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Phytochemical Screening ,Characterization and Antimicrobial Activity of *Foeniculum Vulgare Mill(Fennel)* Seeds And Its Volatile oil

المسح الكيميائي والتشخيص والنشاط البكتيري لبذور الشمر وزيته الطيار

A Thesis Submitted in Partial fulfillment for the Requirements of Master Degree in Chemistry

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وَهُوَ ٱلَّذِى أَنزَلَ مِنَ ٱلسَّمَاءِمَاءَ فَأَخْرَجْنَا بِهِ مَبَاتَ كُلِ شَىءٍ فَأَخْرَجْنَا مِنْهُ خَضِرًا نَخْرَجُ مِنْهُ حَبَّامٌ تَرَاحِبًا وَمِنَ ٱلنَّخْلِمِن طَلْعِهَا قِنْوَانُ دَانِيَةٌ وَجَنَّتِ مِنْ أَعْنَابِ وَٱلزَّيْتُون وَٱلرُّمَّانَ مُشْتَبِهَا وَغَيْرَ مُتَشَبِهِ الْنَصْرُوَا إِلَى تَمَرِهِ عَإِذَا أَنْمَرَ وَيَنْعِهِ عَالَهُ فَا نَعْذَالِ حُمْر لَاَيَتِ لِقَوْمِ يُؤْمِنُونَ شَ

Dedication

I dedicate this work to,

My beloved parents,

Family and

Teachers

Acknowledgments

First and foremost, praises and thanks to Allah for given me health and patience throughout my research work to complete the research successfully. I would like to express my deep and sincere gratitude to my research supervisor Dr/ Mohammed Sulieman Ali for giving me the opportunity to do this research, for his guidance, patience and support .I am very thankful to technical staff of Chemistry labs at Sudan University of Science and Technology for their great assistance .I would like to thank the technicians at National Research Centre for their help to extract the Volatile oil of *Foeniculum vulgare mill* . Finally I am extremely grateful to my parents for their love ,prayers ,caring ,support and encourage me during the study.

Abstract

The Foeniculm vulgare mill belongs to the family Apiaceae known as fennel plant, its widespread annual or perennial plant. fennel is well known to ancient Indians, Chinese, Egyptians and Romans. In this study, Essential oil of fennel seeds was isolated using soxhlet and hydrodistillation methods. From the determined oil content it was found that soxhlet method is better than the hydrodistillation in extraction of the volatile oil. The result showed that the highest yield of essential oil by using soxhlet extraction(12%) than hydrodistillation extraction (0.75). The extracted oil was subjected to analysis using GC-MS techniques, the results reveal that the oil contain 15 components and the volatile oil rich in D-Carvone(66.33%) and D-Limonene(31.93%). The seeds was extracted using n-hexane, Chloroform, Ethyl acetate ,Methanol, and water as solvents ,then each extract was phytochemicalty screened which indicated that the extracts were rich in alkaloids ,saponins, tannins, steroids, reducing sugar glycosides and flavonoids, phytochemical constituents were found more in methanolic extract than other extracts. Also the extract was examined to some microbe such Bacillus subtilis. *Staphylococcus* Candida as aureus. albicans, Escherichia coli, pseudomonas aeruginosa using Disc diffusion method, the highest inhibition zone was found against *Pseudomonas*

المستخلص

الشمر نبات ينتمي إلى عائلة الخيمية المعروفة باسم نبات الفينل ،نباتها السنوي او المعمر واسع الانتشار ومعروف قديما لدى الهنود والصينيين والمصريين والرومانيين . في هذة الدراسة تم عزل الزيت العطري لبذور الشمر عن طريق السوكسليت و التقطير المائي. وعلى حسب محتوى الزيت وجد أن السوكسليت أفضل طريقة التقطير المائي لاستخلاص الزيت الطيار ،أظهرت النتائج أن أعلى إنتاجية للزيت باستخدام استخلاص السوكسيت (12%) من التقطير المائى (0,75%). كما خضع الزيت المستخلص للتحليل باستخدام تقنيات GC-MS . أظهرت النتائج 15 مكونا وأن الزيت الطيار غني بالكارفون بنسبة (66,33%)و الليمونين (33,93%)، استخلصت البذور باستخدام الهكسان، الكلوروفورم، ،خلات الايثيل، الميثانول و الماء كمذيبات ثم اختبر كل مستخلص لاختبار المسح الكيميائي وأشير الفحص أن المستخلصات غنية بالقلويدات، الصابونين ، التانينات ، الاستيرويدات ،الجليكوسيدات ،السكريات المختزلة والفلافونيدات ،كذلك وجد أن المكونات في المستخلص الميثانولي أكثر من المستخلصات الأخرى. وأيضا عرض المستخلص على بعض الميكروبات مثل العصية الرقيقة،المكورات العنقودية الذهبية المبيضات البيضاء الإشريكية القولونية والزائفة الزنجارية باستخدام طريقة انتشار القرص وأظهرت النتيجة أن أعلى منطقة تثبيط وجدت ضد الزائفة الزنجارية.

