figure 3.5

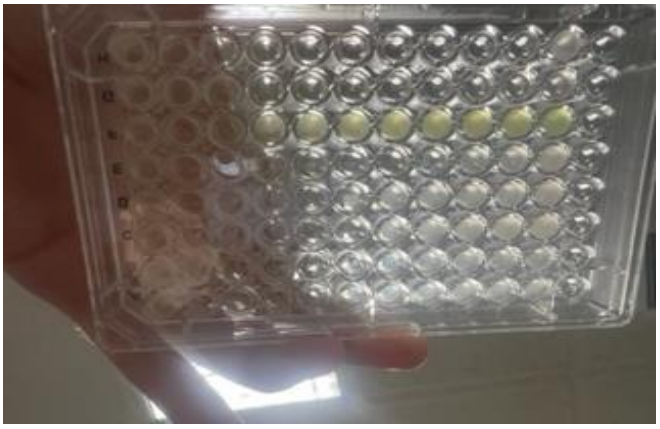
Antibacterial activity of C.incana oil



a. Antibacterial activity of plants extracts by MIC determination. Trail 1.



b. Antibacterial activity of plants extracts by MIC determination.Trail 2.



c. Antibacterial activity of plants extracts by MIC determination.Trail 3.

Table 3.5

Antibacterial activity results MIC values of essential oil of C. Incana compared with Ampicillin and Ciprofloxacin

MIC values (ug/mL) Sources	MRSA	*S. aureus*	*K. pneumonia*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*
Plant oil	26.041	31.25	1.302	2.604	0.48825	62.5	0.2441
Ampicillin	0	3.12	1	3.12	18	0	0
Ciprofloxacin	12.5	0.78	0.125	1.56	15	3.12	0

3.5.2 Anti-fungal activity

The following formula was used to determine the percentage of mycelial inhibition:

% mycelial inhibition = (Dc-Ds/Dc) * 100% (3.5)

Colony diameter of the control (Dc), colony diameter of the sample (Ds).

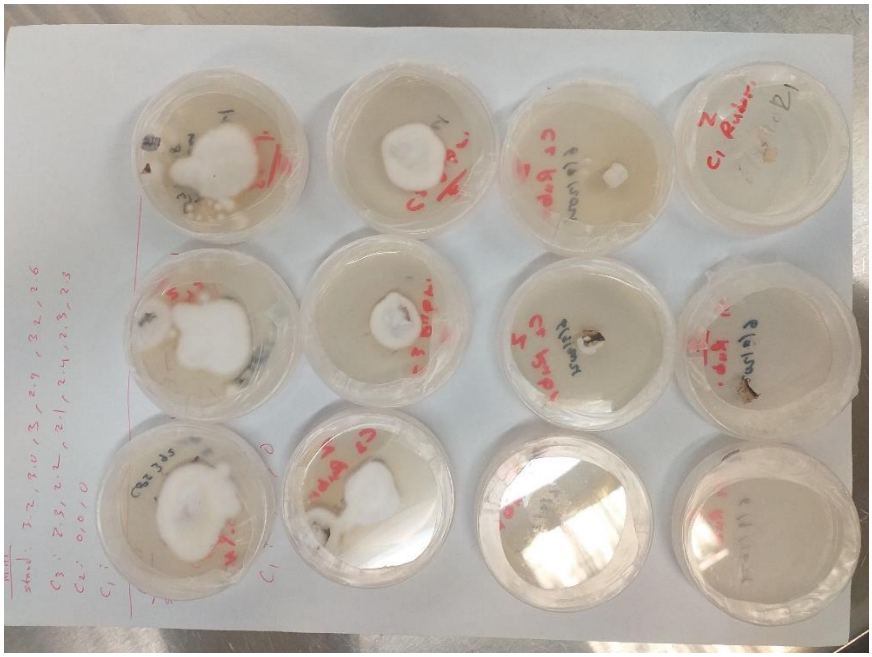
Table 3.6

Percent inhibition of dermatophytes at different concentration of C.incana oil

	%inhib.		
Conc..µL/ml	T. ment.±SD	M. canis±SD	T. rubrum ±SD
0	0±0	0±0	0±0
0.25	92.5 ±0.37	29.1 ±2.24	20.5 ±5.28
0.5	100 ±0.0	100 ±0.0	68.8 ±2.96
1	100 ±0.0	100 ±0.0	100 ±0.0

Figure 3.6

Anti-fungal activity of C.incana oil



a. Anti-fungal activity of *C.incana* oil against *T.rubrum* .

