Sudan University of Science and Technology

College of Graduate Studies

Chemical Constituents of Some Phytochemicals From *Ammomum sublatum, Foeniculum vulgare* and *Psidium guajava* and their Biological Activity

المكونات الكيميائية لبعض النواتج الطبيعية لنباتات الهبهان الاسود, الشمار و الجوافة وفعاليتها البيولوجية

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

By

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الاستـــهـــــلال

قال تعالي :

(وَإِذْ قُلْتُمْ يَا مُوسَىٰ لَن نَّصْبِرَ عَلَىٰ طَعَامٍ وَاحِدٍ فَادْعُ لَنَا رَبَّكَ يُخْرِجْ لَنَا مِمَّا تُنبِتُ الْأَرْضُ مِن بَقْلِهَا وَقِثَّائِهَا وَفُومِهَا وَعَدَسِهَا وَبَصَلِهَا ۖ قَالَ أَتَسْتَبْدِلُونَ الَّذِي هُوَ أَدْنَىٰ بِالَّذِي هُوَ خَيْرٌ ۚ اهْبِطُوا مِصْرًا فَإِنَّ لَكُم مَّا سَأَلْتُمْ ۗ وَضُرِبَتْ عَلَيْهِمُ الذِّلَّةُ وَالْمَسْكَنَةُ وَبَاءُوا بِغَضَبٍ مِّنَ اللَّهِ ۗ ذَٰلِكَ بِأَنَّهُمْ كَانُوا يَكْفُرُونَ بِآيَاتِ اللَّهِ وَيَقْتُلُونَ النَّبِيِّينَ بِغَيْرِ الْحَقِّ ۗ ذَٰلِكَ بِمَا عَصَوا وَّكَانُوا يَعْتَدُونَ .)

سورة البقرة الاية (٦١)

صدق الله العظيم

Dedication

To ,

my parents,

husband , children

and brothers .

Acknowledgement

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

I would like to express my gratitude to my supervisor Prof. Mohamed Abdelkarim for his careful supervision , valuable advice , kind treatment and effort during this work .

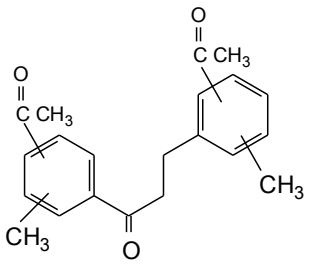
I would like to express my gratitude to all those who helped me during this work , also I would like to express my heartfull gratitude to my family .

Also thanks are extended to University of Medical Sciences and Technology , for GC-MS measurements .

Thanks to The Medicinal and Aromatic Plants Research Institute for all facilities .

Abstract

Extraction of the fruit of *Amomum sublatum* with 90% ethanol gave a crude product , which was fractionated over silica gel plates to afford a chromatographically pure flavonoid- compound I. The structure of the isolated flavonoid was partially elucidated by spectral tools (UV and H^1NMR) and the following partial structure was proposed:



Compound I

The aqueous extract of *Ammomum sublatum* was assessed for antimicrobial activity against five standard bacteria. It showed significant activity against the bacterial strains : *Pseudomonas aeruginosa* and *Bacillus subtilis* . It also showed good anticandidal activity

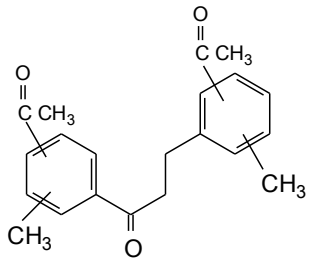
The oils from *Foeniculum vulgae* and *Psidium guajava* were studied by GC-MS , furthermore the oils have been assessed for antimicrobial activity. The GC-MS analysis of *Foeniculum vulgae* oil revealed the presence of 32 components. *Foeniculum vulgae* oil showed excellent activity against *Staphylococcus aureus* in the concentration range :

100-25mg/ml. It also exhibited significant activity against the yeast *Candida albicans* at 100mg/ml.

GC-MS analysis of *Psidium guajava* oil revealed the presence of 30 constituents. The oil showed activity against all test organisms,but it was partially active against the fungus *Candida albicans*. Significant activity against *Staphylococcus aureus* was observed.

مستخلص البحث

تم الحصول علي ناتج خام عند استخلاص النبات بالايثانول وعند اجراء كروموتوغرافيا الطبقة الرقيقة التي استخدمت فيها السيليكا جل تم فصل فلافونويد رئيسي واحد . وقد حدد تركيب مبدئ لهذا الفلافونيد بناء على البيانات الطيفية UV و H^1NMR واعطى التركيب المبدئ التالى :



Compound I

اخضع المستخلص الكحولى لهذا النبات لاختبار مضاد الميكروبات حيث اعطى فعالية جيدة ضد : *Pseudomonas aeruginosa* و *Bacillus subtilis*

درست زيوت نباتى الشمار والجوافة بتقنية GC-MS والتى اوضحت وجود ٣٢ مكونا بزيت الشمار و ٣٠ مكونا بزيت الجوافة . وفى اختبار مضاد الميكروبات ابدى زيت الشمار فعالية ممتازة عند 100-25mg/ml ضد *Staphylococcus aureus* و *Candida albicans* .

اما زيت الجوافة فقد اتضح ان به ٣٠ مكونا . وفى اختبار مضاد الميكروبات ابدى الزيت فعالية عالية ضد *Staphylococcus aureus*.

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Introduction

1.1-The target plant species

1.1.1- *Amomum subulatum*

Amomum subulatum

Amomum subulatum **Roxb**. (Large Cardamom) is a perennial herbaceous crop, cultivated in swampy places across hills around water streams. It has been a well known spice since time immemorial; used as flavouring agent to various dishes indigenous to the Eastern Himalayan region particularly Nepal, Bhutan, and India. Sikkim State of India is the largest producer of cardamom that is around 50% of the world's production. Large cardamom contains 8.6% moisture, 5% total ash value, 1.5% ash insoluble in acid, 3.5% water soluble ash value, 4.88% alcohol extract, 4% non-volatile ether extract and 91.4% of total solid. It

contains 1.95 to 3.32% of essential oil having characteristic aroma and possesses medicinal properties. It is reported as an official drug in Ayurvedic Pharmacopoeia due to its curative as well as preventive properties for various ailments. The major constituent of large cardamom essential oil is 1,8-cineole. The monoterpene hydrocarbon content is in the range of 5 to 17% of which lamonene, sabeinene, and pinenes are significant components. The terpinols comprise approximately 5 to 7% of the oil. Due to the presence of these compounds, it has pharmacognostic properties such as analgesic, antimicrobial, cardiac stimulant, carminative, diuretic, stomachic etc.[1]

1.1.2-Fennel

Foeniculum vulgare

Foeniculum vulgare seeds

Fennel (*Foeniculum vulgare* **Mill.)** is a perennial herb, with feathering leaves, in the family Apiaceae. The plant is cultivated worldwide for its economic value as a flavouring agent in baked foods[2,3]. The plant contains : protein(9.5%) ; fats (10%); minerals(Ca , K , Na, Fe and P),13.4%) ; fibre(18.5%) beside niacin, riboflavin and thiamine[4].

Fennel is added to purgatives to allay their side effects.Seeds are claimed to improve eyesight if taken raw while seed extract has been tested against glfaucoma in model animals. Seeds are also diuretic and hypotensive[5].

The potential pharmacological effects of fennel seems to be associated with its volatile oil which contains, among others, anethole, fenchone, estragol, p- anisaldehyde and α-phellandrene. Anisole is claimed to possess estrogenic properties[7].

Some quercetin and kaempferol conjugates have been isolated from from fennel[7-9]. Such phenolics are responsible for the free radical scavengaging capacity of fennel. Sterols , sugars , acetylated

kaempferol and some benzoisofuranone derivatives were identified in fruits[10,11].

Beside its health promoting properties , a main constituent of fennel-leugenol- has become a cause of concern since the structurally related ,methylleugenol was reported as a potential carcinogenic agent[12].

1.2-The flavonoids

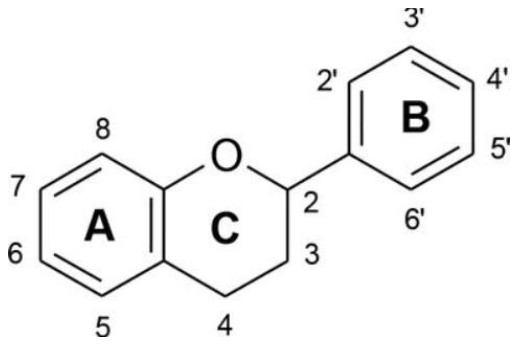
The flavonoids have structure based on a benzopyran skeleton characterized by two phenyl rings joined by a 3 carbon bridge. These natural products are isolated from a wide variety of plants, and are responsible for much of the colouring found in vascular plants. A single plant may contain dozens of different flavonoids , and the distribution of flavonoids within a plant family can yield useful classifying information about that family [13].These phenolics constitute one of the most characteristic class of phenolics in higher plants. Many flavonoids are known to constitute flower pigments in most angiosperm families (flowering plants). However, flavonoids may occur in different parts of the plant [14]. They also play a crucial role in plant development, growth and defense.

Flavonoids possess many interesting biological properties including : antioxidant, anti-inflammatory, and antiviral properties. They also help to maintain the health of small blood vessels and connective tissue, and some are under study as possible treatments of cancer [15].

Over 8000 structurally unique flavonoids have been identified in plants[16] .Flavonoids occur naturally-in fruit, vegetables, and beverages such as tea . For example the flavonol quercetin and the flavone apigenin are found in many fruits and vegetables, including onions, apples, broccoli, and berries. Naringehin is a citrus flavanone. Catechin and other catechins are abundant in green tea. Cyanidin and other anthocyanidins are largely responsible for the deep colors of berries and grapes. Genistein is an isoflavone found predominantly in legumes. The flavonoid consumed most, in general, is quercetin, and the richest sources of flavonoids consumed in general are tea, onions, and apples .Research in the field of flavonoids has rapidly increased. The major actions of flavonoids are those against cardiovascular diseases, ulcers, viruses, inflammation, osteoporosis, diarrhea and arthritis. Brief description about the disease causing effect of free radicals was given and ways by which flavonoids neutralize free radicals has also been mentioned[16].

Plants containing flavonoids have historically been used in traditional eastern medicine[16]. Flavonoids exhibit a wide range of biological activities, and currently are of particular interest as potential anticancer, antiallergic, antithrombotic antibacterial, antifungaland antitumoral agents[16,17.]

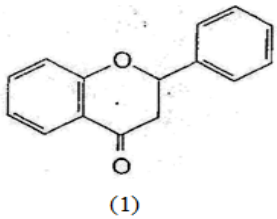
The basic skeleton of the majority of flavonoids consist of two fused six-membered rings (an aromatic A-ring and a heterocyclic C ring) connected to an aromatic B-ring as shown below:



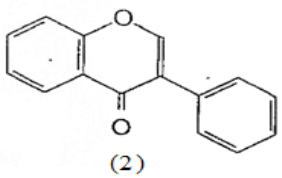
However, flavonoids may be divided into three major classes:

- Flavonoids(1)
- Isoflavonoids(2)
- Neoflavonoids (3)

These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related [14].



(1)



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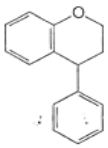
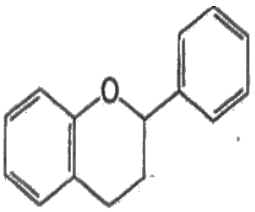


(3)

Furthemore the classification depends on the degree of oxidation and saturation present in the heterocyclic C-ring. When considering these factors, flavonoids may be divided into: flavans(4), flavanones (5), flavones(6) and flavonols (7)[14].



