بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Chemical Studies on *Brassica nigra* Fixed Oil by GC-MS

دراسة كيميائية للزيت الثابت لنبات الخردل بالتحليل الكروموتوغرافي الغازي-طيف الكتلة

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

By

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November , 2016

Sudan University of Science & Technology

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كلية الدراسات العليا

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كلية الدراسات العليا

Ref: SUST/ CGS/A11

Approval Page

(To be completed after the college council approval)

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Thesis title: ..Chemical..studies..on..... ..Brassica..nigra..Fixed..oil..by ..GC-MS..... .....

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قال تعالى:

﴿ والأرض مددناها وألقينا فيها رواسي وأنبتنا فيها من كل شيء موزون ﴾

"سورة الحجر : الأية 19 "

Dedication

To

The soul of my father,

Great mother ,

sisters and beloved uncle

Acknowledgment

First of all our sincere thanks to Allah Almighty for helping me to complete this work. It is a pleasure to record my deep appreciation, and thanks to Prof.Mohamed Abdul kareem for his wise guidance, which helped me to present this project in this shape. I am very grateful to the staff of the Department of Chemistry –Sudan University of Science and Technology for all facilities.

Thanks to my family for their continual support.

Abstract

In this study the fixed oil of the medicinally important species- *Brassica nigra* was investigated. Phytochemical screening revealed the presence of flavonoids, tannins, alkaloids and saponins. The antimicrobial potential was measured via the cup plate agar diffusion assay against six standard human pathogens and significant results were obtained .

The oil was analyzed by GC-MS where 31 constituents were detected being dominated by: 13-docosenoic acid methyl ester(33.53%),9,12-Z,Z-octadecadienoic acid methyl ester(16.20%),11-eicosenoic acid methyl ester(12.12%),9,12,15-octadecatrienoic acid, methyl ester (10.75%), 9-Z--octadecenoic acid methyl ester(5.90%) and hexadecanoic acid methyl ester(4.26%).

مستخلص البحث

في هذه الدراسة اخضع نبات الخردل للمسح الفيتوكيميائى حيث اتضح وجود الفلافونيدات , القلويدات , التنينات والصابونيات وهى مواد ذات فعالية بيولوجية معروفة.

ثم اختبرت فعاليه زيت النبات ضد بعض انواع البكتريا القياسية وكانت الفعاليه لهذا الزيت هامة. ثم حددت مكونات الزيت بتقنيه الكروموتوغرافيا السائله الغازية- طيف الكتله حيث اتضح وجود واحد وثلاثون مكونا اهمها :

13-docosenoic acid methyl ester(33.53%),9,12-Z,Z-octadecadienoic acid methyl ester(16.20%),11-eicosenoic acid methyl ester(12.12%),9,12,15-octadecatrienoic acid, methyl ester (10.75%), 9-Z--octadecenoic acid

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Chapter one
Introduction

1-Introduction

1.1- Gas chromatography- mass spectrometry

GC-MS is essentially an analytical method that combines the feature of gas liquid chromatography and mass spectrometry to identify different substance within a test sample . Applications of GC-MS include during 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 .

GC-MS has been widely heralded as"gold standard" forensic substance identification because it is used to perform specific test . A specific test positively identifies the actual presence of a particular substance in a given sample .A non –specific test

merely indicates that a substance falls into a category of substance .

Although a non specific test could statistically suggest the identify of the substance ,this could lead to false positive identification .

1.2- Instrumentation

The GC-MS is composed of two major buliding blocks: the gas chromatograph and the mass spectrometer . the gas chromatograph utilizes a capillary column which depends on the column`s dimensions (length, diameter, film thichness) as well as the phase properties (e.g. 5% phenyl polysiloxane) .

The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column . The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture , ionize , accelerate , deflect , and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass -to -charge ratio .

These two components , used together , allow a much fine 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 process which 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 gas chromatograph and mass spectrometer .Therefore ,when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis , it typically increases certainly that the analyte of interest is in the sample .

For the analysis of volatile compounds a purge and tap (P and T) 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 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 volatile interface , which is a split inlet system .P and T- GC-MS is particularly suited to volatile organic compounds (VOCs)and aromatic compounds associated with petroleum)[1].

1.3- Types of mass spectrometer detectors

The most common type of mass spectrometer (MS)associated with a gas chromotograph (GC) is the quadrupole mass spectrometer , sometimes referred to by the Hewlett -Packard (now Agilent); trade name ((Mass selective Detector)) (MSD) . Another relatively common detector is the ion trap mass spectrometer . Additionally one may find a magnetic sector mass spectrometer , however these particular instruments are expensive and bulky and not typically found in high – throughput service laboratories . Other detectors may be encountered such as time of flight (TOF) , tandem quadrupoles (MS – MS) , or in the case of an ion trap MS^n where (n) indicates the number mass spectrometry stages[1] .

1.3.1- GC- Tandem MS

When a second phase of mass fragmentation is added , for example using a second quadrupole in a quadrupole instrument , it is called tandem MS (MS/MS) .MS/MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background[1] .

The first quadrupole (Q_1) is connected with a collision cell (q_2) and anther quadrupole (Q_3) . Both quadrupoles can be used in scanning or static mode , depending on the type of MS/MS analysis being performed. Types of analysis include : production scan ,selected reaction monitoring (SRM) (sometimes referred to multiple reaction monitoring (MRM) and neutral loss scan . For example : when Q_1 is in static mode (looking at one mass only as in SIM) , and Q_3 is in scanning mode , one obtains the so –called product ion spectrum (also‟ daughter spectrum ”). From this spectrum , one can select a prominent production which can be the production for the chosen precursor ion . The pair is called a‟transition ” and forms the basis for SRM .(SRM) is highly specific and virtually eliminates matrix background[1] .

