بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduates Studies

GC- MS Studies on *Spinacia oleracea* Seed Oil

دراسة زيت بذور نبات سبانخ بتقنية كروماتغرافيا الغاز – طيف الكتلة

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

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Oct.,2016

بسم الله الرحمن الرحيم

وَإِن كُنتُمْ فِي رَيْبٍ مِّمَّا نَزَّلْنَا
عَلَىٰ عَبْدِنَا فَأْتُوا بِسُورَةٍ مِّن
مِّثْلِهِۦ وَٱدْعُوا شُهَدَآءَكُم مِّن دُونِ
ٱللَّهِ إِن كُنتُمْ صَٰدِقِينَ

Dedication

To the soul of my father,

My beloved mother,

And to my brothers and sisters.

Acknowledgement

I would like to thank Allah Almighty for giving me strength to do this work.

And to express my deepest thanks and gratitude to my supervisor Prof. Mohammed Abd Alkarim, Professor of chemistry at Sudan University of Science and Technology for his valuable guidance and continuous supervision and support till completion this work.

Also I would like to thank University of Medical Science and Technology laboratories for carrying out the GC-MS analysis, and university of Khartoum for the chemical analysis.

Special thanks for my company, TABUK Pharmaceuticals Company staff for their great support.

Abstract

In this study fixed oil from *Spinacia oleracea* seed was extracted using hexane as solvent, the percentage of the oil was(4.7%). The oil was studied by GC-MS . 24 components were identified, the main components were: Linoleic acid (38.5%) Oleic acid (24.9%), and Palmitic acid (16.7%).

Some physicochemical properties were measured : peroxide number (10.4), acid value (22.1), density (0.93), refractive index (1.48). The oil was screened for antimicrobial activity against six standard human pathogens and promising results were obtained.

الخلاصة

في هذه الدراسة تم استخلاص زيت من بذور نبات السبانخ باستخدام تقنية الاستخلاص بالمذيب (نورمال هكسان) ووجد ان نسبة الزيت كانت 4.7%. وتم دراسة الزيت باستخدام تقنية كروموتوغرافيا الغاز – طيف الكتلة. كانت المركبات الأساسية المكونة للزيت هي حمض اللينيولك (38.5%) وحمض الاوليك (24.9%) وحمض البالمتيك (16.7%).

بعض الخواص الفيزيوكيميائية تم قياسه الرقم البيروكسيدي (10.4%) والرقم الهيدروجيني (22.1%) والكثافة (0.93%) معامل الإنكسار (1.84%).

أيضاً تم قياس النشاطية المضادة للميكروبات وتم التحصل على نتائج مرضية.

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Introduction

1. General approach

Human kind has used plants for healing for many thousands of years, and it is from this tradition that the use of aromatic plant compounds in medicine began. Oils were used in the treatment of human disorders since time immemorial. There are also over 200 references to aromatics and ointments in the old and new testaments; noted for being used for healing of the sick.

Centuries of practical use of oils have been confirmed, and we now know that the 'fragrant pharmacy' contains compounds with an extremely broad range of biochemical effects. There are about three hundred essential oils in general use today by professional practitioners. With the continual bombardment of viral, bacterial, parasitic and fungal contamination in our world, oils are a great benefit to help protect our bodies and homes from this onslaught of pathogens. Immune systems need support and oils can give it. Oils have long been revered for their healing and aromatherapy properties. What many people do not realize is that several oils kill bacteria, fungi, and viruses. This means that these valuable oils not only can fight and prevent infection in your body, but they also prevent illness, treat skin conditions, disinfect the surfaces of your home, and eliminate microbes floating in the air of your environment.

Because of the enormous amount of raw product used to make wholly natural oils, lots of products on the market have been polluted with lower quality, commercial – grade oils or contain other chemical substances to reduce the cost or increase the profit margin – a fact not usually revealed on the label. This is why it is important to study the chemical composition of the natural oil extracted. This is characterized by the complexity in the separation of its components, which belong to various classes of compounds and which are present in a wide range of concentrations. Therefore it is complicated to establish a composition profile of the oils. The gas chromatographic combined with mass spectroscopy method (GC-MS) is almost exclusively used for the qualitative analysis of oils. Also study of various physicochemical characteristics explores the practical importance of herbal oils in daily life. Physicochemical properties of oil like color, odor, peroxide value, density, specific gravity, refractive index, optical rotation, acid value, iodine value, saponification value etc indirectly influence the quality of both essential and fixed oils. The commercial importance of oils mostly depends on these physicochemical properties, which provide baseline data to determine its suitability for consumption.

1.2- Spinach (*Spinacia oleracea*)

Spinach (*Spinacia oleracea*) is an edible flowering plant in the family Amaranthaceae native to central and western Asia[1]. It is an annual plant (rarely biennial), which grows up to 30 cm tall. Spinach may survive over winter in temperate regions. The leaves are alternate, simple, and ovate to triangular and very variable in size from about 2–30 cm long and 1–15 cm broad, with larger leaves at the base of the plant and small leaves higher on the flowering stem. The flowers are inconspicuous, yellow-green, 3–4 mm in diameter, maturing into a small, hard, dry, lumpy fruit cluster 5–10 mm across containing several seeds[1].

Spinach is considered a super-food due to its high nutritional value. First, spinach contains a high amount of beta-carotene, the inactive stage of vitamin A. Beta-carotene is also found in many other leafy greens and yellow, red, and orange fruits and vegatables, and it contributes to good vision, healthy skin, a strong immune system, and reproductive system health. When beta-carotene is converted to vitamin A, more moisture is retained in the skin to keep it looking smooth and young[1].

Another important group of ingredients found in spinach is antioxidants. Antioxidants are believed to reduce the risk of getting cancer, heart disease and premature blindness. In spinach, vitamin E, vitamin C, zinc, beta-carotene, selenium, and manganese are all powerful antioxidants[1].

Spinacia oleracea plant and seeds

1.3- Oils

An oil is any neutral, nonpolar chemical substance that is a viscous liquid at ambient temperatures and is both hydrophobic (immiscible with water, literally "water fearing") and lipophilic (miscible with other oils, literally "fat loving"). Oils have a high carbon and hydrogen content and are usually flammable and slippery[2].

The general definition of oil includes classes of chemical compounds that may be otherwise unrelated in structure, properties, and uses. Oils may be animal, vegetable, or petrochemical in origin, and may be volatile or non-volatile. They are used for food, fuel, lubrication, and the manufacture of paints, plastics, and other materials. Specially prepared oils are used in some religious ceremonies as purifying agents. Organic oils are produced in remarkable diversity by plants, animals, and other organisms through natural metabolic processes. Lipid is the scientific

term for the fatty acids, steroids and similar chemicals often found in the oils produced by living things, while oil refers to an overall mixture of chemicals. Organic oils may also contain chemicals other than lipids, including proteins, waxes (class of compounds with oil-like properties that are solid at common temperatures) and alkaloids[2].

Lipids can be classified by the way that they are made by an organism, their chemical structure and their limited solubility in water compared to oils. They have a high carbon and hydrogen content and are considerably lacking in oxygen compared to other organic compounds and minerals; they tend to be relatively nonpolar molecules, but may include both polar and nonpolar regions as in the case of phospholipids and steroids[2].

1.4- Fixed oils

Fixed oils are often extracted from vegetable origin, such as nuts or seeds of the botanical source. Seed oil is a vegetable oil that is obtained from the seed (endosperm) of some plants, rather than the fruit (pericarp)[3].