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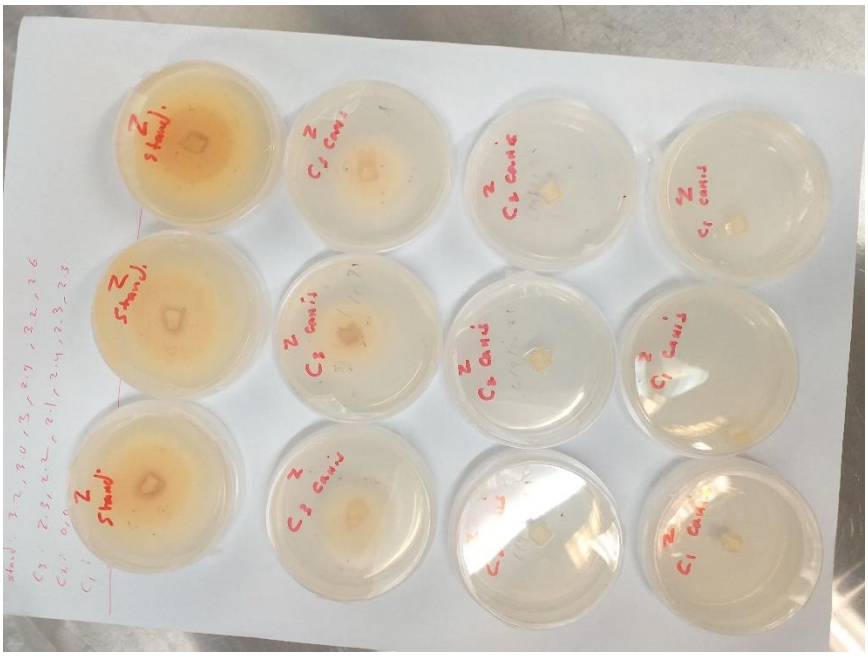
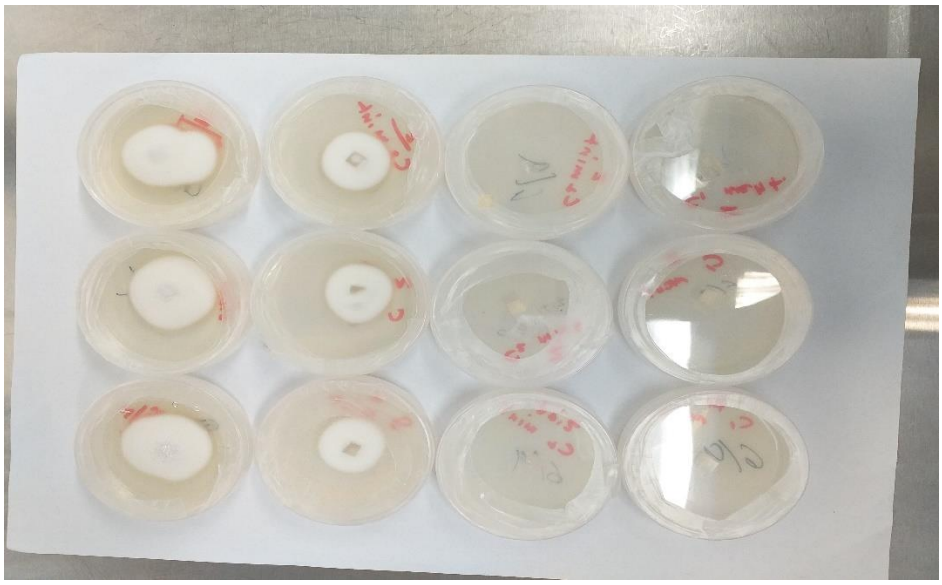


b. Anti-fungal activity of *C.incana* oil against *M.canis*.



c. Anti-fungal activity of *C.incana* oil against *T.ment.*

Anti-cancer activity

The oil of *C. incana* was tested against HeLa cells and showed promising results. According to Figure 3.6, the IC_{50} is 50 µg/mL. This leads to a search for the active component in the oil.

Table 3.7

Percent inhibition of HeLa cell at different concentration of C.incana

Concentration µg/ ml	% of inhibition
15.6	11.7
31.3	39.7
62.5	62.7
125	72.4
500	78.8

Chapter Four

Discution and Conclusion

4.1 Chemical composition

The essential oil of *C. incana* components was characterised by MS analysis (Table 3.1). The plant oil analysis showed about 60 components with different percentages. The components with the highest percentages were piperitenone oxide (41.18%), pulegone (29.762%), limonene (8.424%), trans-2,4-dimethylthiane, s,s-dioxide (8.28%) and a piperitenone derivative, cyclohexanone, 5-methyl-2-(methylethyl) (4.122%), while the other components were present in the remaining percentage. These results are slightly different from other studies held in Turkey and Jordan. “The following were the primary elements of the research previously undertaken in Turkey, from the Kestel (Bursa) forest: trans-pipertone oxide (41.37%), piperitenone oxide (34.47%) and piperitenone (6.67%). The conclusions of the previous research undertaken in Jordan's Ajloun Forest were as follows: benzenamine, 4-methyl-3-nitro (34.11%), (2S, 4R)p-mentha-6,8-diene-2-hydroperoxide (31.48%), cis-piperitone oxide (7.72%), menthone (4.05%) and azulene (3.57%)” [40].

“The essential oil composition can vary from one plant to another, even in the same species. These variations may critically influence the yield, composition, and biological activities of the essential oils, such as genetic background, environmental conditions, habitat, developmental stage, time of harvesting, and extraction methods” [82].

Trans piperitone oxide was found only in *C. incana* essential oil when compared to essential oils from other *Calamintha* species. nepeta subsp. glandulosa, whereas the stereoisomer cis-piperitone oxide has been found in several *Calamintha* species, including *C. sylvatica* subsp. *Sylvatica* and *C. vardarensis* [83]-[84].

Piperitone oxide is a substance that exhibits antinociceptive, cardiovascular, analgesic and sedative actions, in addition to fungicidal and insecticidal activities, suggesting it is an attractive option for the alternative management of agricultural pests [85].