1.4- Ionization

After the molecules travel through the column , pass through the transfer line and enter into the mass spectrometer they are ionized by various method being used at any given time . Once the sample is fragmented it will then be detected , usually by an

electron multiplier diode , which essentially turns the ionized mass fragment into an electrical signal that is then detected .The ionization technique chosen is independent of using full scan or SIM[1] .

1.4.1 Electron ionization

By far the most common and perhaps standard form of ionization is electron ionization (EI) . The molecules enter into the MS (the source is a quadruple or the ion trap itself in an ion trap MS) where they are bombarded with free electrons emitted from a filament , causing the molecule to fragment in a characteristic and reproduced way . This "hard ionization" technique results in the creation of more fragments of low mass to charge ratio (m/z) and few , if any , molecules approaching the molecular mass unit .Hard ionization is considered by mass spectrometrists as the employ of molecular electron bombardment , whereas "soft ionization" is caused by molecular collision with an introduction gas . The molecular fragmentation pattern is dependent upon the electron energy applied to the system , typically 70ev (electron volt) . The use of 70ev facilitates comparison of generated spectra with library spectra using manufacturer –supplied software or software developed by the national Institute of standards (NIST-USA). Spectral library searches employ matching logarithms such as probability based matching[2] and dot – product matching that are used with methods of analysis written by many method

standardization agencies . Sources of libraries include NIST[3] Wilely[4], the AAFS[5], and instrument manufacturers .

1.4.2- Cold electron ionization

The "hard ionization" process of electron ionization involves the cooling of molecules before their ionization , resulting in mass spectra that are richer in information[6,7]. In this method - named cold electron ionization (cold - ET) . the molecules exit the GC column , mixed with added helium make up gas and expand into vacuum through a specially designed supersonic molecular beam (SMB) . Collisions with the make up gas at the expanding supersonic jet reduce the internal vibrational (and rotational)energy of the analyte molecules , hence reducing the degree of fragmentation pattern , thus making cold –Et mass spectra compatible with library search identification probabilities of known compounds , amplify isomer mass spectral effects and enable the use of isotope abundance analysis for the elucidation of element formulae[8].

1.4.3- Chemical ionization

In chemical ionization a reagent gas , typically methane or ammonia is introduced into the mass spectrometer . Depending on the technique (positive Cl or negative Cl)chosen , this reagent gas will interact with the electrons and analyte and cause a (soft) ionization of the molecule of interest . Benefits of using chemical ionization is that a mass fragment closely

corresponding to the molecular weight of the analyte of interest is produced[8] .

In positive chemical ionization (PCI) the reagent gas interacts with the target molecule , most often with a proton exchange . This produces the species in relatively high amounts . In negative chemical ionization (NCI)the reagent gas decreases the impact of the free electrons on the target analyte . this decreased energy typically leaves the fragment in great supply[8] .

1.5- Analysis

A mass spectrometer is typically utilized in one of two ways : full scan or selected ion monitoring (SIM). The typical GC – MS instrument is capable of performing both functions either individually or concomitantly , depending on the setup of the particular instrument[8] .

The primary goal of instrument analysis is to quantify an amount of substance . This is done by concentrations among the atomic masses in the generated spectrum . two kinds of analysis are possible , comparative and original . Two kinds of analysis are possible: comparative and original .In comparative, the given spectrum goes to spectrum library to see if its characteristics are present for some sample in the library . This is best performed by a computer because visual distortions may take place due to variation in scale . Computers can also

simultaneously correlate more data (such as the retention times identified by GC), to more accurately relate certain data[8] .

Another method of analysis measures the peaks in relation to one another . In this method , the tallest peak is assigned 100% of the value , and the peaks being assigned proportionate values . All values above 3% are assigned . The total mass of the unknown compound is normally indicated by the parent peak and can be used to fit with a chemical formula containing the various elements which are believed to be in the compound[8] .

The isotope pattern in the spectrum , which is unique for elements that have many isotopes , can also be identified , and must be consistent with the characteristics recorded by GC-MS .Typically , this identification is done automatically by programs which come with the instrument, given a list of the elements which could be present in the sample[8] .

A "full spectrum" analysis considers all the "peaks" within a spectrum .Conversely , selective ion monitoring (SIM) only monitors selected ions associated with a specific substance .This is done on the assumption that at a given retention time , a set of ions is characteristic of certain compound . this is a fast and efficient analysis , especially if the analyst has previous information about a sample or is only looking for a few specific substances . When the amount of information collected about the ions in a given gas chromatographic peak decreases , the

sensitivity of the analysis allows for a smaller quantity of a compound to be detected and measured , but the degree of certainty about the identity of that compound is reduced[8] .

1.5.1-Full scan MS

When collecting data in the full scan mode , a target range of mass fragments is determined and put into the instrument‘s method . An example of a typical broad range of mass fragments to monitor would be m/z 50 to m/z 400 . The determination of what range to use is largely dictated by what one anticipates being in the sample while being mixed of the solvent and other possible interferences . A MS should not be set to look for mass fragments too low or else one may detect air (found as m/z 28 due to nitrogen), carbon dioxide (m/z 44) or other possible interferences . Additionally if one is to use a large scan range then sensitivity of the instrument is decreased due to performing fewer scans per second since each scan will have to detect a wide range of mass fragments[8].