Most vegetable oils are seed oils. Some common example is sunflower. Fixed oils contain a lot of helpful nutrients such as minerals, antioxidants, and fat- soluble vitamins. The type of nutrient you acquire depends on the type of fixed oil used. Most fixed oils lack their own scent or aroma. And if they do contain such aromas, it is usually very

subtle. This quality is important since fixed oils are often combined with essential oils during aromatherapy application to avoid competing with the latter's aroma oil, canola oil, and sesame oil. Fixed oils are also commonly referred to as vegetable or base oils. Despite the application on other industries, it is generally limited to the practice of aromatherapy. This is due to the properties contained in fixed oils that enable them to provide natural skin care treatment. There are various methods undergone in order to extract the fatty portion of the plant to be used as fixed or carrier oil in aromatherapy. However, experts suggest using the cold pressed method since it is the one that would enable the preservation of the oil quality due to the lack of heat element. Fixed oils play an important role in aromatherapy. However, it is also largely used in other industries such as food, toiletries, or when making aromatherapy oil blends. In aromatherapy, fixed oils are combined with essential oils so it is safe to use during massage or any method of application that involved direct skin contact[3].

1.5- Aromatherapy

Aromatherapy is the use of aromatic essential oils combined with fixed oils to benefit the body , for example, the treatment of anxiety or minor medical conditions by rubbing pleasant smelling natural oils into the skin or breathing in their smell. Fixed oils act as “carrier oil” in aromatherapy that will enable the properties of the essential oil to be easily absorbed by your body and produce the healing action. In

addition to that, carrier oils are important in diluting the concentration of the essential oil to avoid developing harsh reactions. Science has discovered that our sense of smell plays a significant role in our overall health. Many common essential oils have medicinal properties that have been applied in medicine since ancient times and are still widely used today. For example, many essential oils and fixed oils have antiseptic properties, though some are stronger than the other. In addition, many have an uplifting effect on the mind, though different essential oils have different properties. An essential oil is inhaled directly by the olfactory system to the limbic system of the brain. In true, the brain responds to the particular scent affecting our emotions and chemical balance. Essential oils are also absorbed by the skin and carried throughout the body via the circulatory system to reach all internal organs[4].

1.6- Essential Oils

Essential oils (volatile oils) are the most important group of chemical molecules of plants that make smells what they are. The origin of these names comes from the word "essence," because the fragrances are the essence of many plants, and "volatile" because of their volatility. Volatile oils contain one or two hundred different carbon- and hydrogen-based compounds called terpenes or hydrocarbons. Each volatile oil is made up of a unique blend of up to one hundred different terpenes, which like an artist's palette, gives the plant the ability to build unique

essential oils each with their biological activity and mood- and emotion-affecting properties. Essential oils are not true oils like almond oil, olive oil, or flaxseed oil — those are called fixed oils. Fixed oils don't vaporize the way essential oils do, and they are much heavier[5].

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. Essential oils are also known as volatile oils, ethereal oils, aetherolea, or simply as the oil of the plant from which they were extracted.

Essential oils are generally extracted by distillation, often by using steam, solvent extraction, absolute oil extraction, resin tapping, and cold pressing. They are used in perfumes, cosmetics, soaps and other products, for flavoring food and drink, and for adding scents to incense and household cleaning products[5].

Essential oils have been used medicinally in history. Medical applications proposed by those who sell medicinal oils range from skin treatments to remedies for cancer and often are based solely on historical accounts of use of essential oils for these purposes. Claims for the efficacy of medical treatments, and treatment of cancers in particular, are now subject to regulation in most countries[7] .

1.6.1- Usage of essential oils

The most effective way to use most essential oils is by external application or inhalation, though some can be very beneficial when

taken internally. The use of essential oils include body oils, compresses, cosmetic lotions, baths, hair rinses, inhalation by steam, perfumes and room sprays. Essential oils are very potent - some will cause skin irritation or have other harmful effects if not used properly. Unless specifically noted, it is best to dilute all essential oils in a carrier of base oil like almond, jojoba or apricot kernel before applying to the skin - appropriate dilution is usually only 1 - 10% essential oil in carrier. For inhalation, a diffuser or oil lamp is effective for releasing essential oils into your environment - a very pleasant way of creating a particular atmosphere[8].

1.6.2- Pharmacological Properties of Essential Oils

Essential oils have antiseptic properties and are active against a wide range of bacteria . Moreover, they are also known to be active against fungi and yeasts (*Candida*).The most common sources of essential oils used as antiseptics are: cinnamon, thyme; clover; eucalyptus; culin savory; lavender; citral, geraniol, linalool and thymol**.**When used externally, essential oils like (L'essence de terebenthine) increase microcirculation and provide a slight local anesthetic action. Till now, essential oils are used in a number of ointments, cream and gels, whereby they are known to be very effective in relieving sprains and other articular pains. Oral administration of essential oils like eucalyptus or pin oils, stimulate ciliated epithelial cells to secrete mucus. On the renal system, these are

known to increase vasodilation and in consequence bring about a diuretic effect.

Essential oils from the Umbellifereae family and specially *Mentha* species are reputed to decrease or eliminate gastrointestinal spasms. These essential oils increase secretion of gastric juices. In other cases, they are known to be effective against insomnia. ([21])

1.7- Usage of Fixed Oils

Many vegetable oils are consumed directly, or indirectly as ingredients in food – a role that they share with some animal fats, including butter, ghee, lard, and Schmaltz. The oils serve a number of purposes in this role:

-Shortening; to give pastry a crumbly texture.

-Texture; oils can serve to make other ingredients stick together less.

-Flavor; while less-flavorful oils command premium prices, some oils, such as olive, sesame, or almond oil, may be chosen specifically for the flavor they impart.

-Flavor base; oils can also "carry" flavors of other ingredients, since many flavors are due to chemicals that are soluble in oil.

Fixed oils can be heated and used to cook other foods. Oils suitable for this objective must have a high flash point. Such oils include the major cooking oils.

Vegetable oils are used as an ingredient or component in many manufactured products. Many vegetable oils are used to make soaps,

skin products, candles, perfumes and other personal care and cosmetic products. Some oils are particularly suitable as drying oils, and are used in making paints and other wood treatment products. Vegetable oils are increasingly being used in the electrical industry as insulators as vegetable oils are not toxic to the environment, biodegradable if spilled and have high flash and fire points. However, vegetable oils are less stable chemically, so they are generally used in systems where they are not exposed to oxygen, and they are more expensive than crude oil distillate. Synthetic tetra esters, which are similar to vegetable oils but with four fatty acid chains compared to the normal three found in a natural ester, are manufactured by Fischer esterification. Tetra esters generally have high stability to oxidation and have found use as engine lubricants. Vegetable oil is being used to produce biodegradable hydraulic fluid and lubricant. Vegetable-based oils, like castor oil, have been used as medicine and as lubricants for a long time. Castor oil has numerous industrial uses, primarily due to the presence of hydroxyl groups on the fatty acid chains. Castor oil, and other vegetable oils which have been chemically modified to contain hydroxyl groups, are becoming increasingly important in the production of polyurethane plastic for many applications. These modified vegetable oils are known as natural oil polyols[11].

Vegetable oils are also used to make biodiesel, which can be used like conventional diesel. Some vegetable oil blends are used in unmodified

vehicles but straight vegetable oil, also known as pure plant oil, needs specially prepared vehicles which have a method of heating the oil to reduce its viscosity. The use of vegetable oils as alternative energy is growing and the availability of biodiesel around the world is increasing[11].

Vegetable oil is used in production of some pet foods. AAFCO defines vegetable oil, in this context, as the product of vegetable origin obtained by extracting the oil from seeds or fruits which are processed for edible purposes. In some poorer-grade pet foods, the oil is listed only as "vegetable oil", without specifying the particular oil[11].

