An-Najah National University
Faculty of Graduate Studies

STUDIES TOWARD ISOLATION AND IDENTIFICATION OF BIOACTIVE SUBSTANCES FROM MEDICINAL PLANTS

By
Derar Issa "Mohammad Hassan" Smadi

Supervisor
Dr. Othman Hamed

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry, Faculty of Graduate Studies, An- Najah National University, Palestine.
2011

STUDIES TOWARD ISOLATION AND IDENTIFICATION OF BIOACTIVE SUBSTANCES FROM MEDICINAL PLANTS

By
Derar Issa "Mohammad Hassan" Smadi

This Thesis was defended successfully on 19/1/2011 and approved by:

Committee Members:	signature
1. Dr. Othman Hamed (Supervisor)	.....
2. Dr. Waheed Jondi (Internal Examiner)	.....
3. Dr. Abdalla Walwil (External Examiner)	.....

DEDICATION

To my father, my mother, my brothers, my sister,

and to all my friends

ACKNOWLEDGMENT

Praise and thanks to Allah, for helping and directing me to the right path. Special thanks to my research supervisor Dr. Othman Hamed for the chance given me to work with his research group. I am deeply grateful to him for his constant presence, and his encouragement throughout this research project.

Great thanks to Dr. Nizar Matar for his help during writing the thesis. Many thanks to Dr. Adham Abu Taha for helping me in measuring of antibacterial activity of the fractions. My thanks to the thesis committee members for their willingness to read the thesis and provide useful suggestions.

Finally, I thank my doctors and all staff in the department of chemistry at An-Najah University, as well as to Mr. Omair Nabulsi for helping me in the lab

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحت عنوان:

Studies toward Isolation and Identification of Bioactive Substances from Medicinal Plants

دراسة عزل وتشخيص مواد نشطة طبياً من اصول نباتات طبية

اقر بأن ما اشتملت عليه الرسالة إنما هي من إنتاجي الشخصي باستثناء ما تمت الإشارة إليه حيثما ورد, وان الرسالة ككل, أو أي جزء منها لم يقدم من قبل لنيل أية درجـــة علميـــة أو بحث علمي أو بحثي لدى أية مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is my research own work and has not been submitted elsewhere for any other degree or qualification.

Student's name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

List of Contents

No.	Contents	Page
	Dedication	iii
	Acknowledgment	iv
	Declaration	v
	List of Contents	vi
	List of Tables	viii
	List of Figures	ix
	List of Appendices	x
	Abstract	xi
	Chapter One: Introduction	**1**
1.1	The Significance of Natural Products as Drugs	1
1.2	Sources of Natural products	3
1.2.1	Natural Products from Plants	4
1.2.2	Natural Products From Animals	6
1.2.3	Natural Products from Microorganisms	7
1.2.4	Natural Products from Marine Organisms	8
1.3	Plant Based Agents Antibacterial Agents	9
1.3.1	Terpenoids	9
1.3.2	Alkaloids	11
1.4	Aromatic compounds	12
1.4.1	Flavonoids	12
1.4.2	Quinones	13
1.5	Plants Used In Traditional Medicine in Palestine	14
1.5.1	Walnut Tree	14
1.5.2	Plumbago	16
1.5.3	Salvia L	19
1.6	Aims and Scope of the Work	21
1.7	Chemical Compounds Extracted from *Inula viscosa*	23
1.7.1	Sesquiterpenes	23
1.7.2	Terpenes	24
1.7.3	Lactone	24
1.7.4	Azulene	24
	Chapter Two: experimental	**26**
2.1	General Procedure for extractions	26
2.1.1	Requirements of Efficient Extraction	26
2.1.2	Plant Material	27
2.1.3	Preparation of the Collected plants for Extraction	27
2.2	Evaluation of Concentrate by Thin Layer Chromatography (TLC)	27
2.3	Separation of Extract by Flash Chromatography	28