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1-Introduction and literature

1.1 Natural products

Natural products are chemical compounds or substances isolated from living organism (Bhat et al., 2005). Natural products can be prepared by chemical synthesis and have played a central role in the development of the field of organic chemistry ,the definition of natural products within the field of organic chemistry is usually restricted to organic compounds isolated from natural sources that are produced by the pathways of primary or secondary metabolism (Hanson JR 2003), but within the field of medicinal chemistry is secondary metabolites restricted to (Lippincott Williams al., et 2016; Williams DA 2002). Naural products are divided into primary and secondary metabolites (Kliebenstein 2004;Karlovsky 2008), A primary metabiltes is directly involved in normal Growth, development and reproduction of an organism, also its essential to the survival of the organism that produces them, Example of primary metabiltes such as carbohydrate, protein, fat and oil, alcohol e.t.c. Secondary metabolites are not directly involved in growth, development and reproduction of an organism, and also are not essential for survival but they have an ecological function. Plant secondary metabolite can be found in the leaves, stem, root or the bark of the plant depending on the type of secondary metabolite that is been produced (Hill 1952). The most bioactive secondary metabolite are the Alkaloids, Tannins, Flavonoids and Phenolic compounds (Hill 1952). Secondary metabolites differ from primary metabolite in having a restricted distribution in the plant kingdom. That is, particular secondary metabolite are found in only one plant species or related group of species, where as primary metabolites are found throughout the plant kingdom. For many years these compounds were thought to be simply functionless end products of metabolism, or metabolic wastes. studies of these substances was pioneered by organic chemist of the nineteenth and early twentieth centuries who were

interested in these substances because of their importance as medicinal drugs,poison,flavor and industrial material(Taiz et al 2005).

Plant secondary metabolites are generic term used for more than 30,000 different substances which are exclusively produced by plants .the importance of these substances has only recently been discovered by scientists. secondary metabolite carry out a number of protective functions in the human body, it can boost the immune system, protect the body from free radicals, kill pathogenic germs and much more keep the body fit .

Plants secondary metabolites can be divided into three chemically distinctive groups namely

- Trepenes
- Phenolic compounds
- Nitrogen containing compounds

i)**Trepenes** are the largest class of secondary product. They are also called terpenoids.Terpenes are volatile organic compounds made up of union of 5 carbon atoms known as isoprene(C5H8 units), .the basic structural element of terpene are sometimes called isoprene unit because terpene decompose at high temperature to give isoprene.terpene are toxic and feeding deterrents to many plants feeding insect and mammals. Thus they appear to have important defensive role in the plant kingdom(Gershenzon 2012).

Terpenoids give plants and flowers their fragrance, they occur widely in the leaves and fruits of higher plant, conifers, citrus and eucalyptus. They are classified into monoterpenes(C10), sesquiterpenes(C15), and diterpenes(C20), The simpler mono and sesqui terpenes are chief constituent of the essential oils obtained from sap and tissues of certain plant and trees, terpenes give essential oils their aromas ,flavors, and medicinal benefits. The di and tri terpenoids are not steam volatile , they are obtained from plant and tree gums and resins. Tertraterpenoids are obtained from a separate group of compounds called "Carotenoids". Monoterpenes are divided into three divisions: acyclic, such as citral, geraniol, monocyclic terpenes such as limonene, pulegone, bicyclic, such as alpha-pinenes and camphene. The bicyclic monoterpenes are divided into three sections depending on the size of the second ring (R.Croteau 1998)Regarding to their chemotherapeutic activities the most investigated monoterpenes are limonene, carvone, citral, pulegone, linalylacetate, linalool, geraniol, alpha-pinene and camphene(K.Wagner 2003).







Alpha pinene

carvone

limonene











Figure (1.1) The structure of the most investigated monoterpenes

Most natural terpenoid hydrocarbon have the general formula (C5H8)n. They can be classified on the basis of value of n or number of carbon atoms present in the structure (Saneena Bano 2007).

No	Number of carbon	Value of n	Class
	atoms		
1	10	2	Monoterpenoids(C10H16)
2	15	3	Sesquiterpenoids(C15H24)
3	20	4	Diterpenoids(C20H32)
4	25	5	Sesterpenoids(C25H40)
5	30	6	Troterpenoids(C30H48)
6	40	8	Tetraterpenoids(C40H64)
7	>40	>8	Polyterpenoids(C5H8)n

 Table (1.1):Classification of Terpenoids

Each class can be further subdivided into subclass according to the number of rings present in the structure (R.Croteau 1998).