1.8- Negative health effects of fixed oils

Hydrogenated oils have been shown to cause what is commonly termed the "double deadly effect", raising the level of LDLs and decreasing the level of HDLs in the blood, increasing the risk of blood clotting inside blood vessels. A high consumption of oxidized polyunsaturated fatty acids (PUFAs), which are found in most types of vegetable oils (e.g. soybean oil, corn oil – the most consumed in USA, sunflower oil, etc.) may increase the likelihood that postmenopausal women will develop breast cancer. A similar effect was observed on prostate cancer and skin cancer in mice. Due to their susceptibility to oxidation from the exposure to oxygen, heat and light, resulting in the formation of oxidation products such as inflammatory peroxides and hydroperoxides,

plant oils rich in polyunsatured fatty acids have a limited shelf-life and regular consumption of rancid oils (from prolonged storage or overheating) may have negative health consequences[12].

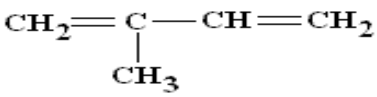
1.9- Antimicrobial Oils

An antimicrobial is an agent that kills microorganisms or inhibits their growth[13]. Plants and their derivatives, such as essential oils, are often used in folk medicine. In nature, essential oils play an important role in the protection of plants. Essential oils contain a wide variety of secondary metabolites that are capable of inhibiting or slowing the growth of bacteria, yeasts and molds. Essential oils and their components have activity against a variety of targets, particularly the membrane and cytoplasm, and in some cases, they completely change the morphology of the cell[14]. The antimicrobial activity of essential oils, similar to all natural extracts, is dependent on their chemical composition and the amount of the single components. Many of the antimicrobial compounds are constitutively expressed by the plants, and others can be synthesized as mechanism of self-defense in response to pathogens. Vegetables, spices and fruits with high level of essential oils are excellent sources of natural elements with activity against microorganisms[15]. These molecules can be naturally present in their active form in the plant or can be activated by specific enzymes when the vegetal organism is subjected to particular biotic or abiotic stress[16]

1.10- Chemical constituents of oils

Fixed oils contain nonvolatile substances ,hydrocarbons, fatty acids, sterols, carotenoids, waxes, and flavonoids[17].

Essential oils consist of compounds that have hydrogen and carbon as their building blocks. Basic hydrocarbon found in plants is isoprene having the following structure[17].



Isoprene

1.10.1- Fatty acids

A fatty acid is a carboxylic acid with a long aliphatic chain, which is either saturated or unsaturated. Most naturally occurring fatty acids have an unbranched chain of an even number of carbon atoms, from 4 to 28. Fatty acids are usually derived from triglycerides or phospholipids. Fatty acids are important sources of fuel because, when metabolized, they yield large quantities of ATP. Many cell types can use either glucose or fatty acids for this purpose[12].

Fatty acids that are required by the human body but cannot be made in sufficient quantity from other substrates, and therefore must be obtained from food, are called essential fatty acids. There are two series of essential fatty acids: one has a double bond three carbon atoms removed from the methyl end; the other has a double bond six carbon atoms removed from the methyl end. Humans lack the ability to

introduce double bonds in fatty acids beyond carbons 9 and 10, as counted from the carboxylic acid side. Two essential fatty acids are: linoleic acid (LA) and alpha-linolenic acid (ALA). They are widely distributed in plant oils. The human body has a limited ability to convert ALA into the longer-chain omega-3 fatty acids — eicosapentenoic acid (EPA) and docosahexaenoic acid (DHA), which can also be obtained from fish[18]. Some saturated fatty acids are displayed in Table 1.1.

Table 1.1 : Some saturated fatty acids

Common Name	**Systematic Name**	**Structural Formula**	**Lipid Numbers**
Propionic acid	Propanoic acid	CH_3CH_2COOH	C3:0
Butyric acid	Butanoic acid	$CH_3(CH_2)_2COOH$	C4:0
Valeric acid	Pentanoic acid	$CH_3(CH_2)_3COOH$	C5:0
Caproic acid	Hexanoic acid	$CH_3(CH_2)_4COOH$	C6:0
Enanthic acid	Heptanoic acid	$CH_3(CH_2)_5COOH$	C7:0
Caprylic acid	Octanoic acid	$CH_3(CH_2)_6COOH$	C8:0
Pelargonic acid	Nonanoic acid	$CH_3(CH_2)_7COOH$	C9:0
Capric acid	Decanoic acid	$CH_3(CH_2)_8COOH$	C10:0
Undecylic acid	Undecanoic acid	$CH_3(CH_2)_9COOH$	C11:0
Lauric acid	Dodecanoic acid	$CH_3(CH_2)_{10}COOH$	C12:0
Tridecylic acid	Tridecanoic acid	$CH_3(CH_2)_{11}COOH$	C13:0
Myristic acid	Tetradecanoic acid	$CH_3(CH_2)_{12}COOH$	C14:0
Pentadecylic acid	Pentadecanoic acid	$CH_3(CH_2)_{13}COOH$	C15:0
Palmitic acid	Hexadecanoic acid	$CH_3(CH_2)_{14}COOH$	C16:0
Common Name	**Systematic Name**	**Structural Formula**	**Lipid Numbers**
Margaric acid	Heptadecanoic acid	$CH_3(CH_2)_{15}COOH$	C17:0
Stearic acid	Octadecanoic acid	$CH_3(CH_2)_{16}COOH$	C18:0
Nonadecylic acid	Nonadecanoic acid	$CH_3(CH_2)_{17}COOH$	C19:0
Arachidic acid	Eicosanoic acid	$CH_3(CH_2)_{18}COOH$	C20:0

Heneicosylic acid	Heneicosanoic acid	$CH_3(CH_2)_{19}COOH$	C21:0
Behenic acid	Docosanoic acid	$CH_3(CH_2)_{20}COOH$	C22:0
Tricosylic acid	Tricosanoic acid	$CH_3(CH_2)_{21}COOH$	C23:0
Lignoceric acid	Tetracosanoic acid	$CH_3(CH_2)_{22}COOH$	C24:0
Pentacosylic acid	Pentacosanoic acid	$CH_3(CH_2)_{23}COOH$	C25:0
Cerotic acid	Hexacosanoic acid	$CH_3(CH_2)_{24}COOH$	C26:0
Heptacosylic acid	Heptacosanoic acid	$CH_3(CH_2)_{25}COOH$	C27:0
Montanic acid	Octacosanoic acid	$CH_3(CH_2)_{26}COOH$	C28:0
Nonacosylic acid	Nonacosanoic acid	$CH_3(CH_2)_{27}COOH$	C29:0
Melissic acid	Triacontanoic acid	$CH_3(CH_2)_{28}COOH$	C30:0
Henatriacontylic acid	Henatriacontanoic acid	$CH_3(CH_2)_{29}COOH$	C31:0
Lacceroic acid	Dotriacontanoic acid	$CH_3(CH_2)_{30}COOH$	C32:0
Psyllic acid	Tritriacontanoic acid	$CH_3(CH_2)_{31}COOH$	C33:0
Geddic acid	Tetratriacontanoic acid	$CH_3(CH_2)_{32}COOH$	C34:0
Ceroplastic acid	Pentatriacontanoic acid	$CH_3(CH_2)_{33}COOH$	C35:0
Hexatriacontylic acid	Hexatriacontanoic acid	$CH_3(CH_2)_{34}COOH$	C36:0
Heptatriacontanoic acid	Heptatriacontanoic acid	$CH_3(CH_2)_{35}COOH$	C37:0
Octatriacontanoic acid	Octatriacontanoic acid	$CH_3(CH_2)_{36}COOH$	C38:0

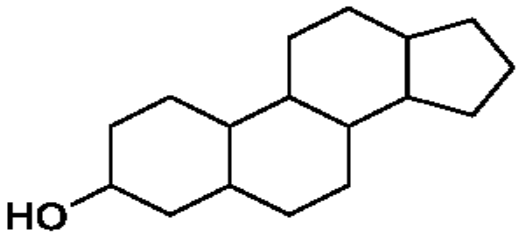
Table 1.2 Examples of Unsaturated Fatty Acids