Full scan is useful in determining unknown compounds in a sample .It provides more information than SIM .When it comes to confirming method development it may be common to first analyze test solutions in full scan mode to determine the retention time and mass fragment fingerprint before moving to a SIM instrument method[8] .

1.5.2- Selected ion monitoring

In selected ion monitoring (SIM) certain ion fragments are entered into the instrument and only mass fragments are detected by the mass spectrometer .

The advantage of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g. three fragments) during each scan .More scans can take place each second. Since only a few mass fragments of interest are being monitored , matrix interferences are typically confirming the likelihood of a potentionally positive result . It is relatively important to be sure that the ion ratios of the various mass fragments are comparable to a known reference standard8 .

1.6- Applications of GC/MS

1.6.1- Environmental monitoring and cleanup

GC –MS is becoming the tool of choice for tracking organic pollutants in the environment . The cost of GC –MS equipment has decreased significantly , and the reliablility has increased at the same time , which has contributed to its increased adoption in environmental studies. There are some compounds for which GC-MS is not sufficiently sensitive ,including certain pesticides and herbicides , but for most organic analysis of environmental samples , including many major classes of pesticides , it is very sensitive and effective[7,8] .

1.6.2 -Criminal forensics

GC-MS can analyze the particles from a human body in order to help link a criminal to a crime . The analysis of fire debris using GC-MS is well established , and there is even an established American Society for Testing Materials (ASTM)standard for fire debris analysis . GC MS/MS is especially useful hereas samples often contain very complex matrices and results , used in court , need to be highly accurate[8] .

1.6.3- Law enforcement

GC-MS is increasingly used for detection of illegal narcotics , and may eventually supplant drug – sniffing dogs [9] . It is also commonly used in forensic toxicology to fin drugs and /or poisons in biological specimens of suspects or victims .

1.6.4- Sports anti –doping analysis

GC-MS is the main tool used in sports anti – doping laboratories to test athletes' urine samples for prohibited performance – enhancing drugs ,for example anabolic steroids[10] .

1.6.5- Security

Explosive detection systems have become a part of all airports. These systems run on a host of technologies , many of them based on GC-MS .There are only three manufacturers certified by the FAA to provide these systems , one of which is thermo

Detection (formerly Thermedics), which produces explosive detectors[10] .

1.6.6- Chemical warfare agent detection

Traditional GC –MS units with transmission quadruple mass spectrometers , as well as those with cylindrical ion trap (CIT – MS)and ion trap (T – ITMS) mass spectrometers have been modified for field portability and near real – time detection of chemical warefare agents (CWA) such as Sarin, Soman and VX . The complex and large GC –MS systems have been modified and configured with resistively heated low thermal mass (LTM) gas chromatographs that reduce analysis time to less than ten percent of the time required in traditional laboratory systems . Additionally , the system is smaller , and more mobile , including units that are mounted in mobile analytical laboratories (MAL). Depending on the system , the analytes can be introduced via liquid injection , desorbed from sorbent tubes through a thermal desorption process , or with solid – phase micro extraction (SPME)[10] .

1.6.7- Food ,beverage and perfume analysis

Food and beverage contain numerous aromatic compounds ,some naturally present in the raw materials and some forming during processing . GC-MS is extensively used for the analysis of these compounds which includes esters ,fatty acids ,alcohols ,aldehydes terpenes etc . It is also used to detect and measure

contaminants from spoilage or adulteration which may be harmful and which is often controlled by governmental agencies , for example pesticides[10] .

1.6.8- Astrochemistry

Several GC-MS have left earth and were brought to Mars by the Vikking program[ ].Venera 11 and 12 and Pioneer Venus analysed the atmosphere of Venus by GC –MS [11,12] . The material in the comet 67 Plchurynmov –Gerasimenko was analyzed by GC –MS in 2014[13] .

1.6.9- Mediciene

Dozens of congenital metabolic diseases also known as inborn error of metabolism are now detectable by newborn screening tests , especially the testing using gas chromatography –mass spectrometry . GC –MS can determine compounds in urine even in minor concentration .These compounds are normally not present but appear in individuals suffering from metabolic disorders . This is increasingly becoming a common way to diagnose IEM for earlier diagnosis and institution of treatment eventually leading to a better outcome . It is now possible to test a newborn for over 100 genetic disorders by a urine test at birth based on GC- MS[13] .

In combination with Isotopic labeling of metabolic compounds , the GC –MS is used for determining metabolic activity .Most applications are based on the use of ^{13}C as the labeling and the measurement of $^{13}C - ^{12}C$ ratios with isotope mass spectrometer (IRMS); an MS with a detector designed to measure a few selections and return values as ratios[14] .

1.7- Essential oils

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 , such as oil of clove . An oil is " essential " in the sense that it contains the " essence of " the plant's fragrance the characteristic fragrance of the plant from which it is derived [15] .

The term essential used here does not mean idispensable as with the terms essential amino acids or essential fatty acids which are so called since they are nutritionally required by a given living organism[16] .

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

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 efficiency of medical treatments , and treatment of cancers in particular , are now subject to regulation in most countries[16] .

As the use of essential oils has declined in evidence – based medicine , one must consult order textbooks for much information on their use[17 ,18] . Modern works are less inclined to generalize ; rather than refer to " essential oils " as a class at all , they prefer to discuss specific compounds , such as methyl salicylate , rather than " oil of wintergreen[19,20] .

