Sudan University of Science and Technology

College of Postgraduate Studies

Constituents and Antimicrobial Activity of Pimpinella anisum oil

زيت اليانسون مضاداً للميكروبات مكونات وفعالية

A Thesis Submitted in Partial Fulfillment of the Requirements of the M.Sc. Degree Chemistry

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الاستهلال

بسم الله الرحمن الرحيم

قال تعالى :

﴿ قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَاعَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ﴾

صدق الله العظيم

سورة البقرة – الآية (32).

Dedication

To

My parents

My husband

Sons

My brothers and sisters

Acknowledgment

Thanks at first and last for the light of our life **Allah** who gave us the strength while doing this project and guided me through the way in this life.

Then I would like to express my special and deep thanks to Prof. Mohammed Abedel Karim for his patience and great efforts of supervising and leading me through this study.

Also I thank the staff members of the chemistry Dept.-Sudan University of Science and Technology .

Abstract

Pimpnella anisum oil was analyzed by GC-MS . Ten constituents were identified by GC-MS analysis dominated by the following major components:

i) 9-Octadecenoic acid methyl ester (14.21%)

ii) Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl) methyl] cyclopropyl]methyl]-, methyl ester (10.64%)

The antimicrobial potential of the oil has been assessed via the cup plate agar diffusion bioassay. *Pimpnella anisum* oil showed partial activity against G+ve *Bacillus subtilis* and G-ve *Escherichia coli*. Ampicilin, gentamicin and clotrimazole were used as positive controls.

المستخلص

فى هذه الدراسه تم استخلاص زيت الينسون ثم تم تحليل الزيت بتقنية الكرموتوغرافيا الغازيه-طيف الكتله . وقد اوضح هذا التحليل ان المكونات الرئيسه لهذا الزيت هي :

i) 9-Octadecenoic acid methyl ester (14.21%).
ii) Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl) methyl]cyclopropyl] methyl]-, methyl ester (10.64%).

ايضا اجرى اختبار مضاد الميكروبات حيث اعطى الزيت نشاطا ضد :
Bacillus subtilis and *Escherichia coli*

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1Introduction

1.1Natural products

Natural products are chemical compounds or substances isolated from living organism[1].the chemistry of the natural product include their biosynthesis extraction identification, quantification, structural elucidation, physical properties and reactions by which they are produced by the pathway of primary or secondary metabolism[2].

Mpounds[3]. Metabolism is defined as series of enzyme-catalyzed biochemical reaction or transformation occurring within the cells of an organism which are mainly required for its growth, development and for proper response to its environment[4].

Metabolism can be usually restricted to small molecules[5].A primary metabolites is directly involved in normal growth development and reproduction. Example carbohydrate, protein, fat and oil, alcohol etc. On the other hand secondary metabolites are not directly involved in growth development and reproduction of an organism ,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[3].

The most bioactive secondary metabolite are the alkaloids, tannins, flavonoids and phenolic compounds.

1.2Classification of plants secondary metabolite

Plants secondery metabolites can be divided into three chemically distinctive groups namely :

i)Terpenes

ii)Phenolic compound

iii)Nitrogen containing compounds

aTerpenes

A derivative of terpene called saponins are steroids and triterpene glycoside,so named because of their soap- like properties .Another derivative of terpene is cartenoids which give the yellow ,red and orange colour in some plants like carrot.examples of plants containing terpenoids include *Polypodium vulgare, Digitalis spine* , peppermint plant, lemon ,basil ,sage ,corn ,*gossypium hispida* [cotton] ,wild tobacco[6].

bPhenolic compounds

Plants produce a large variety of secondary products that contain a phenolic group – a hydroxyl functional group on an aromatic ring .These substances are classified as phenolic compounds. Plant phenolics are a chemically heterogenous compound, some soluble only in organic solvents, some are water soluble, while others are insoluble polymers. Some simple phenolic are activated by ultra violet light. Phenolics are widely spread in vascular plants and appear to function in different capacities. The derivatives of phenolic compounds include simple phenyl propanoid, benzoic acid derivatives, anthocyanin, isoflavone, tannins, lignins and flavonoid compound beginning with phenyl alanines. Lignan is generally formed from three different phenyl propanoid alcohols namely, coniferyl, coumaryl and sinapyl7. The flavonoids are one of the largest classes of plant phenolics and the basic structure contains 15 carbon arranged in two aromatic ring connected by a three carbon bridge[5] .The basic function of the flavonoids is for pigmentation and defence .the red, pink, purple and blue colours observed in plants parts are as result of a class of flavonoids known as the anthocyanins. The purple colour of *Commelina commenis* was found to consist of six anthocyannin molecule[8].