Common name	**Chemical structure**	**Δx**	**C: D**
Myristoleic acid	$CH_3(CH_2)_3CH=CH(CH_2)_7COOH$	cis-Δ9	14:1
Palmitoleic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	cis-Δ9	16:1
Sapienic acid	$CH_3(CH_2)_8CH=CH(CH_2)_4COOH$	cis-Δ6	16:1
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	cis-Δ9	18:1
Elaidic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	trans-Δ9	18:1
Vaccenic acid	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$	trans-Δ11	18:1
Linoleic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	cis,cis-Δ9,Δ12	18:2
Linoelaidic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	trans,trans-Δ9,Δ12	18:2
α-Linolenic acid	$CH_3(CH_2)CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	cis,cis,cis-Δ9,Δ12,Δ15	18:3
Arachidonic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH2)_3$ COOHNIST	cis,cis,cis,cis-Δ5Δ8,Δ11,Δ14	20:4
Eicosapentaenoic acid	$CH_3(CH_2)CH=CHCH2CH=CHCH2CH=CHCH_2CH=CHCH2C$ H=CH(CH2)$_3$COOH	cis,cis,cis,cis,cisΔ5,Δ8 ,Δ11,Δ14,Δ17	20:5

Erucic acid	$CH_3(CH_2)7CH=CH(CH2)11COOH$	cis-Δ13	22:1
Docosahexaenoic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$	cis,cis,cis,cis,cis,cis-Δ4,Δ7,Δ10,Δ13,Δ16,Δ19	22:6

1.10.2- Sterols

Sterols, also known as steroid alcohols, are a subgroup of the steroids and an important class of organic molecules. They occur naturally in plants, animals, and fungi, with the most familiar type of animal sterol being cholesterol. Sterols of plants are called phytosterol and sterols of animals are called zoosterol. Plant sterols are substances found naturally in vegetables, fruits, nuts, grains and vegetable oils. Phytosterols, more commonly known as plant sterols, have been shown in clinical trials to block cholesterol absorption sites in the human intestine, thus helping to reduce cholesterol in humans[19].



Chemical structure of Sterols

1.10.3- Carotenoids

Carotenoids are the pigments that give fruits and vegetables such as carrots, cantaloupe, sweet potato, and kale their vibrant orange, yellow, and green color. Beta-carotene, lycopene, and lutein are all different

varieties of carotenoids. They all act as antioxidants with strong cancer-fighting properties. Carotenoids are a class of hydrocarbon compounds consisting of 40 carbon atoms (tetraterpenes), with a structure characterized by an extensive conjugated double-bond system that determines the color (it serves as a light-absorbing chromophore): as the number of conjugated double-bond increases, color changes from pale yellow, to orange, to red[20].

1.10.4-Flavonoids

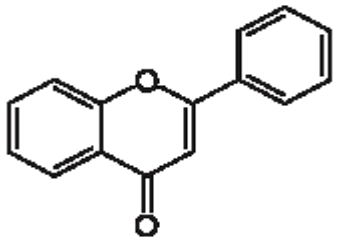
Flavonoids are a group of plant metabolites thought to provide health benefits through cell signaling pathways and antioxidant effects, these molecules are found in a variety of fruits and vegetables. Flavonoids are polyphenolic molecules containing 15 carbon atoms and are soluble in water. Flavonoids are a class of plant and fungus secondary metabolites. Chemically, they have the general structure of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and heterocyclic ring (C). This carbon structure can be abbreviated C6-C3-C6. According to the IUPAC nomenclature, they can be classified into[21]:

i)Flavonoids or bioflavonoids

ii) Isoflavonoids, derived from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure

iii) Neoflavonoids, derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure

The three flavonoid classes above are all ketone-containing compounds, and as such, are anthoxanthins (flavones and flavonols). This class was the first to be termed bioflavonoids. The terms flavonoid and bioflavonoid have also been more loosely used to describe non-ketone polyhydroxy polyphenol compounds which are more specifically termed flavanoids. Research shows that flavonoids are poorly absorbed in the human body (less than 5%), with most of what is absorbed being quickly metabolized and excreted These findings suggest that flavonoids have negligible systemic antioxidant activity, and that the increase in antioxidant capacity of blood seen after consumption of flavonoid-rich foods is not caused directly by flavonoids, but is due to production of uric acid[22].



Flavone backbone (2-phenyl-1, 4-benzopyrone)

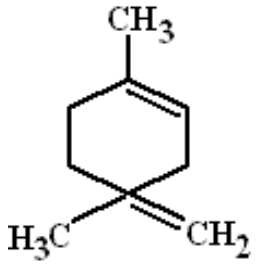
Pure essential oils are mixtures of more than 200 components, normally mixtures of terpenes or phenylpropanic derivatives, in which the chemical and structural differences between compounds are minimal[22].

1.10.5- Terpenes

Terpenes generally have names ending in "ene" For examples: 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 , 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[23].

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

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. Menthol is an example of a terpene with two isoprene units[24].



Menthol

The branched-chain C_{10} hydrocarbons comprises two isoprene units and are widely distributed in nature with more than 400 naturally

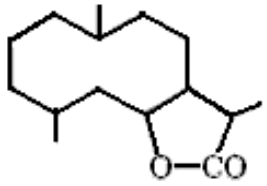
occurring monoterpenes identified. Moreover, besides being linear derivatives (geraniol, citronellol), the monoterpenes can be cyclic molecules (menthol – monocyclic; camphor – bicyclic; pinenes (**α** and **β**). Thujone (a monoterpene) is the toxic agent found in *Artemisia absinthium* (wormwood) from 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 antipyretic[24,25].

ii)Sesquiterpenes

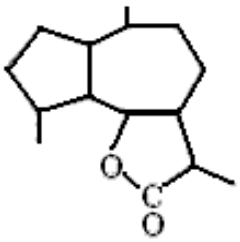
Sesquiterpenes are biogenetically derived from farensyl pyrophosphate and in structure may be linear, monocyclic or bicyclic. They constitute a very large group of secondary metabolites, some having been shown to be stress compounds formed as a result of disease or injury.They are claimed to possess antiinflammatory, antiseptic, antiallergic and analgesic properties.

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.

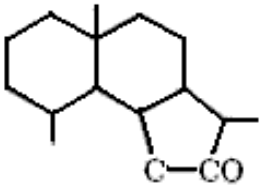
Chemically these compounds can be classified according to their carboxylic skeletons; thus, from the germacranolides can be derived the guaianolides, pseudoguaianolides, eudesmanolides, eremophilanolides, xanthanolides, etc.



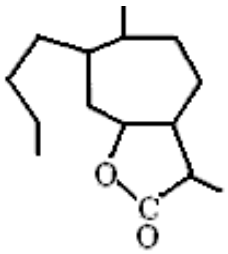
Germacranolides



Guaianlides



Eudesmanolides



Xantnanolides

A structural feature of all these compounds, which appears to be associated with much of the biological activity, is the α, β - unsaturated- γ- lactones.

iii) Diterpenes

Many biological activities were attributed to diterpenes including**:** anti-fungal, expectorant, hormonal balancers, hypotensive. Diterpenes are made of up four isoprene units. These molecules are too heavy to allow for evaporation with steam in the distillation process, so they are rarely

found in distilled essential oils. Diterpenes occur in all plant families. 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.

When terpenes are attached to an oxygen atom, and hydrogen atom, the result is an alcohol. These molecules possess anti-septic, anti-viral, bactericidal and germicidal properties . 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. Terpenols have a very low or totally absent toxic reaction in the body or on the skin. Therefore, they are considered safe to use.