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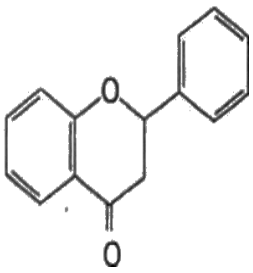

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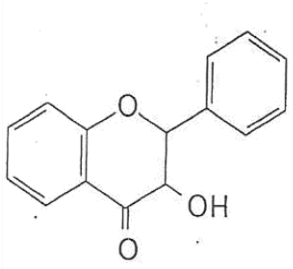

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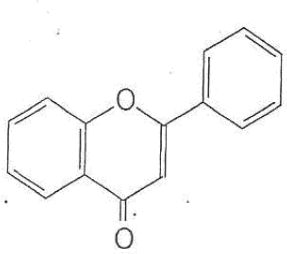


(7)

Being mainly water-soluble, the flavonoids may be extracted with 70% ethanol and remains in the aqueous layer. Flavonoids dissolve in alkalis , giving yellow solutions and with addition of acid the solutions become colourless.Thus they could easily be detected on TLC plates or in solution. Since these phenolics possess conjugated aromatic system they usually give intense absorption bands in UV and visible regions of the spectrum.

It is known that flavonoids exist in plants bound to sugar as glycosides and flavonoid aglycones may occur in a single plant

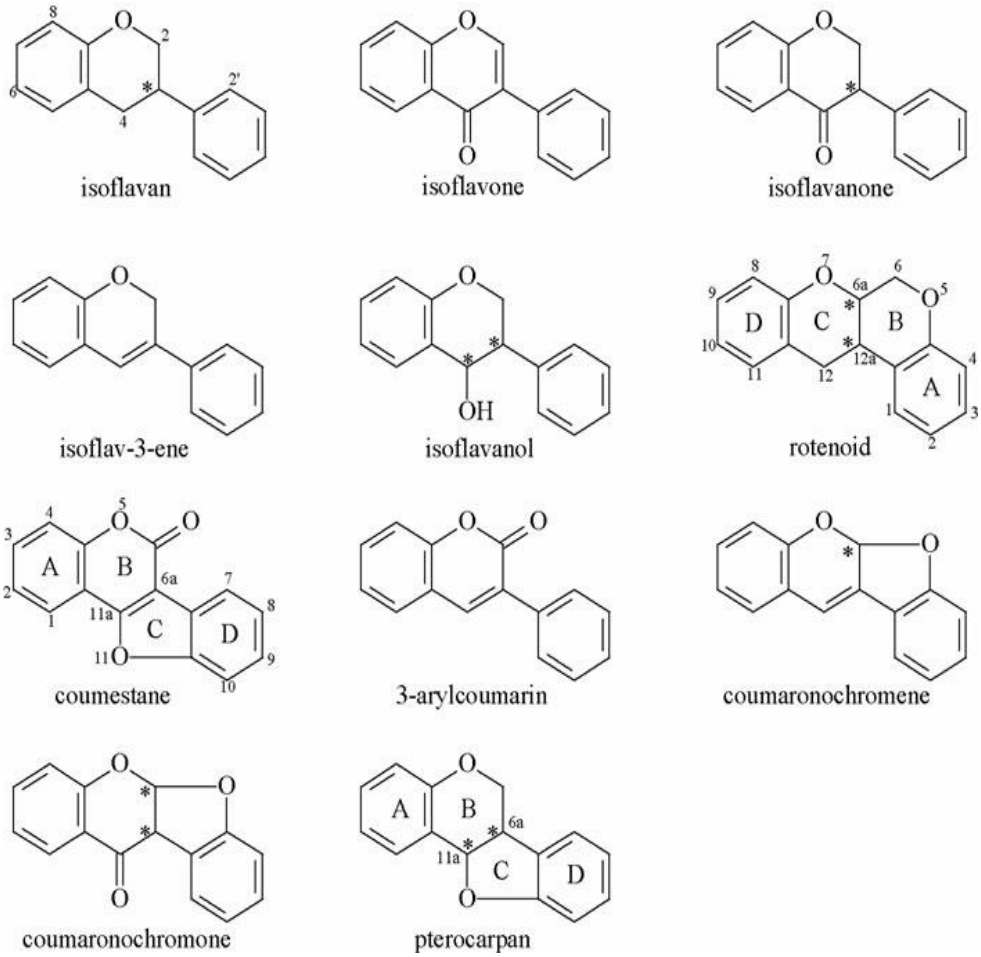
in several glycosidic combination and this is exactly the reason why separating flavonoids it is usually better to examine the aglycones present in hydrolyzed plant extracts before considering the glycosides that may be present in original extract[16].

As far as the distribution of flavonoids is concerned, flavones are known to occur in all vascular plants, but some classes are more widely distributed than others. However , flavonols are found in only a few plant families [16]. Also it is very rare to find only a single flavonoid component in a plant tissue since such tissues usually contain a mixture of flavonoids . In addition there are often mixtures of different flavonoid classes. Research [17] has established that flavones are important co- pigments being essential for the full expression of anthocyanin colour in floral tissues The coloured anthocyanin in flower petals are almost invariably accompanied by colourless flavones or flavonols.

If the classification of flavonoid type in plant tissue is to be considered , then it is based initially on a study of solubility properties and colour reactions. This is usually accompanied by one-dimensional chromatographic examination of hydrolyzed plant extract and two-dimensional chromatography of the alcoholic extract. The flavonoids can then be separated by the usual chromatographic techniques and the individual flavonoids may be identified by some spectral tools (UV,IR , NMR and MS).[15]

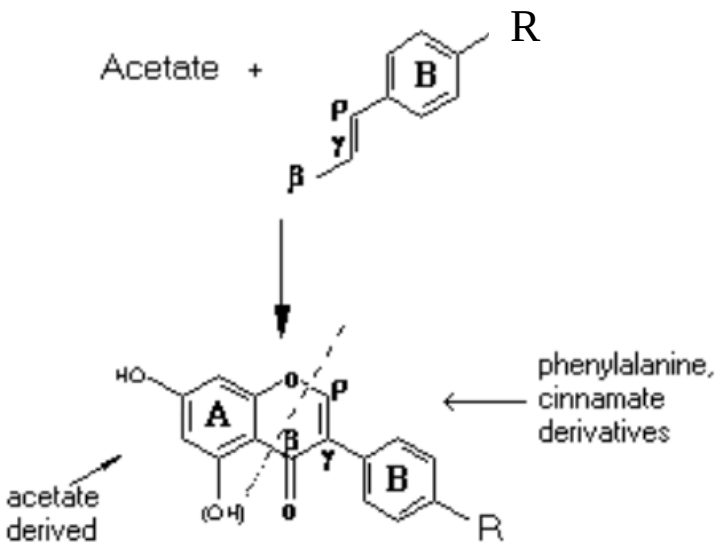
A distinctive class of flavonoids is represented by the isoflavonoids. These plant phenolics possess a 3-phenylchroman skeleton that is biogenetically derived by 1,2-aryl migration in a 2-phenylchroman precursor.

Despite being of very limited distribution in the plant kingdom, the isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises from number and complexity of substituents on the basic 3-phenylchroman system as well as the different oxidation levels' and the presence of additional heterocyclic rings[14]. Isoflavonoids are subdivided into isoflavans, isoflavones, isoflavanones , isoflavan-3-enes , isofilavanols , rotenoids , coumestanss ,3-aryl coumarin , coumaronochromasne and pterocarpans .



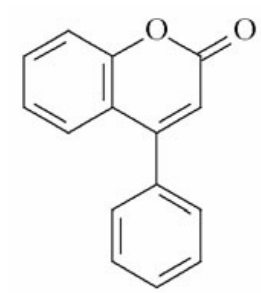
It has been reported that isoflavonoids have a rather limited taxonomic distribution, mainly within the *Leguminosae*. However, knowledge about the biosynthesis of isoflavonoids mainly originates from studies with radioactive isotopes by feeding labelled ^{13}C –cinnamates[17].

As far as colour is concerned, all isoflavonoids are colourless and it has been reported that acetate gives rise to ring A and that phenylalanine, cinnamate and cinnamate derivatives are incorporated into ring B and C-2, -3, and -4 of the heterocyclic ring[17].

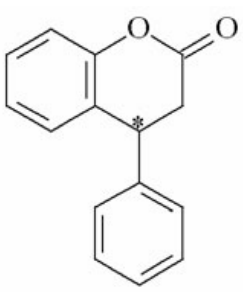


Different studies indicated that chalcones and flavanones are efficient precursors of isoflavonoids and the required aryl migration of ring B from the former 2 or beta position to the 3 or alpha position of the phenylpropanoid precursor must take place after formation of the basic C^{15} skeleton[17].

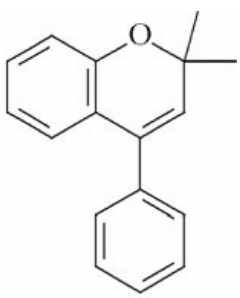
Neoflavonoids represent another class of flavonoids and these phytochemicals are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins.



4-arylcoumarin



3,4-dihydro-4-arylcoumarin



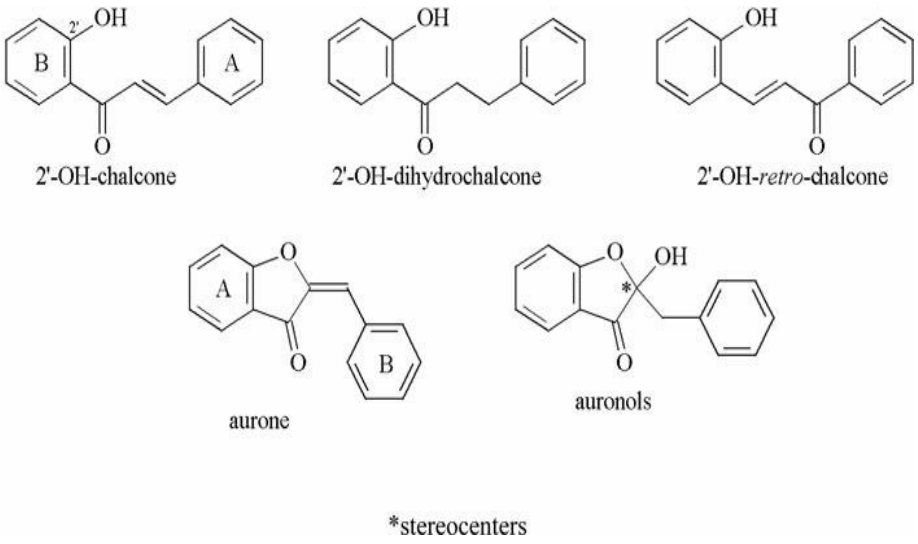
neoflavene

*stereocenters

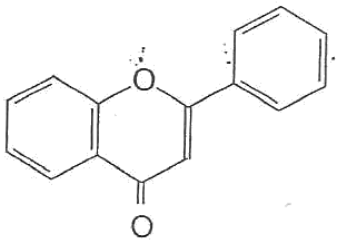
The isoflavonoids and neoflavonoids can be regarded as abnormal flvonoids[17].

1.3- Minor flavonoids

Among compound possessing a C_6-C_3-C_6 backbone are the chalcones and aurones which are considered as minor flavonoids. Such group of phenolics include: the 2' – hydroxychalcones , 2'-OH – dihydrochalcones , retro chalcone , aurones 2-benzylidenecoumaranone and auronols[14].



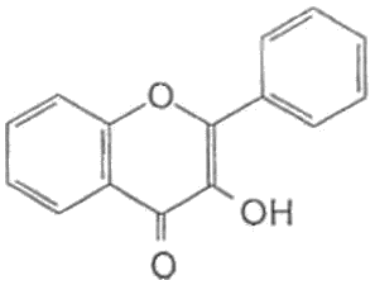
Examples of flavones include the widespread apigenin and luteolin. Isoflavones. are isomers of flavones in which the B ring of the flavonoisd nucleus is attached to C_3[18].