No	Contents	Page
2.3.1	Flash Column Chromatography	28
2.4	Evaluation of fractions for Antibacterial Activity	30
2.4.1	Materials	30
2.4.2	Microorganisms used	30
2.4.3	Screening for Antimicrobial Activity	30
2.4.4	Determination of Minimum Inhibitory Method	31
	Chapter Three: Results and Discussion	**32**
3.1	Analysis of the separated fractions	32
3.1.1	Fraction Three	34
3.1.2	Fraction Four	34
3.2	Analysis the fractions	39
3.2.1	Screening Results	39
3.2.2	Determination of Minimum Inhibitory Concentration (MIC)	40
3.2.3	Determination of Minimal Bactericidal Concentration (MBC)	41
	References	**43**
	Appendix	**52**
	الملخص	**ب**

List of Tables

No.	Tables	Page
Table (2.1)	A summary of the separated fractions from Inula viscose extract (about 10.0 g)	29
Table (2.2)	Screening results (Zone of inhibition in mm)	31
Table (2.3)	MIC results of fraction 4	321
Table (2.4)	MBC results for S.aureus bacteria	32
Table (3.1)	LC/MS Analysis Results of Fraction 2	34
Table (3.2)	LC/MS Analysis Results of Fraction 4	34
Table (3.3)	Summary of the ^{13}C chemical shifts of 3,3'-di-*O*-methylquercetin (61).	39
Table (3.4)	Screening results (Zone of inhibition in mm)	40
Table (3.5)	A Summary of MIC Results of Fraction 4	41
Table (3.6)	MBC and MIC results of fraction four against *S. aureus* bacteria	41

List of Figure

No.	Figure	Page
Fig. (1.1)	Examples on natural materials with anaesthetic properties	2
Fig. (1.2)	Examples on bioactive materials extracted from various plants	3
Fig. (1.3)	Natural products with anticancer and antimalarial activities	4
Fig. (1.4)	Commercial anticancer with natural origin	5
Fig. (1.5)	bioactive material extracted from animals	7
Fig. (1.6)	Examples on Bioactive compounds extracted from Bacteria	7
Fig. (1.7)	Marine source natural products with bioactivities	8
Fig. (1.8)	Terpenoids with antifungal activities extracted from leaves of pinus radita	10
Fig. (1.9)	Example on aromatic compounds with antibacterial activity	12
Fig. (1.10)	Example on flavonoids with antibacterial activities	13
Fig. (1.11)	Example on isoflavonoids with antibacterial activities	13
Fig. (1.12)	Structures and name of materials extracted from walnut tree	15
Fig. (1.13)	Natural materials available in plumgbago europea L	18
Fig. (1.14)	Chemical compounds that are available in Salvia Fruticosa	21
Fig. (3.1)		35
Fig. (3.2)	^{1}H NMR of major component of fraction 4 of *I. Viscosa*	36
Fig. (3.3)	^{1}H NMR of major component of fraction 4 of *I. Viscosa*, Showing the coupling constants between major peaks	37
Fig. (3.4)	^{13}C NMR of major component of fraction 3 of *I. Viscosa*	38

List of Appendices

No.	Appendix	Page
Fig a1	^{1}H NMR of fraction 3 of *I. Viscosa*, showing the presence of a mixture of two components	52
Fig a2	GC/MS of crude of *I. Viscosa*.	53
Fig a3	LC/MS of fraction 4 of *I. Viscosa*, showing the presence of a mixture of two components	54
Fig a4	LC/MS of component 1 of fraction 4 after purification	55
Fig a5	MS of component 1 of fraction 4 after purification	56
Fig a6	MS of component 2 of fraction 4 after purification	57
Fig a7	LC/MS of fraction 2 of *I. Viscosa*, showing the presence of a mixture of five components	58

