Sudan University of Science and Technology

College of Graduate Studies

Extraction, Antioxidant and Biological Activity of The Oil from *Mentha spic ata.L* (*Spearmint*) Leaves

A Dissertation Submitted in Partial Fulfillment for the Requirements of Master Degree in Chemistry

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الاستهلال

(اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (1) خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ (2) اقْرَأْ وَرَبُّكَ الْأَكْرَمُ (3) الَّذِي عَلَّمَ بِالْقَلَمِ (4) عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ (5)

صدق الله العظيم

سورة العلق الآيات 1-5

Dedication

This study is dedicated to

My Parents,

Brothers,

and Sisters.

Acknowledgment

First and foremost my endless praise is due to Allah Almighty for giving me health strength and success to complete this research.

My great thanks go to my supervisor prof. Mohamed El mubark for his continuous encouragement and help during his supervision of the work.

My thanks are extend to the members of the National Center For Research for technical support, also to my colleagues in work and friend for their moral support.

Abstract

The aim of this study was to investigate the chemical composition, the antimicrobial and antioxidant activities of the essential oil from dried leaves of *Mentha spicata* (viridis). Moreover the antimicrobial activity of the oil against four bacterial species: Gram-positive bacteria, *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923) , Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and fungal strains*, Candida albicans* (ATCC 7596) using Agar plate diffusion method was also assessed. Antioxidant activity was performed using DPPH radical-scavenging method.

The result of extraction shows that oil yield was 1.23% .From GC-MS analysis, 28 components were identified, accounting for 100.00% of the essential oil composition. The main constituents of the oil were D-carvone (57.20%) and D-Limonene (19.41%) .The essential oil exhibited moderate level of antibacterial activity against all test microorganisms, and didnot show antifungus activity. In general Gram-positive bacteria were more susceptible to *Mentha spicata* essential oil than Gram-negative bacteria. staphylococcus aureus was the most sensitive of the microorganisms to the antibacterial activity of *Mentha spicata* essential oil (inhibition zone = 13 mm). The results show this essential oil has clearly good antioxidant activity DPPH activity compared to Propyl gallate (as standard).

المستخلص

الهدف من هذه الدراسة هو التحقق من التركيب الكيميائي والفعالية المضادة للميكروبات ومضادات الاكسدة للزيت العطري من اوراق النعناع البلدي المجفف .بالاضافة ، تم ايضا تقييم الفعالية المضادة للميكروبات للزيت ضد أربعة انواع من البكتريا : البكتريا الموجبة بيسلس (NCTC 8236) واستافيلوكوكوس أوريس (ATCC25923) ، البكتريا السالبة ايشريكيا كولاي (ATCC2592) وبسيدومونس ايرفينوزا (ATCC 27853) ، وفطر كانديدا البكانس (ATCC7596) باستخدام طريقة انتشار لوحة اجار . تم اجراء الفعالية المضادة للاكسدة باستخدام طريقة .DPPH

اظهرت نتيجة الاستخلاص ان نسبة انتاج الزيت كانت 23. 1%. من تحليل GC-MS تم تحديد 28 مكون تمثل 100 % من تركيبة الزيت العطري . المركبات الرئيسية للزيت هي : الكارفون بنسبة (20% . 57) ومركب الليمونين بنسبة (41 . 19 %).

اظهر الزيت العطري مستوي متوسط للفعالية البكتيرية ضد جميع الكائنات الحية الدقيقة المختبرة ، ولم يظهر فعالية فطرية . وعموما البكتريا الموجبة اكثر حساسية تجاه زيت النعناع البلدي من البكتريا السالبة . بكتريا الاستافيلوكوكوس أوريس كانت اكثر الكائنات الحية الدقيقة حساسية للفعالية البكتيرية في زيت النعناع البلدي (منطقة التثبيط = 13مم) ،واظهرت النتائج ان هذا الزيت العطري له فعالية مضادة جيدة للأكسدة مقارنة بمركب بروبايل جاليت (كمعيار قياسي) .

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List of Abbreviations

ISO	International Standards Organization
SFE	Supercritical Fluid Extraction
MAHD	Microwave-Assisted Hydrodistillation
UAE	Ultrasound-assisted extraction
SFME	Solvent-free microwave extraction
MHG	Microwave hydro diffusion and gravity
FMC	Food Machinery Corporation
BOEs	Brown Oil Extractors
GC-MS	Gas Chromatography – Mass Spectrometry
B.P	Boiling Point
SOD	Superoxide Dismutase
GSH-Px	Glutathione peroxidase
CAT	Catalase
TBARS	Thiobarbituric acid- reactive substance
B.s	*Bacillus subtilis*
S.a	*Staphylococcus aureus*
E.c	*Escherichia coli*
P.a	*Pseudomonas aeruginosa*
C.a	*Candida albicans*
NCTC	National Collection of Type Culture
ATCC	American Type Culture Collection
MHA	Mueller Hinton Agar

M.D.I.Z	Mean diameter of growth inhibition zone
DPPH	2-2-diphenyl-1-picril-hydrazyl
DMSO	Dinethyl sulfoxide
NIST	National Institute of Standards and Technology
RSA	Rabical Scavenging Activity
SD	Standard definition
M.	*Mentha*
Mm	Millimeter
G	Gram
Cfu	Colony forming Unit
Ml	Milliter
Ul	Microliter
H	Hour
℃	Centigrade

Chapter One

Introduction and literature review

Chapter One

Introduction and literature review

1.1 Introduction

Essential oils are plant-based volatile oils with strong aromatic components that are made up of different chemical compounds. For example, alcohols, hydrocarbons, phenols, aldehydes, esters and ketones, they are predominantly, extracted using steam distillation. Essential oils are used in perfumery, aromatherapy, cosmetics, incense, medicine, household cleaning products. They are valuable commodities in the fragrance and food industries for flavoring food and drink (Hesham et al., 2016).

Family of *Lamiaceae* are well known for the essential oils common to many members of the family. Members of this family are used for is different purposes. however, the main use can be medicinal, ornamental and aromatic as perfume. (Adel Nadjib et al., 2011).

Mentha is a genus of aromatic perennial herbs. It is one of the most important genera of the family *Lamiaceae*, and the tribe *Mentheae*, is divided into five sections and consists of approximately 25 species , distributed mostly in temperate and sub-temperate regions of the world ((Harley and Brighton, 1977**,** Savithri et al, 2002), which are originally used as a medicinal herb to treat stomach ache and chest pains, and during the middle Ages, powdered mint leaves were used to whiten teeth. Mint tea is a strong diuretic. It also in aids digestion, in a way that it breaks down the fats. In recent years, it has been often recommended for treating obesity (Bohloul et al., 2009).

Essential oils from mint species have strong antioxidant activity and good antimicrobial activity (Mkaddem et al., 2009), they are used to flavor foods, in dental as toothpastes and mouthwashes, in fragrances and they are used in chewing gums, alcoholic beverages, cosmetics.

The taxonomy of the genus *Mentha* is still not definite but on the basis of a phylogenetic analysis of morphology, chromosome numbers, and major constituents of essential oil, the genus *Mentha* is now redefined to include 18 species and 11 hybrids placed in four sections: *Pulegium, Tubulosae*, *Eriodentes* and *Mentha* (Tucker & Naczi, 2007).

Mentha spicata L. (spearmint) is a perennial herb, it is one of the *Mentha* species native species of Europe, North Africa and in Asia Minor and near East (Boukhebti et al, 2011), witch is used with spices to give the food a special flavor and fragrance. It is also used for flavoring chewing gums, toothpaste, confectionery and pharmaceutical preparations (Saleem et al., 2000). Essential oil are used as antimicrobial and antioxidant agents (Mata, 2007). The main constituents of the *M.spicata* essential oil are phenolic compounds such as carvone and limonene (Mkaddem et al,2009).

the *spearmint* extract is a matrix rich in phenolics. The major phenolic compounds in the spearmint extract were represented by rosmarinic acid and its derivatives (Martina et al, 2016).

1.2 Essential oils

Essential oils are complex mixtures of volatile compounds produced by living plants and isolated by physical only (pressing and distillation) from a whole plant or plant part of known taxonomic origin**.**

An essential oil as defined by the ISO in document ISO 9235.2—aromatic natural raw materials—vocabulary is as follows , “Product obtained from vegetable raw material—either by distillation with water or steam or—from the epicarp of *Citrus* fruits by a mechanical process, or—by dry distillation” (Hüsnü and Gerhard, 2010).

An alternative definition of essential oils, established by Professor Dr. Gerhard Buchbauer of the Institute of Pharmaceutical Chemistry, University of Vienna, includes the following suggestion: “Essential oils are more or less volatile substances with more or less odorous impact, produced either by steam distillation or dry distillation or by means of a mechanical treatment from one single species” (Hüsnü and Gerhard, 2010).

All plant possess principally the ability to produce volatile compounds, quite often however, only in traces. Essential oils plants in particular are those plant species delivering an essential oil of commercial interest (Hüsnü and Gerhard, 2010).

Aromatic oils can be found in all the various parts of a plant, including seeds, bark, root, leaves, flowers, wood, balsam and resin (Julia Lawless, 2013).

All parts of aromatic plants may contain essential oils as follows:

_ Flowers, of course, including: orange, pink, lavender, and the (clove) flower bud or (ylang-ylang) bracts,

_ Leaves, most often, including: eucalyptus, mint, thyme, bay leaf, savory, sage, pine needles, and tree underground organs, e.g., roots (vetiver)

_ Rhizomes (ginger, sweet flag),

_ Seeds (carvi, coriander),

_ Fruits, including: fennel, anise, Citrus epicarps.