Furthermore, pulegone is an oxygenated monoterpene that extends back to *C. nepta*. An antimicrobial effect was established by Pulegone, specifically against all *Salmonella* species. All *Calamintha* species contain limonene, such as *C. sylvatica*, *C. vardarensis*, *C. nepeta* and *M. menthifolia*, in minor percentages; however, it was discovered in a significant amount in the sample used in our study (8.424%). Limonene is used in pharmaceutical products to enable medical creams and ointments to enter the skin more effectively. It was discovered that limonene had a strong antifungal impact on *T. rubrum* [86].Thymol has been identified in very small amounts and has anti-oxidant properties.

Additionally, trans-2,4-dimethylthianes s,s-dioxide is expected to have medicinal properties such as catechol-O-transferase inhibition, glutamate oxaloacetate transaminase (GOT) inhibition, glucosyl-transferase inhibition and reverse transcriptase inhibition, as well as increasing glyoxalate transamination [87]. By inhibiting the enzyme catechol-O-methyl-transferase, levodopa's peripheral breakdown is inhibited, allowing a larger concentration to pass across the blood-brain barrier. This inspired us to develop a new class of Parkinson's drugs [88].

The inhibition of GOT sensitises cancer cells to glucose deprivation, which is partially counteracted by oxaloacetate and phosphoenol pyruvate, metabolic intermediates downstream of GOT. Oxaloacetate should be supplied by GOT in order to maintain an intact redox equilibrium at low glucose levels. According to this research, GOT might be a viable target for cancer treatment [89].

The glycosyltransferase inhibitor inhibits the synthesis of the lipid-linked pyrophosphate oligosaccharide precursor, which is essential for peptidoglycan polymerisation. This explains the strong effect of this oil against bacteria; thus, it is possible to find a "lead compound" that acts as an "active component" of potential drugs that cause bacterial death [90].

4.2 Anti- oxidant DPPH

The free radical scavenging activity of the oil extract was investigated using the DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) assay, which is used when DPPH (a free radical) combines with antioxidant compounds, which may give hydrogen to DPPH and decrease it to a more stable form. This reaction creates a colour shift (from deep purple

to light yellow). The absorbance was measured at 519 nm, and both the oil extract and the positive control (Trolox) displayed dose-dependent free radical scavenging activity. Because more scavengers were needed to accomplish a 50% scavenging response, a higher IC_{50} value implies a poorer scavenging activity. Scavengers are therefore less efficient at removing DPPH.

The data revealed in Table 3.1 and Figure 3.2 show that the percent inhibition increased with increasing concentrations. At a concentration of 1000 μg/mL, the plant's essential oil revealed 96% inhibition, which is nearly the same as Trolox and higher than gallic acid. However, the percent inhibition of the oil is higher than that of gallic acid (82% at 630 μg/mL), despite the fact that the IC_{50} for the essential oil was 390 μg/mL. The antioxidant effect of the essential oil is primarily due to its main components, such as thymol, piperitone oxide and polygon, although there is the possibility of other phenomena, such as synergy or antagonism, with its minor components.

Numerous phytochemicals have been investigated for their capacity to scavenge reactive species and prevent oxidative stress. Many studies have found a link between oxidative stress and the development of conditions including Alzheimer's disease, Parkinson's disease, chronic inflammation, rheumatoid arthritis, and some kinds of cancer. *C. incana* essential oil can be a good natural source of antioxidants, whether used as a nutraceutical, preventative measure or cosmeceutical [91].

4.3 α-amylase inhibition

Using starch as a substrate and acarbose as a positive control, *C. incana* essential oil was subjected to an *in vitro* assessment of α-amylase inhibitory activity. The findings in Table 3.3 demonstrate that the levels of inhibition varied in the essential oil. As the IC_{50} of the essential oil increased, this indicated weak inhibition of the enzyme. Table 3.2 and Figure 3.3 show that the IC_{50} value of the oil was 120 μg/mL, while that of acarbose was 7.5 μg/mL. The control of blood glucose levels requires the α-amylase enzyme to be inhibited, as this is involved in the breakdown of polysaccharides. The restricting power of *C. incana* essential oil was moderate. The major constituents of *C. incana* essential oil, such as carvacrol and thymol, have been connected to its antidiabetic properties. Some studies on the Lamiaceae plant family have been conducted in this area [92].

When compared to plants known for their antidiabetic characteristics, *C. incana* essential oil was shown to be a more potent inhibitor of α-amylase than both *Origanum vulgare* subspecies (subsp. vulgare and subsp. hirtum) essential oil [92, 93]. As a result, the inhibitory action of *C. incana* essential oil against α-amylase suggests that it might be used to treat type 2 diabetes.

4.4 Pancreatic anti-lipase inhibition

The influence of the synthetic acid esters on the PPL enzyme was assessed by measuring the hydrolysis of PNPB to p-nitrophenol. The test calculates the percentage of inhibition of the drugs in comparison to the standard, orlistat. The data in Table 3.3 and Figure 3.4 show that the fraction of the lipase enzyme that was inhibited increased as the oil content increased. In comparison to orlistat, the IC_{50} of the essential oil was less than 800 µg/mL. On the other hand, noticeable values were shown at 40%. The values were 200 µg/mL and 300 µg/mL for orlistat and the essential oil, respectively. This indicates that the essential oil of *C. incana* has an effect on the lipase enzyme. Inhibition of the lipase enzyme results in reduced fatty acid absorption, which reduces the accumulation of fatty acids in the body. It also lowers blood LDL cholesterol levels and raises HDL levels.