Interest in essential oils has revived in recent decades with the popularity of aramothereby , a branch of alternative medicine that claims that essential oils and other aromatic compounds have curative effects . Oils are volatilized or diluted in a carrier oil , diffused in the air by a nebulizer , heated over a candle flame , or burned as incense .

The earlist recorded mention of the techniques and methods used to produce essential oils is believed to be that of Ibn al-Baitar (1188 -1248) , and Andalusian physician , pharmacist and chemist[21] .

1.8 –Extraction of essential oils

1.8.1- Distillation

Today most common essential oils – such as lavender , peppermint , tea tree oil and eucalyptus - are distilled . Raw plant material , consisting of the flowers , leaves , wood , bark , roots , seeds ,is put into a distillation apparatus over water . As the water is heated , the steam passes through the plant material , vaporizing the volatile compounds . The vapors flow through a coil , where they condense back to liquid , which is then collected in the receiving vessel[21] .

Most oils are distilled in a single process . One exception is *Cananga odorata* oil , which takes 22 hours to complete through a fractional distillation[21] .

The recondensed water is referred to as a hydrosol ,herbal distillate or plant water essence , which may be sold as another fragrant product .

Popular hydrosols include rose water, lavender water , lemon balm , clary sage and orange blossom water . The use of herbal distillates in cosmetics is increasing . Some plant hydrosols have unpleasant smells and are therefore not sold[21] .

1.8.2- Expression

Most citrus peal oils are expressed mechanically or cold – pressed (similar to olive extraction)[22] .Due to the relatively large quantities of oil in citrus peel and low cost to grow and

harvest the raw materials , citrus – fruit oils , are cheaper than most other essential oils . Lemon or sweet orange oils that are obtained as by-products of the citrus industry are even cheaper . Before the discovery of distillation , all essential oils were extracted by pressing[23].

1.8.3- Solvent extraction

Most flowers contain too little volatile oil to undergo expression , their chemical compounds are too delicate and easily denatured by the high heat used in steam distillation . Instead , a solvent such as hexane or supercritical carbon dioxide is used to extract the oils . Extracts from hexane and other hydrophobic solvents are called concretes , which are a mixture of essential oil , waxes , resins , and other lipophilic (oil – soluble) plant material[23] .

Highly fragrant concretes contain large quantities of non – fragrant waxes and resins ,hence , another solvent , such as ethyl alcohol ,which is more polar in nature ,is used to extract the fragrant oil from the concrete .

The alcohol solution is chilled to -180^0 ($0\ ^0F$) for more than 48 hour which causes the waxes and liquids to precipitate out . The precipitates are then filtered out and the ethanol is removed from the remaining solution by evaporation , vacuum purge ,or both ,leaving behind the absolute oil[23].

Supercritical carbon dioxide is used as a solvent in supercritical fluid extraction . This method has many benefits including

avoiding petrochemical residues in the loss of some " top notes " when steam distillation is used . It does not yield absolute oil directly . The supercritical carbon dioxide will extract both waxes and essential oils that makeup the concrete[23] .

Subsequent processing with liquid carbon dioxide , achieved in the same extractor by merely lowering the extraction temperature , will separate the waxes from the essential oils . This lower temperature process prevents the decomposition and denaturing of compounds . When the extraction is complete , the pressure is reduced to ambient and carbon dioxide reverts to a gas , leaving no residue . Supercritical carbon dioxide is also used for making decaffeinated coffee . Although it uses the same basic principles, it is a different process because of the difference in scale[23] .

1.8.4- Florasols extraction

Florasol (1,1,1,2-tetrafluoroethane) , a refrigerant , was developed to replaced Freon . Although it is an " ozone – friendly " product , it poses significant danger to the environment due to its global warming potential[24] . The European Union has banned its use ,with a phase –out process that began in 2011[25]. One advantage is that the extraction of essential oils occurs at or below room temperature so degradation through high temperature extremes does not occur .

The essential oils are mostly pure and contain little no foreign substances .

1.9- Production quantities

Estimates of total production of essential oils are difficult to obtain . One estimate, compiled from data in 1989 , 1990 and 1994 from various sources , gives the following total production , in tones , of essential oils for which more than 1,000 tones were produced[26] .

Oil	Tones
Sweet orange	12,000
Menth arvensis	4,800
Peppermint	3,200
Cedarwood	2,600
Lemon	2,300
Eucalyptus globulus	2,070
Litsea cubebe	2,000
Clove(leaf)	2,000
Spearmint	1,300

1.10- Antimicrobails

An antimicrobial is an agent that kills microorganisms or inhibits their growth[27]. Antimicrobial medicines can be grouped according to the microorgasims they act primarily against . For example , antibiotics are used against bacteria while antifungals are used against fungi .They can also be classified according to their function . Agents that kill microbes are called microbicidal , while those that merely inhibit their growth are called biostatic . The use of antimicrobial medicines to treat infection is known as antimicrobial chemotherapy , while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis[27] .

The main classes of antimicrobial agents are : disunfectants (“ non selective antimicrobials ” such as bleach) which kill a wide range of microbes on nonliving surfaces to prevent the spread of illness ; antiseptics (which are applied to living tissue and help reduce infection during surgery) and antibiotics (which destroy microorganisms within the body) . The term “antibiotic ” originally described only those formulations derived from living organisms but is now also applied to synthetic antimicrobials , such as the sulphonamides , or fluoroquinolones . The term is also used to be restricted to antibacterials (and is often used as a synonym for them by medical literature) ,but its context has

broadened to include all antimicrobials . Antibacterial agents can be further subdivided into bactericidal agents , which kill bacteria , and bacteriostatic agents , which slow down or stall bacterial growth .Use of substances with antimicrobial properties is known to have been common practice for at least 2000 years[27] .

