Sudan University of Science and Technology

College of Graduate Studies

GC-MS Analysis of Capsicum annum Fixed Oil and Assessment of Antimicrobial Activity

دراسة الزيت الثابت لنبات الشطة بالتحليل الكروموتوغرافي الغازي

طيف الكتلة ونشاطه المضاد للمايكروبات

A Thesis Submitted in partial Fulfillment of the

Requirement of the M.Sc. Degree in chemistry

By

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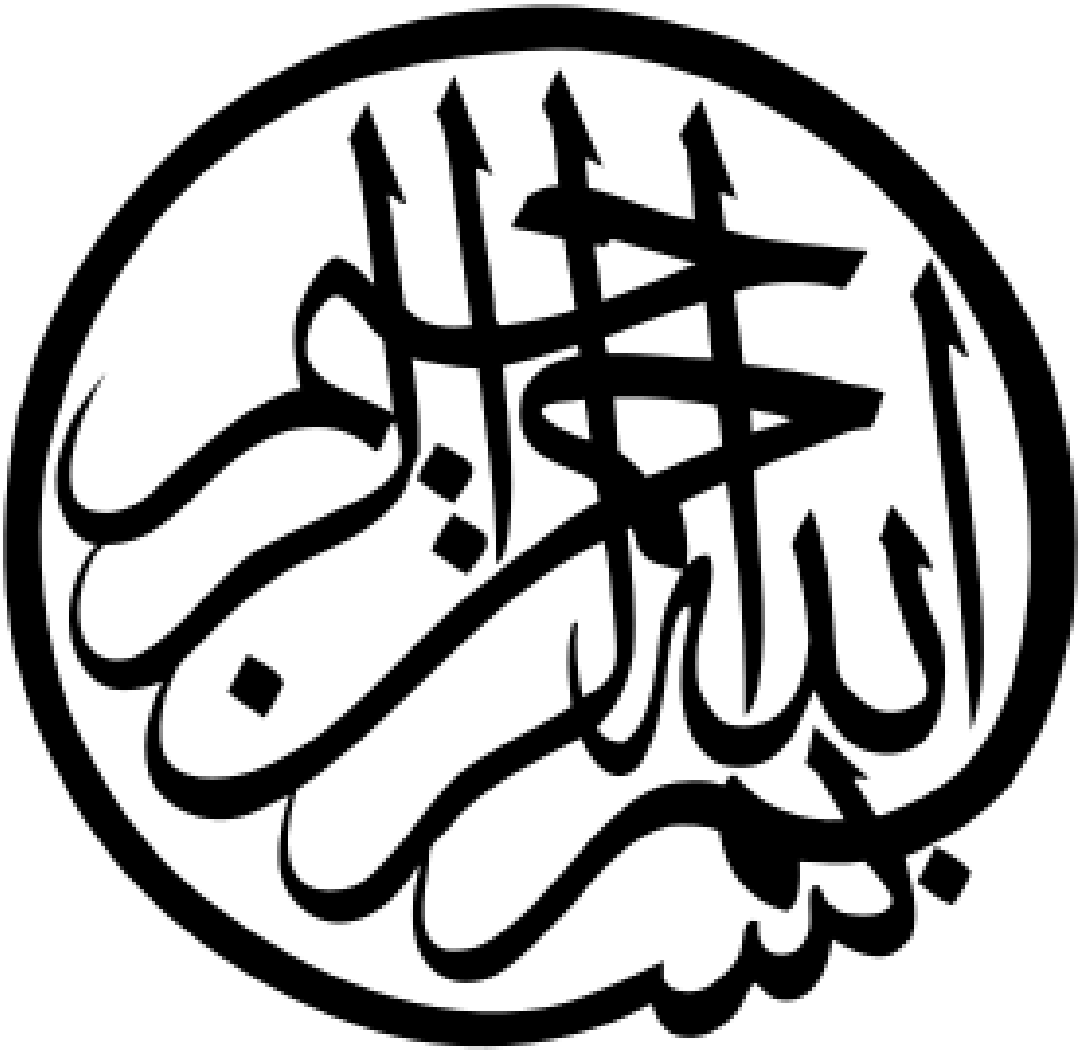

الآيـــة

قال تعالى :

(إِنَّ اللَّهَ لَا يُغَيِّرُ مَا بِقَوْمٍ حَتَّى يُغَيِّرُوا مَا بِأَنْفُسِهِمْ)

صدق الله العظيم

(الرعد : الآية "11")

DEDICATION

To

My mother

My father

My sister

My grand mother

Acknowledgment

I am most grateful to Allah for helping throughout my life; I would like to extend my thanks to my supervisor professor Mohamed Abdel Karim Mohamed for this continuous encouragement, excellent supervision, and valuable advice.

I would like to thanks my family for their support, also thanks to all those who help and encouragement me to do this study.

Abstract

In this study, the fixed oil of Capsicum annum was extracted from seeds with n-hexane at room temperature. The oil was analyzed by gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis of the studied oil revealed the presence of 27 components.