Essential oil may contain a formyl function. Medicinally, essential oils containing aldehydes are effective in treating Candida and other fungal infections.They also possess anti-inflammatory, antiseptic, anti-viral, bactericidal, disinfectant, sedative properties.

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[24].

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

Essential oils may contain ketones 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[25].

Essential oils may contain lactones 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[26].

1.11- Physicochemical characteristics of oil

Physicochemical characteristics provide a baseline for suitability of oils. The physicochemical properties of the oil comprise: color, odor, % yield, density, optical activity, refractive index, specific gravity, carbon residue, absolute viscosity, viscosity index, kinematic viscosity, total acid number, iodine number and saponification value.

Viscosity is a measure of resistance of a fluid to deform under shear stress. It is commonly perceived as thickness, or resistance to pouring. Viscosity describes a fluid's internal resistance to flow and may be thought of as a measure of fluid friction. It determines the rheological proprieties of these oils.

The refractive index is the degree of the deflection of a beam of light that occurs when it passes from one transparent medium to the other. It increases with the length of chains and with the number of carbon atoms present. Therefore, the refractive index determines evidences that the sample might be unsaturated long carbon chain[27] .

The iodine value is a useful tool in predicting the drying properties of oils. The high iodine value of oils indicate the high content of unsaturation and suggests that the oils may be used as drying agent for the manufacturing of oil paints, varnishes, cosmetics and also as cocking oil manufacturing index. The iodine value is also an index of assessing the ability of oil to go rancid. It is also used for determining the level of

oxidative deterioration of the oil by enzymatic or chemical oxidation[28] .

i)**Acid value**

Acid value is an important physicochemical property index of oil which is used to determine the quality, age, edibility and suitability of oil for industrial use such as paint. This value is used to measure the extent of glycerides in the oil, which have been decomposed by lipase and other physical factors such as light and heat[29] .

ii)Saponification value

Saponification value is an index of average molecular mass of various fatty acids in oil samples. The lower value of saponification means molecular weight of fatty acids is lower and has lower limit of use in industry. The saponification value suggests the use of oil in production of liquid soap, shampoos and lather shaving creams[30].

iii)The peroxide value

The peroxide value is defined as the amount of peroxide oxygen per 1 kilogram of fat or oil. Detection of peroxide gives the initial evidence of rancidity in unsaturated fats and oils. Other methods are available, but peroxide value is the most widely used. It gives a measure of the extent to which an oil sample has undergone primary oxidation, extent of secondary oxidation may be determined from p-anisidine test. The double bonds found in fats and oils play a role in autoxidation. Oils with a high degree of unsaturation are most susceptible to autoxidation. The

best test for autoxidation (oxidative rancidity) is determination of the peroxide value. Peroxides are intermediates in the autoxidation reaction. Autoxidation is a free radical reaction involving oxygen that leads to deterioration of fats and oils which form off-flavors and off-odors. Peroxide value, concentration of peroxide in an oil or fat, is useful for assessing the extent to which spoilage has advanced[31].

1.12-Extraction of the oils

Oils can be extracted via two key methods: Distillation (includes hydrodistillation) and Expression. Also, can be extracted via solvent extraction or enfleurage, although enfleurage is rarely performed in nowadays.

i)The Distillation Process

During distillation the plant material is placed upon a grid inside the still. Once inside, the still is sealed, and, depending upon the above methods, steam or water/steam slowly breaks through the plant material to remove its volatile constituents. These volatile constituents rise upward through a connecting pipe that leads them into a condenser. The condenser cools the rising vapor back into liquid form. The liquid is then collected in a vehicle below the condenser. Since water and essential oil do not mix, the essential oil will be found on the surface of the water where it is siphoned off. Occasionally an essential oil is heavier than water and is found on the bottom rather than the top[31].

The three types of distillation include:

ii)Water Distillation

The plant material comes into direct contact with the water. This method is most often employed with flowers (rose and orange blossoms), as direct steam causes these flowers to clump together making it difficult for steam to pass through.

iii)Water and Steam

This method can be employed with herb and leaf material. During this process, the water remains below the plant material, which has been placed on a grate while the steam is introduced from outside the main still (indirect steam)[31].

iv)Steam Distillation

This method is the most commonly used. During this process, steam is injected into the still, usually at slightly higher pressures and temperatures than the above two methods[31].

v)Percolation or Hydrodiffusion

This is a relatively recent method and is very similar to steam distillation except that the steam comes in through the top rather than the bottom, and there is a shorter distillation time. It is useful in extracting essential oils from woody or tough material or seeds.

Hydrosols, also known as hydrolats, are the by-product or product (depending on the distiller purpose) of the distillation process. Hydrosols contain the water-soluble constituents of the aromatic plant and retain a small amount of essential oil. Every liter of hydrosol contains between 0.05 and 0.2 milliliter of dissolved essential oil, depending on the water solubility of the plant's components and the distillation parameters[32].

vi)Expression Extraction

Expression, also referred to as cold pressing, is a method of extraction specific to citrus essential oils, such as tangerine, lemon, bergamot, sweet orange, and lime. In older times, expression was done in the form of sponge pressing, which was literally accomplished by hand. The zest or rind of the citrus would first be soaked in warm water to make the rind more receptive to the pressing process. A sponge would then be used to press the rind, thus breaking the essential oil cavities, and absorb the essential oil. Once the sponge was filled with the extraction, it would then be pressed over a collecting container, and there it would stand to allow for the separation of the essential oil and water/juice. The essential oil would finally be siphoned off[32].

vii)Solvent Extraction

Some plant material is too fragile to be distilled and an alternative method must be employed. Solvent extraction is the use of solvents, such as petroleum ether, methanol, ethanol, or hexane, to extract the

odoriferous lipophilic material from the plant. The solvent will also pull out the chlorophyll and other plant tissue, resulting in a highly colored or thick/viscous extract. The first product made via solvent extraction is known as a concrete. A concrete is the concentrated extract that contains the waxes and/or fats as well as the odoriferous material from the plant. The concrete is then mixed with alcohol, which serves to extract the aromatic principle of the material. The final product is known as an absolute.

After the solvent extraction process has been completed, the resulting absolute will have an extremely low concentration of solvent residue, approximately 5 to 10ppm (parts per million). The current European Union standards are for less than 10 parts per million solvent residues in a finished absolute. However, even with such a potentially small residue (less than .0001%), many aromatherapists disagree with the use of absolutes for individuals with a compromised immune system due to the potential effect of the residual pesticide[33].

1.14- Chromatography

Chromatography from Greek chroma which means "color" and graphein "to write" is the collective term for a set of laboratory techniques for the separation of mixtures. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the

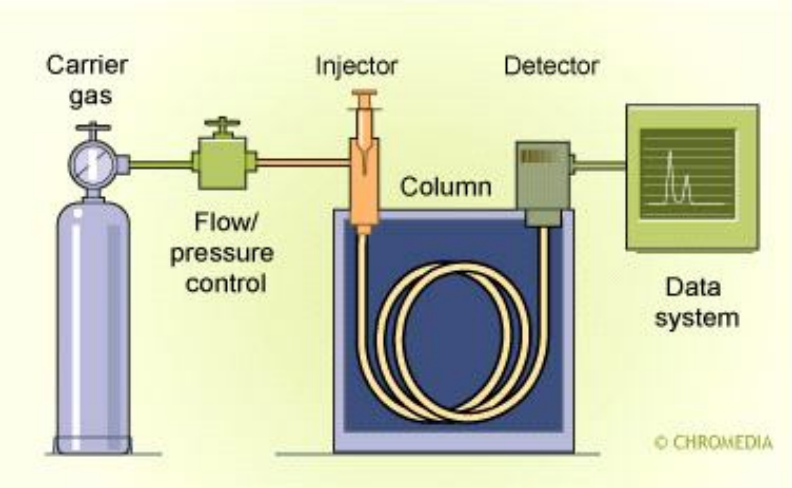
mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture.