In most flavones the A-ring is derived from phloroglucinol and the B- ring is oxygenated in the 4' or 3',4',-or 3',4',5',positions as established from their acetate - shikimate biosynthetic origin[18]. Flavones are mainly found in cereals and herbs. In the West, the estimated daily intake of flavones is in the range 20-50 mg per day.

Due to their beneficial biological effects against atherosclerosis, osteoporosis, diabetes mellitus and certain cancers[20] , flavones have been extensively researched in recent years and public interest in flavones has grown enormously. Flavones intake in the form of dietary supplements and plant extracts has been steadily increasing. Flavones have effects on CYP (P450) activity [21,22] .

1.4- Flavonols



As far as structure is concerned flavonols are similar to flavones. They possess the same backbone. However, flavonols are distinguished by a 3-OH function.

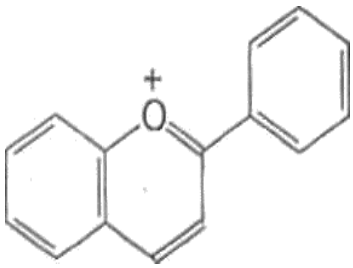
Extensive research indicates that flavanols have beneficial influences on vascular health. These phytochemicals tend to lower blood pressure beside improving blood flow to the brain and heart, making bipod platelets less sticky and they also lower cholesterol [23]. Since flavonols are simply flavones in which the 3-position is substituted by a hydroxyl; both classes of pigments have so far been considered together [18].

Flavanones are described as a : 2 – phenyl – benzopyran – 4 – one. Flavanones are isomeric with chalcones from which they can be obtained synthetically and from which they arise biosynthetiacally The unsubstiuted flavanone is not yet known in nature and the simplest plant flavanone has a single hydroxyl group [19]..

These phytochemicals do possess a chiral carbon, namely C-2, so that naturally occurring members are often optically active. Since flavanones obligate intermediates in flavonoid biosynthesis they have attracted interest .

Upon dehydrogenation, flavanones yield flavones or can undergo hydroxylation at position -3 to yield dihydroflavonols (3- hydroxyl flavanoids) .

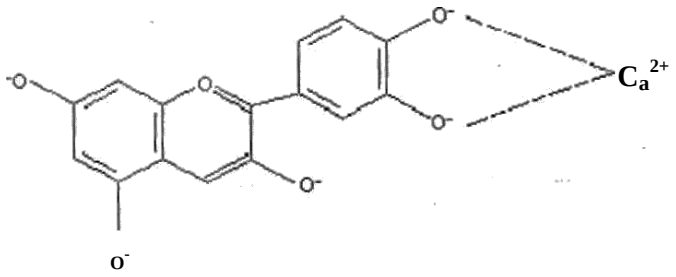
The dihydroflavonols are 2 – pheny l– 3– hydroxybenzo – pyran -4-one and the 7-hydroxydihydroflavonol is the simplest known naturally occurring member of this series . Dihydroflavonols have two asymmetric carbons ,C-2 and C-3, the streochemical implications of which will be mentioned in a separate section .Dihydroflavonnols are interesting compounds, since they are obligated in flavonoid biosynthesis. dihydroflavonls, in turn can yields several other types; dehydrogenation yields flavonols .Reduction of the carbonyl function affords flavan-3,4-diols where enolization and oxidation yieids anthocyanidins[13] .



An extended conjugation made up of the aglycone of the glycoside anthocyanins is displayed in anthocyanidin . With the exception of chlorophill , the anthocyanins are the most important natural pigments visible to the human eye.These phytochemicals possess diverse colours and occur in complex mixtures in all parts of most higher plants.

Anthocyanins are economically important phytochemicals.They are of great economic importance as fruit pigments and they are employed to colour fruit juices and some beverages[17].

Anthocyanins tend to form chelates with some ions like Ca^{2+} and Mg^{2+} under alkali conditions[17].This process may result in extensive conjugation as displyed below:



Chalcones are precursors of all flavonoids .Chalcone is derived from three acetate units and cinnamic acid [17].These natural products are open chain flavonoids in which the two aromatic rings are joined by a three carbon chain[17].

Chalcones can be considered to be derivatives of phenyl styryl ketones .Naturally occurring chalcones are all hydroxylated to a greater or lesser extent .The parent compound chalcone itself is not known as a natural product[17] .

A common chalcone is butein : 2-,4-,3,4–tetra– hydroxychalcone Butein occurs free in the wood or bark of several trees e.g. Acacia, Adenanthera and Rhus [17].

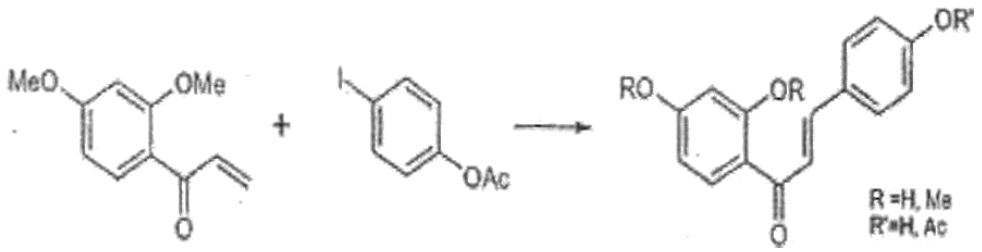
The synthesis of flavonoids have been subject of great number of studies. Although there are several types of skeletons (flavones, isoflavonoid, aurones, etc.), it is the the flavones whose synthesis has been more widely studied. The most used strategy is the reaction of substituted acetophenones with corresponding substituted benzaldehydes either in basic or acidic media[24] .

In the field of microwave - assisted organic reactions, flavonoids have also been studied . However, in this field there was a lack of data for the two-component reaction of acetophenones and benzaldehydes. Although synthesis of chalcones by reaction of these components[25] were reported, all of the acetophenones studied, lacked the 2-OH-substituent that could allow the subsequent cyclization to close the pyrane ring present in flavanones. Otherwise there have been reported cyclizations of chalcones to 3-substituted-flavanones[26], diphenyl - p - diketones to flavones[27] and 2-aminochalcones to 2 – aryl –l,2 , 3 ¸ 4 – tetrahydro - 4-quinolones[28]. But it seemed that nobody had studied (or at least reported) the above mentioned approach of two component addition-cyclization without the addition of a second molecule of aldehyde to position 3 of the flavanone[29].

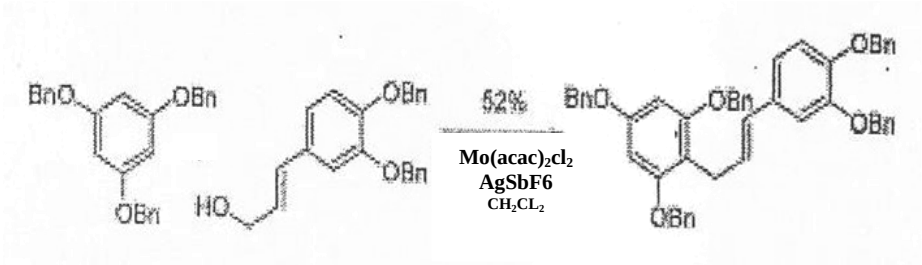
Among the wide variety of reaction conditions for the classical reaction , Chang's reagent (Si02 /H_3BO_3/ piperidine /DMF)was attempted[30]. It was observed that shorter irradiation time lead to better yield[31].

Many flavonoids have been synthesized following a new proposed method based on the use of the Heck reaction. The key step involves

the coupling of an aryl vinyl ketone with an aryl iodide. This procedure affords the flavonoid moiety in a single step [32].



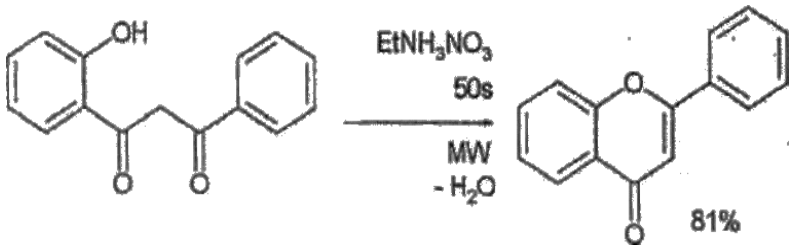
The above mentioned method deals with the formal total synthesis of flavonoids bearing the hydroxylation pattern of the catechin series based on an access to the fully functionalized skeleton via the alkylation of phloroglucinol tribenzyl ether by 3,4-dibenzyloxycinnamyl alcohol. This reaction was revealed to be most successful when catalyzed by the Mo $(acac)_2$,$(SbF_6)_2$ complexes. In addition, the underlying concepts to the different ways that can be used in this C_6-C_3+C_6 strategy are discussed[33].



Interest in the biological properties of flavones has resulted in intense synthetic efforts towards the synthesis of various flavones. There are a number of methods reported for the synthesis of flavones:

- Allan-Robinson reaction
- Auwers synthesis
- Baker-Venkataraman rearrangement
- Algar-Flynn-Oyamada reaction

The basic skeleton of flavonoids is also available via dehydrative cyclization of certain 1,3-diaryl diketones. The effect of an ionic liquid solvent and microwave irradiation on the yield of this process was studied [34].

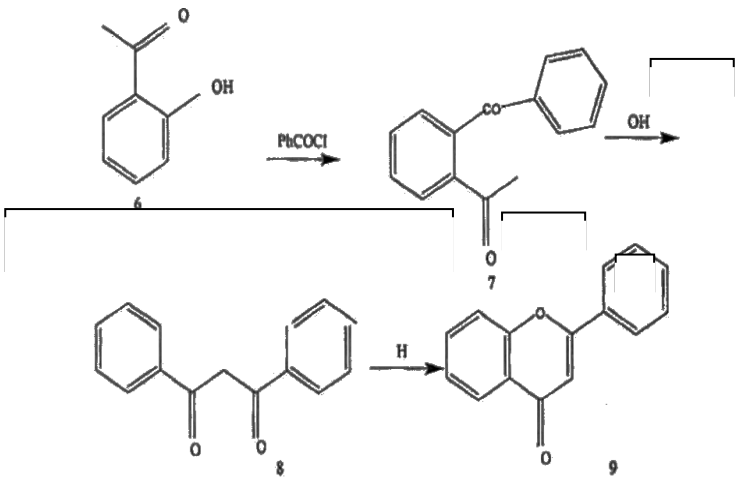


An important tool in structure elucidation of flavonoids is the Wessely-Moser rearrangement [35]. It involves the conversion of 5,7,8-trimethoxyflavone into 5,6,7-trihydroxyflavone on hydrolysis of the methoxy groups to phenol groups. It also has synthetic potential .[36]

Such rearrangement takes place in several steps: **(A)** ring opening to the diketone. (B) bond rotation with formation of a favourable acetylacetone (**C**)hydrolysis of two methoxy groups and ring closure[37].

A general method for synthesizing flavones is the Von-Konstanecki method, which. involves a reaction of o-methoxybenzoate and acetophenone in the presence of sodium [38] .