The major constituents were: octadecadienoic acid methyl ester (56.57%), hexadecanoic acid methyl ester (17.60%).Minor constituents were octadecenoic acid methyl ester (5.62%), methyl stearate (5.50%), and stigmasterol (2.37%).

The oil was screened for antibacterial and antifungal activities using six standard human pathogens. Agar well diffusion technique used to assess the antimicrobial activity of the oil against two Gram positive (Bacillus subtili and Staphylococcus aureus), two Gram negative (Escherichia coli and Pseudomonas aeroginosa) bacteria and two fungal species (Aspergillusniger and Candida albicans).

The results indicate that pithecellobium dulce oil possess bioactive compounds having significant antimicrobial properties.

المستخلص

استخلص الزيت الثابت لنبات الشطة ودرس الزيت بتقنية الكروماتوغرافيا الغازية – طيف الكتلة حيث اتضح ان الزيت به المكونات الرئيسية التالية:-

Octadecadienoic acid methyl ester (56.57%), hexadecanoic acid methyl ester (17.60%).

والمركبات الثانوية هي:

Octadecenoic acid methyl ester (5.62%), methyl stearate (5.50%), and stigmasterol (2.37%).

ثم اخضع الزيت لإختبارات بيولوجية كمضاد للميكروبات وقد أستخدمت اربعة انواع من البكتريا القياسية

Gram positive (Bacillus subtili and Staphylococcus aureus), Grams negative (Escherichia coil and Pseudomonas aeroginosa)

ونوعان من الفطريات:

Aspergillusniger and Candida albicans.

وقد أوضحت نتائج البحث البيولوجي ان الزيت يحتوي على مواد مثبطة لعمل الميكروبات بصورة واعدة.

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1.1.1- Classification

Kingdom: Plantae

- Subkingdom
- Tracheobionta
- Superdivision
- Spermatophyta
- Division
- Magnoliophyta
- Class: Magnoliopsida
- Subclass: Asteridae
- Order: Solanales
- Family: Solanaceae
- Genus: Capsicum
- Species: C. annum

Capsicum annum includes: bell pepper, paprika, pimento, jalapeno, cascabel, cayenne, Chile, chilies, chili pepper, red pepper, red chili, tabasco pepper, African pepper, and bird pepper[1].

The genus Capsicum comprised all the varied forms of fleshy fruited peppers grown as herbaceous annuals - the red, green, and yellow pepper rich in vitamins A and C that are used in seasoning and as a vegetable food. It includes paprika, chili pepper, red pepper (cayenne), and bell peppers. The capsicums under each category vary tremendously and the species designation is not always possible. In general, paprika belongs to C. annum and the red peppers and chili peppers belong to the C. frutescens species[1].

Peppers originate from Central America where most of the main varieties were developed by local Indians. The genus Capsicum is native of South America which emerged probably in the area bordering Southern Brazil and Bolivia. The three species C. annum, C. frutescens and C. Chinese evolved from a common ancestor located in the North of the Amazon basin (NW-Brazil, Columbia). The wild form of this species is native from Florida and the Bahamas to Arizona and down though Central America to Colombia.

As paprika plants tolerate nearly every climate, the fruits are produced all over the world. A fairly warm climate is, however necessary for strong aroma; therefore, in Europe, Hungarian paprika has best reputation; the best comes from the Kalocsa

region. In the Unites States, California and Texas are the main producers[1].

Capsicum annum is a species of the plant genus Capsicum native to southern North America and northern South America. This species is the most common and extensively cultivated of the five domesticated capsicums.

The species encompasses a wide variety of shapes and sizes of peppers, both mild and hot, ranging from bell peppers to chili peppers. Cultivars are descended from the wild American bird pepper still found in warmer regions of the Americas. In the past some woody forms of this species have been called C. **frutescens**, but the features that were used to distinguish those forms appear in many populations of C. annum and there is no consistently recognizable C. frutescens species[7].

Although the species name annuum means "annual (from the Latin annus "year"), the plant is not an annual and in the absence of winter frosts can survive several seasons and grow into a large

perennial shrubs[8]. The single flowers are an off-white (sometimes purplish) color while the stem is densely branched and up to 60 centimeters (24 in) tall. The fruit are berries that may be green, yellow or red when ripe[8]. While the species can tolerate most climates, C. annuum is especially productive in warm and dry climates.

Capsicum annum

The parts used are berry fruits. Removal of seeds and veins results in a less pungent and more brightly coloured product. The pungent constituents found in Cayenne are the capsaicinoids, present only in the fruit of the plant in small amounts, as low as 0.001 to 0.005% in "mild" and 0.1% in "hot' cultivars.

Capsaicin is a phenolic compound.

Apart from capsaicin, the taste of paprika is mostly due to essential oil; paprika scent is mostly due to a range of alkyl methoxypyrazines. Paprika also contains sizable amounts (0.1%) of vitamin C Paprikas derive their colour in the ripe state mainly from carotenoid pigments, which range from bright red (capsanthrine, capsorubin and more) to yellow.