- Acyclic Terpenoids: They contain open structure.
- Monocyclic Terpenoids : They contain one ring.
- **Bicyclic Terpenoids** : They contain two ring.
- **Tricyclic Terpenoids** : They contain three rings.
- **Tetracyclic Terpenoids** : They contain four rings.

ii) **Phenolic compounds** are a chemically heterogeous compound contains a phenol group- a hydroxyl functional group on an aromatic ring. Some phenolic compounds are soluble only in organic solvents, some soluble in water, while others are insoluble polymers .phenolic are wide spread in vascular plants and appear to function in different capacities. The derivatives of phenolic compounds include simple phenyl propanoid, benzoic acid

derivatives, anthocyannin, isoflavones, tannins, lignin, and flavonoid compound beginning with phenyl alanines.

The largest classes of plant phenolic compounds are flavinoids, the basic strcture contain 15 carbon arranged in two aromatic ring connected by a three carbon bridge (Taiz et al 2005). The basic function of the flavonoids is for pigmentation and defence the red, pink, purple and blue colours observed in plants parts are as a result of anthocyannin.

Tannins were first used to describe compound that could convert raw material hides into leather in the process of tanning.tannin are generally toxic that significantly reduce the growth and survivorship of many herbivores when added to their diets. tannins can be seen in fruits like apple, black berries,tea and red wine (Taiz et al 2005). Tannins are mainly constituent of woody plants especially heart wood. Some derivatives of tannin include Gallic acid.

iii)Nitrogen containing compounds A large variety of secondary metabolites have nitrogen in their structure .these include the alkaloids, cyanogenic glucoside, glucosinate (Taiz et al 2005). Alkaloids are large family of more than 15,000 nitrogen containing secondary metabolites found in approximately 20% of the species of vascular plant.the nitrogen atom in these substances is usually part of the heterocyclic ring.

The first example of an alkaloid use in field was morphine, its isolated in 1805 from opium poppy *papaver somniferum* (Fessenden 1907). the function of most alkaloid are now as defence against especially mammals, because of the general toxicity and deterrence capacity (Hartnann 2013).

1.2 Volatile oils

Volatile oils are mixture of hydrocarbon terpenes, sesquiterpenes and polyterpenes and their oxygenated derivatives obtained from various parts of plant. They are extracted from plant to produce essential oils. The essential oils of the plants are the principle of their aromas, They are called volatile

oils because they vaporize rapidly when exposed to the air at ordinary temperatures. Volatile oils are responsible for the odour of the plant. Also they are slightly soluble in water but are soluble in ether, alcohol and most of organic solvents.

In general, the essential oils consist of many mixtures including different sorts of molecules. These chemical constituents are divided into two broad classes: terpenes and phenylpropanoids. Most essential oils consist mainly of monoterpenes, main chemical constituents of the essential oils were found as mixtures of aromatic components and can be obtained by steam distillation or solvent extraction from a large variety of aromatic plants.Essential oil composition depends upon some factors affecting the plant such as :climate ,environmental ,age of plants and the stage of ripening fruits (Bernath J 1999;Radulescu V 2009;Piccaglia R 20001).

1.3 Extraction of volatile oils

Monoterpenes are the main constituents of volatile oils as they consist mainly of hydrocarbons or derivatives derived from the process of monoterpenes or sesquiterpenes containing phenyl terpenoids (Laxminarain 2013;Sadgrovve 2005;J.Sharifi-rad). The volatile oils are obtained from the plant parts in several ways Classical and conventional methods such as Hydrodistillation Method is the most common method used to extract essential oil. It is a traditional method for extraction of essential oils. There are three types of hydrodistillation processes; viz. Water distillation, Water distillation distillation. The and steam and Steam advantages Hydrodistillation is include: the processing can be done with finely powdered material or plant parts, inexpensive, easy to construct and suitable for field operation (H.A.Heshan 2016).

Steam distillation is one of the conventional methods for the isolation of essential oil from plants. The principle of this technique is that when vapor pressure equals the ambient pressure at about 100 _C, the volatile components with the boiling points ranging from 150 to 300 _C can be

evaporated. This method could be improved by carrying out under pressure depending on the chemical nature of essential oil (D.Smith et al., 2001).

Ahydrocarbon solvent is added to the plant material in solvent extraction method to help dissolve the essential oil. When the solution is filtered and concentrated by distillation, a substance containing essential oil remains. It is one of the methods used in the preparation of oils from flowers, in which flower petals are mixed with volatile solvents such as petroleum ether or benzene until all the volatile oils dissolve in the solvent, the solution is evaporated and the solvent is evaporated at low pressure (D.Smith et al., 2001). The stability of essential oils depends on several factors . The internal factors include chemical structure and impurities present in the essential oil. The external factors include presence of oxygen, exposure to light, humidity and temperature (T.Claudia 2013).