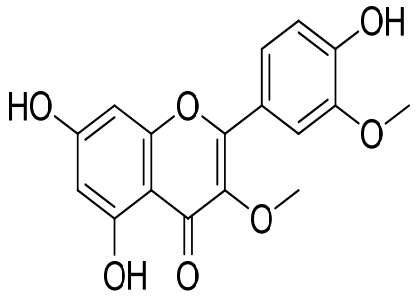
STUDIES TOWARD ISOLATION AND IDENTIFICATION OF BIOACTIVE SUBSTANCES FROM MEDICINAL PLANTS

By
Derar Issa Smadi
Supervisor
Dr. Othman Hamed

Abstract

More than 600 plant species have been used in the Palestinian traditional medicine to treat various diseases. About fifty of these plants are used to treat various skin diseases. This work is a continuation of existing effort to find new medicine from plants grows in Palestine. Medicinal plant Tayoon was chosen for this work. It was chosen because it is an important plant in the Palestinian folklore, it has unlimited number of medical applications. Three main stages were used in separation and identification of tayoon extracts. In the first stage, tayoon was subjected to extraction with ethyl acetate. In the second stage, tayoon extracts were fractionated using flash chromatography, four fractions were separated. In the third stage, the four separated fractions were evaluated for antibacterial activities. Results of the antibacterial study showed that only fraction 4 has activity against bacteria S.aureus. Base on these results fraction 4 was further fractionated by flash chromatography and two components were collected. The major component which was identified to have the bioactivity was analyzed by various spectroscopic techniques and its structure was determined. The major compound of fraction four was identified to be 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-

methoxy-4*H*-chromen-4-one which is known as 3,3’-di-*O*-methylquercetin, its structure is shown in the following figure.



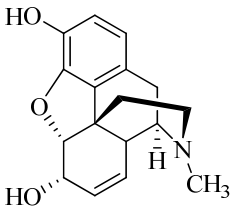
CHAPTER ONE
INTRODUCTION

1.1 The Significance of Natural Products as Drugs

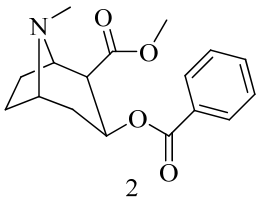
Since the old time, natural products have played a very important role in curing human diseases. The ancient civilizations such as Chinese, Egyptian, Indians, Greek, and North Africans provide written evidence for the use of natural products for treatment of various diseases .[1]

The earliest known written medical prescription is about four thousand year old, Sumerian clay tablet that shows remedies for various illnesses .[2] For instance, mandrake was prescribed for pain relief, turmeric possesses blood clotting properties, roots of the endive plant were used for treatment of gall bladder disorders, and raw garlic was prescribed for circulatory disorders. These treatments are still being used in several countries as affordable drugs.

However, it was not until the nineteenth century that scientists isolated active components from various medicinal plants. Friedrich Sertürner isolated morphine (**1)** (Fig **1.1**) from *Papaver somniferum* in 1806. In 1860, a German chemist Carl Koler isolated cocaine (**2)** (Fig **1.1**), the chemical responsible for certain biological activity. He found that cocaine could act as a local anaesthetic in eye surgery. As the years passed, scientists observed that cocaine paralyzed nerve endings responsible for transmitting pain. As a local anaesthetic, it revolutionized several surgical and dental procedures and since then natural products have been extensively screened for their medicinal purposes.



Morphine
1



Cocaine
2

Fig. (1.1): Examples on natural materials with anaesthetic properties

More examples on bioactive materials extracted from natural sources are shown in **Fig 1.2**. Atropine(**3**) obtained from *Atropa belladonna*, strychnine (**5**), a CNS stimulant, identified from a cone snail, *Conus magus*, and Taxol® (**4**)obtained from the bark of the Pacific yew tree are examples of bioactive materials available in plants.