Capsicum or Cayenne (Capsicum Frutescens) is rich in vitamins A, C, iron and calcium. It contains vitamin G, magnesium,

phosphorus, and Sulphur; it also has some B-complex, and is rich in potassium[1].

The pungency for the red peppers and the colour value for the paprikas are the most important parameters. The hot flavour of chilies is caused by the substance capsicin which is concentrated mainly in the placenta (i.e. the connective tissue between the fruit and the seeds) and the seeds.

Hot peppers, used as relishes, pickled or ground into a fine powder for use as spices, derive their pungency from the compound capsaicin (8-methyl-N-vanilly l-6-enamide), a substance characterized by acrid and burning taste, that is located in the internal partitions of the fruit. The capsaicin stimulates gastric secretions and, if used in excess, causes inflammation. It is a tasteless, odorless white crystalline substance. Its level varies widely in capsicum peppers, from less than 0.05% in the mildly pungent types to as high as 1.3% in the hottest chilies. Most of the capsaicin in a pepper is found in the interior ribs that divide the chambers of the fruit, and to which the seeds are attached[1].

Capsinoid chemicals provide the distinctive tastes in C. annuum variants. In particular, capsaicin creates a burning sensation ("hotness"), which in extreme cases can last for several hours after

ingestion. A measurement called the Scoville scale has been created to describe the hotness of peppers and other foods[1].

1.2- Medicinal uses of Capsicum annum:

Hot peppers are used in medicine as well as food in Africa and other places around the world.

In his book "Flora Medica' the English botanist John Lindley described C. annuum thus[1]:

"It is employed in medicine, in combination with Cinchona in intermittent and lethargic affections, and also in atonic gout, dyspepsia accompanied by flatulence, tympanitis, paralysis etc. Its most valuable application appears however to be in cynanche maligna (acute diphtheria) and scarlatina maligna (malignant scarlet fever) used either as a gargle or administered internally[1].

- Cayenne is stomachic, carminative, stimulant antispasmodic, analgesic, alterative, astringent, hemostatic, and antiseptic in nature.
- Cayenne is widely considered to be the most potent and safest stimulant known.
- Cayenne has a strong effect upon the circulation, initially acting upon the heart and the large arteries, followed by a stimulant activity upon the arterioles and then the

capillaries.

- It exhibits a protective effect on the respiratory system.
- The capsaicin has substantial antigen toxic and ant carcinogenic effects and is an important dietary phytochemical with potential chemo preventive activity.
- The powder is used in any catarrhal affliction as in colds, cough, asthma and urinary catarrh.
- Capsaicin is used primarily as a topical cream for pain caused by conditions such as arthritis and general muscle Soreness.
- In cases of dyspepsia, flatulence and constipation, Cayenne promotes the digestive secretions and stimulates peristalsis.
- Cayenne is also indicated in tired, painful muscles, joint stiffness, and coldness in the extremities.
- Cayenne is applied topically as a powder on wounds to arrest bleeding, working rapidly to form a clot and seal off the wound.
- Cayenne is of great use in the treatment of indolent ulcers, abscesses, and sores.
- Cayenne is also an important among remedy to stop the process of mortification and gangrene, arresting decomposition and decay through its antiseptic properties.

- It is also helpful in menstrual cramps[1].

Alicia et.al. Characterized and quantified[9] some constituents of C. annum at four maturity stages (Immature green, green, immature red and red). Individual hydroxycinnamic acids flavonoids, vitamin C and individual; carotenoids were characterized and quantified. Twenty three flavonoids and 5 hydroxycinnamic derivatives were identified from pericarp by HPLC-diode array detection-electrospray ionization-mass spectrometry.

Hydroxycinnamic acid derivatives C-glycosides of quercetin, leteolin and chrysoeriol and a large number of C- glycosyl flavones have been characterized. Difference in the individual and total phenolic content was observed between different mature stages[9].

Aneta et.al, determined[10]the content and chemical composition of capsaicinoids a group of alkaloids responsible for the pungency of the capsicum fruit. Capsaicinoids were extracted with hexane and analyzed by GC/MS the alkaloids: capsaicin, dihydrocapsaicin, nonivamide were detected as major constituents.

The carotenoid composition of thirteen types of Capsicum annum have been evaluated by Keiko e.al the same author discussed the ratio of B-carotene to capsanthin. Highest values of total carotenoids content and capsanthin were recorded for two varieties[11].

Rosa et.al. claimed[12] that antioxidant systems from Capsicum annum are involved in response to temperature changes in ripe fruits.

In brain- in vitro studies, Oboh et.al. demonstrated[13] the ability of aqueous extracts of Capsicum annum to inhibit Fe^{2+} - induced lipid peroxidation in rat brain[5].

Four new cyclic diterpenes glycosides (named Capsianosides) together with 12 known compounds were isolated from Capsicum annum fruits. Structures of the new isolates were deduced on the basis of their spectral data. The known capsidol - showed significant in vitro bacteriostatic activity comparable to that of standard (metrnidazole). Some of the isolated compounds were evaluated for antioxidant potential[14].