_ Wood and bark, including: cinnamon, sandalwood , rosewood

(Wissal et al., 2016).

Essential oils are generally a pale to clear or slightly yellowish liquids, mostly insoluble in water, with specific gravities between 0.80 to

1.20. The odour of an essential oil will resemble the source flora, made up of a large number of constituents, sometimes (Hunter, 2009).

1.2.1 Essential oils extraction methods

The choice of extraction method for the recovery of essential oils and other volatile materials from plants has great bearing on the composition and quality. True essential oils are extracted through various distillation methods. However a number of volatile constituents of plants are very fragile and susceptible to heat, reactive to moisture during extraction and are difficult to liberate from the surrounding plant material, thus other more situational effective methods of extraction are also utilized (Hunter, 2009).

Essential oils are obtained from plant raw material by several extraction methods .There are two types of extraction methods:

1- Classical methods (Traditional methods).

They include Hydrodistillation, Steam Distillation, Solvent extraction, Soxhlet Extraction, Cold Pressing method.

2- Innovative Techniques Methods or Modern (Non-Traditional) Methods.

One of the disadvantages of conventional techniques is related with the thermolability of essential oils components which undergo chemical alterations (hydrolyse, isomerization, oxidation) due to the high applied temperatures.

These are the methods most widely used on a commercial scale. However, with technological advancement, new techniques have been developed which may not necessarily be widely used for commercial production of essential oils but are considered valuable in certain situations, such as the production of costly essential oils in a natural state without any alteration of their thermosensitive components or the extraction of essential oils for micro-analysis

These techniques are as follows:

- Supercritical fluid extraction (SFE).
- Microwave-assisted hydrodistillation (MAHD).
- Ultrasound-assisted extraction (UAE).
- Solvent-free microwave extraction (SFME).
- Microwave hydrodiffusion and gravity (MHG) (Hesham et al., 2016).

The common extraction methods of essential oils are three methods in use. Expression is probably the oldest of these and is used almost exclusively for the production of Citrus oils. The second method, hydrodistillation or steam distillation, is the most commonly used one of the three methods, while dry distillation is rarely used in some very special cases.

1.2.1.1 Expression

This method was used almost exclusively for the production of *Citrus peel* oils. *Citrus* and the allied genus *Fortunella* belong to the large family *Rutaceae*.**,**the reason for extracting citrus oils from fruit peel using mechanical methods is the relative thermal instability of the aldehydes contained in them. Fatty, for example, aliphatic, aldehydes, terpenoid aldehydes, terpenic hydrocarbons and esters contained in the peel oils are sensitive to heat and oxygen (Hüsnü and Gerhard, 2010).

Until the beginning of the twentieth century, industrial production of cold-pressed citrus oils was carried out manually. First of all, the fruit had to be peeled and soaked in warm water washed and cut into two halves. The pulp was then removed from the fruit, The fruit peel was, manually, turned inside out so that the epicarp was on the inside, squeezed by hand to break the oil glands and the oil soaked up with a sponge. The peel was now turned inside out once again and wiped with the sponge and the Parts of a citrus fruit. Production of Essential Oils 97 sponge squeezed into a terracotta bowl, the “concolina.” After decantation, the oil was collected in metal containers (Hüsnü and Gerhard, 2010).

Alater improvement of the fruit peel expression process was the “scodella” method. The apparatus was a metallic hemisphere lined inside with small spikes, with a tube attached at its center. The fruit placed inside the hemisphere was rotated while being squeezed against the spikes thus breaking the oil cells. The oil emulsion, containing some of the wax coating the fruit, flowed into the central tube was collected, and the oil was subsequently separated by centrifugation (Hüsnü and Gerhard, 2010).

Today the only systems of significance in use for the industrial production of peel oils can be classifi ed into four categories: “sfumatrici” machines and “speciale sfumatrici,” “Pellatrici” machines, “FMC whole fruit process,” and “Brown oil extractors (BOEs)”. The machines used in the “sfumatrici” methods consist in principle of two parts, a fixed part and a moveable part. The fruit is cut into two and the flesh is removed. In order to extract the oil, the citrus peel is gently squeezed, by moving it around between the two parts of the device, and rinsing off the squeezed-out oil with a jet of water. The oil readily separates from the liquid on standing and is collected by decantation (Hüsnü and Gerhard, 2010).

1.2.1.2 Distillation

Distillation is the process of vaporizing a liquid, condensing the vapor, and collecting the condensate in another container. This technique is very useful for separating a liquid mixture when the components have different boiling points, or when one of the components will not distill (Donald et al., 2002).

It is most likely the Arabs inherited their knowledge of distillation techniques from the Syrian Empire. However, almost all distillation until midway, through the Nineteenth Century, was water or hydro distillation.

the Germans and French in Grasse began experimenting to improve the distillation process. However it was only in the beginning of the Twentieth Century that steam from an external source to the charge bin was introduced, bringing in the method of steam distillation.

distillation enables the separation of volatile constituents contained in some form of plant material, through a parent carrier vapour (water) capturing other volatile materials from the plant material in the charge (Hunter, 2009).

The apparatus is used in distillation there are six pieces of specialized glassware are used distilling flask, distillation head, thermometer adapter, water condenser, vacuum takeoff adapter, receiving flask (Donald et al., 2002).

The Fundamental Principals of distillation requires an understanding of the principals of the laws of thermodynamics and physical chemistry. the practice of distillation on herbaceous materials is influenced by latent heat, the behaviour of gases, vapour pressure, and steam.

Heat is a form of energy which converts water into vapour. In distillation, heat is therefore converted energy in the form of steam, Liquids will change into a gaseous state at a specific temperature according to a certain pressure. Below boiling point a liquid stores energy as heat. When a liquid is heated, its molecules become more active until a point where they separate from the parent liquid into vapour. If the surrounding space is closed, the new vapour molecules will exert pressure. This is called vapour pressure.

Steam is a two-phase mixture of air gases and moisture molecules. Saturated steam carries microscopic particles of liquid which give the gas a ‘cloudy’ appearance. Wet steam will carry more of these particles than dry steam.

The aim of passing steam through a charge bin of plant material is to capture and carry the volatile compounds with the steam through the charge to the condenser (Hunter, 2009).

There are three types of hydrodistillation for isolating essential oils from plant materials:

- Water distillation.
- Water and steam distillation.
- steam distillation.(Hunter, 2009).

1.2.1.2.1 Water distillation

Water distillation was the only method used before the Twentieth Century,it involves distilling plant material totally immersed in water. Depending upon the specific gravity and charge mass in the still, the material will either float or sit totally immersed in the water. Heat is introduced by direct heating of the sides of the vat, a steam jacket, a closed system coil or in some cases a perforated steam coil.

Essential oils contain a number of oxygenated constituents that are relatively soluble in water. This would include phenols, alcohols and some aldehydes. During the early stages of water distillation, these compounds would dissolve in water and become part of the boiling mixture and resulting mixed vapour.

As water boils and converts to steam and rises, it will come into contact with the plant material. The oil on the surface of this material will be vaporized by the rising steam as it comes into contact with the plant leaves surface. This steam carrying some volatile vapour will rise to the surface and carryover into the vapor space above the water until it reaches the still condenser (Hunter, 2009).

The boiling temperature of water in water distillation is slightly less than the boiling point of water, due to the mixed liquid of solublised volatiles and water. Heat applied to the still will cause the creation of a small bubble of saturated mixed vapour from the liquid phase, where upon formation it rises to the top of the water. During the rise, it's the bubble's pressure, temperature and proportion of oil in water decreases. The condensing volatiles are less dense than water, float to the top of the water and form a film on the surface of the water in the vessel. This lost oil tends to remain on top of the surface and cannot revaporise easily due to its higher boiling point and the generally cooler temperatures at the water surface. Most of the oil recovered in water distillation is the portion of the oil that does not condensate through this action.

In water distillation, plant material is placed in a sealed vessel or retort that directly connected to a condenser. From the condenser the distillate runs into a separator. The rate of distillation is controlled by the intensity of heat, the pressure of the vessel or retort and/or the rate of introduction of steam. Another method to recover the dissolved aromatic materials from the water distillate is to add a solvent. The mixture is then vigourously shaken to pick up dissolved constituents from the water into the solvent. These materials are then recovered through vacuum distillation of the solvent which results in a secondary essential oil . Another method that will contribute to minimizing oil loss due to constituent solubility in water during the separation phase is to control the outgoing distillate temperature from the condenser (Hunter, 2009).

1.2.1.2.2 Water and steam distillation

Water and steam distillation involves the storing of the plant material above a water bath situated in the bottom of the charge vessel and heating the water either through direct fire, a The extraction of essential oils 193 steam jacket or a closed or open steam coil. Water and steam distillation produces saturated wet steam at the prevailing vessel pressure, which is usually atmospheric pressure. Within this configuration, unlike water distillation, only steam comes in contact with the plant material. Water and steam distillation is not very suitable for fine materials as steam will find a low resistant path and tend to create channels. This means that steam will not flow through the whole mass of plant material and an incomplete distillation will take place. If the plant material is loosely packed, the same effect will happen, as the material will offer no resistance to the steam. Water and steam distillation may take a long period of time to reach operating temperature as the plant material needs to be heated up with only saturated steam. This may cause early condensation and wetting of the plant material. Due to the limits on pressure that can be built up in the charge vessel, water and steam distillation will have only a limited effect on extracting high boiling materials from plant materials. However there is less opportunity for hydrolysis to occur than with water distillation. Water and steam distillation has another advantage over steam distillation as there are fewer decomposed products during the process due to less chance of plant material drying out. However water and steam distillation will take a lot longer. Water and steam distillation can produce very good results under reduced pressure. Water and steam distillation is much cheaper to set up than steam distillation facilities and lends itself to portable stills that can be transported from place to place (Hunter, 2009).