A recent study conducted by the World Health Organization (WHO) shows that, about 80% of the world's population relies on traditional medicine .[3] More than one hundred drugs prescribed in USA today come from natural sources, more than 90% of which come either directly or indirectly from plant sources .[4] About 50% of the anticancer drugs in the market come from natural products or natural products derivatives .[5] More than a hundred anticancer drugs have been developed between the years 1981-2006, 25% of which are natural product derivatives, eighteen are natural product mimics, eleven candidates are derived from a natural product pharmacophore, and nine are pure natural products .[6] Thus natural products make a very significant contribution to drug discovery.

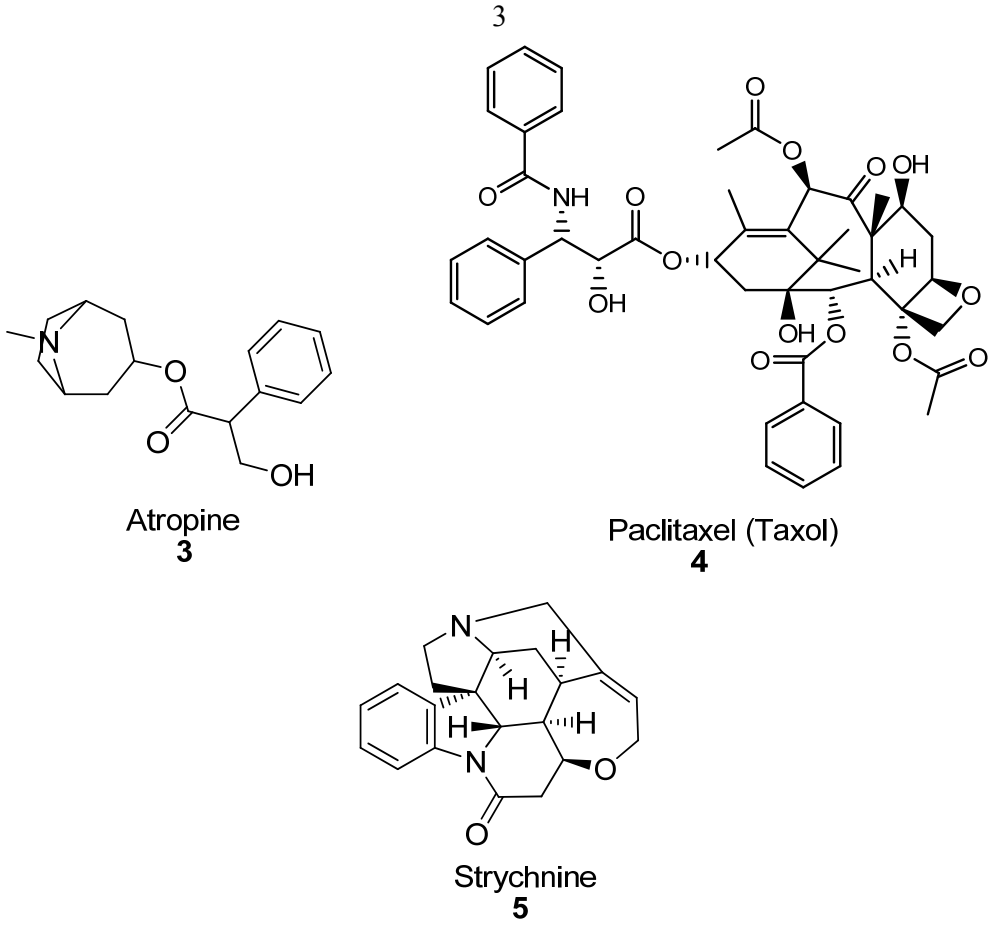


Fig. (1.2): Examples on bioactive materials extracted from various plants

1.2 Sources of Natural Products

Naturally products could be classified into four types based on their sources.