1.3 Gas chromatography:

The technique of gas chromatography is a widely applied technique in many branches of science. For a long time, GC has

played fundamental role in detecting how many compounds and in what proportion they exist in a mixture .But the ability of this technique to establish the nature and chemical structure of these separated and quantified compounds is ambiguous and reduced and requires a spectroscopic method of detection system. The most used is the mass spectrometric detector (MSD) which allows obtaining the "fingerprint" of the molecule i.e. its mass spectrum. Mass spectra provide information on the molecular weight, elemental composition if high resolution mass spectrometer is used, functional groups and spatial isomers of the molecules[15]. The sample in gas chromatographic system may be a liquid solution or a collection of molecules adsorbed on a surface e.g. the solid phase micro extraction system. During the transfer into the GC, sample is volatilized by rapid exposure to a zone kept at relatively high temperature (200-300℃) and mixed with a stream of carrier gas (Ar,He,Ne of H). The resulting gaseous mixture enters the separation section, chromatographic column which in its current version is a fused -silica tubular capillary coated internally with a thin film.Upon their displacement through the column, the analyte molecules are partitioned between the gas carrier stream(mobile phase) and the polymer coating (stationary phase), to an extent which dependsmainly on their chemical

structure. At the end of the separation section, the molecules reach a detection system in which a specific physical property) thermalconductivity) or a physicochemical process (ionization in a flame electron capture) gives rise to an electron signal which is proportional to the amount of molecules of the same identity. A data system permits the processing of these data to produce a graph of the variation of detector system with time) chromatogram). Four principal sections are distinguishable in the chromatograph: introduction(injector),separation) chromatographiccolumn), detection and data handling unit. Each section has its own function and its responsibility for the quality of the analysis and the results obtained[15].

The injection system for example, should ideally transfer the sample to the column quantitatively without discrimination on molecular weight or volatility and without chemical alteration (decomposition or isomerization). It is a critical step, especially for quantitative analysis. For correct GC operation, among other conditions, this gateway to the column should remain unpolluted, clean, inert and leak-free the main requirement the analyte in GC is that it should be volatile enough to be present in detectable amount in the mobile phase. Substances with low vapour pressure

will not enter the chromatographic column and will accumulate at the injection System[15].

Highly polar, thermlabile, ionic and high molecular weight compounds are not compatible with regular GC analysis. Depending on the molecular structure of the analyte and the functional groups available, it is possible in some cases to obtain chemical derivatives which has a high vapour pressure.

An important characteristics of the chromatographic column is its resolution, or its ability to separate components with very similar distribution constants between the mobile and stationery phases (K_D). Chromatographic resolution is a function of many operational parameters. Among them, the nature of the stationery phase, mobilephase, temperature, the size of column (length, inner diameter and the thickness of the stationery phase). As the number of components in the mixture increases and the structure similarity between its components grows (isomerism) longer column are required for complete separation of components. Alternatively, for the same purpose one can employ smaller internal diameter columns. Obviously, increasing the length of the column markedly increases the analysis time. So the analysis of polyromantic

hydrocarbons and controlled drugs is regularity accomplished by using a 30m long column[15].

Sample preparation for GC involves technologies which preferentially isolate volatile and semi-volatile substances and prevents the presence of ionic and high molecular weight species in the mixture to be injected in the GC. These procedures can be divided roughly into three major groups: distillation, extraction and headspace methods. The resulting extracts or distillates are volatile mixtures suitable for GC and GC-Ms analysis, but the mixture may need drying (anhydrous sodium sulphate) prior to injection into the chromatograph[15].

However, these techniques, in general, are not suitable for studying and isolating compounds at trace level.

To improve extraction efficiency and substantially reducing distillation time, microwave - assisted hydro distillation[16,17] is a common example of a laboratory-scale technique for essential oils and other volatile mixtures isolation. Such microwave- assisted distillation requires quarter the time employed for conventional heating.

1.4- Volatile oils (essential oils):

Essential oils (EOs) are aromatic oily liquids obtained from plant material. They can be obtained by expression, fermentation effleurage or extraction but the method of steam distillati on is most commonly used for commercial production of Eos. The term 'essential oil' is thought to derive from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug Quinta essential[19]. An estimated 3000 EOs are known, of which about 300 are commercially important - destined chiefly for the flavours and fragrances market[18]. It is has long been recognized that some EOs have antibacterial properties[19,20] and these have been reviewed in the past[21,22] as have antibacterial properties of spices[22] but the relatively recent enhancement of interest in "green" consumerism has lead to a renewal of scientific interest in these substances[21]. Besides antibacterial properties[23], EOs or their components have been shown to exhibit antiviral[24], antimycotic[25], antitoxigenic[26], antiparasitic[27], and insecticidal[28] properties. These characteristics are possibly related to the function of these compounds in plants[19].