4.5 Anti-microbial

4.5.1 Antibacterial activity

The plant's oil was tested against six bacteria isolates, including four gram-negative bacteria (*K. pneumoniae, E. coli, P. vulgaris and P. aeruginosa*) and two gram-positive bacteria, (MRSA (clinical strain) and *S. aureus*). The broth microdilution technique was employed to assess antibacterial effectiveness *in vitro*. All types of bacteria were affected by the plant's oil at different concentrations, ranging from 62.5 µg/mL against *P. aeruginosa* to 0.4883 µg/mL against *P. vulgaris*, which were resistant to all positive standards, according to the MIC values (Table 4-5).

MRSA and *S. aureus* have MICs of 26.041 µg/mL and 31.25 µg/mL, respectively. This gives us an indication that the essential oil of *C. incana* has a weak effect on gram-positive bacteria. The MICs for *K. pneumoniae, E. coli* and *P. vulgaris,* were 1.302 µg/mL, 2.625 µg/mL and 0.4883 µg/mL, respectively. This indicates that gram-negative bacteria are more sensitive to the essential oil of *C. incana*, except for *P.*

aeruginosa with a MIC of 62.5 µg/mL, which is known for its resistance to a majority of clinically employed antibiotics. It can be concluded that the oil is bacteriostatic.

In comparison to antibacterial substances, few antifungal substances are known and commercially available. According to the data in Table 3.6, the essential oil of *C. incana* has a good effect on *C. albicans*, with a MIC of 0.2441 µg/mL, which is due to the presence of different components of the *C. incana* essential oil, such as pulegone, which has twice the effect on *C. albicans* as nystatin, and limonene [94].

The detection of the components responsible for the antibacterial action is extremely challenging due to the chemical constituents of the essential oil, which includes several different chemical compounds. Frequently, the synergistic or antagonistic interactions between many components lead to the antibacterial activity. The antibacterial activity of the essential oil is primarily due to its main components, such as pulegone, piperitenone and limonene [94-96] .

4.5.2 Antifungal activity

The oil was also tested for its antifungal activity against *M. canis*, *T. rubrum* and *T. mentagrophyte*. Table 3.6 and Figure A2 show the percent inhibition of *C. incana* at different concentrations. The oil reveals complete inhibition against all dermatophytes at 1 µL/mL, while the IC_{50} ranges from 0.15 µL/mL against *T. mentagrophyte* to 0.37 µL/mL against *T. rubrum*. These results are considered a revolution in antifungal science, and the *C. incana* essential oil can be used as a lead compound for antifungal medication.

Identification of the component responsible for the antifungal effect is extremely challenging due to the essential oil's complicated chemical components, which include dozens of different compounds. Antifungal activity is frequently caused by synergistic or antagonistic interactions between multiple components. Although additional phenomena, such as synergy or antagonistic interactions with smaller components, are probable, the antifungal activity of essential oils is generally attributed to their primary constituents, phenolic compounds like thymol, pulegone and limonene. There are many inhibitory mechanisms that *C. incana* essential oil exerts on fungi. The inhibition of cell wall synthesis, malfunction of the fungal mitochondria, and inhibition of efflux pumps are some of these effects [97].

4.6 Anticancer activity

The plant oil was tested against the HeLa cell line, which is a cancer cell line, and showed considerable results. The IC_{50} value of the oil was 50 μg/mL, as shown in Table 3.7 and Figure A3. On the other hand, at a concentration of 500 μg/mL, the oil revealed 79% inhibition.“ The nonspecific activity explains that this essential oil is a complex compound and may have synergistic effects. In this case, more than one chemical component in this oil may be contributing to the cytotoxic activity”. Therefore, further studies are required to emphasise which components of the oil candidate selectively inhibit cancer cell growth [98].

4.7 Conclusion and Recommendation

This study shows that *C. incana oil contains different percentages of phytochemicals:* piperitone oxide was the most abundant component (41.18%), followed by pulegone (29.762%), limonene (8.424%), trans-2,4-dimethylthiane, s,s-dioxide (8.28%) and a piperitenone derivative, cyclohexanone, 5-methyl-2-(methylethyl) (4.122%). These components represent the main constituents of *C. incana oil* and *provide different potential biological activities.*

C. incana essential oil has a strong antioxidant effect and a moderate enzymatic screening (lipase and α-amylase enzymes). It also has a potent effect on gram-negative bacteria like *E. coli, K. pneumoniae* and *P. vulgaris*, as well as on *C. albicans.*

C. incana has shown promising results on dermatophytes like *M. canis, T. rubrum and T. mentagrophyte*. The oil reveals complete inhibition against all dermatophytes at 1 μL/mL.

C. incana essential oil has an anticancer effect against HeLa cells, but we still need more study to search for the active component in the oil that is responsible for this effect.

To assess the potential pharmacological activity, safety and toxicity of this plant extract, more *in vivo* investigations are needed. Furthermore, additional research is required to separate, recognise and describe the primary elements accountable for the potential pharmacological effects.