1.2.1.2.3 Direct steam distillation

Steam distillation employs an external steam generation system, external to the charge vessel. This configuration provides much more control (depending upon the boiler capacity) than water and steam distillation. This is because in steam distillation the wetness fraction, temperature and pressure can be manipulated according to needs and conditions. However, it is a misconception that greater steam volumes and increased pressures have positive effects on the process in all cases. As mentioned previously, dry and superheated steam has the effect of drying out plant material, which potentially halts distillation through the stopping of hydrodiffusion process. Faster steam flow rates do not necessary mean quicker recovery times. Fabricated steam boilers cost much more to run than water baths and may lead to high distillation costs, especially if they require petrochemical feed stocks. However if steam distillation facilities are designed and built with the correct steam ratings, they are much more economical to run than water and water and steam distillation systems (Hunter, 2009).

1.2.2 Chemical constituents of essential oils

pure essential oils are mixtures of more than 200 components, normally mixtures of terpenes or phenylpropanic derivatives, in which the chemical and structural differences between compounds are minimal. They can be essentially classified into two groups:

Nonvolatile residue that comprises 1–10% of the oil, containing hydrocarbons, fatty acids, sterols, carotenoids, waxes, and flavonoids.

Volatile fraction Essential oil contains of 90–95% of the oil in weight, containing the monoterpene and sesquiterpene hydrocarbons, as well as their oxygenated derivatives along with aliphatic aldehydes, alcohols, and esters (Abd Elmoez, 2011).

1.2.2.1 Hydrocarbon

Essential Oils consist of Chemical Compounds that have hydrogen and carbon as their building blocks. Basic Hydrocarbon found in plants are isoprene having the following structure (Abd Elmoez, 2011)

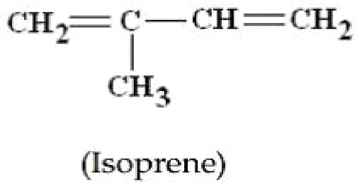


Figure 1.1: Molecular structure of Isoprene

1.2.2.2Terpenes

Generally have names ending in " **ene**."

<u>For examples</u>: Limonene, Pinene, Piperene, Camphene, etc. Terpenes are anti-inflammatory, antiseptic, antiviral, and bactericidal. Terpenes can be further categorized in monoterpenes, sesquiterpenes and diterpenes. Referring back to isoprene units under the Hydrocarbon heading, when two of these isoprene units join head to tail, the result is a monoterpene, when three join, it's a sesquiterpene and four linked isoprene units are diterpene (Abd Elmoez, 2011).

1.2.2.2.1Monoterpenes [C10H16]

Are analgesic, bactericidal, expectorant, and stimulant.

Monoterpenes are naturally occurring compounds, the majority being unsaturated hydrocarbons (C_{10}).But some of their oxygenated derivatives such as alcohols, Ketones, and carboxylic acids known as monoterpenoids.

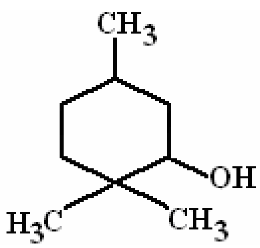


Figure 1.2: Molecular structure of Menthol

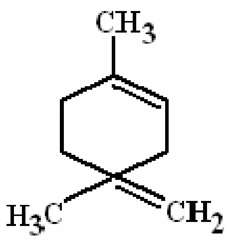


Figure 1.3: Molecular structure of Limonene

The branched-chain C_{10} hydrocarbons comprises of two isoprene units and is widely distributed in nature with more than 400 naturally occurring monoterpenes identified. Moreover, besides being linear derivatives (Geraniol, Citronellol), the monoterpenes can be cyclic molecules (Menthol – Monocyclic; Camphor – bicyclic; Pinenes (**α** and **β**) – Pine genera as well. Thujone (a monoterpene) is the toxic agent found in Artemisia absinthium (wormwood) from which the liqueur, absinthe, is made. Borneol and camphor are two common monoterpenes. Borneol, derived from pine oil, is used as a disinfectant and deodorant. Camphor is used as a counterirritant, anesthetic, expectorant, and antipruritic, among many other uses e.g camphene and pinene in cypress oil, camphene, pinene and thujhene in black pepper (Abd Elmoez, 2011).

1.2.2.2.2Sesquiterpenes

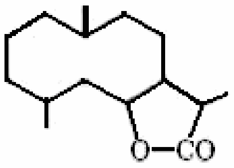
Are anti-inflammatory, anti-septic, analgesic, anti-allergic.

Sesquiterpenes are biogenetically derived from farensyl pyrophosphate and in structure may be linear, monocyclic or bicyclic. They constitute a very large group of secondary metabolites, some having been shown to be stress compounds formed as a result of disease or injury (Abd Elmoez, 2011).

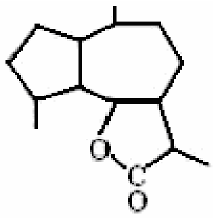
Sesquiterpene Lactones

Over 500 compounds of this group are known; they are particularly characteristics of the Compositae but do occur sporadically in other families. Not only have they proved to be of interest from chemical and chemotaxonomic viewpoints, but also possess many antitumor, anti-leukemia, cytotoxic and antimicrobial activities. They can be responsible for skin allergies in humans and they can also act as insect feeding deterrents.

Chemically the compounds can be classified according to their carboxylic skeletons; thus, from the germacranolides can be derived the guaianolides, pseudoguaianolides, eudesmanolides, eremophilanolides, xanthanolides, etc.

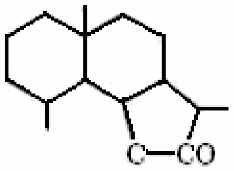


Germacranolides

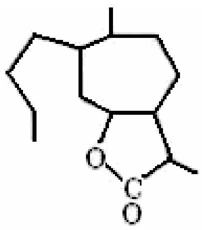


Guaianlides

Figure 1.4: Molecular structure of Germacranolides and Guaianlides



Eudesmanolides



Xantnanolides

Figure 1.5: Molecular structure of Eudesmanolides and Xantnanolides

Astructural feature of all these compounds, which appears to be associated with much of the biological activity, is the α, β -unsaturated-γ- lactones e.g farnesene in chamomile and lavender, beta-caryophyllene in basil and black pe pper (Abd Elmoez, 2011).

1.2.2.2.3Diterpenes

Are anti-fungal, expectorant, hormonal balancers, hypotensive .

Diterpenes are made of up four isoprene units. This molecule is too heavy to allow for evaporation with steam in the distillation process, so is rarely found in distilled essential oils. Diterpenes occur in all plant families and consist of compounds having a C20 skeleton. There are about 2500 known diterpenes that belong to 20 major structural types. Plant hormones Gibberellins and phytol occurring as a side chain on chlorophyll are diterpenic derivatives. The biosynthesis occurs in plastids and interestingly mixtures of monoterpenes and diterpenes are the major constituents of plant resins. In a similar manner to monoterpenes, diterpenes arise from metabolism of geranyl geranyl pyrophosphate (GGPP).

Diterpenes have limited therapeutical importance and are used in certain sedatives (coughs) as well as in antispasmodics and antoxiolytics e.g sclareol in clary sage is an example of a diterpene alcohol (Abd Elmoez, 2011).

1.2.3.3 Alcohols

Are anti-septic, anti-viral, bactericidal and germicidal. Alcohols are the compounds which contains Hydroxyl groups. Alcohols exist naturally, either as a free compound, or combined with a terpenes or ester. When terpenes are attached to an oxygen atom, and hydrogen atom, the result is an alcohol. When the terpene is monoterpene, the resulting alcohol is called a monoterpenol. Alcohols have a very low or totally absent toxic reaction to the body or on the skin. Therefore, they are considered safe to use e.g linalool found in ylang-ylang and lavender, geraniol in geranium and rose, nerol in neroli (Abd Elmoez, 2011).

1.2.2.4 Aldehydes

Are anti-fungal, anti-inflammatory, anti-septic, anti-viral, bactericidal, disinfectant, sedative.

Medicinally, essential oils containing aldehydes are effective in treating Candida and other fungal infections e.g citral in lemon, lemongrass and lemon balm, citronellal in lemongrass, lemon balm and citrus eucalyptus (Abd Elmoez, 2011).

1.2.2.5 Acids

Are anti-inflammatory. Organic acids in their free state are generally found in very small quantities within Essential oils. Plant acids act as components or buffer systems to control acidity e.g cinnamic and benzoic acid in benzoin, citric and lactic acids. (Abd Elmoez, 2011).

1.2.2.6 Esters

Esters are formed through the reaction of alcohols with acids. Essential oils containing esters are used for their soothing, balancing effects. Because of the presence of alcohol, they are effective antimicrobial agents. Medicinally, esters are characterized as antifungal and sedative, with a balancing action on the nervous system. They generally are free from precautions with the exception of methyl salicylate found in birch and wintergreen which is toxic within the system e.g linlyl acetate in bergamot and lavender, geranyl formate in geranium (Abd Elmoez, 2011).

1.2.2.7 Ketones

Are anti-catarrhal, cell proliferant, expectorant, vulnery. Ketones often are found in plants that are used for upper respiratory complaints. They assist the flow of mucus and ease congestion. Essential oils containing ketones are beneficial for promoting wound healing and encouraging the formation of scar tissue. Ketones are usually (not always) very toxic**.** The most toxic ketone is Thujone found in mugwort, sage, tansy, thuja and wormwood oils. Other toxic ketones found in essential oils are pulegone in pennyroyal, and pinocamphone in hyssops. Some non-toxic ketones are jasmone in jasmine oil, fenchone in fennel oil, carvone in spearmint and dill oil and menthone in peppermint oil e.g fenchone in fennel, carvone in spearmint, menthone in peppermint (Abd Elmoez, 2011).