Although spices have been used for their perfume, flavour and preservative properties since antiquity[29] of the known EOs, only oil of turpentine was mentioned by Greek and Roman historians[19]. Distillation as a method of producing EOs was first used in the East (Egypt, India and Persia)[19] more than 2000 years ago and was improved in the 9th century by the Arabs[29].

The first authentic written account of distillation of essential oil is ascribed to Villanova (c.1235-1311), a Catalan physician[19].

By the 13th century EOs were being made by pharmacies and their pharmacological effects were described in pharmacopoeias[29] but their use does not appear to have been widespread in Europe until the 16th century, from which time they were traded in the city of London. Publishing separately in that century on the distillation and use of EOs, two Strassburg physicians, Brunschwig and Reiff, mention only a relatively small number of oils between them: turpentine, juniper wood, rosemary, spike (lavender), clove, mace nutmeg, anise and cinnamon[19]. According to the French physician, Du Chesne (Quercetanus), in the 17th century the preparation of EOs was well known and pharmacies generally stocked 15-20 different oils[19]. The use of tea tree oil for medicinal purposes has been documented since the colonisation of

Australia at the end of the 18th century, although it is likely to have been used by the native Australians before that. The first experimental measurement of the bactericidal properties of the vapours of EO is said to have been carried out by De la Croix in 1881[20]. However, in the course of the 19th and 20th centuries the use of EOs in medicine gradually became secondary to their use for flavour and aroma[19].

Currently, the greatest use of EOs in the European Union (EU) is in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their functional properties)[18]. The well-known use of EO in aromatherapy constitutes little more than 2% of the total market. Individual components of EOs are also used as flavourings, either extracted from plant material or synthetically manufactured.

The antibacterial properties of essential oils and their components are exploited in such diverse commercial products as dental root canal sealers, antiseptics[30] and feed supplements for lactating sows and weaned piglets. A few food preservatives containing EOs are already commercially available. 'DMC Base Natural' is a food preservative bb and comprises 50% essential oils from rosemary, sage and citrus and 50% glycerol[31]. 'Protecta One' and 'Protecta

Two' are blended herb extracts produced in (USA) and are classed as safe food additives in the U.S.A. Although the precise contents are not made known by the manufacturer, the extracts probably contain one or more EOs. Further physiological effects of EOs are made use of in widely differing products such as commercial potato sprout suppressants and insect repellents[32].

Commonly used method for producing EOs on a commercial basis is steam distillation. Extraction by means of liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile but is much more expensive[33]. The difference in organoleptic profile indicates a difference in the composition of oils obtained by solvent extraction as opposed to distillation and this may also influence antimicrobial properties. This would appear to be confirmed by the fact that herb EOs extracted by hexane have been shown to exhibit greater antimicrobial activity than the corresponding steam distilled EOs[34]. EOs are volatile and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes. Numerous publications have presented data on the composition of the various Eos. The major components of the economically interesting EOs are summarised by Bauer et.al[29]. Detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the

EO or its headspace[35]. EOs can comprise more than sixty individual components[36]. Major components can constitute up to 85% of the EO whereas other components are present only as a trace[29]. The phenolic components are chiefly responsible for the antibacterial properties of EOs. There is some evidence that minor components of essential oils have a critical part to play in/antibacterial activity, possibly by producing a synergistic effect between other components. This has been found to be the case for sage, certain species of Thymus and oregano[37]. Usually composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources[38]. This can be explained, at least in part, by the formation of antibacterial substances from their precursors. P-Cymene (1-methy 1-4 (1-methylethy1) – benzene) and y-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the precursors of carvacrol (2-methyl-5-(l-methylethyl phenol) and thymol (5-methyl- 2- (1-methylethyl)phenol) in species of Origanum and Thymus (35. The sum of the amounts of these four compounds present in Greek oregano plants has been found to be almost equal in specimens derived from different geographical regions and to remain stable in plants harvested during different seasons. The same is true of T. vulgaris from Italy. This indicates that the four

compounds are biologically and functionally closely associated and supports the theory that thymol is formed via p-cymene from y-terpinene in Thymus vulgaris[39]. Generally, EOs produced from herbs harvested during or immediately after flowering possess the strongest antimicrobial activity[40]. Enantiomers of EO components have been shown to exhibit antimicrobial activity to different extents. The composition of EOs from different parts of the same plant can also differ widely. For example, EO obtained from the seeds of coriander (Coriandrum sativum L.) has a quite different composition to EO of cilantro, which is obtained from the immature leaves of the same plant[41].

Chapter Two
Materials and Methods

2- Materials and methods:

2.1- materials:

2.1.1. Plant material:

Seeds of Capsicum annumwere purchased from the local market-Khartoum and kindly authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum-Sudan.

2.1.2. Instruments:

A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm dimeter; 0.25um, Thickness) was used.

2.1.3. Test Organisms:

The standard micro organisms shown in table (2.1) were used evaluating the antibacterial and antifungal activitoil.es of Capsicum annum seed.