List of Abbreviations

Abbreviation	Meaning
GC	Gas chromatography
GC-MS	Gas chromatography –mass spectrometry
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNSA	Di-nitrosalicylic acid
PL	Pancreatic lipase
MRSA	Methicillin-resistant *staphylococcus aureus*
DMSO	Dimethyl sulfoxide
IC_{50}	The half maximal inhibitory concentration
EO	Essential oil
MIC	Minimal inhibitory concentration
ID	Identification
LC/MS	Liquid chromatography-mass spectrometry
LC/NMR	Liquid chromatography –Nuclear Magnetic Resonance
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
SM	Secondary metabolite
UV	Ultraviolet
XO	Xanthine oxidase
COX	Cyclo-oxygenase
NADPH	Nicotin amide adenine dinucleotide
CH	Chalcone synthase
CHI	Chalcone isomerase
CHKP	Chalcone ketide reductase
C-GTF	Chalcone 2'-O-glucosyltransferase
TAL	Tyrosine ammonia lyase
HCQT	Hydroxycinnamoyl-coenzyme a quinate transferase
COMT	Caffeic acid o-methyltransferase
F5H	Ferulic 5-hydroxylase
DM	Diabetes mellitus
WHO	World health organization
NEC	New chemical entities
CO2	Carbon dioxide

C.incana	*Calamentha incana*
$CaCl_2$	Calcium chloride
λmax	Maximum wave length of absorption
Č	Celsius
KV	Kilo volt
M/Z	Mass to charge ratio
(w/w)%	Weight by weight %
TIC	Total ionic concentration
Nm	Nanometer
Mg/ml	Milligram per millileter
VF	Volumetric flask
Soln	Solution
PNPB	Para-nitrophenyl byturate
ATCC	American type culture collection
NA	Nutrient agar
CFU	Colony forming unit
MHB	Mueller Hinton broth
SDA	Sabouraud dextrose agar
EDTA	Ethylene diamine tetra acetic acide
MTS	Tetrazolium inner salt
IU	International unit
SD	Standard deviation
GOT	glutamate oxaloacetate transaminase

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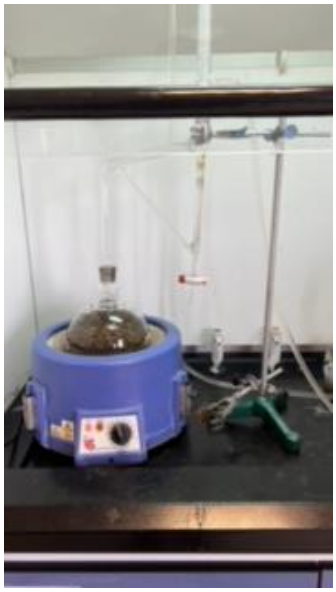
Appendices

Appendix A

Figures of Syudy

Figure A1

Hydro-distillation process



a :Hydro-distillation process

b : Yield of Oil from of Process

Figure A2

Anti-fungal activity of the essential oil against M.crains , T.rubrum, T.ment

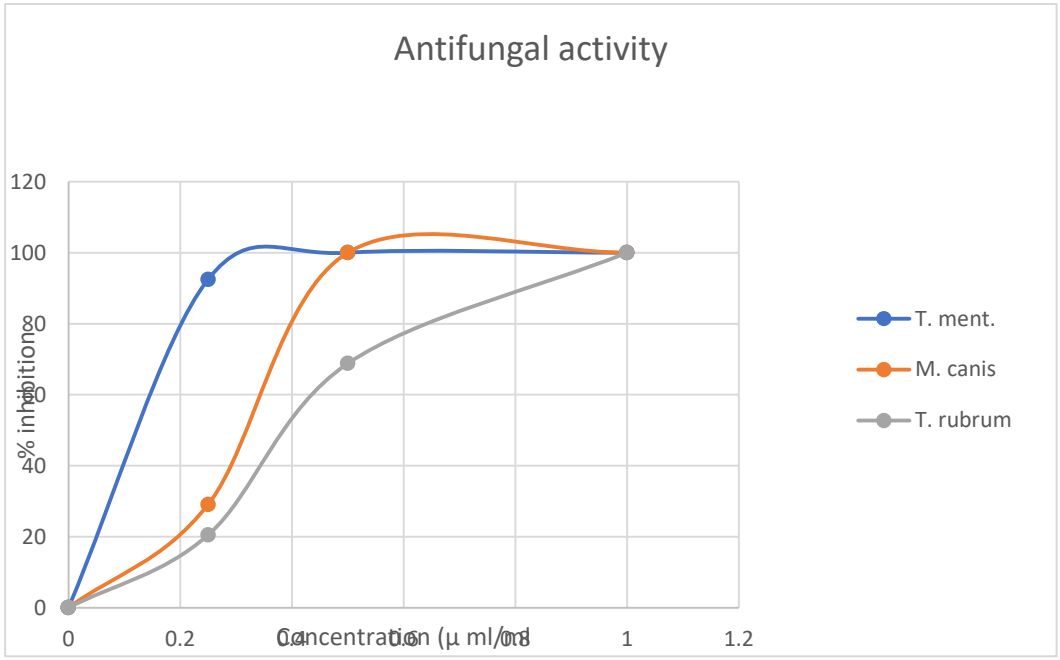
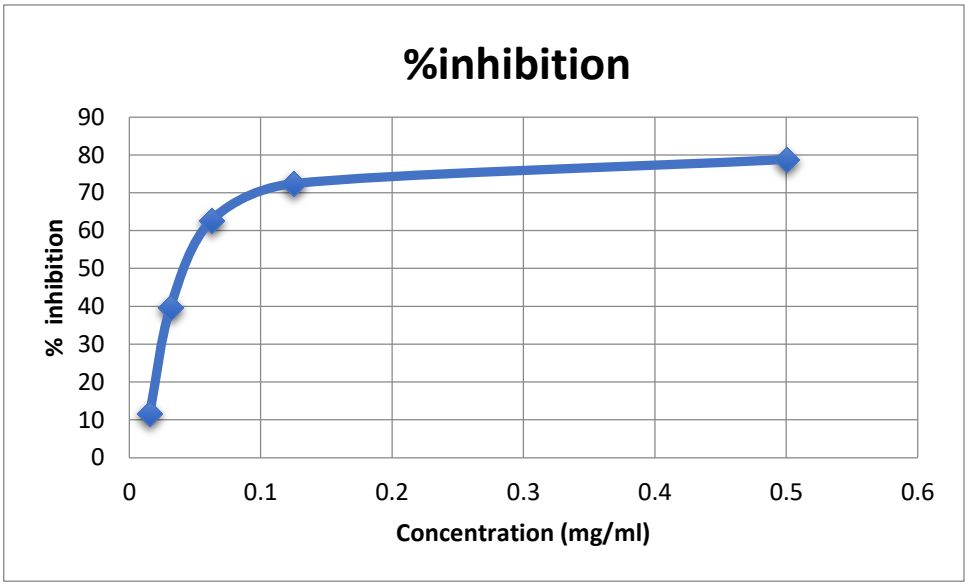