1. Natural products from plants
2. Natural products from animals
3. Natural products from microorganisms
4. Natural products from marines

1.2.1 Natural Products from Plants

The efficiency of medicinal plants in treatment of various diseases is known and proved thousands of years ago. Farnsworth, N. R *et. al* [7] showed in a documented article that up to date, 35,000-70,000 plant extracts have been screened for their medicinal use.

The earliest known records for using plants as medicine are from Mesopotamia about 2600 B.C., and these are still significant part of traditional medicine and herbal remedies .[8] Important drugs such as Taxol® **(4) (Fig 1.2)**, camptothecin**(7) (Fig 1.3)**, morphine **(Fig 1.1)** and quinine**(6) (Fig 1.3)** have been isolated from plant sources. The first two are widely used as anticancer drugs, while the remaining are analgesic and antimalarial agents, respectively.

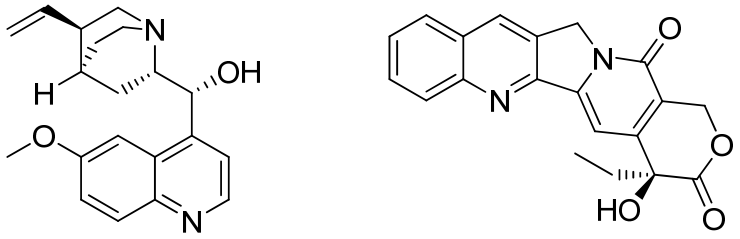


Fig. (1.3): Natural products with anticancer and antimalarial activities

Other anticancer agents available in the market today derive their origin from plants are: podophyllotoxin **(11)**, Etoposide **(10)**, teniposide**(9)**, *Catharanthus roseus,* vincristine, **(8)** and vinblastine **(8)**.