Table 2.1: Test Organisms

Ser. No	Micro organism	Type
1	Bacilius subtilis	G+ve
2	Staphylococus aureus	G+ve
3	Pesiudomonasaeroginosa	G-ve
4	Escherichia coli	G-ve
5	Aspergillusniger	Fungi
6	Candida abicans	Fungi

2.2. Methods:

2.2.1. Preparations of reagents for phytochemical screening:

i) Flavonoid test regents:

- Aluminum 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: 1.36g in 60ml. water.

- Potassium iodide solution: 5g in 10 ml water

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

- Wagner regent

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

2.2.2. **Preparation of plant extract for phytochemical screening**

(150g) of powdered shade – dried seeds of Capsicum annum were 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 formajor secondary constituents.

i) **Test for unsaturated sterols andfor triterpenes**

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

ii) Test for flavonoids:

(20ml) of the (PE) were evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then

dissolved in 30ml 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 3ml of the filtrate few drops of aluminum chloride solution were added.
- To 3ml of the filtrate few drops of potassium hydroxide solution were added.

iii) Test for alkaloids:

(10ml) 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 19 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

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

iv) Test for tannins:

(10ml) 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 (10ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water0 were added. The mixture was cooled, filtrated and the volume adjusted to 10ml. with more saline solution. (5ml) 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. (10ml) 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 Capsicum annum seeds:

Powdered seeds of Capsicum annum (200g) were exhaustively extracted with n-hexane at room temperature. The solvent was removed under reduced pressure and theoil was kept in the fridge at 4^0 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 methanolicsulphuric 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:

Capsicum annum 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 um, 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 Table2.3.

Table 2.2: oven Temperature program

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

Table 2.3: Chromatographic conditions:

Column oven temperature	150.0^{o} C
Injection temperature	300.0^{o}C
Injection mode	Spilt
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.
Split 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^{o}C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100ml of normal saline to produce a suspension containing about 10^{8}-10^{9} colony forming units per ml. The suspension was stored in the refrigerator at 4^{o}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 to dry, and then incubated at 37oC for 24 hours.

2.6.2.2. Preparation of fungal suspensions:

Fungal cultures were maintained on dextrose agar incubated at 25oC 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 200ml of sterile molten nutrient agar which was maintained at 45oC in a water bath. (29ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plated which were divided into two halves, two cups in each half (10mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for a sample. Separated 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^{o}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

Results and Discussion

3-Results and Discussion

3.1-Phytochemical screening

Phytochemical screening of*Capsicum annum* fruits gave positive reactions for: steroids, flavonoids, tannins, terpenes and glycosides.

Table 3.1: Phytochemical screening of *Capsicum annum* fruits

Species	Flavonoids	Tannins	Steroids	Terpenes	Glycosides
Capsicum annum	+ve	+ve	+ve	+ve	+ve

3.2-The GC-MS analysis of *Capsicum annum* essential oil

Identification of the constituents of *Capsicum annum* seed oil was initially accomplished by comparison with the MS library (NIST) and further confirmed by interpreting the observed fragmentation pattern resulting from GC-MS analysis. Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.1- Constituents of oil

GC-MS analysis of the studied oil revealed the presence of 27components (Table: 3.2).The typical total ion chromatograms (TIC) of hexane extract are shown in Fig.3.1.

Table: 3.2: Constituents of *Capsicum annum* fixed oil

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	11.399	225139	0.22	Butylated Hydroxytoluene
2	11.415	26265	0.03	Dodecanoic acid, methyl ester
3	13.740	548655	0.54	Methyl tetradecanoate
4	14.423	22723	0.02	Hexadecanoic acid, 15- methyl-, methyl est
5	14.516	17921	0.02	Tridecanoic acid, 12-methyl-, methyl ester
6	14.550	8467	0.01	5-Octadecenoic acid, methyl ester
7	14.655	15260	0.01	4-Octadecenoic acid, methyl ester
8	14.815	70728	0.07	Pentadecanoic acid, methyl ester
9	15.650	475543	0.47	9-Hexadecenoic acid, methyl ester, (Z)-
10	15.852	17995440	17.60	Hexadecanoic acid, methyl ester
11	16.551	258541	0.25	Hexadecanoic acid, 14-methyl-, methyl est
12	16.611	159310	0.16	Methyl 5,13-docosadienoate
13	16.820	239813	0.23	Heptadecanoic acid, methyl ester
14	17.199	60264	0.06	7-Octadecenoic acid, methyl ester
15	17.439	340421	0.33	Heptadecanoic acid, 16-methyl-, methyl es
16	17.534	57832952	56.57	9,12-Octadecadienoic acid (Z,Z)-, methyl e
17	17.558	5745752	5.62	9-Octadecenoic acid (Z)-, methyle ester
18	17.596	2121173	2.07	9-Octadecenoic acid, methyl ester, (E)-
19	17.759	5620948	5.50	Methyl stearate
20	19.314	329897	0.32	11-Eicosenoic acid, methyl ester
21	19.511	1140360	1.12	Methyl 18-methylnonadecanoate
22	21.131	1068595	1.05	Methyl 20-methyl-heneicosanoate
23	21.442	828329	0.81	Cholesterol
24	21.894	265395	0.26	Tricosanoic acid, methyl ester
25	22.633	1042327	1.02	Tetracosanoic acid, methyl ester
26	25.562	3349781	3.28	Ergost-8(14)-en-3-ol, (3.beta.)-
27	23.835	2424281	2.37	Stigmasterol
		102233280	100.00	