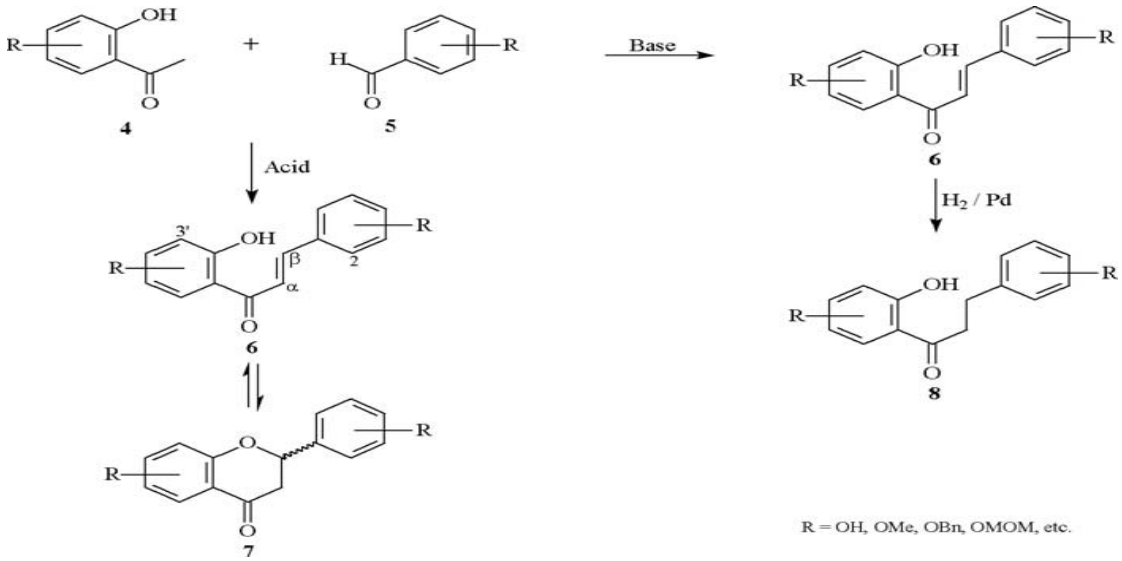
The most convenient route to the synthesis of flavones is the Baker-Venkataraman approach. In this reaction,2 - hydroxyacetophenone is converted to ester , which then undergoes rearrangement by intramolecular Claisen condensation in the presence of potassium hydroxide and pyridine to afford 1,3-diketone which is then cyclized to flavone under rather harsh conditions either by treatment with concentrated sulfuric acid or heating with glacial acetic acid [39].



1.5-Synthesis of chalcones

Chalcones and dihydrochalcones constitute important intermediates in the synthesis of flavonoids[14]. They are considered to be the primary C_6-C_3-C_6 precursors . Chalcones can be synthesized via two well-established routes comprising a base-catalyzed aldol condensation or acid-mediated aldolization of 2-hydroxyacetophenones and benzaldehydes[27].

A Preferred route towards chalcone formation, is the based-catalyzed Aldol condensation. Dihydrochalcones are generally obtained via reduction (H_2/Pd) of the preceding chalcones [2].Conventional base-catalyzed aldol condensation usually employs NaOH or KOH, but, other bases have also been utilized to produce chalcones in up to 89 % yield. These compounds can also be obtained in high yields (75-96%) by Lewis acid catalysis, e.g. borontrifluoride-etherate [40].



1.6- Distribution of flavonoids

Flavonoids are widely distributed among the plant kingdom [18]. Flavonoids are found in vegetables, fruits, nuts , seeds , stems, flowers , tea , wine etc. these are an integral part of our daily food. The dietary intake of flavonoids is estimated to be 1-2 g/day [7]. The average intake of flavonols and flavones was found to be 23 mg/day among which the flavonol quercetin contributed 16mg/day[20].

Anthocyanin pigment present in flowers provides colour to it contributing to pollination [20].Flavonoids present in leaves promote physiological survival of plant. In addition, flavonoids are involved in photosensitization, energy transfer , respiration and photosynthesis control, morphogenesis , sex - determination, energy transfer [18].

1.7- The antioxidant activity of flavonoids

The flavonoids are antioxidants against free radicals and are described as free - radical scavengers [21].This activity is attributed to their hydrogen - donation ability, indeed, the phenolic group of flavonoids serve as a source of readily available "H" atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure[13].However, the free radical scavenging capacity is primarily attributed to high reactivty of hydroxyl substituents that participated in the reaction [20] .Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals.They terminate chain radical reaction by donating hydrogen atom to a peroxy radical , forming flavonoids radical , which further

reacts with free radical thus terminating propagating chain. Naturally , the organism has developed a defiance against toxic superoxide dismutase (SOD) which converts two superoxide anions to H_2O_2 and O_2.

1.8- Extraction of flavonoids

Flavonoids (Particularly glycosides) and be degraded by enzyme action when collected plant material is fresh or non-dried . It is thus advisable to use dry, lyophilized, or frozen samples , when dry plant material is used , it is generally ground into a powder.

For extraction , the solvent is chosen as a function of the type of flavonoid required where polarity is an important consideration here . Less polar flavonoids (e.g., Isoflavonols) are extracted with chloroform , dichloromethane , diethyl ether , or ethyl acetate , while alcohols and more polar aglycones are extracted with alcohols or alcohol water mixtures . Glycosides have increased water solubility and aqueous alcoholic solutions are suitable .The bulk of extractions of flavonoid containing material are still performed by simple dried solvent extraction.

Powdered plant material can also be extracted in a soxhlet apparatus, first with hexane, for example to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat - sensitive compounds .

Sequential solvent extraction is also extremely useful .A First step is extraction with dichloromethane, for example. This will extract

flavonoid aglycone and less polar material . A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents.

The process of extraction is typically performed with magnetic stirring or shaking but other methods have recently been introduced to increase the efficiency and speed of extraction procedure .One of such methods is called pressurized liquid extraction (PLE) . Here extraction is accelerated by using high termperature and high pressure . There is enhanced diffusibity of the solvent and , at the same time , there is the possibility of working under an inert atmosphere and with protection from light. Application of PLE gave better results than maceration and shorter extraction time and smaller amount of solvent were required[22].

Another methodis supercritical fluid extraction (SFE) which relies on the solubilizing properties of supercritical fluid .The lower viscosities and higher diffusion rates of liquids , make them ideal for the extraction of diffusion controlled matrices, such as plant tissues , Advantages of the method are lower solvent consumption , controllable selectivity and less thermal or chemical degradation than methods such as soxhlet extraction.

Extensive applications in the extraction of natural products have been reported, with supercritical carbon dioxide being the most widely used extraction[23,24]. However , to allow for the extraction of polar compounds such as flavonoids . In this method, polar solvents have to be added as modifiers. There is consequently a

substantial reduction in selectivity , this explains why there are relatively few application to poly phenols in the literature.

Another widely used technique is ultrasound-assisted extraction. This technique is a rapid technique and it can also be used with mixtures of immiscible solvent. Another technique , namely - microwave -assisted extraction (MAE) has been described for the extraction of various compounds from different matrices . This is a simple technique that can be completed in a few minutes. Microwave energy is applied to the sample suspended in solvent either in closed vessel or in an open cell. The latter allows larger amounts of sample to be extracted. A certain degree of heating is involved[26].

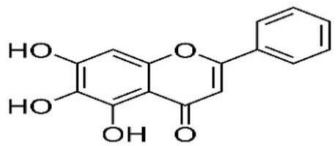
1.9- Biological activity

Flavonoids possess diverse biological and medicinal properties .The contribution of flavonoids is warding off microbial infection and protecting plants from herbivory is well kown . The biological properties of flavonids are considered in an evalution of the medicinal and nutritional values of these compounds. Certain plans and spices containing flavonoids have been used for thousands of years in traditional medicine .The wide range of biological activities of these compounds include antimicrobial, anticancer, antiviral etc.

1.9.1 – antimicrobial activity

Flavonoids and related poly phenols are known to protect plants from microbial invasion. They function as antifungal, antibacterical and antiviral agents where the phenol group in the flavonoids provide antimicrobial activity and this activity is farther enhanced by additional phenolic groups. There is growing interest in their use against fungal pathogens of man. they are considered for treating human disease and especially for controlling the Hiv virus .

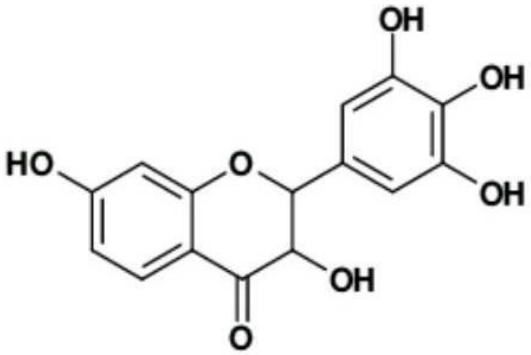
Flavonoids are not only present in plants as constitutive agents but are also formed in plant tissues in response in microbial attack.The flavone -baicaline is reported to largely responsible for plant's antimicrobial effects.



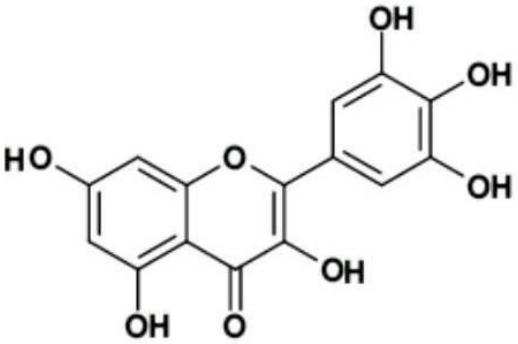
Baicaline

Flavonoids are also known to possess antibacterial activity. The activity of flavonoids quercetin , has been attributed to in hibition of DNA gyrase. Research groups have also isolated and identified the structure of various flavonoids possessing antibacterial activity other flavonoids whose mechanisms of action have been investigated include apogenin, rutin,robinetin, myricetin and galangin . The study of these compounds may help the

development of pharmacologically acceptable antimicrobial agent or class of agents.



Robinetin

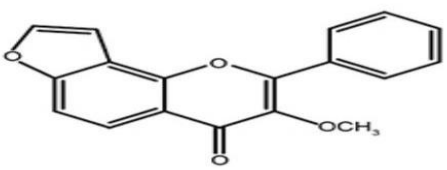


Myricetin

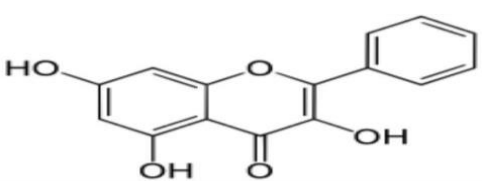
1.9.2- Antifungal activity

Flavonoids have been proposed for use against fungal pathogens of man [29] .Karanjin a furanoflavonol obtained from the seeds of *pongamia glabara* Vent showed promising antifangal activity.

Galangin, a flavonol commonly found in propolis species was first isolated in 1881 from root of *Alpinia officinarum* .Galargin is a broad spectrum antimicrobial agent it can inhabit the growth of Gram positive bacteria, Gram negative bacteria and a number of fungal species [41].



Karnjin



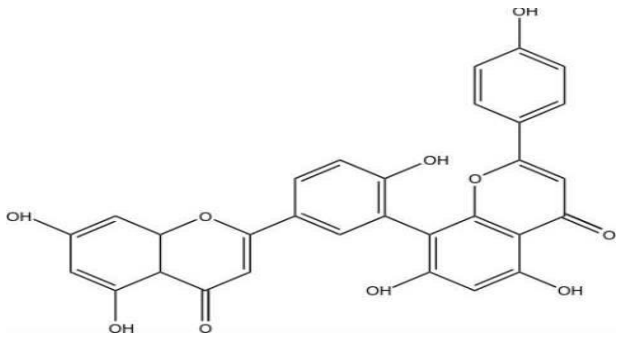
Galangin

1.9.3- Antiviral

Recently researchers have been investigating the inhibitory activity of some flavonoids agaisnt human immunodeficiency virus (HIV). *In vitro* studies have shown that baicalin inhibits (HIV-1) infection and replication .Flavonoids can inhibit reverse transcriptase's of different origin and can act as anti-retroviral agents.