1.3Economic importance of plant secondary metabolites

The secondary metabolites have a lot of economic importance in the plant breeding, plant defence, pollination, ecological effect and others.

i) Plant breeding

Plant breeders try to select varieties which provide maximal yields in combination with optimal quality and resistance against pathogens, herbivores and other environmental stress[9].

ii) Antimicrobials

Some herbs like thyme (*Thymus vulgaris*) contain caffeic acid ,which is effective against viruses , bacteria and fungi[10]. Phenolic compounds may be found in some essential oils .For example- eugenol is found sOmium gratucimum.This phenolic compound is considered bacteriostatic against both fungi and bacteria[11].

iii) Health and pharmaceuticals

Secondary metabolite has been used in the formulation of various drugs. For instance the alkaloids are used as antimalarial drugs, anagestics and other secondary metabolites are used as antibiotics to prevent bacterial infections and other health related issues. *Solanum khasianum* mixed with other alkaloid may be useful against HIV infection as well as intestinal infection associated with AIDS[12] . Other secondary metabolites are used in pharmaceutical industries including atropine from *Atropa* sp , scopolamine from *Datura* sp , quinine from *Chinchona* sp , codeine from *Papaver* sp.

iv) Plant defence against herbivores and pathogens

In conifers such as pine and fir pine, monoterpenes accumulate in resin duct found in the needles, twigs and trunks .These compounds are toxic to insects like bettle .marjorly . Terpenes are generally toxic to plant herbivore and this is the mechanism some plant uses in defending itself from its pray .Also plant can cry for help. In this case the feeding of the plant by the insect will make the plant to excret secondary metabolites which attract carnivores that will come and feed on the insect[13].

v) Toxicity

Many of the plant secondary metabolite are toxic both to man and animals. For example coniine from *Conium* sp , strychnine from *Strychnos* sp. both are toxic to man and animals[4]. Cyanogenic glycosides seen in cassava are also toxic to man and animals. Of special interest is the phytotoxicity of certain coumarins called furanocomarins , which have an attached furan ring . These compound which are not toxic until they are activated by light. Phytotoxic furanocoumarin are especially abundant in members of the Umbelliferae family including celery. These chemical can cause cancers and skin related diseases.

vi) Fragrance , repellance and attractant

Secondary metabolites - terpenes are used as fragrance for insect pollination.In general sense, secondary metabolite function as an attraction to animal for pollination (fragrance and color) or for seed dispersal . Secondary metabolite are often not directed against a single organism but generally against a variety of potential enemies or they may combine the role of both repellent and attractant eg (anthocyanin or volatile terpenes can be attractant in flowers but are also insecticidal and antimicrobial[5] .

1.4Gas chromatography

In 1901 Mikhail Tswett invented adsorption chromatography during his research on plant pigment. He separated different coloured chlorophyll and carotenoid pigments of leaves by passing an extract of the leaves through a column of calcium carbonate, alumina and sucrose eluting them with petroleum ether/ethanol mixtures. He coined the term chromatography in a 1906 publication, from the Greek words *chroma* meaning “colour” and *graphos* meaning “to write”.

The two principal types of chromatography are: gas chromatography (**GC**) and liquid chromatography (**LC**). Gas chromatography separates gaseous substances based on partitioning in a stationary gas phase. Liquid chromatography includes techniques such as size exclusion (separation based on molecular size), ion exchange (separation based on charge) and high-performance liquid chromatography (HPLC separation based on partitioning from a liquid phase).

While the mechanisms of retention for various types of chromatography differ, they are all based on the dynamic distribution of an analyte between a fixed stationary phase and a flowing mobile phase. Each analyte will have a certain affinity for each phase.

There are two types of GC:

- Gas-solid (adsorption) chromatography
- Gas-liquid (partition) chromatography

In every case, successive equilibria determine to what extent the analyte stays behind in the stationary phase (adsorption chromatography) or are coated with a thin layer of liquid phase (partition chromatography).