Ancient Egyptians and ancient Greeks used specific molds and plant extracts to treat infection[28] . More recently , microbiologists such as Louis Paster and Jules Francois Joubert observed antagonism between some bacteria and discussed the merits of controlling these interactions in medicine[29].

In 1928 Alexander Fleming became the first to discover a natural antimicrobial fungus known as *penicillium rubens* . The substance was extracted from a fungus was named- penicillin and in 1942 it was successfully used to treat a *streptococcus* infection [30] . Penicillin also proved successful in treatment of many other infections such as gonorrhea and pneumonia , which were potentially fatal to patients up until then .Many antimicrobial agents exist now for use against a wide range of infections .

1.11-The target species - *Brassica nigra*

-Family: Brassicaceae

-Common /English names

Abyssinian Mustard ,Black Mustard ,Brown Mustard ,Cadlock ,Indian Mustard , Scurvy ,Senvil , Shortpod Mustard , Ture Mustard , Warlock

-Mustard seeds

Are the small round seeds of various mustard plants. The seeds are usually about 1 to 2 millimetres (0.039 to 0.079 in) in diameter and may be colored from yellowish white to black. They are important herbs in many regional foods and may come from one of three different plants: black mustard (Brassica nigra), brown Indian mustard (B. juncea), or white mustard (B. hirta/Sinapis alba)[31].

Mustard seeds generally take three to ten days to germinate if placed under the proper conditions, which include a cold atmosphere and relatively moist soil. Mature mustard plants grow into shrubs[31].

Mustard grows well in temperate regions. Major producers of mustard seeds include Canada, Hungary, England, India, Pakistan and the United States. Brown and black mustard seeds return higher yields than their yellow counterparts[32].

In Pakistan, rapeseed-mustard is the second most important source of oil, after cotton. It is cultivated over an area of

307,000 hectares with annual production of 233,000 tones and contributes about 17% to the domestic production of edible oil[32].

Mustard seed is a rich source of oil and protein. The seed has oil as high as 46-48%, and whole seed meal has 43.6% protein.

In Ethiopia , where it is cultivated as a vegetable, the shoots and leaves are consumed cooked and the seeds used as spice [33] .

Ground seeds of the plant mixed with honey are widely used in eastern Europe as cough suppressant . In Eastern Canada , the use of "mouche demoutarde" to treat respiratory infections was popular before the advent of modern medicine . It consisted in mixing ground mustard seeds with flour and water , and creating a cataplasm with the paste . This cataplasm is put on the chest or the back and left until the person felt a stinging sensation[33] .

The plant itself can grow from two to eight feet tall , with racemes of small yellow flowers . These flowers possess four petals each . The leaves are covered in small hairs ; they can wilt on hot days , but recover at night [34] .

Black mustard is insect - pollinated . Bees collect the copious mustard nectar and produce a mild – flavored , light – colored honey . Mildews appear on the leaves causing malformation of flower heads and pods , a situation encontrolled by sulfur-dusting or spraying with Bordeaux Mixture . Main insect pest is mustard sawfly (Athalia lugensproxima), larvae which feed on the leaves[34] .

Findings of present *in vitro* study confirmed the folkloric claims of the antibacterial effectiveness of spices domestically consumed in India . Results suggested that essential oils of reference spices most effectively inhibited bacterial strains , followed by powdered forms , while aqueos extracts were found to be ineffective . Essential oils of *B.nigra* and powdered form of *B.nigra* may be considered for further *in vitro* and *in vivo* studies against other harmful pathogens and may be considered for food preservation[35] .

Aims of this study

This study was designed to:

1. -extract of essential oil from the medicinally important *Brassica nigra* plant.
2. -Conduction GC-MS studies on the extracted oil.
3. -to test the antimicrobial activity of the oil.

Chapter two
Materials and methods

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2. Material and Methods

2.1. Materials

2.1.1 Plant material

The seeds of *Brassica nigra* were collected from Nyala, west Sudan in October 2015. The plant was authenticated by direct comparison with a herbarium sample.

2.1.2- Instruments

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

2.1.3-Test organisms

Brassica nigra oil was screened for antibacterial and antifungal activities using the standard microorganisms shown in table(2.1).

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

Flavonoid and phenolic test reagents

- Aluminium chloride solution

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

- Potassium hydroxide solution

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

-Ferric chloride solution

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

Alkaloid test reagents

Maeyer reagent

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

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

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

-Wagner reagent

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

2.2.2.Preparation of plant extract for phytochemical screening

(100 g) of powdered shade - dried seeds of *Brassica niger* were extracted with 80% aqueous methanol (soxhlet) until exhaustion. This prepared extract(PE) was used for phytochemical screening.

2.2.3. Phytochemical screening

The prepared extract (PE) was used for following tests:

2.2.3.1- 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 sodium sulphite anhydrous. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

2.2.3.2- Test for flavonoids

(20 ml) of the (PE) was 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. Red colour was observed.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added. A dark yellow colour was formed.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.

2.2.3.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for minutes, then cooled and divided into two portions:

To one portion a few drops of Maeyer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

2.2.3.4- Test for tannins

(10 ml) of (PE) was 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 was treated with few drops of ferric chloride solution. A dark blue colour was observed.