1.2.2.8 Lactones

Are anti-inflammatory, antiphlogistic, expectorant, febrifuge.

Lactones are known to be particularly effective for their anti-inflammatory action, possibly by their role in the reduction of prostaglandin synthesis and expectorant actions. Lactones have an even stronger expectorant action then ketones (Abd Elmoez, 2011).

1.2.3 analysis of essential oils

Most of the methods applied in the analysis of essential oils rely on chromatographic procedures, which enable component separation and identification (Husnu and Gerhard, 2010).

The primary objective in any chromatographic separation is always the complete resolution of the compounds of interest, in the minimum time . to achieve this task the most suitable analytical column (dimension and stationary phase type) are used and adequate chromatographic parameters must be applied to limit peak enlargement phenomen. Chromatography and mass spectrometry are two of the most important techniques for the analysis of essential oils (Husnu and Gerhard, 2010).

In gas chromatographic analysis, the compounds to be analyzed are vaporized and eluted by the mobile gas phase, (the carrier gas, through the column). The analytes are separated on the basis of their relative vapor pressures and affinities for the stationary bed (Husnu and Gerhard, 2010).

Gas chromatography-mass spectrometry (GC-MS) added further improvements to essential oil analysis where broad categories of monoterpenes and varying isomers closely resembling each other in terms of chemical structure could be individually identified. The GC-MS allows recognition of overlapping peaks on a GC and separately identify constituents(Hunter, 2009).

1.2.4 Uses of essential oils

Essential oils are used in perfumery, aromatherapy, cosmetics, incense, medicine, household cleaning products and for flavoring food and drink (Hesham et al., 2016).

the pharmacological of essential oils have been reported as bioactive secondary metabolites. Biological activities, comprising analgesic, antiseptic, sedative, spasmolytic, anesthetic and anti-inflammatory effects have been all reported for essential oils extracted from various plants (Sanaa et al, 2018).

The antibacterial properties of essential oils and their components are exploited in such diverse commercial products as dental root canal sealers, antiseptics and feed supplements for lactating sows and weaned piglets. Afew food preservatives containing, they are already commercially available (Boukhebti et al., 2011).

1.3 Mentha

Mentha is a genus of aromatic perennial herbs belonging to subfamily *Nepetoideae* of the family *Lamiaceae*, distributed mostly in temperate and sub-temperate regions of the world (Hefendehl and Murray, 1972; Nese Okut et al., 2017).

The word “mint” descends from the Latin word menthe, which is rooted in the Greek word minthe, mentioned in Greek mythology as Minthe, a nymph who was transformed into a mint plan (Umberto, 1947).

Species within *Mentha* have a sub cosmopolitan distribution across Europe, Africa, Asia, Australia, and North America. Several mint hybrids commonly occur (Davi dson, 1999).

1.3.1 Classification

Mentha (mint) is a genus of about 25 species (and many hundreds of varieties) of flowering plants in the family *Lamiaceae.* The popular and most common mints for cultivation are *spearmint (M. spicata*) , *peppermint (M. piperita*), and (more recently) *apple mint (M. suaveolens*) (Bohloul et al., 2009).

It is a taxonomically difficult genus because of extensive hybridization, vegetative propagation, polyploidisation and cultivation (Gul et al., 2013). The taxonomy of genus *Mentha* is complicated and within the genus, more than 3000 names have been published from species to formae since 1753 which is the starting date of modern nomenclature (Linnaeus, 1753). The taxonomy of the genus *Mentha* is still not definite but on the basis of a phylogenetic analysis of morphology, chromosome numbers, and major constituents of essential oil. The genus comprises 18 species and 11 hybrids placed into four sections, namely *Pulegium, Tubulosae, Eriodontes* and *Mentha* according to the latest taxonomic treatment (Tucker and Naczi, 2007).

According to the latest taxonomic classification, plants in the genus *Mentha* are divided into four sections (*Tubulosae, Eriodontes, Pulegium, Mentha)* and 18 species on the basis of the number of chromosomes and morphological features (Tucker and Naczi, 2006).

Table 1.1 Infrageneric classification of the *Mentha* plants into the sections *Tubulosae, Eriodontes, Pulegium, Mentha* (Tucker and Naczi, 2006)

Mentha	***, Pulegium***	***Eriodontes***	***Tubulosae***
M.spicata L.	*M.requienii Benth.*	*M.australis*	*M.diemenica Spreng*
M.suaveolens Ehrh.	*M.pulegium L.*	*M.cervina L.*	*M.repens (Hook.f.)Briq.*
M.longifolia (L.)	*M.grandiflora Benth.*	*M.satureoides R.Br.*	
M.japonica (Miq.)Makino		*M.laxiflora Benth.*	
M. dahurica Fisch. ex Benth		*M.gattefossei Maire*	
M.Canadensis L.			
M.arvensis L.			
M.aquatica L.			

Most of the commercially important mints are hybrids or amphiploids, *M.piperita* the *peppermint* is a sterile first generation hybrid between *M.spicata* and *M.aquatica* (Hefendehl and Murray, 1972). The *spearmint M.spicata* is a hybrid of *M.longifolia* and *M.rotundifolia*. Morphological, cytological and biochemical data have shown that the tetraploid species of *M. spicata* (2n=48) originated by chromosomal doubling of hybrids between the two closely related and inter-fertile diploids, *M. longifolia* and *M. suaveolens* (Harley and Brighten, 1977).

Interspecies hybridization exists in the section *Mentha* that includes eight species: *M. suaveolens Ehrh*., *M. longifolia (L.) L., M. spicata L., M. arvensis L., M. canadensis L., M. aquatica L., M. dahurica Fisch*. *ex Benth.* and *M. japonica (Miq.) Makino* (Tucker and Naczi, 2006). The systematics of the latter species is especially difficult because of the ease of hybridization, which is further favoured by polymorphism, polyploidy and vegetative propagation.

Table1. 2 The five species *M. arvensis L., M. aquatica L., M. spicata L., M. longifolia (L.) L.and M. suaveolens Ehrh*. have produced 11 naturally occurring hybrids (Tucker and Naczi, 2006).

Hybrid	Species of originated
M.carinthiaca Host	*M. arvensis L. M. suaveolens Ehrh.*
M.dalmatica Tausch	*M. arvensis L. M. longifolia (L.)*
M.dumetorum Schultes	*M. aquatica L. M. longifolia (L.)*
M.gracilis Sole	*M. arvensis L. M. spicata L.*
M. maximilianea F. W. Schults	*M. aquatica L. M. suaveolens Ehrh.*
M.piperita L.	*M. aquatica L. M. spicata L.*
M.rotundifolia (L.) Huds.	*M. longifolia (L.) M. suaveolens Ehrh.*
M. smithiana R. Graham	*M. aquatica L. M. arvensis L. M. spicata*
M. verticillata L.	*M. aquatica L. M. arvensis L*
M. villosa Huds.	*M. spicata L. M. suaveolens Ehrh.*
M. villoso-nervata Opiz	*M. longifolia (L.) L. M. spicata L*

alternatively mints were classified based on the dominant monoterpene compound prevailing in the essential oil reflected by three metabolic pathways menthol Pathway, carvon Pathway, linalool Pathway (saric-kundalic et al., 2009).

1.3.2 Botanical description of *Mentha*

Mints are aromatic, almost exclusively perennial, rarely annual, herbs. They have wide-spreading underground rhizomes and erect, branched stems, they grow best in wet environments and moist soils. *Mints* will grow 10 - 120 cm tall and can spread over an indeterminate sized area. The leaves are arranged in opposite pairs, from simple oblong to lanceolate, often downy, and with a serrated margin. Leaf colors range from dark green and gray-green to purple, blue, and sometimes pale yellow (Brickell and Zuk, 1997 ; Bohloul et al., 2009). The flowers are produced in clusters ('verticils') on an erect spike, white to purple, the corolla two-lipped with four sub equal lobes, the upper lobe usually the largest. The fruit is a small, dry capsule containing one to four seeds. While Huds. as a synonym of M.suaveolens Ehrh (Hendriks et al., 1976).

1.3.3 Essential oil of *Mentha*

The essential oils of *Mentha* plants are colourless, pale yellow or greenish-yellow liquids consisting mainly of hydrocarbons, alcohols, esters, ketones, ethers and oxides (Appendix 3). Major hydrocarbons found include limonene in *M.arvensis L.* (Lawrence, 2006; Malingre, 1971). The main oils of the *Mentha* plants that have achieved high economic importance are *M. canadensis L., M. piperita L., M. spicata L.* and *M. gracilis sole*. In addition, to a lesser extend the oils of *M. citrata* and *M. pulegium L.* are of commercial importance (Tucker and Naczi, 2006).

large variety of aromatic chemicals in the oil contains , the essential oil composition different greatly in members of the genus are reflected in the number of commercial constituents obtained (menthol, menthone, carvone, limonene, linalool, menthyl acetate, piperitone, and pulegone)

(Savithri et al., 2002; Gracindo et al., 2006).

1.3.4 Uses of *Mentha* spices

The *mint* species have a great importance, both medicinal and commercial. it was originally used as a medicinal herb to treat stomach ache and chest pains, and it is commonly used in the form of tea as a home remedy to help alleviate stomach pain. During the middle Ages, powdered mint leaves were used to whiten teeth. Mint tea is a strong diuretic. It is also aids digestion, in a way that it breaks down the fats. In recent years, it has been often recommended for treating obesity (Quattrocchi, 1974).