Most common form today is a capillary column, in which a virtual liquid phase, often polymer, is coated or bonded on the wall of the capillary tube. Gas solid chromatography (GSC) is for separation of small gaseous species such as H_2, N_2, CO_2, CO, O_2, NH_3 and CH_4 and volatile hydrocarbons, using high surface area inorganic packings such as alumina or porous polymer. The gases are separated by their size due to retention by adsorption on the particles.

The solid support for a liquid phase have a high specific surface area, chemically inert, thermally stable and have uniform sizes. The most commonly used supports are prepared from diatomaceous earth, a spongy siliceous material. Particles have diameters in the range of 60 to

80 mesh (0.18 to 0.25 mm), 80 to 100 mesh (0.15 to 0.18 mm) or 100 to 120 mesh (0.12 to 0.15 mm).

1.5Essential oils (EOs)

Essential oils are very interesting natural plant products and among other qualities they possess various biological properties. The term “biological” comprises all activities that these mixtures of volatile compounds (mainly mono- and sesquiterpenoids, benzenoids, phenylpropanoids, etc.) exert on humans, animals, and other plants aspects.

On account of the complexity of these natural products, the toxicological or biochemical testing of an EO will always be the sum of its constituents which either act in a synergistic or in an antagonistic way with one another. Therefore, the chemical characterization of the EO is very important for the understanding of its biological properties. The constituents of these natural mixtures upon being absorbed into the blood stream of humans or animals get metabolized and eliminated. This metabolic biotransformation leads mostly in two steps to products of high water solubility which enables the organism to get rid of these “xenobiotics” by renal elimination ,

The phytotherapeutic uses of EOs is another overview about scientific papers in peer-reviewed journals over the last 30 years, so to say the medical use of these natural plant products excluding aromatherapeutical treatments and single case studies . Another contribution only deals with antimicrobial activities of those EOs that are monographed in the European Pharmacopoeia. "*In Vitro* Antimicrobial Activities of Essential Oils Monographed in the European Pharmacopoeia 6th Edition" : more than 81 tables show the importance of these valuable properties of Eos[4].

EOs and aromachemicals are low-volume high-value products used in perfumery, cosmetics, feed, food, beverages, and pharmaceutical industries. Enzymes in microorganisms and tissues metabolize EO constituents by adding mainly oxygen function to molecules to render them water soluble to facilitate their metabolism. This is also seen as a means of detoxification for these organisms. Many interesting and valuable novel chemicals are biosynthesized by this way. These products are also considered as natural since the substrates are natural[4].

The EO industry is highly complex and fragmented and the trade of EOs is rather conservative and highly specialized. EOs are produced and utilized in industrialized as well as in developing countries worldwide[4].

Storage and transport of EOs are crucial issues since they are highly sensitive to heat, moisture, and oxygen. Therefore, special precautions and strict regulations apply for their handling in storage and transport[14] .

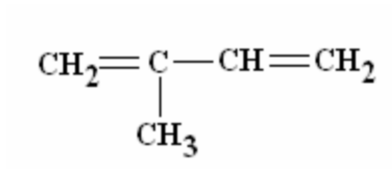
1.6Compostion of essential oils

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[1]:

- Volatile fraction: essential oil which are constituting of 90–95% of the oil in weight and containing the monoterpene and sesquiterpene hydrocarbons, as well as their oxygenated derivatives along with aliphatic aldehydes, alcohols, and esters.
- Nonvolatile residue: that comprises 1–10% of the oil, containing hydrocarbons, fatty acids, sterols, carotenoids, waxes, and flavonoids.

1.6.1Hydrocarbon

Essential oils consist of chemical compounds that have hydrogen and carbon as their building blocks. Basic hydrocarbon found in plants are built of isoprene units having the following structure[1].



(Isoprene)

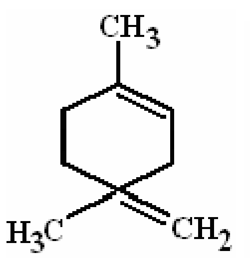
1.6.2 Terpenes

Terpenes are anti-inflammatory, antiseptic, antiviral, and bactericidal. Terpenes can be further categorized in monoterpenes, sesquiterpenes, diterpenes , triterpenes and polyterpenes. 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 diterpenes[1].

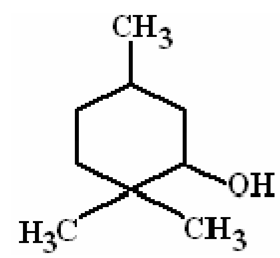
i) Monoterpenes [$C_{10}H_{16}$]

The main properetes of monotepense are analgesic, bactericidal, expectorant, and stimulant.