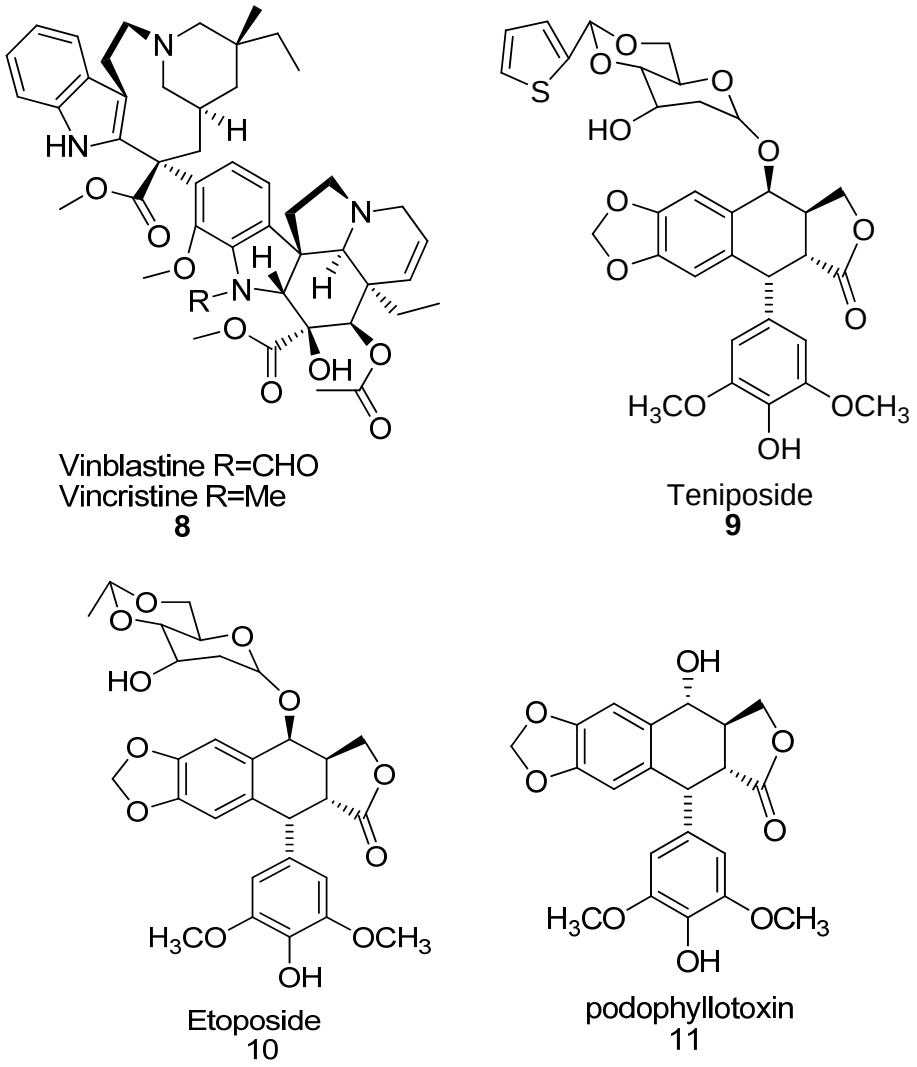


Fig. (1.4): Commercial anticancer with natural origin

Podophyllotoxin is one of the early compounds isolated as an anticancer agent from *Podophyllum peltatum*. It was initially used therapeutically as a purgative and in the treatment of venereal warts .[9] Later, in 1974, it was shown that it acts as an anticancer agent by binding irreversibly to tubulin .[10] Etoposide (**10**) and teniposide (**9**) are modified analogs of podophyllotoxin .

Vincristine (**8**) and vinblastine (**8**) extracted from Madagascar periwinkle, *Catharanthu,* [11] a member of the Apocynaceae. These natural products seem to have a diverse medicinal property. They have anticancer and antihypertensive activities. Both vinblastine and vincristine are now known to prevent cell division by inhibiting mitosis in the cell cycle. They irreversibly bind to tubulin, thereby blocking cell multiplication and eventually causing cell death.[12]

The anticancer agent Paclitaxel (Taxol®) was extracted from Pacific yew tree, Taxus brevifolia ,[13] it has been used in the treatment of several types of cancer, but most commonly for ovarian and breast cancers as well as non-small cell lung tumors .[14] It had sales of $750 million in 2002 and $1.0 billion in 2003 .[15]

1.2.2 Natural Products from Animals

Animals are also source of biomaterials that can be used as drugs. For instance, Epibatidine **(12) (Fig 1.5)**, obtained from the skin of an Ecuadorian poison frog, is ten times more potent than morphine .[16] Other interesting bioactive materials obtained from animals and played a significant role in designing a multitude of cures for several diseases are Venoms , toxins, and Teprotide . Teprotide for example, extracted from a Brazilian viper, has led to the development of cilazapril **(14)** and captopril **(13)** which are effective against hypertension .[17]

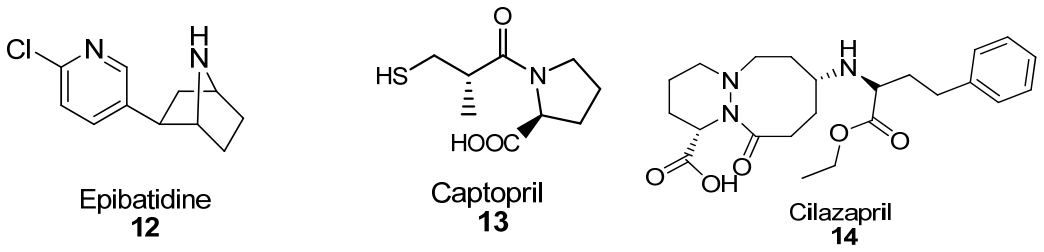


Fig. (1.5): bioactive material extracted from animals

1.2.3 Natural Products from Microorganisms

Microorganisms have been a source of potential drug candidates. The first drug that was obtained from bacteria is penicillin (**17**), which was discovered in 1929. Since then, microorganisms have been screened for bioactive compounds. That led to the discovery of valuable drugs such as for instance, the antibacterial agents cephalosporins (**18**), antidiabetic agents acarbose (**16**), and anticancer agents epirubicin (**15**) .[18]