In commercial spice mixtures for many foods to offer aroma and flavor, cosmetics and pharmaceuticals (Ludwiczuk et al, 2016). In addition , *Mentha* spp. has been used as a folk remedy for treatment of nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints due to its antinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, and anticatharrhal activities, flatulence, vomiting in pregnancy, jaundice, dental caries, rheumatism, hysteria, infantile troubles, dysmenorrhea, amenorrhea, skin diseases, and fever. In addition, they have been consumed as a vermifuge and antidote (Jagetia and Baliga, 2002; Gursoy et al., 2009; Hajlaoui et al., 2009), antiseptic and emmenagogue effects (Edris et al., 2003).

Mentha can be used for common cold, cough, sinusitis, fever, bronchitis, nausea, vomiting, indigestion, intestinal colic and loss of appetite (Akdogan et al., 2007).

The essential oils of the *Mentha* genus have a high commercial value, because they usually contain the monoterpene menthol in their constitutions, and menthol is widely used in pharmaceutical, cosmetic, personal care and food products(Silva et al., 2015), also they used in dental as toothpastes and mouthwashes , in fragrances and they are used in chewing gums, alcoholic beverages, cosmetics, perfumes (Gracindo et al., 2006, Baytop, 1984). they have strong antioxidant activity and good antimicrobial activity (Mkaddem et al., 2009).

Mints are extensively cultivated for their oils and terpenoid components of the oil, such as menthol, carvone, linalyl acetate and linalool, for use in pharmaceutical, cosmetic, food, flavour, beverage and allied industries (Savithri et al., 2002).

1.4 *Mentha spacta.L* (*viridis* or *spearmint*)

Mentha spicata L commonly called *spearmint* is a perennial herb , creeping rhizomatous, and glabrous. It is well known in Sudan is „Nanaa baladi‟, and in many Arab countries, for its medicinal, aromatic with a strong aromatic odor and flavoring (Salim, 1997; Znini et al., 2011). it has formed from cross breeding of *M. longifolia* and *M. rotundifolia* (Hornok, 1992).

1.4.1 Botanical description of *Mentha spicata*

A perennial herbaceouss rhizomatous with erect square stems and green opposite short stalked and with variably hairless to hairy stems and foliage. Length of the plant is between 15 to 50 cm tall and can reach 90 cm. The leaves are 5 - 9 cm long and 1.5 - 3 cm broad, with a serrated margin.Flowers are borne in whorls in dense terminal spike with a tubular calyx and pale violet bell-shaped (conical) corolla it is in slender spikes, each flower pink or white, 2.5 - 3 mm long and broad. This species is widely cultivated as well as grown naturally, and a wide-spreading fleshy underground rhizome (Blamey and GreyWilson, 1989; Adel Nadjib et al., 2011).

1.4.2 Scientific classification of *Mentha virids*

Table.3 The scientific classification of *M.spicata*

(Chawla & Thakur, 2013)

Kingdom	Plantae
Subkingdom	*Tracheobionta*
Superdivision	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Asteridae*
Order	*Lamiales*
Family	*Lamiaceae*
Genus	*Mentha*
Species	*Spicata*

1.4.3 Geographical distribution

Mentha spicata (*Spear mint or spearmint*) is a species of mint native to much of Europe and southwest Asia, though it's exact natural range is uncertain due to extensive early cultivation, It grows in wet soils (Huxley, 1992), it is also native to North Africa, Egypt and Morocco. it was first sighted in 1843 (http://www.mountainroseherbs.com/spearmint.php, 2010). *spearmint* cultivation is localized in Indiana and Michigan. Spearmint is also cultivated in France, the United Kingdom, Italy, Yugoslavia, Hungary, Bulgaria, Russia, South Africa, Thailand and Vietnam (Savithri et al., 2002).

1.4.4 Chemical composition of *Mentha spicata* and oil of its

M. spicata contains about 0.21–2.1% volatile oil, 29–74% carvone, 4–24% limonene and 3–18% cireole. (Akdogan et al., 2004; Baser, 1993) The oil contain oxygenated monoterpenes, monoterpene hydrocarbons and sesquiterpene hydrocarbons (Mejdi et al., 2015).

M. spicata essential oil contains monoterpenoids like carvone, limonene, menthone, menthol, pulegone, and dihydrocarveol. Some of them were found to possess high antioxidant activity (Elmasta and other, 2006). which are extensively exploited for their biological properties (Ringer et al., 2005).

M.viridis (*M.spicata*) is rich in carvone, the main components are carvone and limonene of essential oil as reported (Znini, 2011, Osman, 2017, Ojewumi, 2018, Mejdi et al., 2015).

The *spearmint* extract is a matrix rich in phenolics. The major phenolic compounds in the spearmint extract were represented by rosmarinic acid and its derivatives (Martina et al., 2016) reported that the extracts of *Mentha* species contained bound phenolic acids and flavonoids. The major phenolic acids reported in water-soluble *Mentha spicata* extract are eriocitrin, luteolin glucoside, rosmarinic acid and caffeic acid (Dorman et al., 2003).

Forty-nine components representing 99.18% of the total *M. viridis* essential oil were identified and characterized mainly by carvone (50.47%), followed by 1,8-cineole (9.14%), limonene (4.87%), camphor (3.68%), and β-caryophyllene (3%). Oxygenated monoterpenes corresponded to the main fraction (77.22%) of the total essential oil (Mkaddem, 2009).

the variations in the chemical composition of *M. spicata* oils produced from plants grown in different countries might be attributed to the varied agroclimatic (climatical, seasonal, geographical) conditions of the regions, stage of maturity, and adaptive metabolism of plants (Abdullah et al., 2010).

1.4.5. *spearmint* oil constituents

Carvone is a major and the most abundant component of *spearmint* oil (Znini et al., 2011, Ojewumi et al., 2018), the family of carvone is terpenoids, classification of its ketone ,it is preferred IUPAC name is 2-Methyl-5-(prop-1-enn-2-yl) cyclohex-2-en-1-one .Chemical Formula $C_{10}H_{18}O$,it is colourless to pale yellow liquid,and B.P. 231°C.(Hunter, 2009; Ojewumi et al., 2018).

The percentage of carvone also varies in the essential oil of *spearmint* growing in different countries, e.g., Egypt (46.4%–68.55%) (Elmasta et al., 2006; Foda et al., 2010), Canada (59%–74%) (Zheljazkov et al., 2010) olombia (61.53%) (Roldán et al., 2010), Turkey (78.35%–82.2%) (Telci et al, 2004 , Telci et al 2005), China (55.45%–74.6%. (Hua et al., 2011), Bangladesh (73.2%) (Chowdhury et al., 2007), lgeria (59.4%) (Boukhebti et al., 2011) and Morocco (29%) (Znini et al., 2011) Lower amounts of carvone were reported in the spearmint essential oil from Iran (22.4%) (Hadjiakhoondi et al., 2000).

1.4.6.Traditional uses

Mentha spicata is used with spices to give the food a special flavor and fragrance. It is also used for flavoring chewing gums, toothpaste, confectionery and pharmaceutical preparations (Saleem et al., 2000).

Traditionally, *M. spicata* has been utilized in the foods as a flavoring agent and as an herbal medicine in folk remedies (Asekun et al., 2006), In countries of the Middle East and Africa, the dry or fresh leaves of spearmint are added during the brewing of tea, where it provides a pleasant aroma and refreshing taste (Fabre, 2003). *spearmint* has a long tradition medicinal use. It was taken as a tea to treat general digestive problems. it is widely used in commercially manufactured product (http://www.mountainroseherbs.com/spearmint.php, 2010).

1.4.7 Pharmacology studies and medicinal profile

spearmint essential oil is a common constituent in hygiene and cosmetic products, and substantial amounts are used in the food and beverage industries (Spirling and Daniels, 2001).

In women with hirsutism, drinking one cup of spearmint tea daily for 5 d ays was associated with a decrease in free testosterone and an increase in luteinizing hormone, folliclestimulating hormone, and estradiol (Akdogan et al., 2007).

M. spicata has become a subject of scientific interest in view of other potential uses of its essential oil and extracts, for the most part, as antimicrobial and antioxidant agents (Mata, 2007) .it possesses several biological activities and is used in folkloric medicine as a carminative, antispasmodic, diuretic, antibacterial, antifungal, and antioxidant agent, and for treatment of colds and flu, respiratory tract problems, gastralgia, hemorrhoids, and stomach ache (Mejdi et al., 2015), which are extensively exploited for their biological properties (Ringer et al., 2005).

1.4.7.1Antioxidant activity

Mentha spicata belongs to the family *Lamiaceae* (*Labiatae)* is a rich source of polyphenolic compounds and hence could possess strong antioxidant properties. (Sweetie et al, 2005). Polar extracts of spearmint leaves characterised mainly by a high content of phenolic compounds such as rosmarinic acid, luteolin, and apigenin derivatives. Some of these components have been shown to have antioxidant properties; therefore, Mentha spicata could also be considered an antioxidant source (Dorman, 2003).

M.spicata essential oil contains monoterpenoids like carvone, limonene, menthone, menthol, pulegone, and dihydrocarveol. Some of them were found to possess high antioxidant activity (Elmasta et al., 2006).

Mentha extract has been found to have antioxidant and antiperoxidant properties due to the presence of eugenol, caffeic acid, rosmarinic acid and α-tocopherol (Ponnan et al., 2006) and it has been found to have antioxidant and antiperoxidant properties due to the presence of eugenol, caffeic acid, rosmarinic acid and a-tocopherol and it could enhance error-free repair for DNA damage and hence could be antimutagenic(naser et al., 2011).