Monoterpenes are naturally occurring compounds, the majority being unsaturated hydrocarbons (C_{10}). Some of their oxygenated derivatives such as alcohols, ketones, and carboxylic acids are known as monoterpenoids.Examples are limonene and menthol[1].



Limonene



Menthol

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.Some of these being linear derivatives (geraniol, citronellol). The monoterpenes can be monocyclic like camphor – bicyclic like pinenes (**α** and **β**) or tricyclic . 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[1].

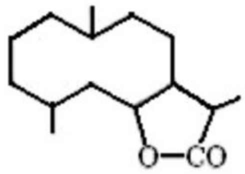
ii) Sesquiterpenes

The main properties of sesquiterpenes are anti-inflammatory, anti-septic, analgesic, anti-allergic.

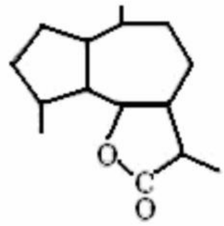
Sesquiterpenes are biogenetically derived from farensyl pyrophosphate and their structure may be linear, monocyclic or bicyclic. They constitute a very large group of secondary metabolites, some have been shown to be stress compounds formed as a result of disease or injury.

Over 500 sesquiterpene lactones 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.

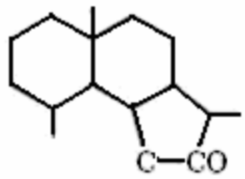
Chemically these lactones can be classified according to their carboxylic skeletons; thus, from the germacranolides can be derived the guaianolides, pseudoguaianolides, eudesmanolides, eremophilanolides, xanthanolides, etc[1].



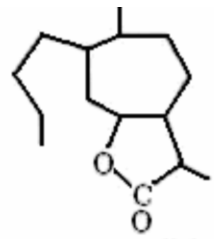
Germacranolides



Guaianlides



Eudesmanolides



Xantnanolides

iii) **Diterpene**

The main properties of diterpense are an 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 almost 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 [1].

iv) Alcohols

The main properties of alcohols are an antiseptic, antiviral, bactericidal and germicidal.

Alcohols are the compounds which contains hydroxyl function. Alcohols exist naturally, either as a free compound, or combined with a terpenes or ester. When the terpene is monoterpene, the resulting alcohol is called a monoterpenol. Alcohols have a very low or totally absent toxic reaction in the body or on the skin. Therefore, they are considered safe to use[1].

v) Aldehydes

The main properties of plant aldehydes are anantifungal, anti-inflammatory, anti-eptic, antiviral, bactericidal, disinfectant, sedative. Medicinally, essential oils containing aldehydes are effective in treating *Candida* and other fungal infections[1].

vi)Acids

The main properties of plant acids are anti-inflammatory.

Organic acids may occur in their free state or are generally found in very small quantities within essential oils. Plant acids act as components or buffer systems to control acidity[1].

vi) Esters

Esters are formed through the reaction of alcohols with acids. Essential oils containing esters are used for their soothing, and balancing effects. 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[1].

vii) Ketones

The main properties of plant ketones are anti-catarrhal, cell proliferant and expectorant.

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[1].

viii) Lactones

The main properties of plant lactone are antiinflammatory, antiphlogistic, expectorant and 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 than ketones[1].

1.7Extraction of natural products

Extraction is the first step to separate the desired natural products from the raw materials. Extraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle. Solvent extraction is the most widely used method.

The extraction of natural products progresses through the following stages:

(i) the solvent penetrates into the solid matrix.

(ii) the solute dissolves in the solvents.

(iii) the solute is diffused out of the solid matrix.

(iv) the extracted solutes are collected.

Any factor enhancing the diffusivity and solubility in the above steps will facilitate the extraction. The properties of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ratio, the extraction temperature and the extraction duration will affect the extraction efficiency[14].Methods such as supercritical fluid extraction (SFC), pressurized liquid extraction (PLE) and microwave assisted extraction (MAE), have also been applied in natural products extraction, and they offer some advantages such as lower organic solvent consumption, shorter extraction time and higher selectivity. Some extraction methods, however, such as sublimation, expeller pressing and enfleurage are rarely used in current phytochemical investigation Extraction efficiency increases with the increase in extraction duration in a certain time range. Increasing time will not affect the extraction after the equilibrium of the solute is reached inside and outside the solid material.