The chromatographic technique is used for the separation of amino acids, proteins and carbohydrates. It is also used for the analysis of drugs, hormones, vitamins. It is helpful for the qualitative and quantitative analysis of complex mixtures. The technique is also useful for the determination of molecular weight of proteins. Types of Chromatography include; Paper Chromatography ,Thin Layer Chromatography(TLC) , Gel Chromatography, Column Chromatography, Ion Exchange Chromatography, Gel Filtration Chromatography, Gas Liquid Chromatography, Affinity Chromatography[34].

1.14 .1-Gas chromatography (GC)

Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound in preparative chromatography; GC can be used to prepare pure compounds from a mixture[35].



Schematic of the GC system

A gas chromatograph is a chemical analysis instrument for separating chemicals in a complex sample. A gas chromatograph uses a flow-

through narrow tube known as the column, through which different chemical constituents of a sample pass in a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature[35].

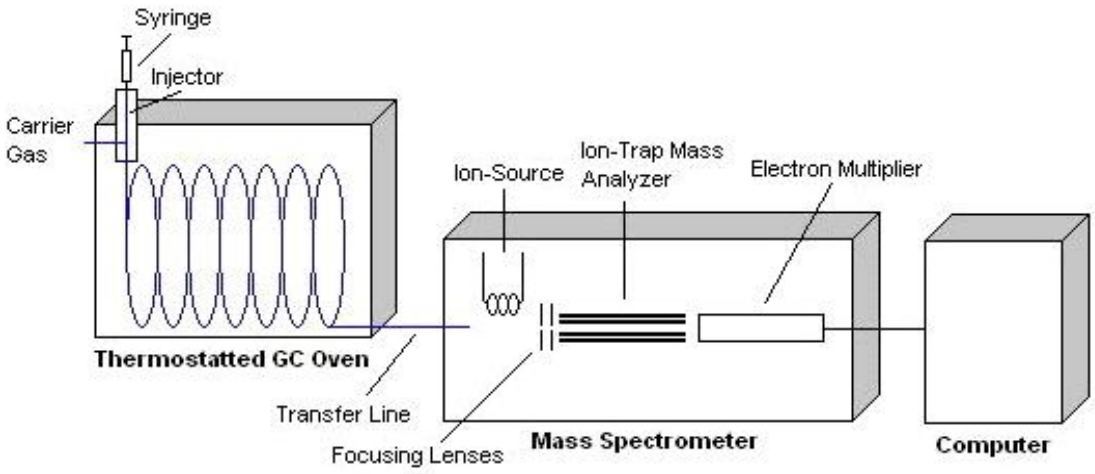
In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. The samples are also required to be salt-free; they should not contain ions. Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

Some gas chromatographs are connected to a mass spectrometer which acts as the detector. The combination is known as GC-MS. Some GC-MS are connected to an NMR spectrometer which acts as a backup detector. This combination is known as GC-MS-NMR. Some GC-MS-NMR is connected to an infrared spectrophotometer which acts as a backup detector. This combination is known as GC-MS-NMR-IR. It

must, however, be stressed this is very rare as most analyses needed can be concluded via purely GC-MS[35].

1.14.3- Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification[36].



Schematic of the GC/MS system

The use of a mass spectrometer as the detector in gas chromatography was developed during the 1950s after being originated by James and Martin in 1952.

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (i.e. have the same retention time), which results in two or more molecules that co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically increases certainty that the analyte of interest is in the sample. For the analysis of volatile compounds, a purge and trap (PT) concentrator system may be used to introduce samples. The target analytes are extracted and mixed with water and introduced into an airtight chamber. An inert gas such as nitrogen (N_2) is bubbled

through the water; this is known as purging. The volatile compounds move into the headspace above the water and are drawn along a pressure gradient (caused by the introduction of the purge gas) out of the chamber. The volatile compounds are drawn along a heated line onto a 'trap'. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. PT/ GC-MS is particularly suited to volatile organic compounds (VOCs) and aromatic compounds associated with petroleum)[37].

Aim of this study

This study was aimed to:

-Screening the target species for secondary metabolites.

-Extraction of fixed oil from **target** species.

-Conducting GC-MS studies on the extracted oil.

Chapter Two

2-Materials and methods

2.1-Materials

2.1.1- Reagents

-For oil extraction:

- n-Hexan

- For GC-MS:

- Sodium hydroxide
- Methanol
- Phosphoric acid
- Sodium chloride
- n-Hexan
- Diethyl ether
- Sodium sulphate
- Helium (carrier gas)

-For physicochemical tests:

- Sodium thiosulphate
- potassium iodide
- Starch solution

- Chloroform
- Acetic acid
- Isopropanol
- Potassium hydroxide
- Phenolphthalein indicator

2.1.2- Instruments

- GC-MS
- Soxhlet extractor
- Incubator
- Autoclave
- Refractometer

2.1.3-Test organisms

The standard microorganisms shown in Table (2.1) were used evaluating the antibacterial and antifungal activities of *Spinacia oleracea* seed oil.

Table 2.1: Test organisms

Ser. No	Micro organism	Type
1	*Bacillus subtilis*	G+ve
2	*Staphylococcus aureus*	G+ve
3	*Pseudomonas aeroginosa*	G-ve
4	*Escherichia coli*	G-ve
5	*Aspergillusniger*	fungi
6	*Candida albicans*	fungi

2.2- Methods

2.2.1- Preperations of reagents for phytochemical screening

i)-Flavonoid test reagents

- Aluminium chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

ii)- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. water.

- **Potassium iodide solution** : 5 g in 10 ml. water

The two solutions were combined and then diluted with water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and(2 g) of potassium iodide in (100 ml) water.

2.2.2- Preparation of plant extract for phytochemical screening

(150 g) of powdered shade- dried whole plant of *Spinacia oleracea* was macerated with n-hexane until exhaustion. This prepared extract(PE) was used for phytochemical screening.

2.2.3- Phytochemical screening

The prepared extract of the plant was screened for major secondary constituents.

i) Test for unsaturated sterols and for triterpenes

(10 ml)of the (PE) was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chlorform solution was dehydrated over anhydrous sodium sulphite . (5 ml) portion of the solution was mixed with(0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

ii)Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

iii)Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Maeyer reagent were added., to the other portion few drops of Wagner reagent were added.

iv) Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

v)Test for saponins

(1g) of dried powdered plant material was placed in a clean test tube. (10 ml) of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand.

2.2.4-Extraction of oil from ***Spinacia oleracea*** **seeds**

Powdered seeds of *Spinacia oleracea* (200g) were exhaustively extracted with n-hexane at room temperature.The solvent was removed under reduced pressure and the oil was kept in the fridge at 4^{o}C for further manipulation.

2.2.4.1-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added, then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes .The Hexane layer was then separated.(5µl) of the hexane extract were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.5- GC-MS analysis

Spinacia oleracea seed oil was analyzed by gas chromatography – mass spectrometry.A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μm, thickness)was used.Helium (purity; 99.99 %) was used as carrier gas.Oven temperature program is given in Table 2.2, while other chromatographic conditions are depicted in Table 2.3.

Table 2.2: Oven temperature program

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

Table2.3 : Chromatographic conditions

Column oven temperature	150.0^{o}C
Injection temperature	300.0^{o}C
Injection mode	Split
Flow control mode	Linear velocity
Pressure	139.3KPa
Total flow	50.0ml/ min
Column flow	1.54ml/sec.
Linear velocity	47.2cm/sec.
Purge flow	3.0ml/min.
Spilt ratio	- 1.0

2.2.6-Antimicrobial assay

2.2.6.1-Preparation of bacterial suspensions

One ml aliquots of 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 sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10^8-10^9colony forming units per ml.The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

2.2.6.2-Preparation of fungal suspensions

Fungal cultures were maintained on dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile

normal saline, and the suspension was stored in the refrigerator until used.