The flavonoid –amentoflavone- possesses anti-reverse transciplace activity. Some studies also revealed that common flanonols and some aurone were strongly active in inhibiting the tomato ring spot virus[42].

The flavonoid- querctin -and other flavonoids appear to interfere with an early event in the virus life cycle. It has been reported that querctin at a concentration of SMG ml^{-1} resulted in 10% inhibition of local lesion development of the virus on the test plant *chenopodium quinoa* [43].



Amentoflavone

1.9.4- Anticancer activity

Several plant species containing flavonoids have been used for thousands of years in traditional Eastern medicine as anticancer . Flavonoids and their derivatives from both natural and synthetic sources have been investigated for various activites. Flavonoids are known to inhibit carcinogensis. They can act by mechanisms that can inhibit tumor and kill cancer cells.

1.10- Essential oils

Essential oils (EOs) are very interesting natural plant products and among other qualities they possess various biological properties. The term "biological" comprises all activities that these mixtures of volatile compounds (mainly mono- and sesquiterpenoids, benzenoids, phenylpropanoids, etc.) exert on humans, animals, and other plants.[44]The term essential oil dates back to the sixteenth century and derives from the drug Quinta essentia, named by Paracelsus von Hohenheim of Switzerland [45]. Essential oils or "essences" owe their name to their flammability. Numerous authors have attempted to provide a definition of essential oils. The French Agency for Normalization: Agence Française de Normalisation (AFNOR) gives the following definition (NF T 75-006): "The essential oil is the product obtained from a vegetable raw material, either by steam distillation or by mechanical processes from the epicarp of Citrus, or "dry"" distillation. The essential oil is then separated from the aqueous phase by physical means [46]. Essential oils are soluble in alcohol, ether, and fixed oils, but insoluble in water. These volatile

oils are generally liquid and colorless at room temperature. They have a characteristic odor, are usually liquid at room temperature and have a density less than unity, with the exception of a few cases (cinnamon, sassafras, and vetiver). They have a refractive index and a very high optical activity. These volatile oils contained in herbs are responsible for different scents that plants emit. All parts of aromatic plants may contain essential oils as follows:

_ Flowers, of course, including: orange, pink, lavender, and the (clove) flower bud or (ylang-ylang) bracts,

_ Leaves, most often, including: eucalyptus, mint, thyme, bay leaf, savory, sage, pine needles, and tree underground organs, e.g., roots (vetiver),

_ Rhizomes (ginger, sweet flag),

_ Seeds (carvi, coriander),

_ Fruits, including: fennel, anise, Citrus epicarps,

_ Wood and bark, including: cinnamon, sandalwood, rosewood.

Essential oils are produced by various differentiated structures, especially the number and characteristics of which are highly variable. Essential oils are localized in the cytoplasm of certain plant cell secretions, which lies in one or more organs of the plant; namely, the secretory hairs or trichomes, epidermal cells, internal secretory cells, and the secretory pockets. These oils are complex mixtures that may contain over 300 different compounds [47]. They consist of organic volatile compounds, generally of low molecular weight below 300. Their vapor pressure at atmospheric pressure and at room temperature

is sufficiently high so that they are found partly in the vapor state[48,49]. These volatile compounds belong to various chemical classes: alcohols, ethers or oxides, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and mainly the terpenes. Alcohols, aldehydes, and ketones offer a wide variety of aromatic notes, such as fruity ((E)-nerolidol), floral (Linalool), citrus (Limonene), herbal (-selinene), etc. Furthermore, essential oil components belong mainly to the vast majority of the terpene family . Many thousands of compounds belonging to the family of terpenes have so far been identified in essential oils [42], such as functionalized derivatives of alcohols (geraniol, _-bisabolol), ketones (menthone, p-vetivone) of aldehydes (citronellal, sinensal), esters (-tepinyl acetate, cedryl acetate), and phenols (thymol). Essential oils also contain non-terpenic compounds biogenerated by the phenylpropanoids pathw*ay* ,such as eugenol, cinnamaldehyde, and safrole. analytical methods applied in the characterization of essential oils have to account for a great number of molecular species. Moreover, it is also of great importance to highlight that an essential oil chemical profi le is closely related to the extraction procedure employed and, hence, the choice of an appropriate extraction method becomes crucial. On the basis of the properties of the plant material, the following extraction techniques can be applied: steam distillation (SD), possibly followed by rectifi cation and fractionation, solvent extraction (SE), fractionation of solvent extracts, maceration, expression (cold pressing of citrus peels), supercritical fl uid extraction (SFE), pressurized-fluid extraction,

simultaneous distillation– extraction (SDE), Soxhlet extraction, among others. Chromatography, especially GC, has evolved into the dominant method for essential oil analysis. This is to be expected because the complexity of the samples must be unraveled by some type of separation, before the sample constituents can be measured and characterized; in this respect, GC provides the greatest resolving power for most of these volatile mixtures. They are widely used in the cosmetics industry, perfumery, and also aromatherapy. The latter is intended as a therapeutic technique including massage, inhalations, or baths using these volatile oils. The last key will serve as chemical signals allowing the plant to control or regulate its environment (ecological role): attraction of pollinating insects, repellent to predators, inhibition of seed germination, or communication between plants (emission signals chemically signaling the presence of herbivores, for example). Moreover, EOs also possesses antifungal or insecticide and deterrent activities.They are many biological activates like: antimicrobial, antioxidant properties, anti-inflammatory activity, cytotoxicity, cancer chemoprotective activities[50].

Aim of this study

This study was aimed to:

-Extraction of the major flavonoid from *Ammomum sublatum*.

-Elucidation of the structure of the isolated flavonoid via UV and NMR experiments.

-Extraction of fennel fixed oil.

-Conducting GC-MS studies on the extracted oil.

-Screening *Ammomum sublatum* ethanolic extract and fennel oil for antimicrobial activity.

Chapter Tow

Materials and methods

Materials and Methods

2.1- Materials

2.1.1- Solvents

All solvents used are of analytical grade(Loba Chemicals-India). Methanol HPLC grade is used for spectroscopic purposes(Sigma-Aldrich , England).

2.1.2- Plant material

Fruits of *Ammomum sublatum* and seeds of fennel were purchased from the local market Khartoum (Sudan) . The plants were identified and authenticated by The Institute of Medicinal and Aromatic Plants, Khartoum, Sudan.

2.1.3- Chromatographic materials

- Glass jars, 10x20x24 cm, fitted with covers, were used for chromatographic fractionation.
- Silica gel G (Loba Chemicals) was used for TLC experiments.

2.1.4- Equipments

Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV -Visible Spectrophotometer(Shimadzu).

When visualizing TLC plates a multiband UV λmax (254 / 365 nm) portable ultraviolet, a product of Hanovia lamps (6 watt S/Y and L/W) was used.. ^{1}HNMR spectra were obtained on a Bruker AM 500 spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d_6. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal

standard and the coupling constants (J) are expressed in Hertz (Hz).

2.1.5-Test organisms

Standard microorganisms used for antimicrobial screening are depicted below:

Table 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	*Candida albicans*	fungi

2.2- Methods

2.2.1- Extraction of plant phenolics

(1 Kg) of powdered shade-dried fruits *Ammomum sublatum* were extracted with 80% methanol for 72 hours. The cooled solution was filtered and evaporated to dryness under reduced pressure.

2.2.2-Test for flavonoids

(50 mg) of the ethanolic extract was defatted with petroleum ether and the residue was dissolved in(30ml) 80% methanol and filtered. The filtrate was used for the following tests:

(1) To (30 ml) of the filtrate few drops of methanolic aluminium chloride were added . A dark yellow colour was observed .

(2) To (30 ml) of the filtrate few drops of potassium hydroxide solution were added . A dark yellow colour was observed .

(3) To (30 ml) of the filtrate few drops of ferric chloride solution were added . A blue coloration was observed .

2.2.3-Isolation of flavonoids

The ethanolic crude extract was applied on TLC plataes (20x20cm) as narrow strips. The bands were irrigated with 15% acetic acid. The developed chromatograms were air-dried and examined under both visible and UV light (ʎ 366,245nm).. The equivalent bands from each plate were then scratched, combined and slurred with methanol. After several hours of contact, with occasional shaking, the liquid was evaporated *in vacuo* to dryness. In this way compounds I(R_f 0.70) was obtained in a chromatographically pure form.

2.2.4-Extraction of oil from fennel seeds

Powdered seeds of fennel(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^oC for further manipulation.

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

Fennel oil was analyzed by a Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 µm, thickness).Helium was used as carrier gas.Oven temperature program is given in Table 2. For other chromatographic conditions see Table 3.

Table 2: Oven temperature program

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

Table 3: Chromatographic conditions

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

2.2.5-Antimicrobial test

Fennel oil ant the ethanolic extract of *Ammomum sublatum* were screened for their antimicrobial activity against five human pathogenic bacterial strains: Gram-positive (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungal species **:** *Candida albicans*. The cup plate agar diffusion bioassay was used.

2.2.5.1- Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline solution to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and

by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.2.5.2- Preparation of fungal suspension

The fungal cultures were maintained on dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.5.3- Testing of antibacterial susceptibility

The cup plate agar diffusion assay was used to screen the antibacterial activity of the sample and performed by using Mueller Hinton agar (MHA).

(2ml) of the standardized bacterial stock suspension were mixed with (200 ml) of sterile molten nutrient agar which was maintained at 45°C. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle . Each plate was divided into two halves. In each half two cups (10mm in diameter) were cut using sterile cork borer (No 4). Each half was designed for a test solution.

Agar discs were removed, alternate cups were filled with(0.1 ml) samples of each test solution and allowed to diffuse at room temperature for two hours. The plates were then incubated at 37^{o}C for 24 hours. After incubation, the diameters of the resultant growth inhibition zones were measured in duplicates and averaged.

2.2.5.4-Testing of antifungal susceptibility

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

Chapter Three

Results and discussion

Results and Discussion

3.1-Flavonoids of *Ammomum sublatum*

The fruits of *Ammomum sublatum* were extracted with 95% ethanol for 72hr. The solvent was removed *in vacuo* gaving a crude product. A Silica gel TLC plates eluted with chloroform:methanol (3:7; v:v) gave a chromatographically pure component – compound I .The structure of this compound has been elucidated via their spectral data(UV and NMR).

3.1.1- Compound I

Usually when considering structural elucidation of flavonoids, the first step is to conduct extensive UV studies ,including the use of UV shift reagents, to know the class to which the flavonoid belongs.

Flavonoids usually exhibit two absorption bands ; band I and II. Band I is due to the absorption of the cinnamoyl system, while band II originates from the benzoyl system.

It is known that flavones, flavonols, chalcones and aurones give both band I and II, due to effective conjugation between the carbonyl function and the aromatic B ring. Flavanones, isoflavones, dihydroflavonols and dihydrochalcones give only band II in the range : 230-290nm.These classes of flavonoids are

known to lack conjugation between the B ring and the carbonyl function and thus afford absorption due to the benzoyl chromophore only.

In the UV compound I absorbs (Fig.1) at λ_{max}234nm. Such absorption is characteristic of :flavanones, isoflavones, dihydroflavonols and dihydrochalcones.