2.2.3.5- Test for saponins

(1 g)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. Honey comb was formed.

2.2.4- Biological activity

2.2.4.2 -Antimicrobial assay

Brassica nigra seed oil was screened for antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*) using the cup plate agar method with some minor modifications.

2.2.4.2.1- Preparation of bacterial suspensions

One ml. aliquots of 24 hours broth culture of the test organisms were distributed onto 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 100 ml of normal saline to produce suspension containing about 10^{8}- 10^{4} colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the

saline 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 volume (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 to dry, and then incubated at 37^{o} C for 24 hours.

2.2.4.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on dextrose agar incubated at 25^{o}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.

2.2.4.2.3 -Testing for antibacterial activity

The cup plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity of the oil.

Two ml of the standardized bacterial stock suspention were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes and the agar was left to settle 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 of the halves was designed for one of the extracts.

The agar discs were removed and cups were filled with(0.1) ml of each extract 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 extracts and the standard antimicrobial

chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.4.2.4 -Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used. Samples were used here by the same concentrations used above.

2.2.5-Extraction of oil from seeds of *Brassica niger*

Powdered seeds of *Brassica niger* (500g) were exhaustively extracted with n-hexane (soxhlet).The solvent was removed under reduced pressure and the oil was kept in the fridge at 4^{o}C for further manipulation.

2.2.5.1-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

The oil of *Brassica nigra* seeds was analysed 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(°C)	Hold Time ($min.^{-1}$)
-	150.0	1.00
4.00	300.0	0.00

Table2.3: Chromatographic conditions

Column oven temperature	150.0°C
Injection temperature	300.0°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

Chapter two
Materials and methods

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3-Results and Discussion

3.1-Phytochemical screening

In the present study, Sudanese *Brassica nigra* was screened for the occurrence of bioactive compounds. The results(Table 3.1) positively showed the presence of flavonoids, saponins, tannins, alkaloids and glycosides in both aqueous and methanolic extract of seeds.

Table 3.1:Phytochemical screening of *Brassica nigra* seed oil

Species	Flavonoids	Tannins	Alkaloids	Glycosides	Saponins
Brassica niger	+ve	+ve	+ve	+ve	+ve

3.2-Antimicrobial activity

*Brassica nigr*a oil was evaluated for antimicrobial activity via the cup plate agar diffusion assay. The average of the diameters of the growth inhibition zones are shown in Table (3.2) .The results were interpreted in terms of the commonly used terms (<9mm: inative;9-12mm:partially active;13-18mm:

active;>18mm:very active) .Tables (3.3) and (3.4) represent the antimicrobial activity of standard antibacterial and antifungal

chemotherapeutic agents against standard bacteria and fungi respectively.

Table 3.2 : Antibacterial activity of *Brassica nigra* seed oil :M.D.I.Z (mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
*Brass*sica *nigra* oil	100	12	10	15	14	11	13

Table 3.3 : Antibacterial activity of standard chemotherapeutic agents :M.D.I.Z (mm)

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

Table 3.4 : Antifungal activity of standard chemotherapeutic agents against standard fungi

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

- Sa.*: Staphylococcus aureus*
- Ec.*: Escherichia coli*
- Pa.: *Pseudomonas aeruginosa*
- An.: *Aspergillus niger*
- Ca.: *Candida albicans*
- *Bs.: Bacillus subtilis*

The oil showed activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Aspergillus niger*.However,it gave partial activity against *Pseudomonas aeruginosa* and *Candida albicans*.

3.3-The GC-MS analysis of *Brassica nigra* oil

GC-MS analysis of *Brassica nigra* oil was conducted and the identification of the constituents was initially accomplished by comparison with the MS library (NIST) and further confirmed by interpreting the observed fragmentation pattern. Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.3.1- Constituents of oil

The GC-MS spectrum of the studied oil revealed the presence of 20 components(Table 3.5).The typical total ion chromatograms(TIC) are shown in Fig.1.