The greater the solvent-to-solid ratio is, the higher the extraction yield is; however, a solvent-to-solid ratio that is too high will cause excessive extraction solvent and requires a long time for concentration. The conventional extraction methods, including maceration, percolation

usually uses and reflux extraction usually use organic solvents and require a large volume of solvents and long extraction time.

i) Maceration

This is a very simple extraction method with the disadvantage of long extraction time and low extraction efficiency. It could be used for the extraction of thermo-labile components[15].lower temperature is applied in maceration with nearly identical extraction yields, which can be translated into economic benefit[16].

ii) Percolation

Percolation is more efficient than maceration because it is a continuous process in which the saturated solvent is constantly being replaced by fresh solvent[17] .

iii) Reflux extraction

Reflux extraction is more efficient than percolation or maceration and requires less extraction time and solvent. It cannot be used for extraction of thermolabile natural products[18] .

iv) Soxhlet extraction

The Soxhlet extraction method integrates the advantages of the reflux extraction and percolation, which utilizes the principle of reflux and siphoning to continuously extract the herb with fresh solvent. The Soxhlet extraction is an automatic continuous extraction method with high extraction efficiency that requires less time and solvent consumption[19] .

v) Pressurized liquid extraction (PLE)

(PLE)applies high pressure in extraction such pressure keep solvents in a liquid state above their boiling point resulting in a high solubility and high diffusion rate of lipid solutes in the solvent, and a high penetration of the solvent in the matrix.

Pressurized liquid extraction has been successfully applied by the researchers in extracting many types of natural products including saponins, flavonoids and essential oil from[20-22].

vii) **Supercritical fluid extraction (SFE)**

Supercritical fluid extraction (SFE) uses supercritical fluid (SF) as the extraction solvent. SF has similar solubility to liquid and similar diffusivity to gas, and can dissolve a wide variety of natural products. Their solvating properties dramatically changed near their critical points due to small pressure and temperature changes. Supercritical carbon dioxide (S-CO_2) is widely used in SFE because of its attractive merits such as low critical temperature (31 °C), selectivity, inertness, low cost, non-toxicity, and capability to extract thermally labile compounds. The low polarity of S-CO_2 makes it ideal for the extraction of non-polar natural products such as lipid and volatile oil. A modifier may be added to S-CO_2 to enhance its solvating properties significantly[23].

vii) Microwave assisted extraction (MAE)

Microwaves generate heat by interacting with polar compounds such as water and some organic components in the plant matrix following the ionic conduction and dipole rotation mechanisms. The transfer of heat and mass are in the same direction in MAE, which generates a synergistic effect to accelerate extraction and improve extraction yield[24,25] .

viii) Ultrasound assisted extraction (UAE)

Ultrasonic-assisted extraction (UAE), also called ultrasonic extraction or sonication, uses ultrasonic wave energy in the extraction, dissolution and diffusion of the solute as well as the heat transfer, which improves the extraction efficiency. The other advantage of UAE includes low solvent and energy consumption, and the reduction of extraction temperature and time[26,27] .

1.8*Pimpinella anisum* (Anise)

Pimpinella anisum **L**. is an annual herb and a grassy plant with white flowers and a small green to yellow seed. The plant is self fertile; prefer light sandy and medium loamy and well- drained soil. When threshed out, the fruit or the so- called seed (part used) may be easily dried in trays, in a current of air in half-shade, out-of-doors, or by moderated heat. The taste is sweet and spicy, and the odour aromatic and agreeable[28]. The plant is indigenous to Near East and widely cultivated in Mediterranean rim (Turkey, Egypt, Syria, Spain, etc.) and in Mexico and Chile. It has been used as an aromatic herb and spice since Egyptian times antiquity and has been cultivated throughout Europe[29].

***Pimpinella anisum* seeds**

Pimpinella anisum seeds

Chemical studies have demonstrated that *Pimpinella anisum* seed contain trans-anethole as the main compound (80-95%) or more[30], estragole[31], eugenole[32], pseudoisoeugenol[33], methylchavicol and anisaldehyde[34], terpene hydrocarbons[35] , polyenes and polyacetylenes[36] as the major components. An unusual compound is the phenol ester 4-methoxy2-(1-propene-yl)-phenol-2-methyl-butyrate, which is characteristic for anise (5%).