Figure A3

Anticancer activity against HeLa cell



Appendix B

Tables of Study

Table B1

Chemicals and reagents used for anti-bacterial and anti-fungal evaluation

Chemicals and reagents	Manufacturer	Country
Mueler Hinton Broth 21.9 g/L	HiMedia Laboratories	Mumbai-India
Normal saline	_____	_____
SDA	Beeton Dickinson company	France
Dimethyl sulfoxide	CARLO ERBA	France
Nutrient agar	Beeton Dickinson company	France

Table B2

Chemical and reagent used for producing EO

Chemicals and reagents	Manufacturer	Country
Calcium chloride	Sigma-aldrich	Usa

Table B3

Chemical and reagent used for cytotoxic screening

Chemicals and reagents	Manufacturer	Country
Trypsin-EDTA solution	Sigma Aldrich	USA
Cell Titer 96 r aqouse one solution	Promega	USA
RPMI media	Life technologes	UK
Fetal bovine serum	Sigma Aldrich	USA
Pen-strep	Biological industries	Israel
L-glutamine solution	Sigma Aldrich	USA

Table B4

Instrumentations used for extraction of EO and chemical screening of EO.

Instrumentations	Manufacturer	Country
Balance	Radwag, AS 220/c/2	Poland
Micropipettes	Finnpipette	Finland
Shaker	Memmert Shaking Incubator	Germany
PH-meter	Bibby scientific LTD	UK
GC-MS (Gas Chromatography Mass Spectrometry	QP-5000 Shimadzu GC-MS	Japan

Table B5

Instrumentation used for anti-oxidant and enzymatic screening

Instrumentations	Manufacturer	Country
Balance maximum capacity 4500 g	Boeco	Germany
UV-Vis (Ultraviolet-Visible) Spectrophotometer	Jen WAY 7315	UK
Water bath	Memert	Germany
water bath sonicator	MRC	Haifa
Heater	Lab-Tech	Korea
Refrigerator	Beko	UK
Single micropipette 100-5000 pL	Nichiryo Nichipet	Japan
single micropipette 100 - 1000 pL and 20-200 ML	Huma pette	Germany
large glass test tubes , volumetric flasks 10, 50, 100 mL, plastic cuvettes		

Table B6

Instrumentation used for antimicrobial screening

Instrumentations	Manufacturer	Country
Balance max 300 g, d-0.001g	Sartorius AY 303	Canada
Hot plate	Lab-Tech	Korea
Autoclave,water and disposed materials	MRC	Haifa
Hood	BIOBASE	China
Incubator	Ari j Levy 300₽L	MRC, Haifa
Multichannel micropipette 1-10 pL	Eppendorf research	Germany
Single micropipette (100-1000 ML)	Microliter , BRAND	Germany
Single micropipette 20-200 ML	Huma pette	Germany
bacterial solutions	Labcon	USA
Microplates 96 Well Cell Culture	Greiner bio-one CELLSTAR	Austria
Autoclave sterilization tapes and parafilm M	Bemis	USA
Loops, disposable sterile pipette volumes 10 mL and Eppendorf- tubes	Nichipet EX	Japan

Table B7

Instrumentation used for cytotoxic screening

Instrumentations	Manufacturer	Country
Biosafty cabinet	MRC	Haifa
CO2 incubater	ESCO	USA
Inverted microscope XD-2	MRC	China
Micro-pipet	Thermo- fisher scientific	USA
Mili-pipet	Heat throw scientific	USA
Micro- plate absorbance rea	Biotek	USA