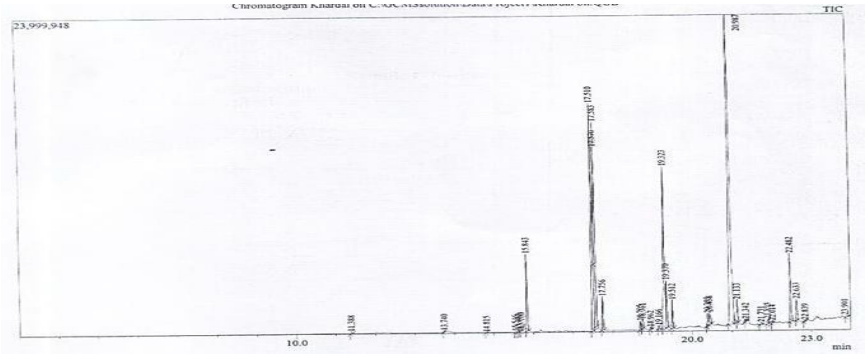

Fig.1:Cromatograms of *Brassica nigra* oil

Table 3.5: Constituents of *Brassica nigra* seed oil

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	11.388	271897	0.12	Butylated Hydroxytoluene
2	13.740	250003	0.11	Methyl tetradecanoate
3	14.815	46627	0.02	Pentadecanoic acid, methyl ester
4	15.546	110510	0.05	7,10-Hexadecadienoic acid, methyl ester
5	15.613	289554	0.13	Methyl 8,11,14-heptadecatrienoate
6	15.650	217067	0.09	9-Hexadecenoic acid, methyl ester, (Z)-
7	15.700	371831	0.16	Adipic acid, isohexyl 2-methoxyethyl ester
8	15.843	9815011	4.26	Hexadecanoic acid, methyl ester
9	17.510	37331401	16.20	9,12-Octadecadienoic acid (Z,Z)-, methyl e
10	17.570	13593022	5.90	9-Octadecenoic acid (Z)-, methyl ester
11	17.583	24772021	10.75	9,12,15-Octadecatrienoic acid, methyl este
12	17.756	4148144	1.80	Methyl stearate
13	18.705	1234092	0.54	Adipic acid, 2-methoxyethyl octyl ester
14	18.761	1164783	0.51	Hexanedioic acid, dioctyl ester
15	18.962	349721	0.15	Octadecanoic acid, 2,3-dihydroxypropyl es
16	19.166	493022	0.21	.gamma.-Linolenic acid, methyl ester
17	19.323	27928201	12.12	11-Eicosenoic acid, methyl ester
18	19.370	6061251	2.63	cis-11-Eicosenoic acid, methyl ester
19	19.512	3539445	1.54	Methyl 18-methylnonadecanoate
20	20.385	1895265	0.82	Adipic acid, decyl 2-methoxyethyl ester
21	20.438	1649677	0.72	Adipic acid, dodecyl 2-methoxyethyl ester
22	20.987	77253719	33.53	13-Docosenoic acid, methyl ester
23	21.133	2896958	1.26	Methyl 20-methyl-heneicosanoate
24	21.342	232642	0.10	1,3-Dioxolane, 4-methyl-2-(1-pentenyl)-, (I
25	21.731	385099	0.17	cis-13-Eicosenoic acid, methyl ester
26	21.895	771707	0.33	Oxiraneoctanoic acid, 3-octyl-, methyl este
27	22.014	344113	0.15	Octadecanoic acid, 9,10-epoxy-, isopropyl
28	22.482	9562918	4.15	15-Tetracosenoic acid, methyl ester, (Z)-
29	22.633	2757562	1.20	Tetracosanoic acid, methyl ester
30	22.839	263431	0.11	9-Octadecenoic acid, 1,2,3-propanetriyl es
31	23.901	403705	0.18	Cyclopropaneoctanoic acid, 2-hexyl-, meth
		230404399	100.00	

The following major constituents were detected in the chromatograms:

13-Docosenoic acid methyl ester(33.53%)

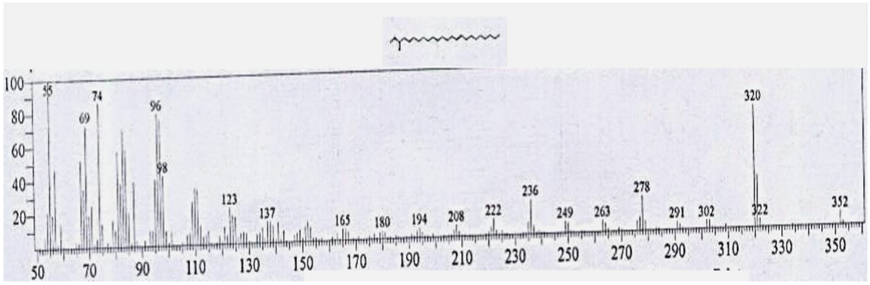

Fig. 2: Mass spectrum of 13-docosenoic acid methyl ester

The EI mass spectrum of 13-docosenoic acid methyl ester is shown in Fig. 2.The peak at m/z 352, which appeared at R.T. 20.987 in total ion chromatogram, corresponds to $M^+[C_{23}H_{44}O_2]^+$ .The peak at m/z320 corresponds to loss of a methoxyl function.

9,12-Z,Z-Octadecadienoic acid methyl ester(16.20%)

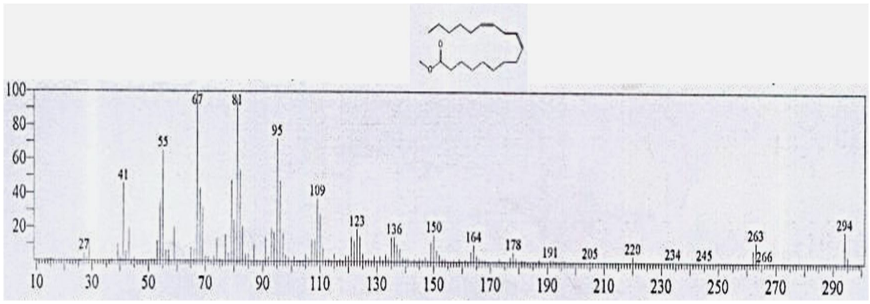

Fig. 3: Mass spectrum of 9,12-Z-Z-octadecenoic acid methyl ester

The EI mass spectrum of 9-octadecenoic acid methyl ester is shown in Fig.3.The peak at m/z 294, which appeared at R.T. 17.51 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.