In folk medicine, anise is used as an appetizer, tranquillizer and diuretic drug and it has been reported that it has several therapeutic effects on several conditions such as digestive, gynaecological, neurologic and respiratory disorders[12] .

The essential oil is used as an expectorant, carminative and in cough mixtures especially in pediatrics, and the important phenyl-propane, such as trans-anethole and estragole, have a stabilizing influence on the autonomic nervous system[29] .

Anise (*Pimpinella anisum L.*) (family: *Umbelliferae*) is native to the Mediterranean region[1]. The major production area in Sudan is northern Sudan, while there is a very limit production in Khartoum state[37] .Anise seed contains 1.5-5% essential oil and is used as flavouring, digestive, carminative, and in relief of gastrointestinal spasms. Consumption of anise in lactating women increases milk and also reliefs their infants from gastrointestinal problem[38] .

The composition of anise varies considerably with origin and cultivation method. Here are typical values for the main constituents; moisture: 9-13%, protein: 18%, fatty oil: 8-23%, essential oil , crude fibre: 12-25%.[6] Essential oil yielded by distillation is generally around 2-3% and anethole makes up 80-90% of the oil[39] . Anethole is a phytoestrogens[40] .

Plants are known to produce a high number of naturally occurring secondary metabolites, many of them with unique pharmacological activities. These metabolites include the flavonoids, phenolic acids, and tannins[41]. Owing to their versatile biological and pharmacological properties, food, nutrition, cosmetics, and pharmaceutical industries have found many applications for these compounds in the production of functional foods, nutritional composites, personal care products, and medicines[42,43].Therefore, modern plant biotechnological tools were used to maximize the production of these secondary metabolites via plant cell cultures

Aim of this study

This study was designed to:

-Extract the oil from the medicinally important species *Pempinella anisum.*

-Conduct a GC-MS analysis to identify constituents of the oil.

-Evaluate the antimicrobial potential of the oil.

2Materials and Methods

2.1Plant material

Pimpnella anisum seeds were collected from around Rhyad-Saudi Arabia. The plant was identified and authenticated by direct comparison with a reference herbarium sample.

2.2Instruments

A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.3Test organisms

Test organisms used in this study are: *Bacillus subtilis (*G+ve) ,*Staphylococcus aureus(*G+ve), *Pseudomonas aeroginosa* (G-ve) ,*Escherichia coli* (G-ve) and *Candida albicans* (fungus).

2.4Extraction of oil

Powdered seeds of *Pimpnella anisum* (300g) were macerated with n-hexane for 48h.The solvent was removed under reduced pressure giving the oil.

2.5Gachromatography-Mass spectrum analysis

(2ml) of the oil was mixed thoroughly with 7mLof alcoholic sodium hydroxide that was prepared by dissolving 2 g in 100 mL methanol. (7 mL) . Alcoholic sulfuric acid (1ml H_2SO_4 in 100 mL methanol) was then added. The mixture was shaked for 5 minutes .The content of the test tube was left to stand overnight. Then (1mL) of supersaturated sodium chloride was added and the tube was shaken for 5 min. (2mL) of normal hexane were added and the contents were shaken thoroughly for 5 minutes. (5 µL) of the n-hexane were diluted with (5mL) of diethyl ether and dried over anhydrous sodium sulphite . (1µL) of the diluted sample was injected in the GC.MS vial.

The qualitative and quantitative analysis of the sample was carried out by using a Shimadzu machine- model (GC/MS-QP2010-Ultra) The sample was injected under the following chromatographic conditions: column oven temperature :150.0^{o}C ; injection temperature:300.0^{o}C ;injection mode : split; flow mode: linear velocity; pressure:139KPa; total flow: 50.0ml/min ; column flow:1.54ml/sec. ; linear velocity: 47.2cm/sec. ;purge flow:3.0 ml/min. ; split ratio: -1.0. Oven temperature program is presented Table 1

Table 1: Oven temperature program

Rate	Temperature(^{o}C)	Hold Time (min.$^{-1}$)
-	150.0	1.00
4.00	300.0	0.00

2.6-Antimicrobial assay

The paper disc diffusion method was used to screen the antibacterial activity of the oil and performed by using Mueller Hinton agar (MHA). 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 (Whitman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µL of a solution of test sample. The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured and recorded as average of two replicates.