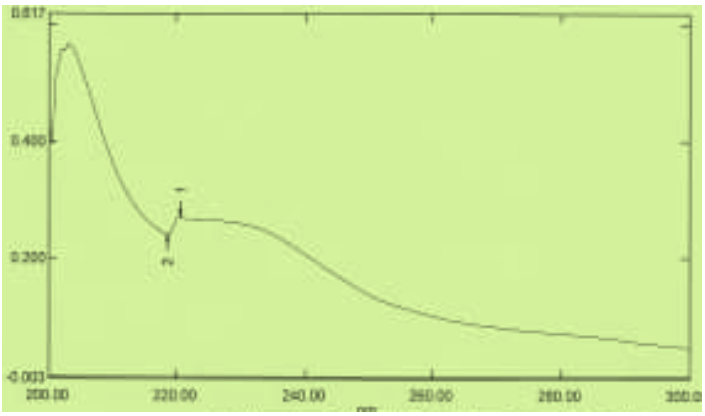
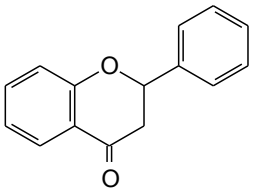
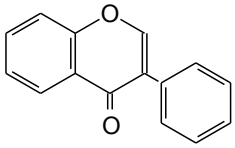


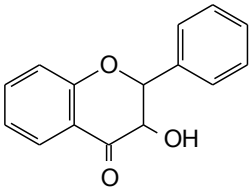
Fig. :UV spectrum of compound I



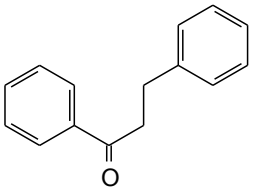
Flavanone



Isoflavone



Dihydroflavonol



Dihydrochalcone

Among the flavonoids ,isoflavones possess a spectrum characterized by a shoulder in the range 400-430nm and such

shoulder was not detected in the UV spectrum(Fig.1) of compound I . Dihydroflavonols are distinguished by a 3-OH function which is detectable by the shift reagent –sodium methoxide(Fig.2). This reagent also detects a 4`-OH function. In both cases the sodium methoxide spectrum shows a bathochromic shift accompanied by a decrease in intensity in case of a 3-OH function.

However, the sodium methoxide spectrum(Fig.2) gave λ_{max} 207nm i.e. it did not show any bathochromic shift indicating absence of 3- and 4` -OH and consequently absence of dihydroflavonols(see Fig. 2). Such argument suggests that the isolated flavonoid is either a flavanone or a dihydrochalcone.

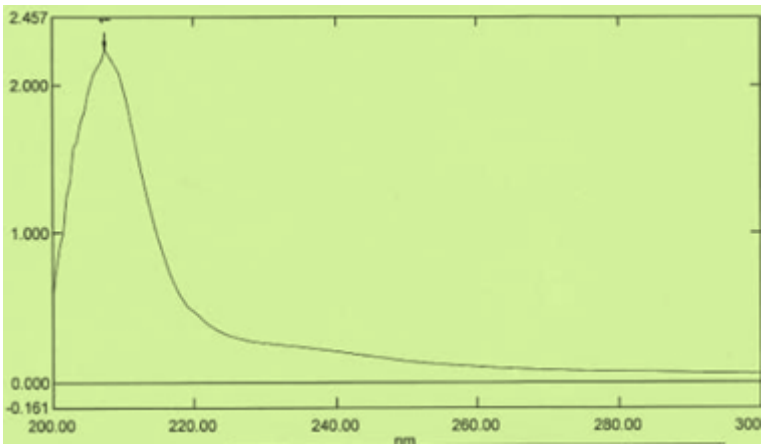


Fig.2 :Sodium methoxide spectrum of compound I

Due to the mutual splitting of the unequivalent C-3 protons in flavanones a double doublet appears around δ2.8ppm. Such doublets are further split into a pair of quartets (usually merging into a multiplet) by C-2 proton. Another multiplet arising from C-2 proton being split by C-3 protons appears around δ5.2ppm.

Thus the ^{1}HNMR can distinguish between flavanones and dihydrochalcones. The ^{1}HNMR of compound I(Fig.3) did not reveal such multiplets indicating that the isolated compound is a dihydrochalcone.

The ^{1}HNMR spectrum(Fig.3) showed : δ1.22 which was assigned for a methyl group. The signal at δ1.73ppm which integrate for 6 protons accounts for two acetyl functions, while the resonance at δ4,30 ppm is due to two methoxyl groups. The multiplets centered around δ6.62 and δ7.25ppm were attributed to the aromatic protons .

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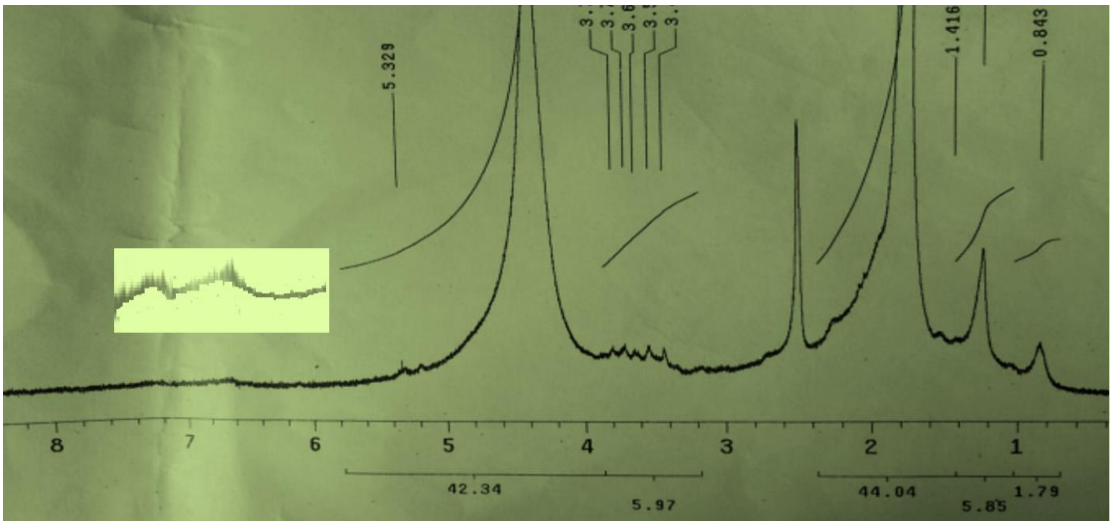


Fig. 3 : ^{1}HNMR spectrum of compound I

Sodium acetate is a useful shift reagent and it is diagnostic of a 7-OH group. In presence of a 7-OH function, the sodium acetate spectrum afford a bathochromic shift .However this spectrum

did not afford any bathochromic shift indicating absence of a 7-OH function (Fig. 4).

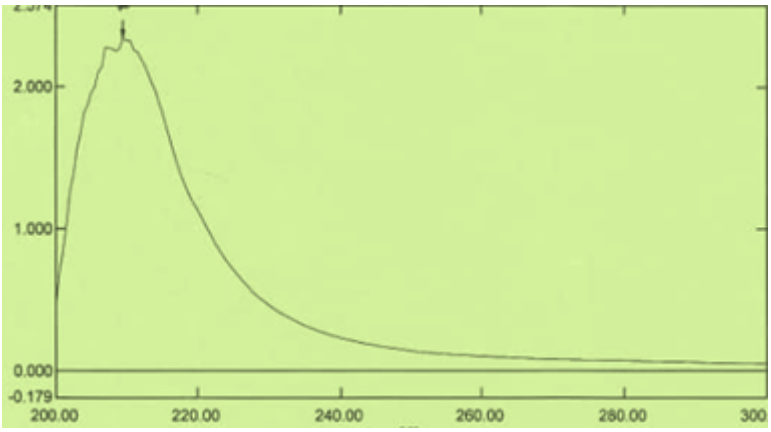
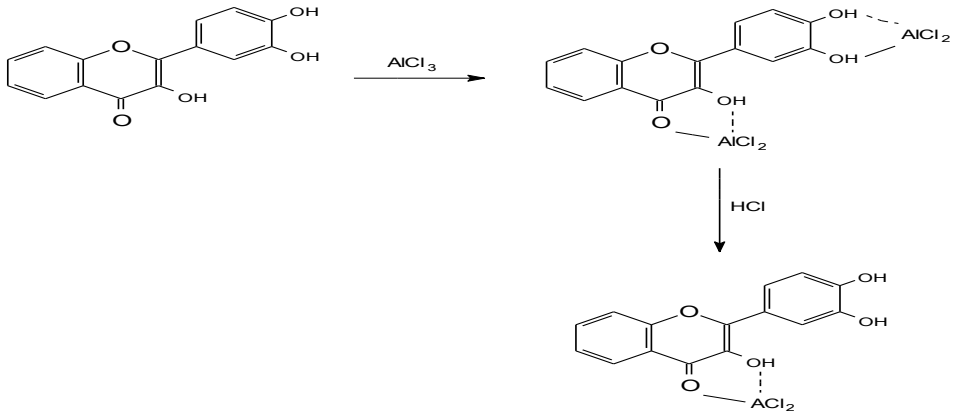


Fig.4 : Sodium acetate spectrum of compound I

Aluminium chloride is another useful UV shift reagent capable of detecting catechol systems as well as 3- and 5-OH functions in the nucleus of flavonoids. This reagent can form complexes with a 3-OH and a 4-keto function; a 5-OH and a 4-keto group . It can also chelates with catechols systems in both aromatic rings. Though the catechol complexes decompose in acidic media, the 3- and 5-OH complexes are quite stable in such media.Such complexes are displayed below:



The aluminium chloride spectrum (Fig. 5) did not give a bathochromic shift indicative of a 5- or a 3-OH functions. The boric acid spectrum(Fig.6) which is diagnostic of catechol systems did not reveal any bathochromic shift indicating absence of such catechols.

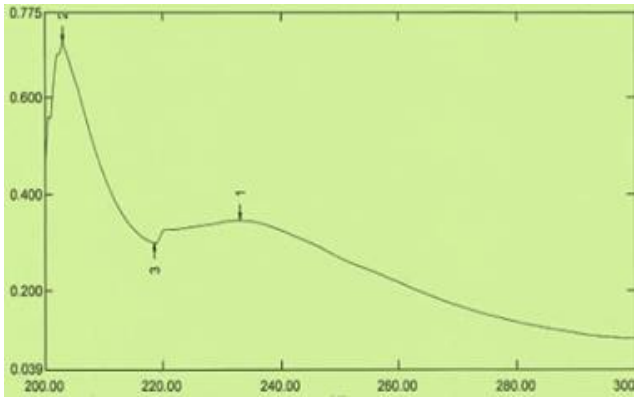


Fig.5 : Alumnium chloride spectrum of compound I

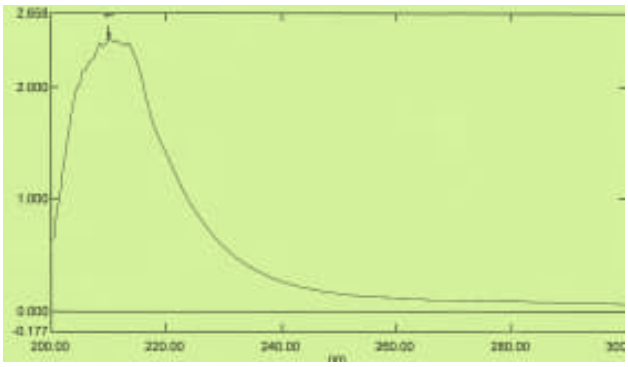
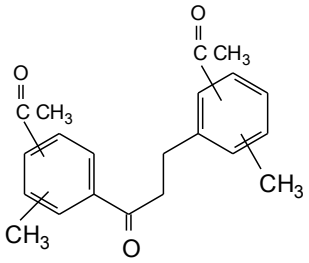


Fig.6 : Boric acid spectrum of compound I

On the basis of the above spectral data the following tentative structure was assigned for compound I:



Compound I

3.1.2-Antimicrobial activity

The aqueous extract of *Ammomum sublatum* was assessed for antimicrobial activity against five standard bacteria. It showed significant activity against revealed activity against the bacterial strains : *Pseudomonas aeruginosa* and *Bacillus subtilis* . It also showed good anticandidal activity (Table3.1).The antimicrobial activities of standard drugs are depicted in Tables 3.2 and 3.3.