11-Eicosenoic acid methyl ester(12.12%)

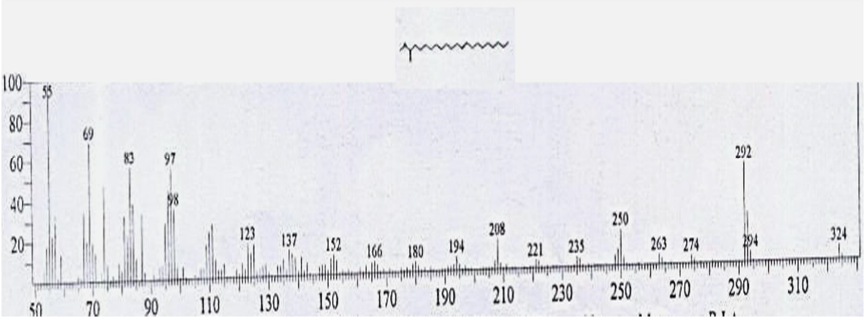

Fig. 4: Mass spectrum of 11-eicosenoic acid methyl ester

The EI mass spectrum of 11-eicosenoic acid methyl ester is shown in Fig.4.The peak at m/z 324, which appeared at R.T. 19.32 in total ion chromatogram, corresponds to $M^+[C_{21}H_{40}O_2]^+$ .The peak at m/z292 corresponds to loss of a methoxyl function.

9,12,15-Octadecatrienoic acid, methyl ester (10.75%)

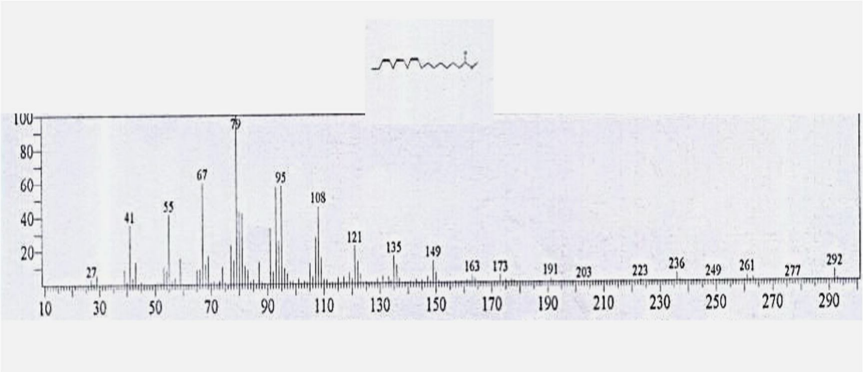

Fig.5: Mass spectrum of 9,12,15-octadecatrienoic acid, methyl ester

The EI mass spectrum of 9,12,15-octadecatrienoic acid, methyl ester is shown in Fig.5.The peak at m/z 292, which appeared at R.T. 17.58 in total ion chromatogram, corresponds to $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z277 corresponds to loss of a methyl group while the signal at m/z261 is due to loss of a methoxyl function.

9-Z--Octadecenoic acid methyl ester(5.90%)

The EI mass spectrum of 9-Z-octadecenoic acid methyl ester is shown in Fig.6.The peak at m/z 296, which appeared at R.T. 17.57 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z266 corresponds to loss of a methoxyl function.

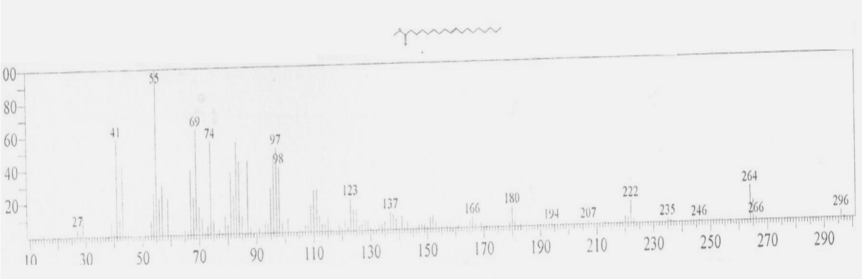

Fig. 6: Mass spectrum of 9-Z-octadecenoic acid methyl ester

Hexadecanoic acid methyl ester(4.26%)

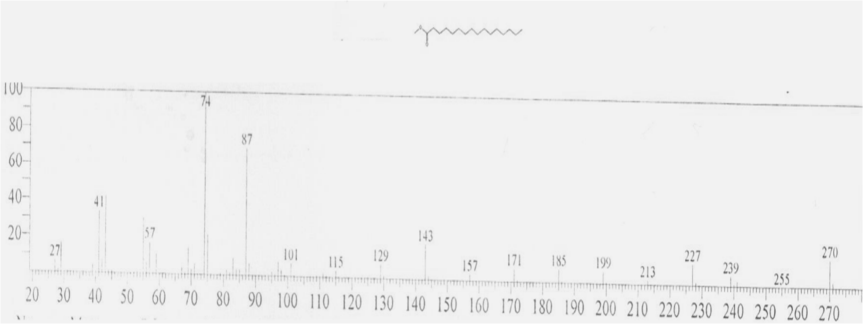

Fig. 7: Mass spectrum of hexadecanoic acid methyl ester

The EI mass spectrum of Hexadecanoic acid methyl ester is shown in Fig.7.The peak at m/z 270, which appeared at R.T. 15.84 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.

Conclusion

Phytochemical screening of *Brassica nigra* revealed the presence of flavonoids, tannins, alkaloids and saponins. The antimicrobial potential was measured via the cup plate agar diffusion assay against six standard human pathogens and significant results were obtained.The oil was analyzed by GC-MS where 31 constituents were detected.

Recommendations

i)Other constituents of *Brassica nigra* may be isolated and studied.

ii)The oil may be screened for other biological activities(anti-inflammatory,antimalarial, antileshmenial…)

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Dedication

To my mother and to my soul of father and sisters and my beloved uncle