3-Results and Discussion

3.1- Gas chromatography-Mass spectrum analysis

Pimpnella anisum oil was analyzed by GC-MS analysis. Retention times and the observed fragmentation pattern were used for identification of constituents. Ten constituents were identified by GC-MS analysis. The typical total ion chromatogram (TIC) is outlined in Fig. (1) and the constituents of the oil are presented in Table 2 .

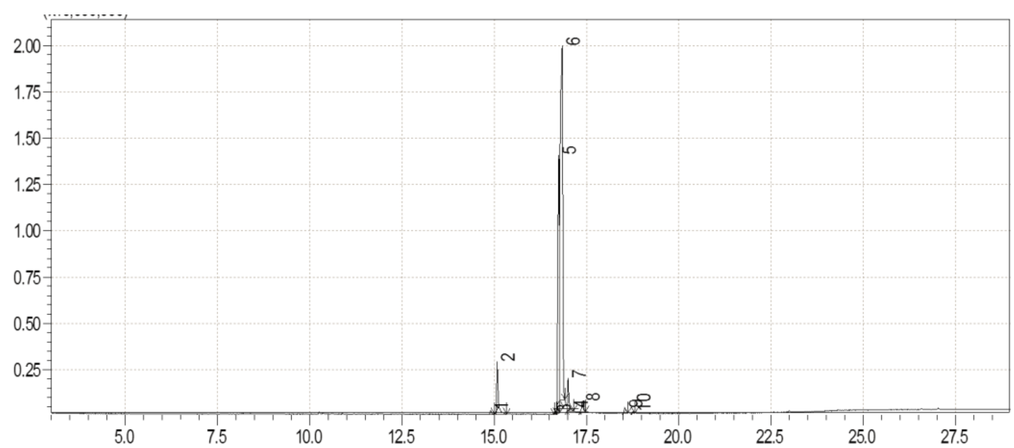


Fig. 1: Total ions chromatograms

Table 2: Constituents of *Pimpnella anisum* oil

No.	Name	Ret.Time	Area%
1.	9-Hexadecenoic acid, methyl ester, (Z)-	14.902	3.64
2.	Hexadecanoic acid, methyl ester	15.082	2.62
3.	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester	16.605	10.64
4.	6,9-Octadecadienoic acid, methyl ester	16.651	0.66
5.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	16.746	0.34
6.	9-Octadecenoic acid (Z)-, methyl ester	16.843	14.21
7.	Methyl stearate	17.004	0.07

8.	Ethyl Oleate	17.385	0.52
9.	cis-11-Eicosenoic acid, methyl ester	18.536	1.76
10	Eicosanoic acid, methyl ester	18.759	0.15

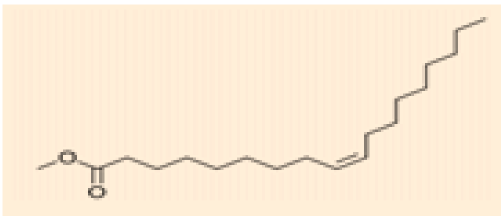
3.2-Major constituents

The following components appeared in total ions chromatogram as major constituents:

i)9-Octadecenoic acid methyl ester(14.21%).

ii)Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester(10.64%).

The mass spectrum of 9-octadecenoic acid methyl ester is shown in Fig.2.The peak at m/z296 (at R.T. 18.319) corresponds $M^+[C_{19}H_{36}O_2]^+$. The signal at m/z266 corresponds to loss of a methoxyl .Fig. 3 illustrates the mass spectrum of cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester. The signal at m/z334 (RT. 16.605) is due to the molecular ion : $M^+[C_{22}H_{38}O_2]^+$.