1.4.7.2Antimicrobial activity

Essential oil of *M. spicata* has also good antibacterial activity against common foodrelated pathogenic bacteria such as *Salmonella enteritidis, Escherichia coli* O157:H7, *Bacillus cereus, Bacillus subtilis, L. monocytogenes,* and *Staphylococcus aureus* (Shahbazi, 2015).

The essential oil of *M. spicata* showed strong insecticidal and mutagenic activity (Franzios et al., 1997). it have been reported by various researchers as a good mosquito repellant and possess antimicrobial activit (Ojewumi et al., 2017).

extensively applied to treatment of various diseases such as nausea, vomiting, and gastrointestinal disorders and also as breath freshener, antiseptic mouth rinse, and toothpaste (Kumar et al., 2011; Tyagi and Malik, 2011) .

The boiled leaves extract has an anti-infectious antiflatulence effect and antiinflammatory action (notably of the digestive system), it was counseled in the viral hepatitis and colitis, gastric acidities, aerophagia, to stimulate the digestion; furthermore, it presents some invigorating and stimulating qualities (Mkaddem, 2009), fresh and dried leaves of *Mentha spicata* is widely used as a stomach pain-relieving agent, antispasmodic, digestive and carminative.

1.4.8 Toxicity Studies

Acute Toxicity The LD50 of orally administered *spearmint* essential oil in rats is 5000 mg/kg . In rats administered spearmint tea for 30 days as the sole source of drinking water, some histopathological changes were observed in the kidneys . Similarly, in female rats administered *spearmint* tea for 30 days as the sole source of drinking water, some histopathological changes were observed in uterine tissues. In rats given spearmint tea ad libitum for 30 days, liver enzyme changes were observed. Decreases in superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) and an increase in thiobarbituric acid-reactive substance (TBARS) were noted, it is showed some mutagenic activity in Drosophila melanogaster (Zoe and Michael, 2013).

Objective

Main objective

It is to investigate the chemical composition and biological properties of *Mentha spicata* essential oil.

Specific objectives

They are:

- To extract the oil from dried leaves of *Mentha spicata (viridis)* .
- To determined the chemical composition of oil extract.
- To investigate the antioxidant and antimicrobial properties of *M.spicata* oil.

Chapter two

Materials and methods

Chapter two

Materials and methods

2.1Materils

- Sample collection and preparation

Mentha viridis plant was purchased from market (Khartoum, Sudan), in october 2019. The plant was authenticated by Medicinal and Aromatic Plants and Traditional Medicine Research Institute (National Center for Research, Khartoum-Sudan). The leaves were separated, washed under tap water repeatedly until clean, dried in the shade at room temperature, and pulverized and stored prior to extraction.

- Distilled water.
- Bacterial microorganisms:

Bacillus subtilis	NCTC 8236 (Gram + ve bacteria)
Staphylococcus aureus	ATCC 25923(Gram +ve Bacteria)
Escherichia coli	ATCC 25922(Gram -ve bacteria**)**
Pseudomonas aeruginosa	ATCC 27853 (Gram -ve bacteria)

National Collection of Type Culture (NCTC), Colindale, England.

American Type Culture Collection (ATCC) Rockville, Maryland, USA.

- Fungal microorganisms:

Candida albicans	ATCC7596

2.2 Methods

2.2.1 Extraction *Mentha spicata* volatile oil

325g of sample were placed in 1000 ml rounded bottom capacity flask. 1000 ml of distilled water was added and the Clevenger receiver (Duran West Germany) and condenser attached to the top of the flask . System was heated at 100 C for about four hours.Oil was above water layer pipetted , dried over anhydrous sodium sulphate and stored in a dark container in refrigerator till used (Sukhdev et al., 2008).

2.2.2 Gas chromatography-mass spectrometry(GC- MS) analysis

The qualitative and quantitative analysis of the sample was carried by using GC\MS technique model (GC\MS – QP2010- Ultra Japans) Shimadzu Company, serial number 020525101565SA and capillary column (Rtx-5ms-30m * 0.25 mm *0.25um).The sample was injected using split mode , instrument operating in EI mode at 70 e V . Helium as the carrier gas passed with flow rate 1.69 ml\min, the temperature program was started from 50 C with rate 7C\min to 180 C then the rate was changed to 10C\min reaching 280 C as final temperature degree, the injection port temperature was 300 C, the ion source temperature was 200 C and the interface temperature was 250 C. The sample was analyzed by using scan mode in the range of m\z 40 -500 charges to ratio and the total run time was 28 minutes. Identification of components for the sample was achieved by comparing their retention index and mass fragmentation patents with those available in the library, the national Institute of Standards and Technology (NIST).

2.2.3Antioxidant Activity

The antioxidant activity of the essential oil based on the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl free radical was determined according to the method of Shimada et al(1992), with some modification .In 96- wells plate , the test samples were allowed to react with 2.2Di (4- tert-octylphenyl)-1-picryl-hydrazyl stable free (DPPH) for half an hour at 37 C .The concentration of DPPH was kept as (300 u M). The test sample were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group .All test and analysis were run in triplicate.

2.2.4 Antimicrobial Activity of the oil

2.2.4.1 Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37° C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used.

The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique (Miles and Misra, 1938). Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.2.4.2 Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100ml of sterile normal saline, and the suspension were stored in the refrigerator until used.

2.2.4.3 Testing of antibacterial susceptibility

2.2.4.3.1 Disc diffusion method

Paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extracts.The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

Chapter three

Results and discussion

Chapter three

Results and discussion

3.1 The preparation of the extract (oil)

The essential oil was extracted by the hydrodistillation of the dried leaves of *M. spicata* collected in Khartoum region, the yield found is 1.23% v/w, with clear, a pale-yellow color and persistent aromatic-spicy odor.

Yield percentage of oil extract = volume of oil \ weight of plant sample *100

= 4 \ 325 *100 = 1.23%.

The extracted yield of essential oil of the dried leaves of *M. viridis* was 1.23%. This study's results are in close agreement with the findings of Sanaa et al. (2018) who reported the yield of oil from the Laghouat region (Algeria) of *M. spicata* was 1.04%.

Isa Telci et al., 2004 from turkey investigated the yield of essential oils contents (14 samples) ranged from 1.15% to 2.00, also In other studies Silva L.F et al., 2015 from state of Minas Gerais (Brazil) found the oil content 1.52% and Kokkini and D.Vokou (1989) who reported the oil content of *M. spicata*, from the 40 samples of northern Greece examined, ranged from 0.3% to 2.2%.

3.2 Chemical composition of essential oil

Essential oils composition was determined by gas chromatography coupled to mass spectrometry (GC–MS) analysis.

The results for the chemical composition of *M. spicata* essential oil are presented in Table 3.1 Using GC/MS, 28 compounds, representing 100.00% of the oil, were identified. The major constituents of the oil were were D-carvone (57.20%), other components present in appreciable contents were: D-Limonene (19.41%) , 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis- (5.20%) , Eucalyptol (4.37%) ,and Cyclohexanone, 2-methyl-5-(1-methylethenyl)- (2.62%) beta.-Myrcene (1.83%), %), (-)-8-p-Menthen-2-yl, acetate, trans(1.41%) Caryophyllene (1.35%).

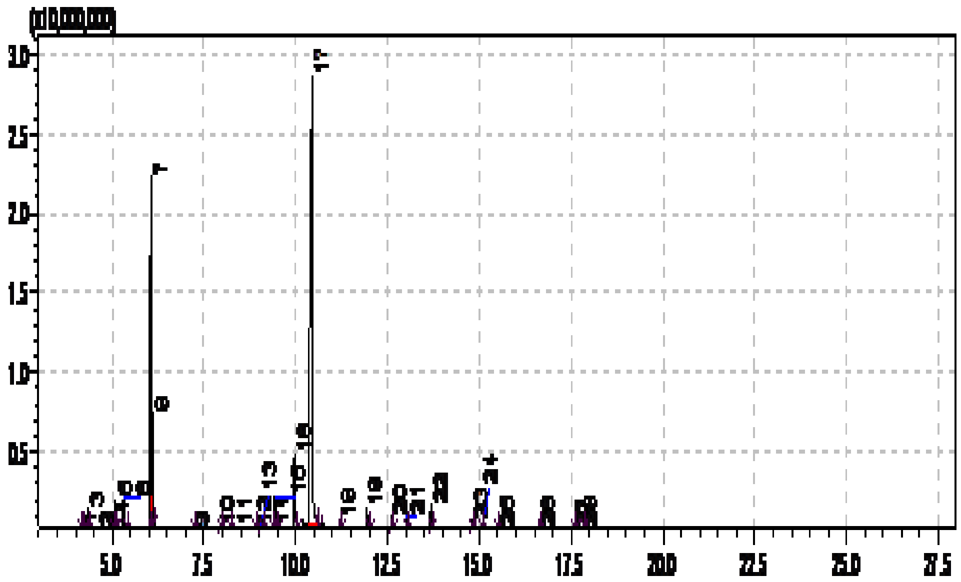


Figure 3.1 : GC-MS spectrum of Total compounds in essential oil from dried leaves of *Mentha spicata*

Table 3.1 shows the chemical composition of the essential oil from dried leaves of *M. spicata*