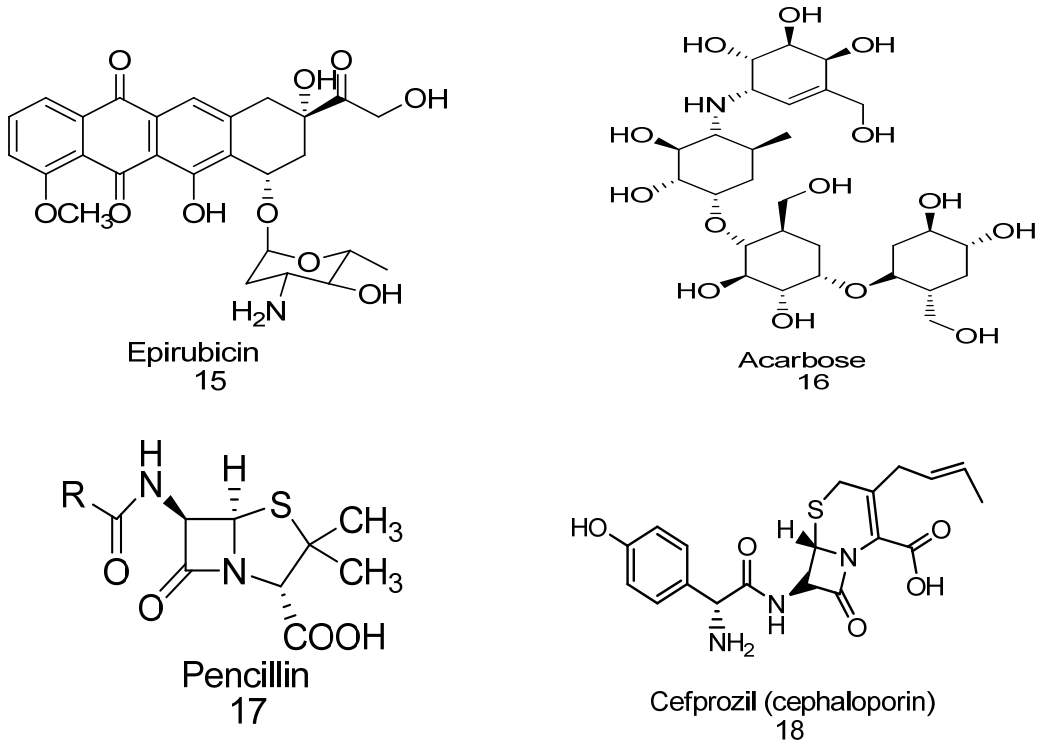
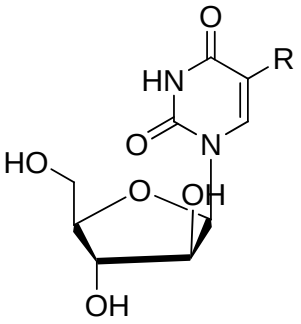


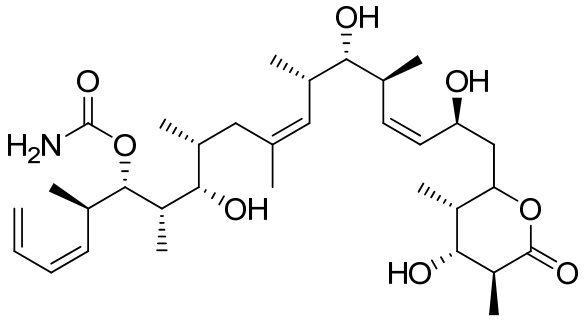
Fig. (1.6): Examples on Bioactive compounds extracted from Bacteria