Table 3.1: Antimicrobial activity of *Ammomum sublatum* fruit extract

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca
Ammomum sublatum	100					
Ethanolic extract	100	15	20	10	20	15

Table 3.2 : 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 3.3 : Antifungal activity of standard chemotherapeutic agent

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*

3.2-*Foeniculum vulgare* oil

3.2.1-GC-MS analysis of *Foeniculum vulgare* fixed oil

GC-MS analysis of *Foeniculum vulgare* fixed oil was carried out. The MS library (NIST) was checked for identification of the constituents (a 90-95% match was observed) . Furthermore, the observed fragmentation pattern was interpreted.

The GC-MS spectrum of the studied oil revealed the presence of 32 components(Table3.4).The typical total ion chromatograms (TIC) is depicted in Fig.7.

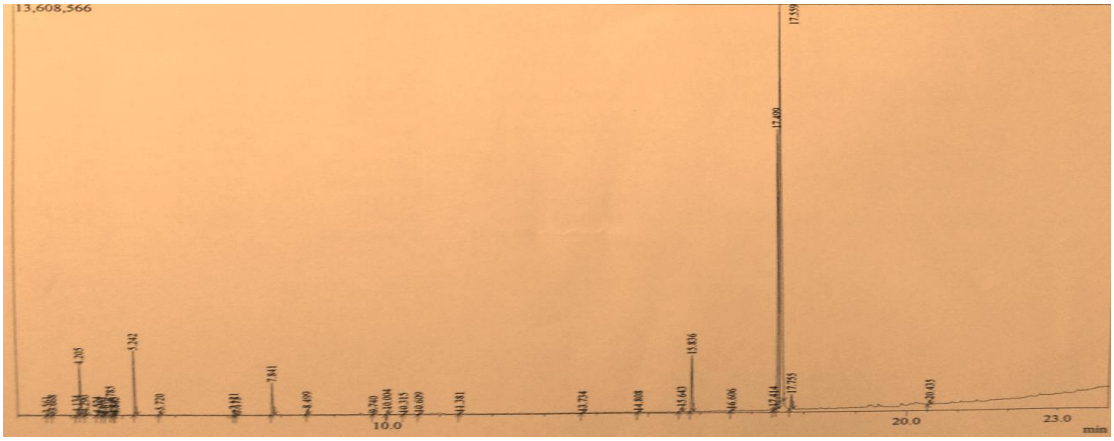


Fig.7: Total ion chromatograms

Table 3.4: Contituents of *Foeniculum vulgae* oil

Peak#	R.Time	Area	Area%	Name
1	3.561	78697	0.13	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-met
2	3.668	149117	0.25	.alpha.-Pinene
3	4.134	163802	0.27	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-me
4	4.205	2134541	3.51	.beta.-Pinene
5	4.290	123090	0.20	.beta.-Myrcene
6	4.524	62880	0.10	.alpha.-Phellandrene
7	4.603	16082	0.03	3-Carene
8	4.679	18436	0.03	(+)-4-Carene
9	4.785	512747	0.84	o-Cymene
10	4.843	48379	0.08	D-Limonene
11	4.865	42651	0.07	.beta.-Phellandrene
12	5.242	3027403	4.98	.gamma.-Terpinene
13	5.720	172319	0.28	Undecane
14	7.121	253023	0.42	Dodecane
15	7.175	86329	0.14	1-Cyclohexene-1-carboxaldehyde, 4-(1-me
16	7.841	1719357	2.83	Benzaldehyde, 4-(1-methylethyl)-
17	8.499	281517	0.46	Tridecane
18	9.740	54609	0.09	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-
19	10.004	210440	0.35	Benzenepropanol, 4-methoxy-
20	10.315	30972	0.05	Caryophyllene
21	10.609	70725	0.12	(E)-.beta.-Famesene
22	11.381	49224	0.08	Butylated Hydroxytoluene
23	13.734	37259	0.06	Methyl tetradecanoate
24	14.808	33780	0.06	Pentadecanoic acid, methyl ester
25	15.643	336214	0.55	9-Hexadecenoic acid, methyl ester, (Z)-
26	15.836	2902997	4.78	Hexadecanoic acid, methyl ester
27	16.606	76436	0.13	Methyl 8-heptadecenoate
28	17.414	159302	0.26	6,9-Octadecadienoic acid, methyl ester
29	17.499	17325881	28.50	9,12-Octadecadienoic acid (Z,Z)-, methyl e
30	17.559	29508900	48.54	9-Octadecenoic acid (Z)-, methyl ester
31	17.755	825729	1.36	Methyl stearate
32	20.435	279634	0.46	Phenol, 2,2'-methylenebis[6-(1,1-dimethyl

Main constituents of the oil are discussed below:

9-Z-Octadecenoic acid methyl ester(48.54%%)

Fig. 8 shows the EI mass spectrum of 9-octadecenoic acid methyl ester.The peak at m/z 296, which appeared at R.T.

17.559 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$, while the peak at m/z266 accounts for loss of a methoxyl function

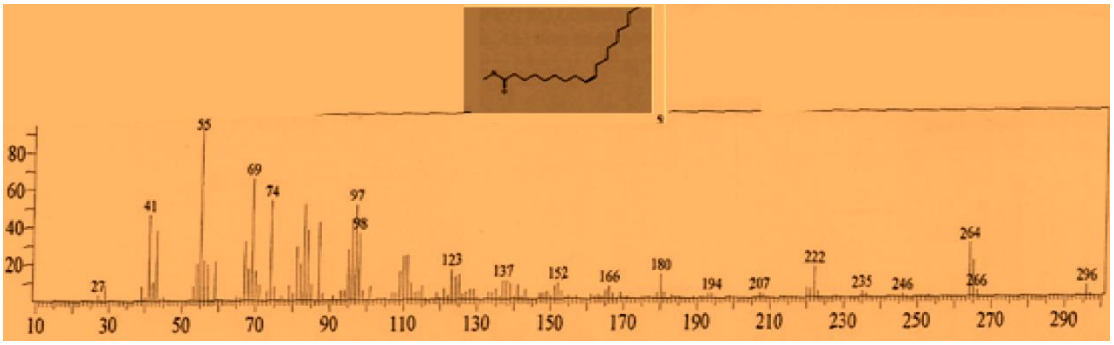


Fig. 8: Mass spectrum of 9-octadecenoic acid methyl ester

9,12-Z,Z-Octadecadienoic acid methyl ester (28.50%)

The mass spectrum of 9,12-octadecadienoic acid methyl ester is displayed in Fig.9.The peak at m/z294(R.T. 17.499-in total ion chromatogram)) corresponds $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z263 corresponds to loss of a methoxyl function.

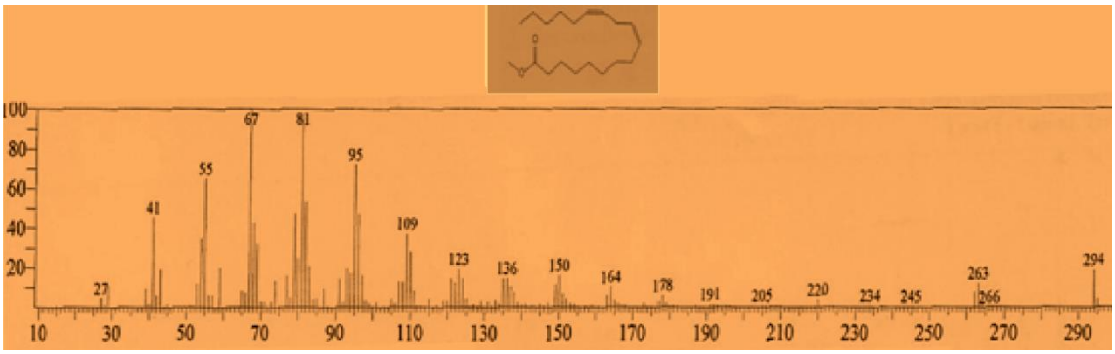


Fig. 9: Mass spectrum of 9,12-octadecadienoic acid methyl ester

γ – Terpinene(4.98%)

Fig. 10 shows the mass spectrum of γ – terpinene .The peak at m/z136 , which appeared at R.T. 17.559 in total ion chromatogram, corresponds $M^+[C_{10}H_{16}]^+$.

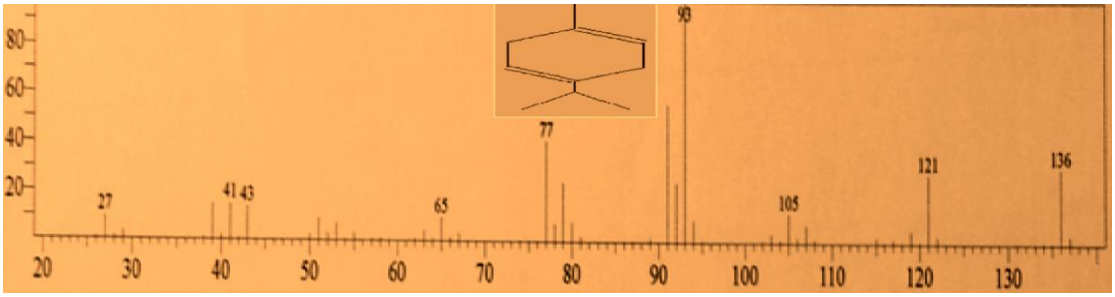


Fig. 10: Mass spectrum of γ – terpinene

β-Pinene(3.51%)

The masss spectrum of β-Pinene is shown in Fig.11. The molecular ion $M^{+}(C_{10}H_{16})$ appeared at m/z136 with RT,4.205 in total ion chromatogram.

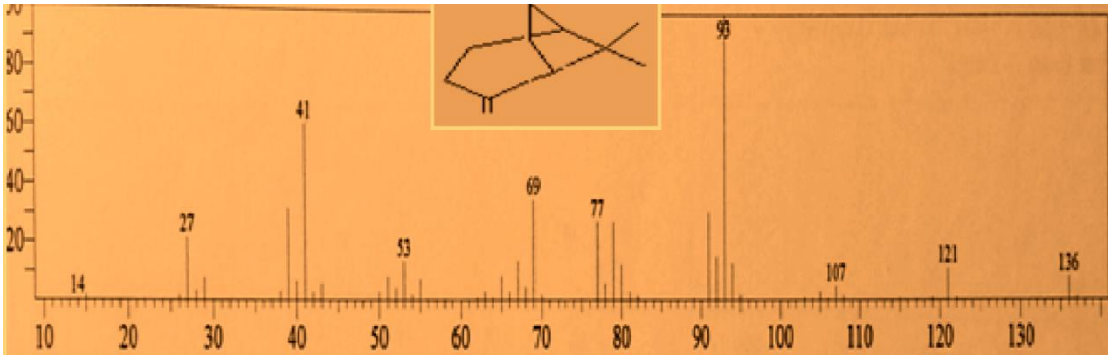


Fig. 11: Mass spectrum of β-pinene

3.2.2-Antibacterial activity

Foeniculum vulgae oil was screened for antimicrobial activity against five standard bacterial strains . The diameters of the growth of inhibition zones are shown in Table (3.5) . Conventional terms were used for interpretation of the results : (<9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .

Table 3.5 : Antibacterial activity of *Foeniculum vulgare* oil

Type	Conc.(mg/ml)	**Sa**	Bs	Ec	Ps	Ca
Oil	100	20	14	15	15	17
	50	18	-	14	14	15
	25	17	-	13	13	10
	12.5	15	-	12	12	9
	6.25	11	-	10	7	-

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

Foeniculum vulgae oil showed excellent activity against *Staphylococcus aureus* in the concentration range : 100-25mg/ml. It also exhibited significant activity against the yeast *Candida albicans* at 100mg/ml.