One of the disadvantages of conventional techniques is related with the thermolability of Essential oil components which undergo chemical alterations (hydrolysis, isomerization, oxidation) due to the high applied temperatures (H.A.Heshan 2016). Non conventional techniques have more advantages over conventional methods such as better quality extracts, reducing energy consumption and operating units, lesser or no CO2 emission, minimize harmful co-extract formation, replacing petroleum based solvents by alternative solvents etc. Non conventional techniques Such as Supercritical Fluid Extraction (SFE). Supercritical Fluid Extraction (SFE) is the process of separating one component from another using supercritical fluids as the extracting solvent. This extraction method produces higher yield, higher diffusion coefficient, and lower viscosity. Many essential oils that cannot be extracted by steam distillation can be obtainable with carbon dioxide extraction (H.A.Heshan 2016).

Microwave-Assisted Hydrodistillation (MAHD). It is an advanced hydrodistillation technique utilizing microwave oven in the extraction process. It is a current technology to extract biological materials and has

been regarded as an important alternative in extraction techniques because of its advantages which mainly are a reduction of extraction time, solvents, selectivity, volumetric heating and controllable heating process (M.Silva et al., 2004;A.Farhat et al., 2009)

Ultrasound-Assisted Extraction (UAE). Ultrasound- Assisted Extraction (UAE) is a good process to reach high valuable compounds, has been used for the extraction of many essential oils especially from the flower, leaves or seeds (H.A.Heshan 2016). This method leads to higher extraction yields with lower energy consumption and reduce time

1.4 The Plant Foeniculum Vulgare Mill(Fennels)

The *Foeniculum* vulgare mill belongs to the family Apiaceae commonly known as fennel, is one of the widespread annual or perennial plants with aromatic odour. Three main categories of Foeniculum vulgare Mill are bitter fennel, sweet fennel Florence fennel, or finocchio (Sadeghian et al., 2005) Bitter fennel is grown for its fruits and essential oil, whilst Florence fennel is cultivated for its fruits and essential oil, Sweet fennel is cultivated for its enlarged leaf base, for its fruits and for the essential oil taken from its fruits (Weiss ea 2002). Fennel seed is a short-lived herb, indigenouns to Europe and cultivated in India, China and Egypt (Wichtel et al., 1994). fennel is well known to the ancient Egyptians, Romans, Indians and Chinese. It is also originated in the southern Mediterranean region and grows through naturalization and cultivation in vegetable and herb gardens for its anise -flavored foliage and seeds due to their use in cooking (Badgujar SB, P214-216). It is widely cultivated throughout the temperate and tropical regions of the world which are used as aculinary spice. Mature fruit and essential oil of Foeniculum vulgare are use as a constituent of pharmaceutical and cosmetic products, As flavoring in food products as well. It is an aromatic herb whose fruits contain essential oil which is used for many purposes by human population (Tanira 1996;Leung et al., 1996)The oil of fennel regulates the peristaltic functions of the gastrointestinal tract, relieves the spasms of intestines (Fathy et al ;Ethernten et al., 2002). Externally, the oil relieves muscular and rhematic pains. The different parts of the plants are used to heal simple and complicated diseases, The Foeniculum vulgare mill is one of the most common medicinal traditionally plants used to treat the uterine and intestinal problems. It is also used to treat a lot of various diseases such as abdominal pains (Savo V 2011), colic in children, diarrhea, fever, flatulence, gastralgia, gastritis constipation, and irritable colon. It is also used for arthritis, cancer (Tene V

2007), for conjunctivitis, insomnia (Olveira et al., 2012), kidney ailments, laxative (Mahmood A 2013), leucorrhoea, liver pain, mouth ulcer (Guarrera PM 2005). Fennel herb, seeds and extractives do not appear to have any significant toxicity. The amount of fennel normally consumed in food is non-toxic. Fennel herbal tea and other preparations do not have any reaction in therapeutic doses. Excess amounts of fennel oil may cause nausea, vomiting and seizures.

According to previous study *Foeniculum vulgare* was used in traditional medicine. It is used to treat various ailments such as intestinal and uterine pain. There is no study had been done on the contractile activity of Foeniculum vulgare. This study was carried out to attempt the contractile activity of Foeniculum vulgare seeds extract on the intestinal and uterine muscles. The ethanolic extract of *Foeniculum vulgare* seeds were tested in *vitro* using the isolated tissue technique. The results showed that, the small doses of the of *Foeniculum vulgare* seeds extract caused an increase in the uterus muscle contractility of the wister rat, while the higher doses caused a relaxant effect. No significant effect had been seen on the ileal muscle contractility(Zainab et al., 2017). In other study the chemical composition of essential oils of fennel was identified by GC-MS technique. Results showed that essential oils of fennel have several organic compounds such as hydrocarbons, alcohols, ketones, aldehydes, ethers, esters and other volatile oil and so on. Essential oil of "fennel" has 32 compounds. T-Anethol, αpinene, D-limonene, α -fenchone and fenchol were the highest percent of compounds. biological activity of Aqueous extract of fennel was evaluated by disk diffusion method and results showed that it has antibacterial properties against Escherichia coli, Bacillus subtilis and Pseudomonas aeruginosa, but have not any antifungal activity (Aspergillus oryzae). The results show the extract of fennel was not a good alternative for antibiotics(Mehrzad et al., 2017). Fennel plants are considered as one of the world's most important medicinal plants. Phytochemical screening, the total