2.2.6.3-Testing for antibacterial activity

The cup-plate agar diffusion method was adopted with some minor modifications, to assess the antibacterial activity of the oil. (2ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for a sample. Separate Petri dishes were designed for standard antibacterial chemotherapeutic, (ampicillin and gentamycin).

The agar discs were removed, alternate cup were filled with 0.1 ml samples using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the test compounds and the standard antibacterial chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

Chapter Three

3- Results and Discussion

The fixed oil of *Spinacia oleracea* seed was obtained by seed maceration. GC-MS analysis of the oil was conducted and the identification of the constituents was initially accomplished by comparison with the MS library (NIST).Comparison of the mass spectra with the database on MS library revealed about 90-95% match. Furthermore identification of constituents was confirmed by interpreting the observed fragmentation pattern.

3.1-Physicochemical properties of the oil

The physicochemical characteristics of *Spinacia oleracea* seed oil are displayed in Table 3.1.

Table 3.1: physicochemical characteristics of *Spinacia oleracea* seed oil

Physicochemical character	Result
Color	Light yellow
Odor	Pleasant
Density	0.938
Refractive index	1.468
Acid value	22.13
Peroxide value	10.42

Determination of the various physicochemical characteristics of oils indicates the practical importance of the oil and provides basis for suitability of the oil in daily life. Properties like color, odor, density,

specific gravity, refractive index, optical rotation, acid value, iodine value, saponification value etc provide valuable information about the quality of oils and its suitability for various industrial and pharmacological applications.

3.2- Constituents of oil

The GC-MS spectrum of the studied oil revealed the presence of 24 components: 17 fatty acids; 3 terpenes; 2 benzene derivatives and 2 hydrocarbons (Table:3.2).

Table:3.2: Constituents of *Spinacia oleracea* seed oil

Peak No	Name	R. Time	Area %
Fatty acids:			
1	9,12-Octadecadienoic acid(Z,Z)-methyl ester	17.499	38.53
2	9-Octadecenioc acid(Z)-methyl ester	17.539	24.93
3	Hexadecenoic acid, methyl ester	15.827	16.68
4	Methyl Stearate	17.735	2.78
5	R-(-)-14-methyl-8-hexadecyn-1-ol	20.941	2.51
6	6,9- Octadecenioc acid, methyl ester	20.339	1.92
7	11-Eicosenoic acid, methyl ester	19.279	2.08
8	Docosanoic acid, methyl ester	21.118	0.73
9	Eicosenoic acid, methyl ester	19.495	0.66
10	13-Docosanoic acid, methyl ester	20.941	0.56
11	Tetracosanic acid, methyl ester	22.622	0.53
12	Heptadecenoic acid, 16-methyl -,methyl	17.499	0.47

	ester		
13	Methyl tetraadecanoate	13.719	0.46
14	Cyclopropaneoctanoic acid, 2-octyl-methyl ester	18.173	0.38
15	9- Hexadecenoic acid, methyl ester	15.631	0.37
16	Nonadecaoic acid, methyl ester	18.395	0.35
17	Pentadecenioc acid, methyl ester	14.794	0.32
Hydrocarbons components:			
18	Tetrapentacontane	23.769	0.95
19	1-dodecyne	4.673	0.10
Terpenes:			
20	Eucalyptol	4.897	0.05
21	Beta-ocimene	5.037	0.04
22	1,6-Octdien-3-ol,3,7-dimethyl	5.755	0.03
Antioxidant:			
23	Butylated hydroxytoluene	11.366	0.68
24	Phenol, 2,2-methylenebis{6-(1,1dimthyl	20.408	0.33

The oil peak report demonstrated that fatty acids constituted more than 90% of the total weight; Major acids were: 9-octadecenioc acid , hexadecenoic acid, 9,12-octadecenioc acid and 6,9- octadecenioc acid.Some important components are discussed below:

i) 9, 12-Octadecadienoic acid methyl ester(38.53%)

9, 12-Octadecadienoic acid (also known as linoleic acid) is a polyunsaturated omega-6 fatty acid. The structure of 9, 12-octadecadienoic acid is displayed below:

$$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$$

Linoleic acid belongs to one of the two families of essential fatty acids, which humans cannot synthesize it from other food components.They are only available from diet.The mass spectrum of linoleic acid as its methyl ester is displayed in Fig.3.1.

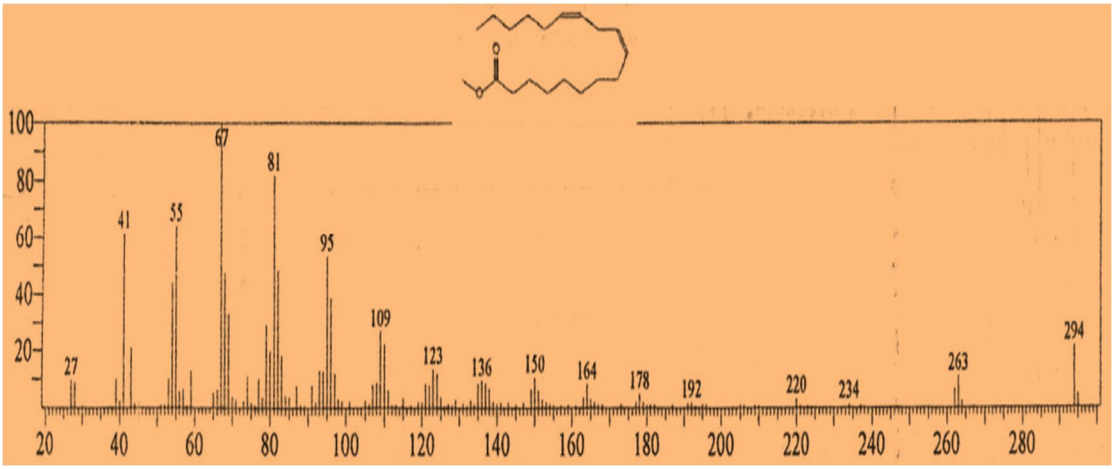


Fig. 3.1: Mass spectrum of 9,12-octadecadienoic acid methyl ester

The peak at m/z 294, which appeared at R.T. 17.499 in total ion chromatogram, corresponds to $M^+[C_{19}H_{34}O_2]^+$ .The peak at m/z263 corresponds to loss of a methoxyl function.

ii)9-Octadecenoic acid methyl ester(24.93%)

9-Octadecenoic acid (also known as oleic acid) is classified as a monounsaturated omega-9 fatty acid. It has the following formula:

$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$.

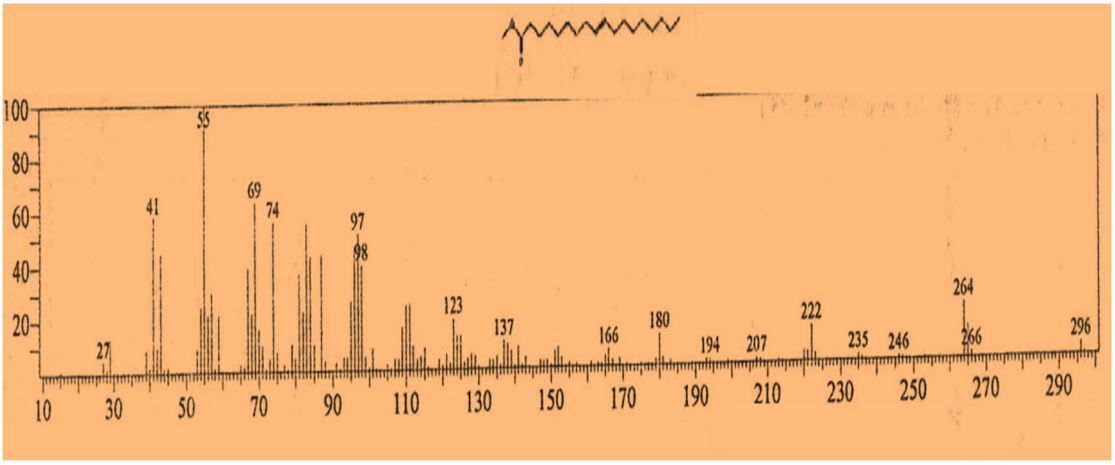


Fig. 3.2: Mass spectrum of 9-octadecenoic acid methyl ester

The EI mass spectrum of 9-octadecenoic acid methyl ester is shown in Fig. 3.2.The peak at m/z 296, which appeared at R.T. 17.539 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z265 corresponds to loss of a methoxyl function.