3.3-psidium guajva

3.3.1-GC-MS analysis of *Psidium guajava* oil

Psidium guajava fixed oil was obtained via maceration of seeds. The oil was studied by GC-MS which revealed the presence of 30 constituents . The total ion chromatograms is displayed in Fig.12 , while the different constituents of the oil are depicted in Table 3.6 .

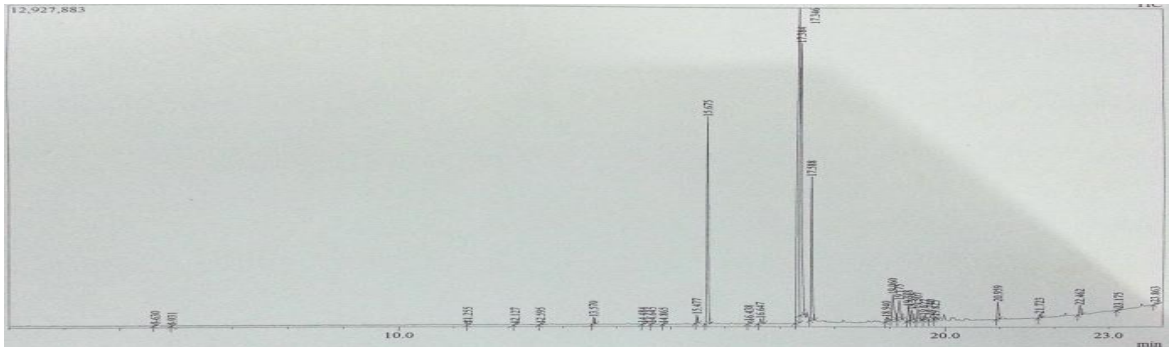


Fig.12 : Total ions chromatograms

Table 3.6 : Costituents of ***psidium guajava*** oil

No.	RT	Area %	Name
1	5.630	0.09	Benzoic acid methy ester
2	5.031	0.02	Octanoic acid methyl ester
3	11.255	0.08	Dodecanoic acid methyl ester
4	12.127	0.06	(-)-Spathulenol
5	12.598	0.08	Apiol
6	13.570	0.45	Methyl tetradecanoate
7	14.484	0.06	5-Octadecenoic acid methyl ester
8	14.645	0.06	Pentadecanoic acid methyl ester
9	14.865	0.04	2- Pentadecanone ,6,10,14-trimethyl
10	15.477	0.53	9-Hexadecenoic acid methyl ester
11	15.675	15.96	Hexadecanoic acid methyl ester
12	16.438	0.16	9,12-Octadecadienoyl chloride
13	16.647	0.33	Heptadecanoic acid methyl ester
14	17.340	36.39	9,12-Octadecadienoic acid(z,z-) methyl ester
15	17.384	23.44	9-Octadecadienoic acid(z-) methyl ester
16	17.588	10.53	Methyl stearate
17	18.940	0.31	9,12-Octadecadienoic acid methyl ester
18	19.060	2.58	9-1-Butyltricyclo[4.2.1.1]decane-1,10-
19	19.175	2.17	E,E,Z-1,3,12-Nonadecatriene=5,14-diol
20	19.338	1.02	Eicosanoic acid methyl ester
21	19.395	0.77	PGHI methyl ester
22	19.507	1.08	1-Naphthalenol decahydro-4α-methyl
23	19.622	0.30	2-Butyl-3-methyl-5-(2-methylpropyl-enyl)
24	19.740	0.49	α- d-Xylopyranoside, methyl-2,3,4-triol
25	19.825	0.33	Methyl-2-octylcyclopropene-1-octanoate
26	20.959	1.20	Docosanoic acid methyl ester
27	21.723	0.31	Tricosanoic acid methyl ester
28	22.462	0.78	Tetracosanoic acid methyl ester
29	23.175	0.14	Pentacosanoic acid methyl ester
30	23.865	0.20	Hexacosanoic acid methyl ester
		100.00	

The following constituents were detected as major components:

i- **9,12-Octadecadienoic acid(36.39%)**

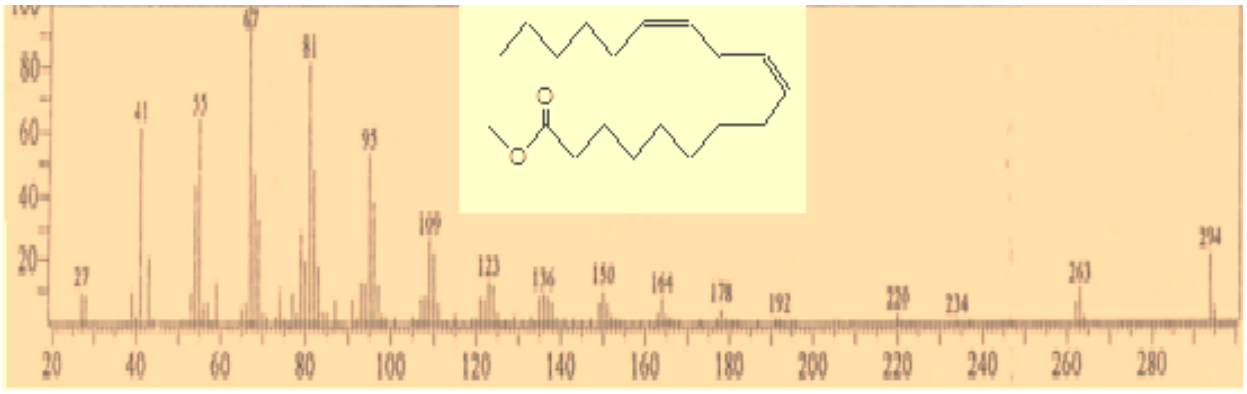


Fig. 13: Mass spectrum of 9,12-octadecadienoic acid methyl ester

The EI mass spectrum of 9,12-octadecadienoic acid methyl ester is shown in Fig. 2.The peak at m/z 294, which appeared at R.T. 17.346 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$ .The peak at m/z263 corresponds to loss of a methoxyl function.

ii-9-Octadecenoic acid methyl ester(20.37%)

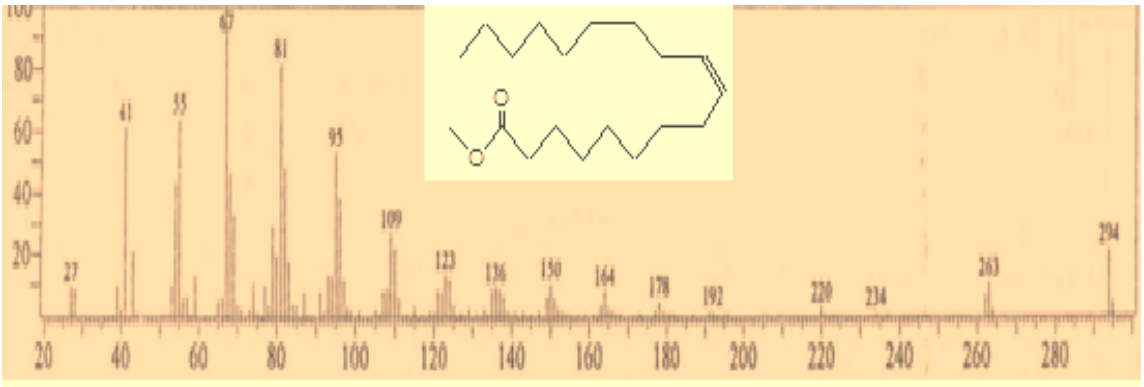


Fig. 14: Mass spectrum of 9-octadecenoic acid methyl ester

Fig. 3 displays the mass spectrum of 9-octadecenoic acid methyl ester . The signal at m/z 294(R.T. 17.384) corresponds to

$M^+[C_{19}H_{34}O_2]^+$, while the peak at m/z263 accounts for loss of a methoxyl function .

iii-Hexadecanoic acid methyl ester(15.96%)

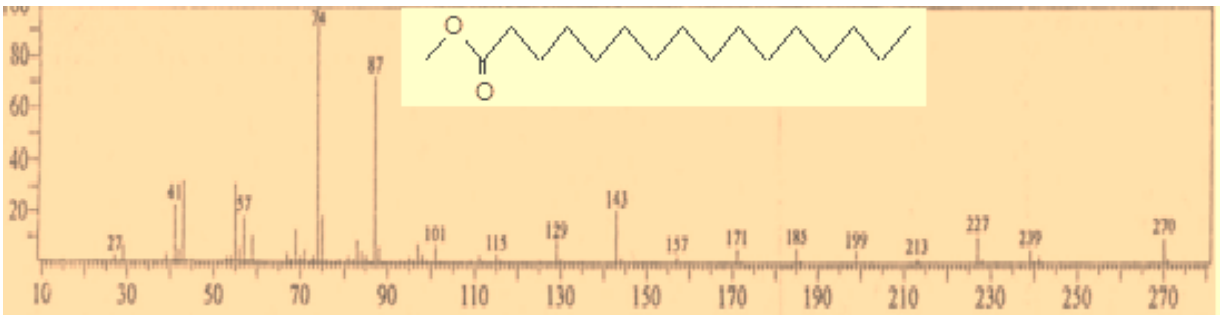


Fig. 15: Mass spectrum of hexadecanoic methyl ester

The mass spectrum of hexadecanoic acid methyl ester is given in Fig.4.The peak at m/z 270, which appeared at R.T. 15.675 corresponds to the molecular ion : $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z239 corresponds to loss of a methoxyl group.

iv-Methyl stearate(10.53%)

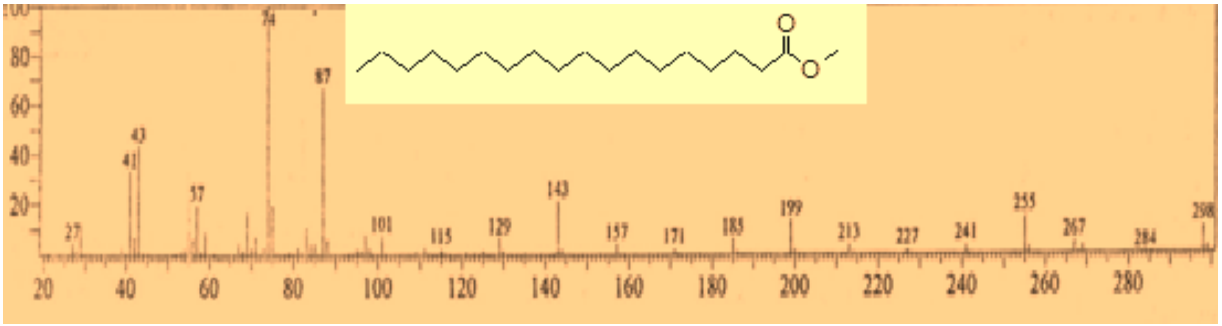


Fig. 16: Mass spectrum of methyl stearate

The mass spectrum of methyl stearate is displayed in Fig. 5. The spectrum showed m/z 298 (R.T. 17.588 in total ion chromatogram) .Appearantly , m/z 298 corresponds to

$M^+[C_{19}H_{38}O_2]^+$ .The fragment at m/z267 is attributed to loss of a methoxyl function.

3.3.2-Antimicrobial activity

Psidium guajava oil was screened for antimicrobial activity against six standard microorganisms. The average of the diameters of the growth inhibition zones are shown in Table (3) .The results were interpreted in terms of the commonly used terms (<9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .

Table 3.7 : Antimicrobial activity of oil :M.D.I.Z (mm)

Drug	Conc .(mg/ml)	Ec	Ps	Sa	Bs	Ca	An
A.digitata oil	100	-	15	24	15	9	13

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

The oil showed activity against all test organisms except for *Escherichia coli* . It was partially active against the fungus *Candida albicans* but significant activity against *Staphylococcus aureus* was observed.

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