phenolic contents (TPC), total flavonoid contents (TFC), in vitro antioxidant, and antimicrobial activities of extracts of *Foeniculum vulgare* seeds were investigated. The antioxidant activity was measured using 1, 1 Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, reducing power and total antioxidant activity. Foeniculum vulgare extracts were also investigated for in vitro antibacterial screening by disc diffusion method against Bacillus megaterium, Entrococcus gallinarium, Escherichia coli and Pseudomona aeruginosa. Methanol extracts contained the highest TPC $[(22.93 \pm 7.17) \text{ mg GAE/g}]$, and TFC $[(8.581 \pm 1.22) \text{ µg CE/mg}]$ and water extract showed the highest DPPH radical scavenging activity (IC50 = 207.94) \pm 83.38 µg/mL). The results also showed that the aqueous: methanol (20:80, v/v) extract had strongest reducing ability (0.711 \pm 0.102 nm) as compared to other extracts. Methanol extract exhibited the highest total antioxidant capacity $(1.63 \pm 0.19 \ \Box g/mL)$ as determined by the phosphomolybdenium method. Of the *Foeniculum vulgare* extracts, the highest inhibition zone was found in aqueous: methanol (20:80, v/v) extracts of the (12.29 \pm 1.34 mm) against Bacillus megaterium. The study revealed that antioxidant and antimicrobial activities of the crude extract of Foeniculum vulgare extracted by different solvents indicating a high potential to be used as natural antioxidants in food preservation as well as for preventing oxidative stress mediated human disorders(Abebie et al., 2017).

In accordance with last study *Foeniculum vulgare* Mill has been used in traditional medicine for a wide range of ailments related to digestive, endocrine, reproductive, and respiratory systems. Additionally, it is also used as a galactagogue agent for lactating mothers. The review aims to gather the fragmented information available in the literature regarding morphology, ethnomedicinal applications, phytochemistry, pharmacology, and toxicology of *Foeniculum vulgare*. It also compiles available scientific evidence for the ethnobotanical claims and to identify gaps required to be filled by future research. Findings based on their traditional uses and

scientific evaluation indicates that Foeniculum vulgare remains to be the most widely used herbal plant. It has been used for more than forty types of disorders. Phytochemical studies have shown the presence of numerous valuable compounds, such as volatile compounds, flavonoids, phenolic compounds, fatty acids, and amino acids. Compiled data indicate their efficacy in several in vitro and in vivo pharmacological properties such as antimicrobial, antiviral, anti-inflammatory, antimutagenic, antinociceptive, antipyretic. antispasmodic, antithrombotic, apoptotic, cardiovascular. antitumor, chemomodulatory, hepatoprotective, hypoglycemic, hypolipidemic, and memory enhancing property. Foeniculum vulgare has emerged as a good source of traditional medicine and it provides a noteworthy basis in pharmaceutical biology for the development of new drugs and future clinical uses (Shamkamt B et al., 2014).

1.5 GasChromatograghy-Mass Spectrometery(GC-MS)

In general, Chromatograpgy is a technique that used to separate complex mixtures of chemicals into its individual components for identification and quantification. Scientist Archer J.P.Martin, Who developed Gas Chromatography(GC) with fellow scientist Anthony T.James. Their invention on GC was awarded the Nobel Prize in Chemistry in 1952 and also they made other developments such as liquid chromatography(LC) and Gas Chromatography-Mass Spectrometery(GC-MS).

GC is chromatographic technique in which the mobile phase is a gas, it is one of the most popular method for separating and analyzing compounds due to high resolution ,low limits of detection ,speed , accuracy and reproducibility. GC use to separate naturally volatile compounds or volatile derivative compounds and this make it useful technique to separate organic and inorganic compounds (Douglas 2007).

Gas chromatography-mass spectroscopy (GC-MS) is an analytical method that combine the features of gas chromatography and mass chromatography to identify different substances (Sparkman 2011).one of

most powerful technique ,its preferred method for the analysis of small and volatile molecules. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals. GC-MS provides specificity and sensitivity, its used to analyze unknown compound and multi-component. GC-MS has many applications today, such as in environmental ,forensic ,food and beverage ,clinical ,pharmaceutical and chemical industries.

Mass spectrometry(MS) is a highly-sensitive detection technique that use to detect and separate ions in the gaseous phase, when coupled to a GC .it immediately ionizes the gaseous eluted compounds, separates the ions in vacuum based on their mass to charge ratios(m/z) and eventually measures the intensity of each ion. These intensities are recorded to give a series of mass spectra which displays the relative ion intensity against m/z.