ID	Name	Ret.Time	Area	Area%
	.beta.-Pinene	4.120	185028	0.07
	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	4.234	43317	0.02
	.alpha.-Pinene	4.355	2169017	0.88
	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	5.029	1397261	0.57
	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	5.095	3108858	1.26
	.beta.-Myrcene	**5.311**	**4525280**	**1.83**
	D-Limonene	**6.047**	**47974948**	**19.41**
	Eucalyptol	**6.100**	**10811767**	**4.37**
	2-Carene	7.210	148379	0.06
	trans-p-Mentha-2,8-dienol	7.917	170343	0.07
	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	8.216	292246	0.12
	L-.alpha.-Terpineol	8.856	346075	0.14
	Terpinen-4-ol	9.040	1022692	0.41
	.alpha.-Terpineol	9.338	428027	0.17
	Cyclohexanone, 2-methyl-5-(1-methylethenyl)-	**9.430**	**6481485**	**2.62**
	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	**9.958**	**12846958**	**5.20**
	D-Carvone	**10.453**	**141354529**	**57.20**

ID	Name	Ret.Time	Area	Area%
	2H-1-Benzopyran, 3,4,4a,5,6,8a-hexahydro-2,5,5,8a-tetramethyl-, (2.alpha.,4a.alpha.,8a.alpha.)-	11.198	993390	0.40
	(-)-8-p-Menthen-2-yl, acetate, trans	**11.932**	**3475420**	**1.41**
	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, acetate	12.597	2141363	0.87
	(-)-.beta.-Bourbonene	13.027	1597025	0.65
	Caryophyllene	**13.678**	**3337402**	**1.35**
	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E)]-	14.787	1316213	0.53
	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	15.063	217372	0.09
	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	15.498	128458	0.05
	Caryophyllene oxide	16.597	293534	0.12
	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1R-(1.alpha.,4.beta.,4a.beta.,8a.beta.)]-	17.560	175832	0.07
	.alpha.-Cadinol	17.781	158833	0.06

This results are similar with those of(Osman et al., 2017) who investigated the of essential oil from Al-kadaro region (Khartoum, Sudan) the major components of *M. viridis L.* oil, which found in extracted sample were D-Carvone (64.63%), D-Limonene (12.27%), (-)-8-p-Menthen- 2-yl, acetate, trans (2.59%), Cyclohexanol, 2- methyl - 5-(1 methylethenyl) (2.36%), Eucalyptol (2.28%), 3-Hexadecyne (1.82%), Caryophyllene (1.72%), Beta– myrcene (1.43%), Trans-Carveyl acetate (1.37%), (-). Beta– Bourbonene (1.08%) are shown in Table 1 and 2 (Osman et al., 2017). also this study agree with those of Suleyman et al., 2010 who reported *M. spicata* oil is rich in L-carvone. Major components of oil were determined as D-limonene (16.47%), 4-terpineol (3.78%), L-carvone (50.33) however 4-terpineol (0.41%).

In other studies (Habiba et al., 2011), carvone (59.40 %) %) is also the major constituent, followed by limonène (6.12%), 1,8-cinéol (3.8%), germacrène-D (4.66%), of the extractable essential oils from this plant. In contrast, in studied *M. spicata* essential oil, 1,8-cinéol (3.8%), germacrène-D (4.66%) is absent.

A Tunisian study show that chemical composition of *M. spicata* essential oil harvested from the Soliman Tunisian locality Mejdi et al., 2015 reported the main constituents were carvone (40.8% ± 1.23%) and limonene (20.8% ± 1.12%) and from Gabes (Mkaddem et al., 2009 reported that the main components were identified as carvone (50.47%).

M. viridis (*M.spicata*) is rich in carvone , the main components are carvone and limonene of *Mentha spicata L*,essential oil as reported by other studies (Znini, 2011; Mejdi et al., 2015; Osman et al., 2017 ; Ojewumi, 2018).

in agreement with other authors, such as (Abdullah et al., 2010) reported carvones (51.70%) as the main components of the essential oil collected from Pakistan.

The yield variability and Variations in the chemical compositions and total numder of its in the essential oil of *Mentha viridis* are explained by the variation in location, genotype differences, time of collection. climatic factors for example temperature moisture, also duration of sunshine, air movement and rainfall.

3.3 Antimicrobial activity

The essential oil from *M. viridis* leaves was screened for antimicrobial activity against four bacterial species: Gram-positive bacteria, *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923), Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and fungal strains , *Candida albicans* (ATCC 7596) using the agar plate diffusion method.

Table 3.2 : Antimicrobial activity of oil *M. viridis* against the standard bacteria and fungi.

Conc. used	Standard tested organisms\ M.D.I.Z(mm)				
	B.s	*S.a*	*E.c*	*Ps.a*	*C.a*
100	10	13	10	-	-

B.s = Bacillus subtilis , S.a = staphylococcus aureus , E.c = Escherichia coli ,

Ps.a = Pseudomonas aeruginosa , C.a = Candida albicans

M.D.I.Z = Mean diameter of growth inhibition zone in (mm)

Key: Interpretation of results:

< 9 mm, inactive; 9-12 mm, partially active; 13-18 mm, active;

>18 mm, very active. Concentration used 100 mg/ml at 0.1ml/cup.

The essential oil of *M. viridis* leaves family (*Lamiaceae*) was screened for antimicrobial activity against four bacterial species: Gram-positive bacteria, *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923), Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and fungal strains, *Candida albicans* (ATCC 7596) using the agar plate diffusion method.

The results showed that the oil is in-active toward *Pseudomonas aeruginosa* . *E.coli* and *Bacillus subtilis* partially active and active against *staphylococcus aureus* . The Activity value of *staphylococcus aureus* is (13) and activity value of *E.coli* is equal to the activity of the *Bacillus subtilis* is (10) but in the case of *Pseudomonas aeruginosa* is in-activite. Antimicrobial activity of the one species of fungal microorganism (*candida albicans*) showed that the oil was in-active.

This result agreed with Osman et al., (2017) who stated that spearmint oil exhibited considerable inhibition capacity against staphylococcus aureus, E.coli and Bacillus subtilis and disagree toward Pseudomonas aeruginosa and candida albicans, also our results are in good agreement with the findings of Habiba et al., (2011) who reported that Gram-positive bacteria are more sensitive to plant essential oils than Gram negative bacteria.

reports from literature on the association between the essential oil phytochemical composition and its antimicrobial activity are found to be in great consistency with the findings of the present study. In particular, oxygenated monoterpenes were evidently reported as potent antimicrobial agent in the composition of several essential oils In addition, 1,8-cineole and sesquiterpenes were shown to exhibit considerable antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria (Sanaa et al., 2018), as will as Cis carveol and carvone, major components of the oil of M. spicata, also exhibited good antibacterial activity (Abdullah et al., 2010).

3.4 Antioxidant activity

The antioxidant activities of essential oil dried leaves of *M. spicata* were determined by DPPH. The results are summarized in Table 3.3 In DPPH, the assessed sample was able to reduce the stable violet DPPH radical to the yellow.

The DPPH (2,2-diphenyl-1-picril-hydrazyl) method is the most used in the quantification of free radical scavenging activity. The reaction is based on the decrease of the purple color that occurs when the nitrogen atom ofthe DPPH is reduced by receiving a hydrogen atom from the antioxidant component (Brand-Williams et al., 1995).

Table 3.3 : Antioxidant Activity of dried leaves of *Mentha spicata*

No.	Sample code	% RSA + SD (DPPH)
1	Oil of *Mentha spicata*	86 +0.07
Standard	Propyl gallate	92+0.01

The essential oil from *M. viridis* shows stronger antioxidant activity antioxidant activity by the DPPH method .The Percentage radical scavenging activity by the oil was found (86%) and for propyle gallate as standard was (92%).

In this study the results are in agreement with Scherer et al., (2013) who reported that methanolic extract showed stronger antioxidant activity by same method but determined antioxidant activity index (AAI).also Mahsan Bayani et al.(2017) repoted *Mentha spicata* as a species with high antioxidant activity in diabetic rats.

The essential oil shows strong antioxidant might be the amounts of phenolic compounds in sample was high.this agreement with (Mimica et al., 1998).who reported The highest antioxidant properties of essential oils might be related to its phenolic contents like phenolic acids, rosmarinic acid and polyphenols as reported in a previous study (Mimica-Dukic et al., 1998). Polar extracts of *spearmint* leaves characterised mainly by a high content of phenolic compounds such as rosmarinic acid, luteolin, and apigenin derivatives. Some of these components have been shown to have antioxidant properties; therefore, *Mentha spicata* could also be considered an antioxidant source (Dorman, 2003).

The essential oil shows strong antioxidant might be test of antioxidant was fallowing after extraction and good storage.

Conclusion

- Hydrodistilation is the best method for extraction essential of *Mentha spicata* (*viridis) oil*.
- The yield percentage is low; it is (1.23%).
- GC-MS analysis, 28 components were identified, accounting for 100.00% of the essential oil were identified.
- Main constituents of the oil were D-carvone (57.20%) and D-Limonene (19.41%), other components present in appreciable contents were, 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis- (5.20%), Eucalyptol (4.37%), and Cyclohexanone, 2-methyl-5-(1-methylethenyl)- (2.62%) beta.-Myrcene(1.83%), (-)-8-p-Menthen-2-yl,acetate, trans(1.41%) Caryophyllene (1.35%).
- The antibacterial activity of oil exhibited moderate level against all tested microorganisms.
- In general, Gram-positive bacteria were more susceptible to *M. spicata* essential oil than Gram-negative bacteria.
- *staphylococcus aureus* was the most sensitive of the microorganisms to the antibacterial activity of *M. spicata* oil (inhibition zone = 13 mm).
- the antioxidant activity of oil is high compared to Propyl gallate (as standard); the results of RSA% for oil of *Mentha spicata* was 86% and for propyl gallate was 92%.