Table B 8

Chemical composition of extracted oil

No.	Common name	R.T* (Min)	R.I*	M.W*	%
1	2-propanoic acid,2-methyl-,octyl ester	7.41	726	198	0.04767
2	2-octanol,2,6-dimethyl	7.41	694	158.28	
3	2-butanoic acid,1-methylpropyl ester	7.41	643	144.2	
4	alpha.-phellandrene	8.42	648	136.2	0.01946
5	bicyclo[3.1.10]hex-2-ene,2-methyl-5-(methylethyl	8.42	664	136.2	
6	1,4-methano-1h-cyclopropa(d)pyridazine,4,4a,5,5a-tetrahydro-6,6-dimethyl	8.42		136.2	
7	1R-.alpha.-pinene	8.69	817	136.2	0.4159
8	Carene	8.69	816	136.2	
9	1,3,6-octatriene,3,7-dimethyl-,(z)-	8.69	817	136.2	
10	spiro(2.4)heptane,1,5-dimethyl-6-methylene	9.36		136.24	0.03434
11	TRICYCLO(3.2.1.0(1,5))OCTANE	9.36		108.18	
12	Camphene	9.36	688	136.2	
13	beta phellandrene	10.34	822	136	0.31184
14	alpha.-phellandrene	10.34	810	136	
15	3-carene	10.52	832	136	0.65445
16	cyclohexene,4-methylene-1-(1-methylethyl)	10.52	807	136.24	
17	alpha.pinene	11.07	774	136	0.46952
18	3-heptanol, 6-methyl	11.47	793	130.23	0.97566
19	3-octanol	11.47	793	130	
20	Limonene	12.7	888	136.24	8.42484
21	cyclohexene,1-methyl-5-(1-methylethyl)5-isopropyl-1-methylcyclohex-1-ene	12.7	871	138.25	
22	Eucalyptol	13	822	154.25	0.00319
23	cyclohexanone,5-methyl-2-(methylethyl)	18.12		154.25	4.12266
24	2-ethyl-5-propylcyclopentanone	18.12		154.25	
25	TRANS-2-METHYL-4-N-BUTYLTHIANE,S,S-DIOXIDE	18.12		204.33	
26	bicyclo(2.2.1)heptane,2-(-1-buten-3-yl)-	18.41		150.27	0.26284

27	cyclopentene,1,2,3-trimethyl	18.41	824	110.2	
28	4-(2-methylcyclohex-1-enyl)-but-2-enal	18.41		136.19	
29	ENDO-BORNEOL	18.41		154.25	
30	cyclohexanone,5-methyl-2-(1-methylethylidene)	20.88		152.24	29.7628
31	PULEGONE	20.88		152.24	
32	trans-2,4-dimethylthiane,s,s-dioxide	21.34	718	162.24	8.28318
33	CIS-2-METHYL-4-N-PENTYLTHIANE,S,S-DIOXIDE	21.34		218.36	
34	cyclohexan,1,3-dimethyl-2-methylene,trans	21.62		124.23	0.13542
35	METHYL11,12-TETRADECADIENOATE	21.62		178.32	
36	CIS-BICYCLO(4.2.0)OCTANE	21.62		110.2	
37	TETRA-METHYLENE-GLUTARIC-ANHYDRIDE	21.71		164.16	0.05097
38	1-ETHYL CYCLOPENTENE	21.71		96.17	
39	PENTALENE,OCTAHYDRO	21.71		110.2	
40	1,1-BICYCLOPENTYL	21.71		138.25	
41	2-CYCLOHEXEN-1-ONE,3-METHYL-6-(1-METHYLETHENYL)	22		150.22	0.47548
42	2-CYCLOHEXEN-1-ONE,2METHYL-5-(1-METHYLETHENYL)	22		150.22	
43	phenol,2-methyl-5-(1-methylethyl)5-isopropyl-2-methylphenol	22.81		150.22	0.38371
44	Thymol	22.81		150.22	
45	phenol,2,3,5,6-tetramethyl	23.1		150.22	0.85183
46	BICYCLO(2.2.1)HEPTANE,7,7-DIMETHYL-2-METHYLENE	23.33		136.24	0.51613
47	CYCLOHEXENE,3METHYL-6-(1-METHYLETHENYL)	23.33		136.24	
48	CYCLOHEPTENE,5-ETHYLIDENE-1-METHYL	23.33		136.24	
49	O-TRIFLUOROACETYL-ISOPULEGOL	23.33		250.26	
50	2,3-dimethylhydroquinone	25.2	739	138.17	41.1784
51	4,6-DECADIENE	25.2		138.25	
52	INDENO(3A,4-B)OXIRENE-2,5(1AH,3H)-DIONE,TETRAHYDRO-4A-METHYL,(1A,4A,7AR)	25.2		180.2	
53	1-METHYLENE-2B-HYDROXYMETHYL-3,3DIMETHYL-4B-(3-METHYLBUT-2-ENYL)-CYCLOHEXAN	27.03		222.37	0.64386
54	CYCLOHEXAN,1,5-DIETHENYL-3-METHYL-2-METHYLENE	27.03		162.28	
55	BICYCLO(7.2.0)UNDEC-4-ENE,4,11,11-TRIMETHYL-8-METHYLENE-	27.03		204.36	