Figure (1.2) Digram of gas chromatograpgy –mass chromatograpgy

1.6 Phytochemical screening Test

Phyto constituents are the natural bioactive compounds found in plants. Phyto chemicals are basically divided into two groups, i.e. primary and secondary constituents; according to their functions in plant metabolism. Primary constituents comprises common sugars, amino acid, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoid, steroids and flavonoids, so on (Thilagavathi.T, Chennai, India)

1.7 Antimicrobial Activity

An antimicrobial is an agent that kills microorganisms or stops their growth (Merrian 2009). Antimicrobial medicines can be grouped according to the microorganisms into antibiotics are used against bacteria, and antifungals are used against fungi. They also classified according to their function into microbicides that kill microbes and bacteriostatic that inhibit the growth. antimicrobial use has been common practice for at least 2000 years. Ancient Egyptians and ancient Greeks used specific molds and plant extracts to treat infection (Wainwright M 1989).

Antimicrobial activity refers to the process of killing or inhibiting the disease causing microbes, various Antimicrobial agents are used for this purpose. Antimicrobial may be anti-bacterial, anti-fungal or antiviral. They all have different modes of action by which they act to suppress the infection.

A variety of laboratory methods can be used to evaluate the in vitro Antimicrobial activity of an extract or pure compound. The most known methods are the disk- diffusion and agar dilution. Disk-diffusion method is based on the principle that antibiotic-impregnated disk, placed on agar previously inoculated with the test bacterium, pick-up moisture and the

antibiotic diffuse radially outward through the agar medium producing an antibiotic concentration gradient. The concentration of the antibiotic at the edge of the disk is high and gradually diminishes as the distance from the disk increases to a point where it is no longer inhibitory for the organism.

The disk diffusion method is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility. Dilution methods are the most appropriate ones for the determination of MIC values, it may be used to quantitatively measure the in vitro antimicrobial activity against bacteria and fungi. They are many approved guidelines for dilution antimicrobial suspceptibility testing of fastidious or non-fastidious bacteria, yeast and filamentous fungi .The most recognized standards are provided by the CLSI and the European committee on antimicrobial Suspceptibility testing (EUCAST).

Objectives of the research

The aim of this study is to

- To extract the volatile oil of fennel seeds
- To test phytochemical screening test of the extracts
- To characterize the volatile oil using GC-MS Technique
- To evaluate antimicrobial Activity of volatile oil of fennel seeds.

2-Materials and Methods

2.1 The Plant Material Collection

The dry fennel (*Foeinculum vulgare mill*) seed used in this research was purchased from local market in Omdurman, The seeds were cleaned physically to remove foreign particles .Then the seeds were ground into a fine powder with the help of mechanical grinder.

2.2 Extraction of Volatile Oil

The volatile oil of fennel seed was extracted by two methods. These methods are hydrodistillation and soxhlet,

The percentage% of volatile oil was calculated as follows in terms of (V/W)according to the following equation

The percentage of volatile oil=

volume(ml)/weight(g)X100

2.3 Chemicals and Reagents

2.3.1Preparation of Reagents

• Mayer Reagent was prepared as follow:

Mercuric chloride 1.36g

Potassium iodide 5.0g

- Distilled water upto 100ml
- Ferric chloride 10%:
- 10 g of ferric chloride in 100 ml distilled water
- Sodium hydroxide (2N):
- 4 g of sodium hydroxide in 50 ml distilled water
- Hydrochloric acid 1%:

1ml of concentrated hydrochloric acid in up to 100 ml distilled water.

2.3.2 Chemicals:

- sulphuric acid conc
- HCL conc
- Methanol
- N-hexane
- Ethyl acetate
- Tap water
- Glacial acetic acid
- Acetic anhydride
- Chloroform
- Distilled water
- Fehling A and B

2.4 Methods of Extraction of Essential oil

2.4.1 Hydrodistillation Extraction

(200 g) of ground fennel seeds were used for essential oil extraction by hydro-distillation method using a Clevenger apparatus (Clevenger 1928) for 3 h. After decanting and drying of the oil over anhydrous sodium sulphate the corresponding mild yellowish coloured oil were recovered and calculated in terms of percentage (V/W) (S.N.Saxena et al., 2018) according to the following equation.

The percentage of volatile oil=

volume(ml)/weight(g)X100

2.4.2 Soxhlet Extraction

50 g of a powdered sample were weighted in celulose thimble and were placed in the extraction chamber of a 125-mL Soxhlet apparatus fitted with a condenser, which was placed on a 500-mL distillation flask containing

500 mL of the n hexane. Powdered seeds were extracted under reflux with nhexane 5 h at 75 °C. After extraction, solvents were evaporated under reduced pressure, using a rotary evaporator at 45 °C. The oils were stored at 4 °C until analysis. The percentage was calculated in terms of percentage (V/W) (S.N.Saxena et al., 2018) according to the following equation.