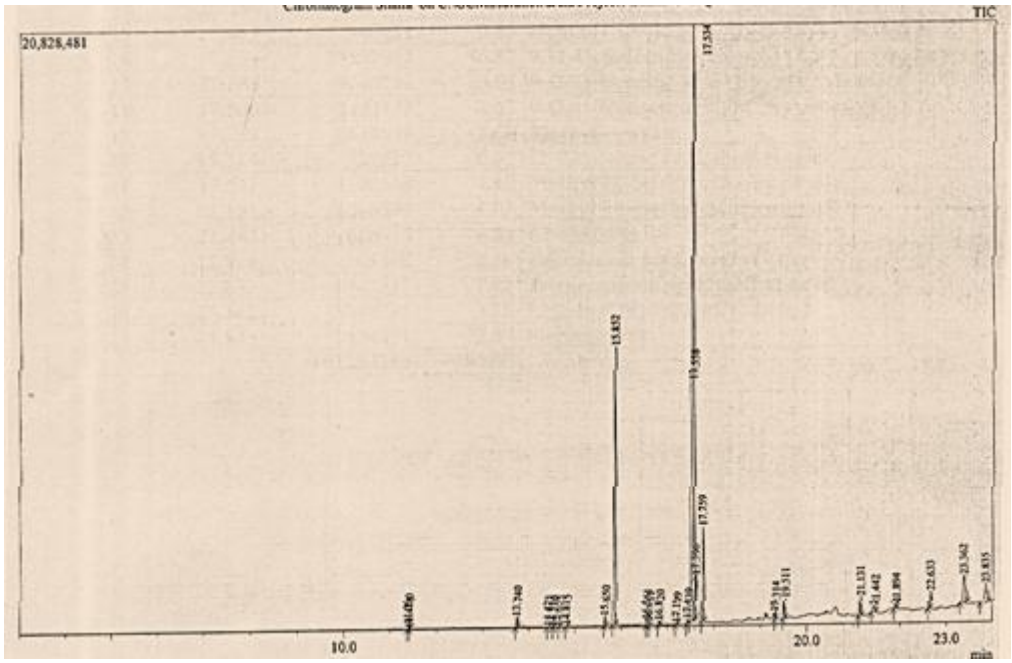

Fig.3.1: Chromatograms of *Capsicum annum*seed oil

The following compounds were detected in the chromatogram as major constituents:

9, 12-Octadecadienoic acid methyl ester (56.57%)

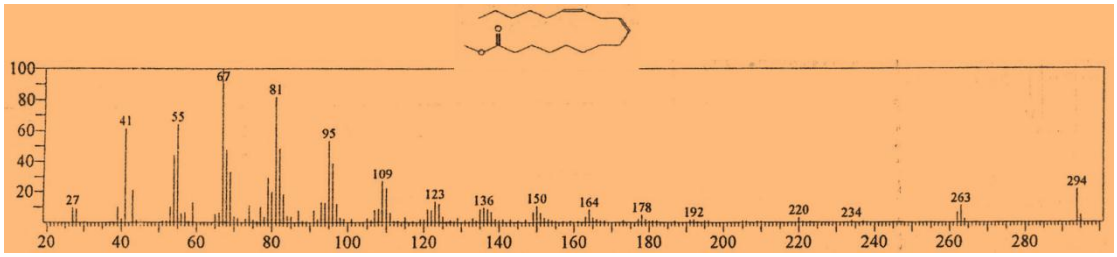

Fig. 3.2: Mass spectrum of9, 12-octadecadienoic acid methyl ester

The EI mass spectrum of 9, 12-octadecanoic acid methyl ester

is shown in Fig. 3.2.The peak at m/z 294, which appeared at R.T. 17.534 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.

Hexadecanoic acid methyl ester (17.60%)

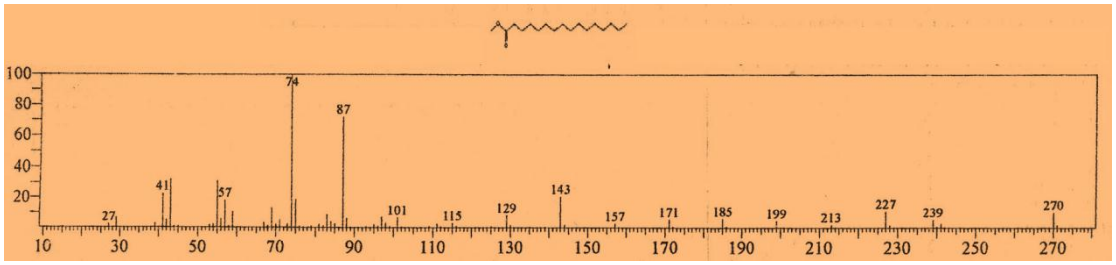

Fig. 3.3: Mass spectrum of hexadecanoic methyl ester

The EI mass spectrum of hexadecanoic acid methyl ester is shown in Fig. 3.3.The peak at m/z 270, which appeared at R.T. 15.852 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.