56	Caryophyllene	27.03	834	204	0.64386
57	alpha-cubene	29	752	204	0.23989
58	1,4METHANOCYCLOOCTA(D)PYRIDAZINE,1,4,4A,5,6,9,10,10A-OCTAHYDRO-11,11-DIMETHYL-,(1A.,4A.)	29.44		204.32	0.63651
59	METHYL 6,8-octadecaiynoate	32	804	289.44	0.4555
60	5,8,11,14,17-icosapentaenoic acid	32		302.4	

R.T : retention time , RI : retention index , M.W : moleculare weight

جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

التركيب الكيميائي، الأنشطة المضادة للميكروبات والخلايا السرطانية للزيت العطري في نبتة كلينوديوم إنسولار من فلسطين

إعداد

زياد بدر سلمان

إشراف

د. نضال جرادات

د. أحمد خساتي

قدمت هذه الاطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس – فلسطين.

2023

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د.احمد خساتي

الملخص

زعتر سيدي موسى (*Clinopodium insulare*) هو واحد من النباتات العطرية الطبية، والتي تهيمن عليها مناطق شرق البحر الأبيض المتوسط بما في ذلك فلسطين، ولها رائحة مميزة تشبه النعناع. تقليديا، تستخدم أوراق زعتر سيدي موسى كتوابل وكشاي عشبي. كان الهدف من العمل الحالي هو فحص المكونات الكيميائية، والخصائص الدوائية المحتملة، والفحص الأنزيمي مثل (amylase)، إنزيمات (lipase) من زيت زعتر سيدي موسى المتطاير الذي تم جمعه من قرية بيت ليد طولكرم في الضفة الغربية – فلسطين.

تم استخراج الزيت العطري للنبات باستخدام تقنية التقطير المائي . تم تحليل الزيت العطري للعينة بحثا عن المكونات الكيميائية باستخدام Gas chromatography Mass spectrometry *(GC-MS)*. تم فحص النشاط المضاد للأكسدة للزيت العطري عن طريق تثبيط 2,2-diphenyl-1-picrylhydrazyl (DPPH). تم استخدام مقايسة 3,5-dinitrosalicylic(DNSA) لتقييم النشاط المضاد Amylase. تم تقييم النشاط المضاد Lipase باستخدام طريقة p-nitrophynel butyrate. تم تقييم النشاط المضاد للميكروبات باستخدام طريقة Microdilution لاختبار مضاد للبكتيريا وطريقة (poisond food technique) لاختبار الفطريات. تم استخدام ست سلالات بكتيرية: أربعة سالبة الجرام: *Escherichia*

coli, Pseudomonas aeruginosa, Proteus vulgaris, and Klebsiella pneumonia; وإيجابية الجرام*(MRSA (Methicillin*-Resistant *Staphylococcus aureus);* وخميرة واحدة *Candida albican;* وثلاثة dermatophytes: *Microsporum canis, Trichophyton rubrum, and Trichophyton mentagrophyte*; تم تقييم النشاط المضاد للسرطان باستخدام مقايسة MTS على خلايا HeLa.

كان محصول الزيت العطري النباتي 0.213(w/w)%. كشف تحليل GC-MS عن وجود ستين مركبا، والتي تمثل 100 ٪ من إجمالي تكوين الزيت. كان المركب الرئيسي هو أكسيد البيبريتون، الذي يمثل 41.178٪. أظهرت *C. incana* نشاطا مضادا للأكسدة مع IC_{50} تبلغ 390مايكروجرام/مل. قد يكون للزيت العطري خصائص مثبطة للإنزيم. ومع ذلك، كان لها تأثير مثبط معتدل amylase بقيمة IC_{50} تبلغ 120 مايكروجرام/مل وتأثير مثبط معتدل مضاد للالايباز بقيمة IC_{50} اقل من 800 مايكروغرام/مل. أظهرت العينة نشاطا واسعا مضادا للميكروبات، قويا ضد البكتيريا سالبة الجرام مع MICs تتراوح بين 0.48825 و 62.5 مجم / مل وبين 26.041 و 31.25 مجم/ مل للبكتيريا إيجابية الجرام، والخميرة مع 0.2441 مجم/ مل. لوحظ النشاط المضاد للفطريات ضد *Microsporum canis* و*Trichophyton rubrum* و *Trichophyton mentagrophyte*، حيث تراوحت MICs من 0.15 ميكرولتر/مل ضد *Trichophyton mentagrophyte* إلى 0.37 ميكرولتر/ مل ضد *Trichophyton rubrum*. تم اختبار النشاط المضاد للسرطان للزيت ضد خلايا HeLa وأظهر نتائج واعدة بقيمة IC50 تبلغ 50 ميكروغرام / مل.

وأظهرت الدراسة أن عينة الزيت المتطاير من (*Clinopodium insulare*) تحتوي على نسب مختلفة من المواد الكيميائية التي وفرت أنشطة بيولوجية محتملة مختلفة على النحو التالي: مضادات الأكسدة ومضادات اللايباز ومضادات السكري ومضادات السرطان والأنشطة المضادة للميكروبات ذات التأثير المتغير حسب نسب المواد فيها.

الكلمات المفتاحية: زيت عطري، مضاد بكتيريا، مضاد فطريات، زعتر سيدي موسى.