The percentage of volatile oil=

volume(ml)/weight(g)X100

2.5phytochemical screening test2.5.1Preparation of plant extracts:

The n-hexane, chloroform, Ethyl acetate, methanol and water: extracts of all were prepared by dissolving 10 g of the seeds fine powder separately in 100 mL each solvent. The contents were kept in orbital shaker for 24 h at room temperature. Thereafter, each extract was filtered using Whatman no.1 filter paper and evaporated to dryness under vacuum at 40°C by using a rotary evaporator (Buchi, 3000 series, Switzerland). The extraction were stored at 4° C . and then used for phytochemical screening test.

2.5.2Phytochemical Screening Test:

A qualitative phytochemical test to detect the presence of alkaloids, flavonoids, saponins, steroids, tannins, and triterpenoids was carried out using standard procedures (Akgul A1988; Zoubiri S 2014;).

Test of Flavonoids

2 ml of the extract were mixed with diluted NaOH to produce yellow coloration. Disappearance of the color upon addition of dil. HCl indicates the presence of flavonoids.

Test of Steroids

2 ml of the extract were evaporated to dryness, the residue was then dissolved in two ml of chloroform and transferred to clean dry test tube, two

ml of acetic anhydride were added followed by addition of concentration sulphuric acid carefully to the wall ofbthe tube. Color development from violet to blue or green indicates presence of a steroids.

Test of Terpenoids

2 ml of the extract were mixed with two ml of chloroform. 3 ml of concentration sulphuric acid were added carefully to form a layer, formation of a reddish brown color at the interface Indicates the presence of terpenoids.

Test of Alkaloids

2 ml of the extract were acidified with 1% HCl, few drops of Mayer's reagent were added, appearance of turbidity indicates the presence of alkaloids.

Test of Saponins

1 ml of extract was mixed with 9 ml distilled water and then the solution was shaken vigorously for 15 seconds and was allowed to stand for 10 min ,Formation of stable foam indicates the presence of saponins .

Test of Tannins

Two ml of the extract were mixed with drops of ferric chloride solution, the result was observed.

Test of Reducing Sugars

Two ml of the extract were heated with equal volumes of Fehling solution A and B. Appearance precipitate indicates the presence of reducing sugars.

Test of glycosides

Few drops of glacial acetic acid and ferric chloride solution were added to 1 ml of extract and then 3-4 drops of concentration sulphuric acid were added ,the appearance of blue-green colour indicates the presence of glycosides.

2.6 Volatile oil analysis

The volatile oil obtained above was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) to determine its chemical constituents.

2.6.1GC-MS Analysis

The qualitative and quantitative analysis of the volatile oil was carried out by using GC/MS technique model(GC/MS-QP2010-Ultra) from japans ,Simadzu company, with serial number 020525101565SA and capillary column(Rtx-5ms-30mx0.25mmx0.25um).The volatile oil was injected by using spilt mode , instrument operating in EI mode at 70eV . Helium as the carrier gas passed with flow rate 1.69ml/min , the temperature program was started from 50c with rate 7c/min to 180c then the rate Was changed to 10c/min reaching 280c as final temperature degree ,the injection port temperature was 300c, the ion source temperature was 200c and the interface temperature was 250c. The volatile oil 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 compounds for the volatile oil was achieved in the library ,The National Institute Of Standards and Technology(NIST), results were recorded.

2.7Determination of Antimicrobial Activity

The microorganisms obtanined from(NCTC), (ATCC) were used to test the anti-microbial activity of fennel essential oil

2.7.1Preparation 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.

2.7.2Preparation 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.

2.7.3Testing of antibacterial susceptibility

2.7.3.1Disc diffusion method

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by 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 10^8 cfu/ 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 $^{\circ}$ C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured (Sana et al., 2012).

3-Results And Discussion

3.1 Extraction of Volatile Oil

The volatile oil of fennel seed was extracted by two methods which have been described in previous section. The two methods are hydrodistillation and soxhlet

The percentage% of volatile oil was calculated as follows:

Hydrodistillation method:

The percentage% of volatile oil =1.5/200x100=0.75%

Soxhlet Method:

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The percentage% of crude volatile oil = 6/50 \times 100 = 12\%
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As per experimental work soxhlet extraction is better method for extraction than hydrodestillation method. The result showed that the highest yield of essential oil by using soxhlet extraction (12%) than hydrodestillation extraction(0.75%).





Figure(3.1) Chromatogram of fennel seeds essential oil

GC-MS test is performed to determine the chemical constituents in the fennel seed oil . The essential oil constituents and retention time of fennel seeds are showed in table 2.Chromatograghic analysis shows that there are 15 components contained in the fennel seeds oil. The three largest components in this sample are: D-limonene ,D-carvone ,limonene oxide.