9-Octadecenoic acid methyl ester (5.62%)

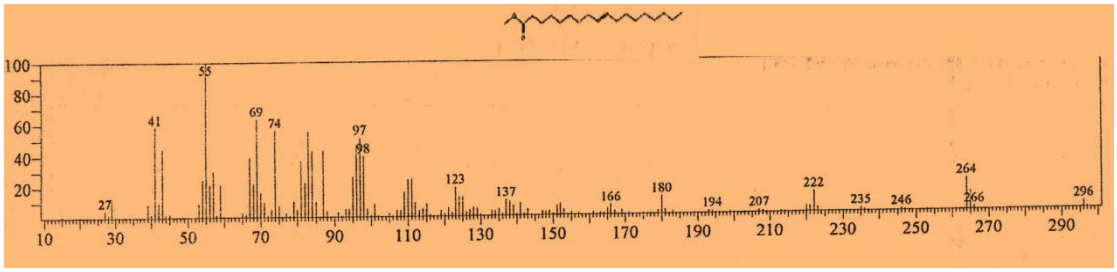

Fig. 3.4: Mass spectrum of9-octadecenoic acid methyl ester

The EI mass spectrum of 9-octadecanoic acid methyl esteris shown in Fig. 3.4.The peak at m/z 296, which

appeared at R.T. 17.558 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 (5.50%)

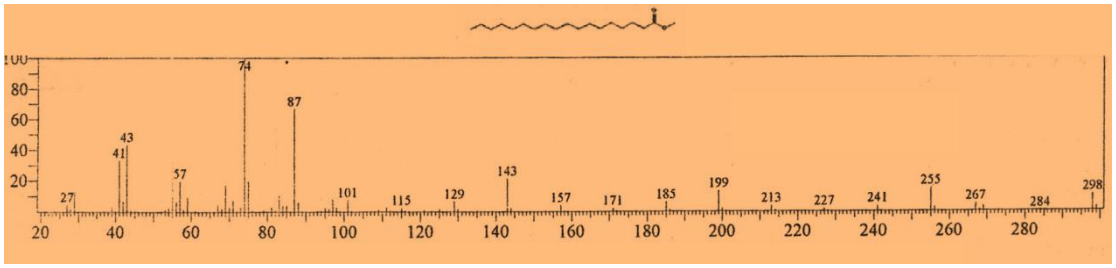

Fig. 3.5: Mass spectrum of methyl stearate

The EI mass spectrum of methyl stearateis shown in Fig. 3.5.The peak at m/z 298, which appeared at R.T. 17.759 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.

Stigmasterol (2.37%)

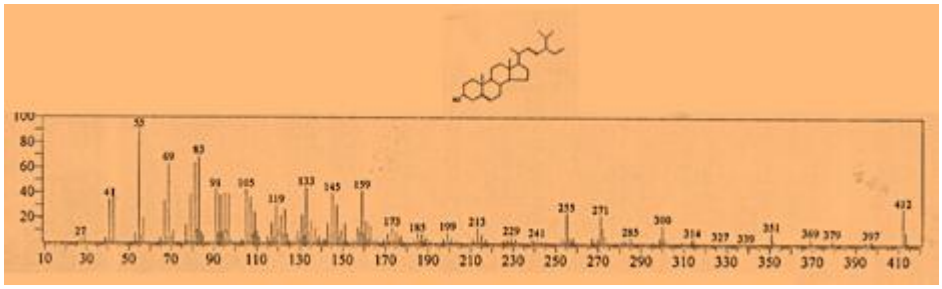

Fig. 3.6: Mass spectrum ofstigmasterol

The EI mass spectrum of stigmasterolis shown in Fig. 3.6.The peak at m/z 412, which appeared at R.T.23.835 in total ion chromatogram, corresponds to $M^{+}[C_{29}H_{48}O]^{+}$.

3.3-Antimicrobial activity

The oil was screened for antimicrobial activity against standard organisms. 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 (<9mm: inactive;9-12mm:partially active;13-18mm: active;>18mm:very active).Tables (3.4) and (3.5) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table (3.3): Antibacterial activity of *Capsicum annum* Oil: M.D.I.Z (mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca
oil	100	15	17	20	20	17

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

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
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

- Sa.*: Staphylococcus aureus*
- Ec.*: Escherichia coli*
- Pa.: *Pseudomonas aeruginosa*
- An.: *Aspergillus niger*
- Ca.: *Candida albicans*
- *Bs.: Bacillus subtilis*
- M.D.I.Z: Mean diameter or growth inhibition zone (mm). Average of two replicates.

The oil showed activity against all test organisms. It showed significant activity against *Staphylococcus aureus*and*Bacillus subtilis*.

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