9-octadecenoic acid methyl ester

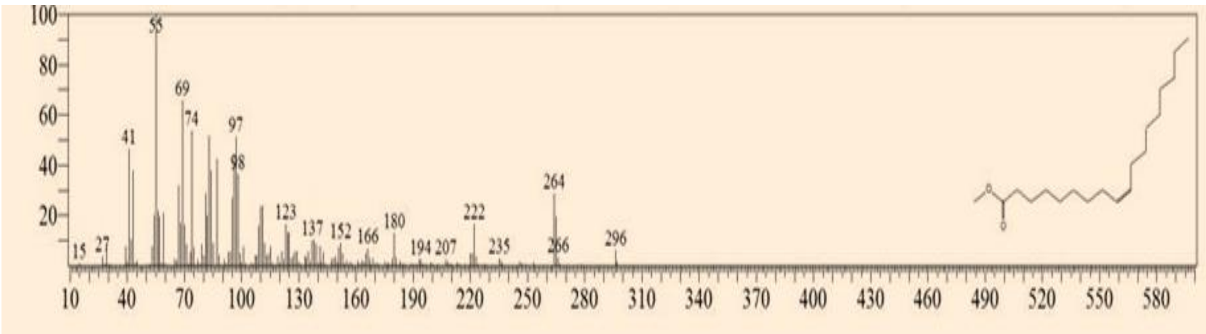
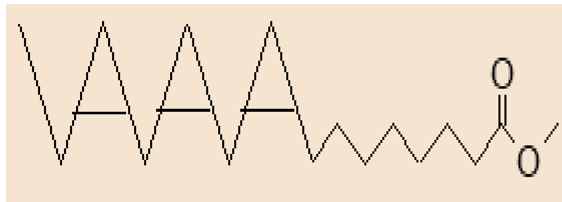


Fig. 2: Mass spectrum of 9-octadecenoic acid methyl ester



Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester

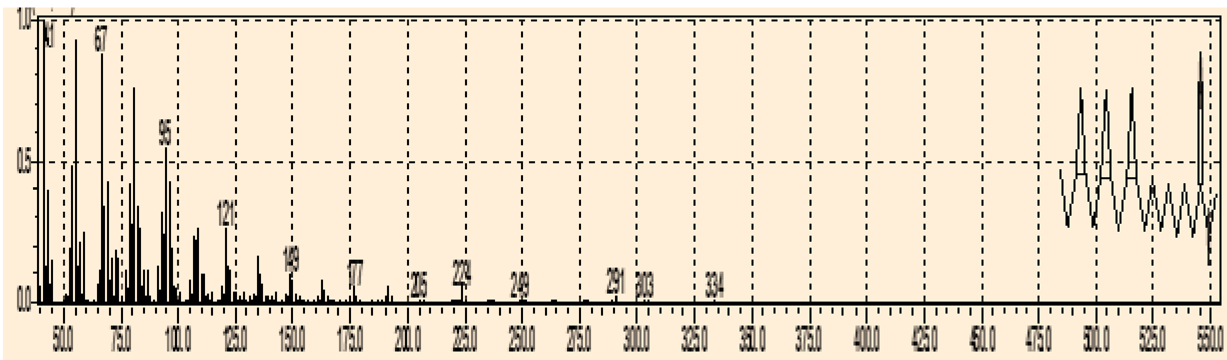


Fig.3: mass spectrum of cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester

3.3-Antimicrobial activity of the oil

Pimpnella anisum oil was evaluated for antimicrobial activity against five standard microbial isolates. The diameters of the growth of inhibition zones are shown in Table 3. Results were interpreted according to the following data :(< 9mm: inactive; 9-12 mm : partially active ; 13- 18 mm : active ; >18mm : very active). *Pimpnella anisum* oil showed partial activity against G+ve *Bacillus subtilis* and *Escherichia coli*. Ampicilin, gentamicin and clotrimazole were used as positive controls(Tables 4 and 5) .

Table 3: Inhibition zones(mm) of *Pimpnella anisum* oil

Sample	Bs	Sa	Ps	Ec	Ca
Oil(100mg/mL)	10	-	7	9	7

Table 4：Table 2: Inhibition zones(mm) of standard antimicrobial drugs

Drug	Conc. (mg/mL)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 5：Table 2: Inhibition zones(mm) of standard antifungal agent

Drug	Conc. (mg/mL)	An	Ca
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

Conclusion

Pimpnella anisum oil was analyzed by GC-MS which revealed the presence of two major constituents: 9- octadecenoic acid methyl ester(14.21%) and cyclopropaneoctanoic acid 2-[2-(2-ethylcyclopropyl)methylcyclopropyl] methyl ester(10.64%).

The oil was evaluated for its antimicrobial potential. It showed moderate activity against *Bacillus subtilis* and *Escherichia coli*.

Recommendations

The following is recommended:

-The extracted oil may be assessed for other biological activities including antimalarial and antiviral activities.

-Other biologically interesting secondary metabolites included in this plant may be isolated and identified. Also their biological activity may be investigated.

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