Table(3.1) Retention time (RT) and Area of the compounds identified inthe volatile oil of fennel by GC-MS

Peak #	R.time	Area	Area%	Name
1	4.132	70961	0.04	Alpha-pinene
2	4.790	79269	0.04	Bicyclo(3.1.0)hexane,4-methylene-1-(1-
				methylethyl)
3	4.846	23552	0.01	Beta-pinene
4	5.130	536188	0.29	Beta-Myrcene
5	5.814	59644644	31.93	D-Limonene
6	7.976	1013252	0.54	Limonene oxide
7	9.212	276316	0.15	Cyclohexanol,2-methyi-5-(1-methyletheyl)-
				(1.alpha.,2.beta.,5.alpha.)
8	9.314	259236	0.14	Cyclohexane,2-methyi-5-(1-methylethenyl)-
				trans
9	9.561	108495	0.06	Neodihydrocarveol
10	9.692	245074	0.13	2-cyclohexen-1-ol,2-methy1,5-
				(1methylethenyl)-cis
11	9.846	345551	0.18	Cyclohexanol,2-methyi- 5-(1-
				methylethenyl)-(1.alpha.,2.beta.,5.alpha.)
12	10.178	123890595	66.33	D-Carvone
13	12.711	24306	0.01	(-)- beta bourbonene
14	12.827	80149	0.04	Cyclohexane,1-ethenyl-1-methy-2,4-bis(1-
				methylethenyl)-,{1S-

				(1.alpha.,2.beta.,5.alpha.)}
15	13.353	189974	0.10	Caryophyllene
		186787562	100.00	

3.3-Phytochemical Screening Test:

The phytochemical test of different extracts of *Foeniculum vulgare mill(fennel)* was done ,five different solvents were used such as,Hexane,chloroform, Methanol Ethyl acetate and water.The results were presented in following Table.

Phytochemical analysis of fennel seed extracts showed the presence of most of the phyto-constituents including alkaloids, flavonoids, saponins, glycosides, reducing sugar, steroids, terpenoids and tannins, table 2 confirm the occurrence of secondary metabolism .phytochemical constituents were more found in methanolic extract than other four extracts of water, ethyiacetate n-hexane and chloroform

Table(3.2):Phytochemical screening test of different Extracts ofFoeniculum vulgare(Fennel)

Phytochemical test	Ethyl	Methanoll	Hexane	Chloroform	water
	acetate				
Alkaloids	-	+	-	+	+
Tannins	-	+	-	-	+
Saponins	+	+	+	+	-
Steroids	+	+	+	+	-
Terpenoids	+	+	+	+	+
Glycosides	-	-	-	-	-
Flavonoids	+	+	+	+	-
Reducing sugar	+	+	-	+	+

3.4 Antimicrobial Activity

Bacillus subtilis : **B.s**Staphylococcus aureus : **S.a**Candida albicans :C.aEscherichia coli : **E.c**Pseudomonas aeruginosa : **Ps.a**

The results were expressed in terms of the diameter of the inhibition zone: < 9 mm, inactive; 9-12 mm, partially active; 13-18 mm, active; >18 mm, very active.

Foeniculum vulgare extracts were investigated for antimicrobial activity against Bacillus subtilis ,Staphylococcus aureus ,Candida albicans,

Escherichia coli ,Pseudomonas aeruginosa, the highest inhibition zone was found against Pseudomonas aeruginosa.

Table (3.3) Antimicrobial activity in terms of inhibition zones of fennel essential oil

Plant	Solvent	concent	E.c	Ps.a	S.a	B.s	C.a
name							
Fennel oil	Dimethyl	100mg\m	12-	13-14	_	8-9	_
	sulphoxide	1	12				

3.5 Conclusion

The qualitative and quantitative analysis of the volatile oil was carried out by GC-MS, in this study we use two methods to extract fennel oil by using soxhlet and hydrodistillation.. The qualitative phytochemicals screening test show the existence of alkaloids ,saponins, tannins, steroids, glycosides and flavonoids. antimicrobial activity test of The *Foeniculm vulgare* oil extract were observed by measuring the diameter of the growth inhibition zone , the result reveal that fennel oil has bacterial effect .

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Appendices

Comparison of Mass spectra of the components of the volatile oil of *Foeniculum Vulgare Mill (fennel)* with standard spectra from the instrument library





10 30 50 70 90 110 130 150 170 190 210 230 250 270 290 310 330 350 370 390 410 430 450 470 490





<< Target >> Line#:4 R.Time:5.125(Scan#:426) MassPeaks:183 RawMode:Single 5.125(426) BasePeak:41.00(19509) BG Mode:5.045(410) Group 1 - Event 1 Scan 100 4

80-60-40-











10 30 50 70 90 110 130 150 170 190 210 230 250 270 290 310 330 350 370 390 410 430 450 470 490

















<<Tanget >> Line#:16 R.Time:7.980(Scn#:997) MassPeaks:249 RawMode:Averaged 7.975-7.985(996-998) BasePeak 43.00(12353) BG Mode:Cal. E from Peak Group 1 - Event I Scan 100 40 41 67 79 94 109 20 41 67 79 94 109 20 50 70 90 110 130 150 170 190 210 230 250 270 290 310 330 350 370 390 410 430 450 470 490

Library search is not complete