Methyl stearate(2.78%)

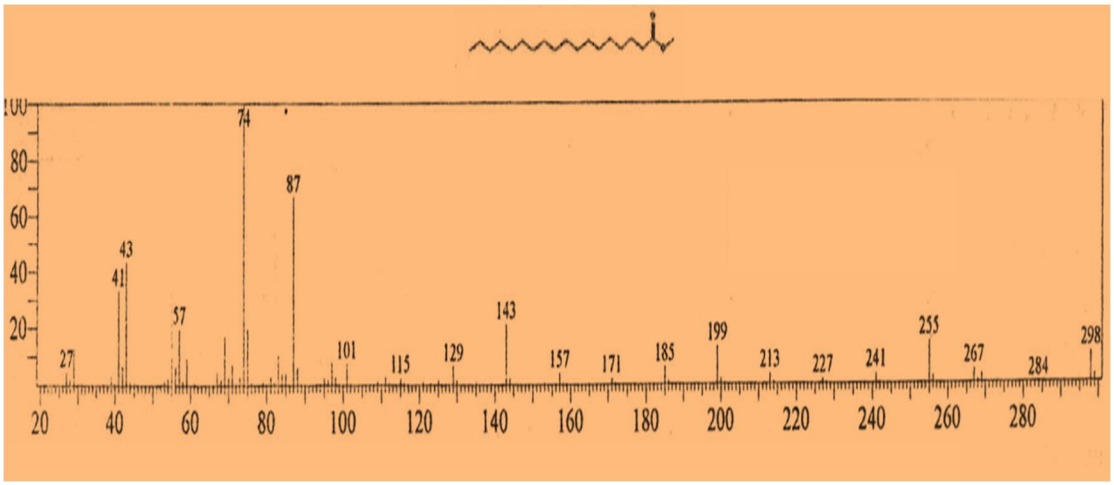


Fig. 3.3: Mass spectrum of methyl stearate

The EI mass spectrum of methyl stearate is shown in Fig. 3.3.The peak at m/z 298, which appeared at R.T. 17.735 in total ion chromatogram, corresponds to $M^+[C_{19}H_{38}O_2]^+$ .The peak at m/z267 corresponds to loss of a methoxyl function

Hexadecanoic acid methyl ester(16.68%)

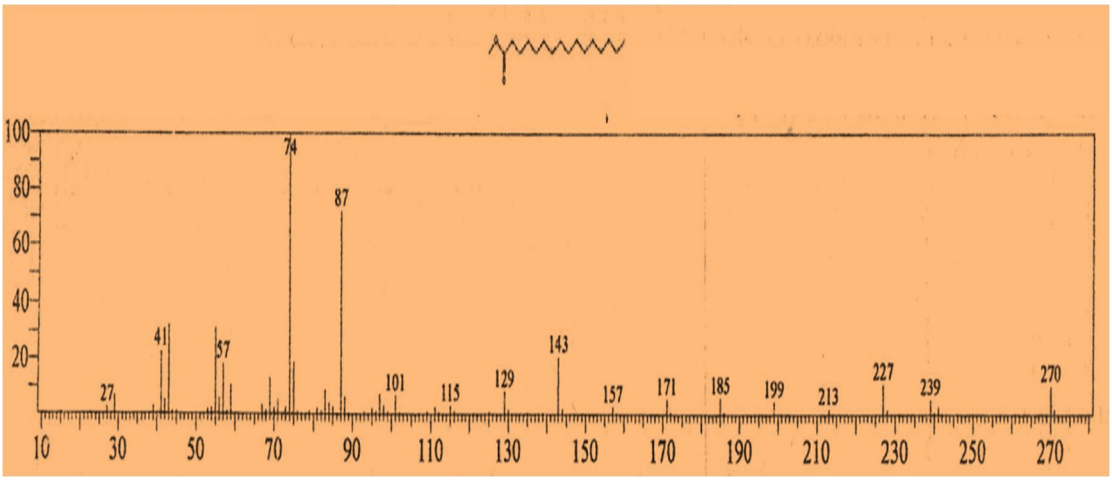


Fig. 3.4: Mass spectrum of hexadecanoic methyl ester

The EI mass spectrum of hexadecanoic acid methyl ester is shown in Fig. 3.4.The peak at m/z 270, which appeared at R.T. 15.827 in total ion chromatogram, corresponds to $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z239 corresponds to loss of a methoxyl function.

Hexadecanoic acid(also called palmitic acid) is the most common saturated fatty acid found in animals, plants and microorganisms. Its chemical formula is:

$$CH_3(CH_2)_{14}COOH.$$

Also the oil was found to contain small portion of hydrocarbons, terpenes, and antioxidant compounds.

3.3- Antimicrobial Activity

The target oil were evaluated for antimicrobial activity using the cup plate agar diffusion assay.The average of the diameters of the growth inhibition zones are shown in Table (3.3) .The results were interpreted in terms of the commonly used terms : 13-18mm growth inhibition zones is considered to be active; more than 18mm: very active. Values less than 9 mm indicate inactivity.Values ranging from 9-12 indicate partial activity. Tables (3.4) and (3.5) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

The oil was active against all test organisms . However, it showed significant activity against the bacterial strains : *Staphylococcus aureus* and *Bacillus subtilis.* It also gave excellent activity against the fungal species : *Spinacia oleracea* .

Table 3.3 : Antibacterial activity of oil :M.D.I.Z (mm)

Organism					
Sample	Ec	Ps	Sa	Bs	Ca
Oil	13	15	18	17	20

Table 3.4 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Pa
Ampicillin	40 20 10	15 14 11	30 25 15	- - -	- - -
Gentamycin	40 20 10	25 22 17	19 18 14	22 18 15	21 15 12

Table 3.5 : Antifungal activity of standard chemotherapeutic agents against standard fungi

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

- S.a*: Staphylococcus aureus*
- E.c*: Escherichia coli*
- P.a: *Pseudomonas aeruginosa*
- C.a: *Candida albicans*
- S.t: *Salmonella typhi*
- *B.a: Bacillus subtilis*

Conclusion:

The oil was successfully extracted from the dry Spinacia oleracea seed using solvent extraction method. And the percent was found to be 4.7%.

GC-MS analysis of the oil was conducted and the identification of the constituents was accomplished by the comparison with the MS library and the comparison with the data bass revealed about 90-95 match, which is found to be 24 components from fatty acids, Terpenes, antioxidant, & hydrocarbons, but the main components were found as fatty acid, which are; (Linoleic acid with peak area percent 38.5, Oleic acid with peak area percent 24.9%, and Palmatic acid 16.6%).

The antimicrobial results for the extracted oil indiacte that, oil is active against all test orgganisms, however it showed signidicance activity against the bacterial strains & excellent activity against the fungal species.

Determination of the various physiochemical properties such as (density, refractive index, peroxide value, acid value) of the oil are tested to indicates the practical importance and provide basis of the suitability of the oil in daily life.

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