Recommendation

It is recommended that, Further studies on the essential oils in order to know the physical and chemical properties , in order to take advantage of the chemical components used in the medical and industrial field application.

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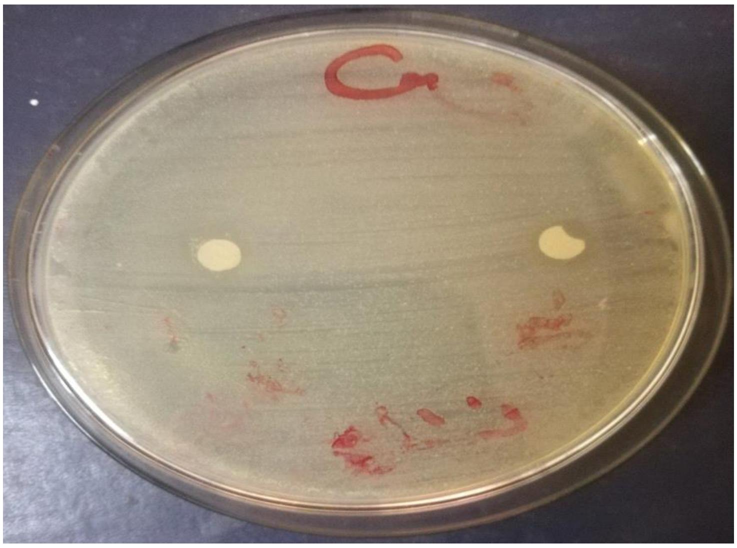
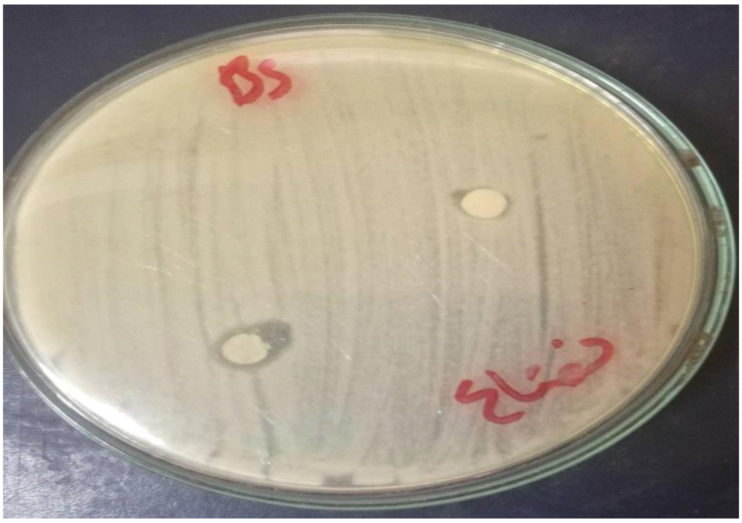
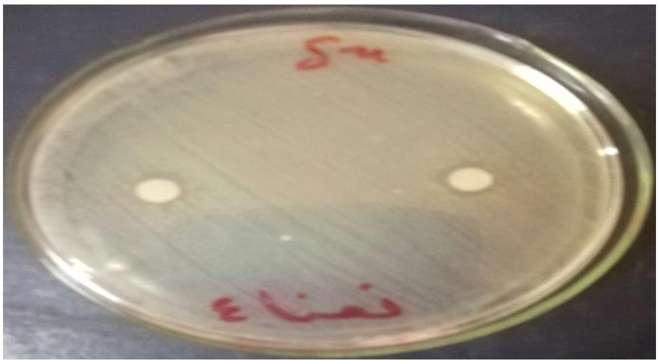
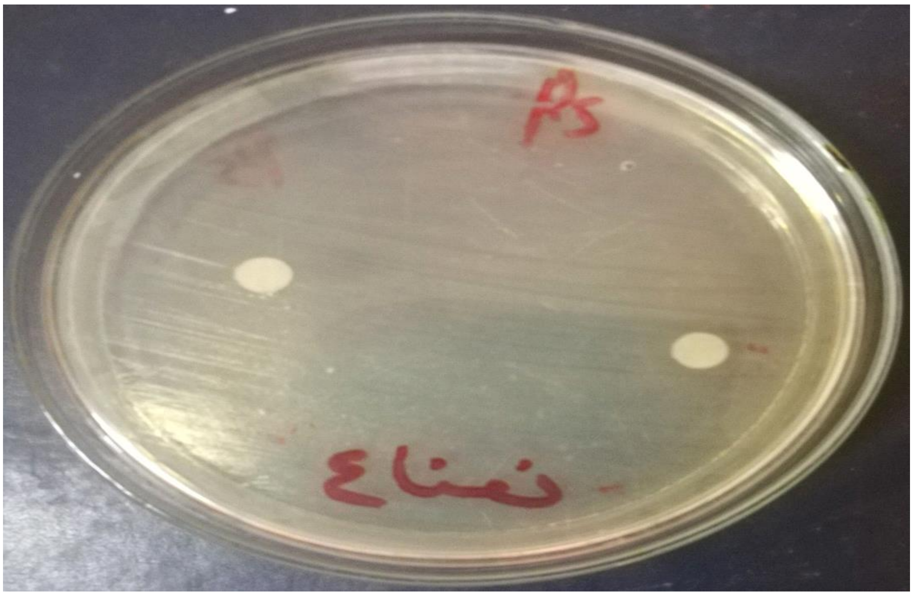
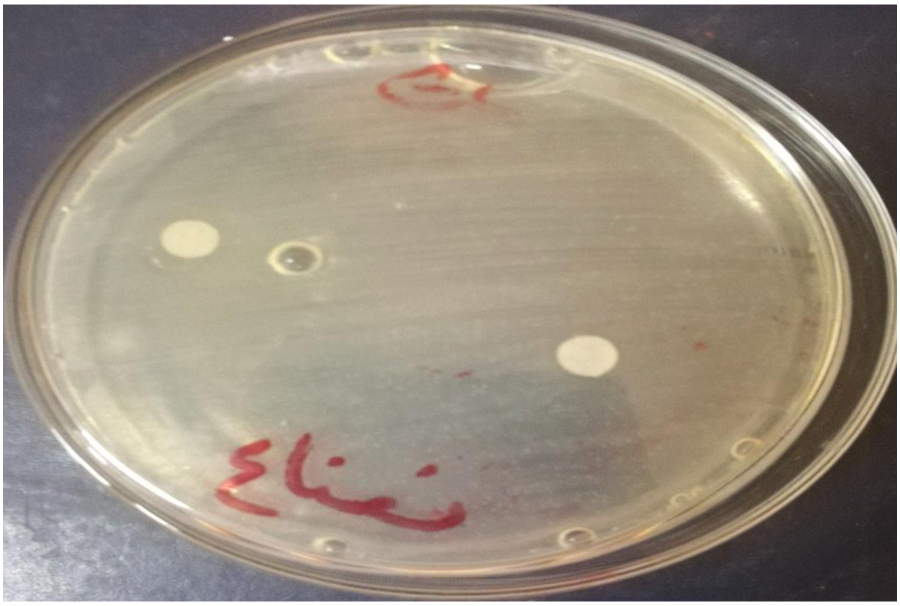
Appendix





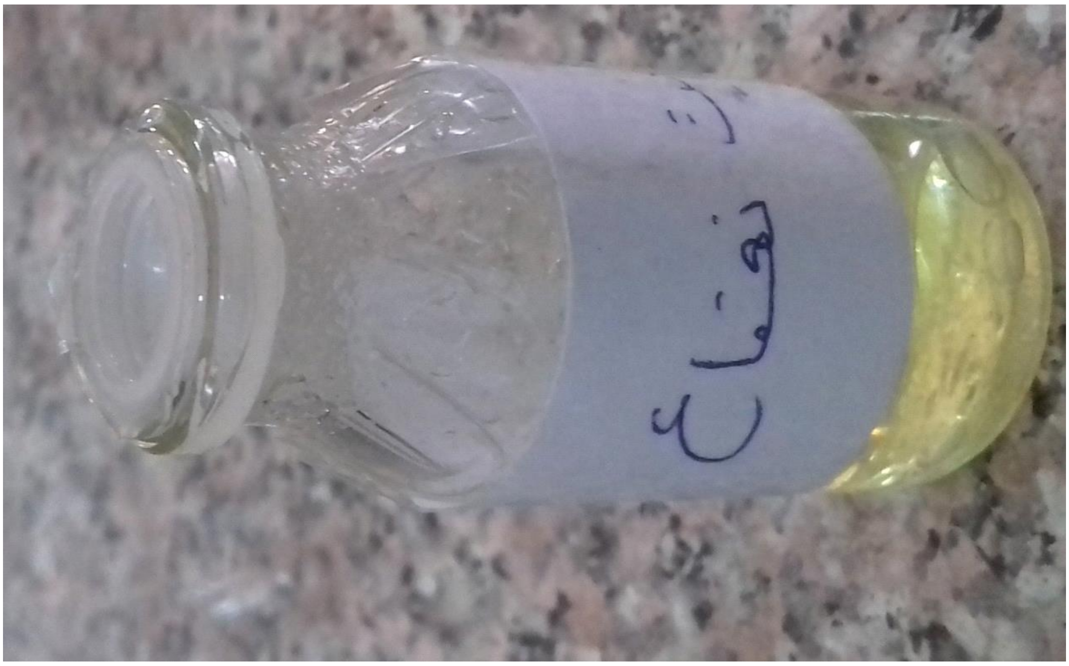






Test microorganisms

***Mentha spicata* plant**



Essential oil of *Mentha spicata*(spearment)