1.2.4 Natural Products from Marine Organisms

Marine organisms are also a potential source of drugs. The first bioctactive compound to be isolated from marine species was spongouridine (**19**) in the 1950s .[19] Since then several others were isolated such as spongothymidine (**19**) from the Carribean sponge *Cryptotheca*. These compounds are nucleotides and show great potential as anticancer and antiviral agents. The discovery of spongouridine led to an extensive research to identify drug candidates from marine sources. This led to the discovery of the anticancer agent discodermolide (**20**), isolated from the marine sponge, *Discodermia dissoluta*, which has similar mode of action to that of paclitaxol® besides possessing a strong antitumor activity. It is unique in that it exhibits better water solubility as compared to paclitaxol®.[20]



Spongouridine R= H
Spongothymidine R= CH_3
19



(+)- Discodermolide
20

Fig. (1.7): Marine source natural products with bioactivities

1.3 Plant Based Antibacterial Agents

In spite of the availability of a large number of antibacterial agents, there is still a great need for more potent antibacterial drugs.

Most of the drugs available is either having toxicity for ecosystem or bacteria developed some kind of resistance against them.

Therefore, researchers are increasingly turning their attention to folk Medicine, looking for new leads to develop better drugs against microbial infections.[22] Plants represent a reservoir of effective chemotherapeutants and can provide valuable sources of drugs which posses antimicrobial properties. During recent years, large number of plants have been screened for their antibacterial activity, as a result of that large numbers of plants have been found to contain ingredients that have some kind of activities toward bacteria .[21] Examples of these are mentioned earlier in this chapter and will be presented in the next few pages with some details.

1.3.1 Terpenoids

The terpeniods, sometimes called isopreniods, are a large diverse class of naturally occurring organic chemicals. They are derived from five-carbon isoprene units assembled and modified in thousands of ways. Some terpenoids have unusual seven-carbon ring structures .[23]

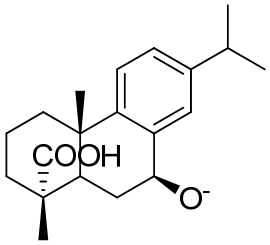
Plant terpeniods are used widely for their aromatic qualities; they play a major role in traditional herbal therapies and are under investigation for antibacterial, antineoplastic and other pharmaceutical functions.

Terpeniods contribute to the scent eucalyptus**,** the flavors of cinnamon, cloves, and ginger and the color of yellow flowers.

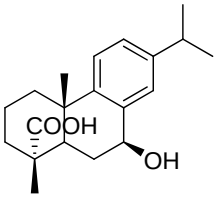
Monoterpenoids are well known of their antimicrobial activity.

Several terpeniods showed antifungal activity against microsporum cookie and trichophyton mentagrophyes and fusaruim .[24]

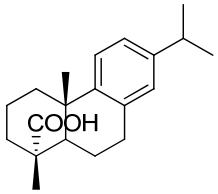
Examples on these tepenoids are shown in Figure **(1.8)** which was isolated from the leaves of pinus radita .[25]



(1*R*,4a*S*,9*S*)-1-carboxy-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthren-9-olate
21



(1*R*,4a*S*,9*S*)-9-hydroxy-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene-1-carboxylic acid
22



(1*R*,4a*S*)-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene-1-carboxylic acid
23

Fig. (1.8): Terpenoids with antifungal activities extracted from leaves of pinus radita.

Another type of biological active terpenoids is saponins, that are a group of triterpenoids which have been isolated from different plant (e.g. rapanea, dolichos, camellia, and primula). Saponins show a wide range of biological activities including antibacterial activity. [26-28]

Saponins are considered to be an exception among antifungal compounds in that their antifungal activity is usually correlated with the sugar moiety glycosylated to the 3-hydroxyl group of the terpenoids and thus a polar part of the molecule, whereas most other antifungal costituents tend to be strongly lipophilic and inactive in glycoside form .[29,30]

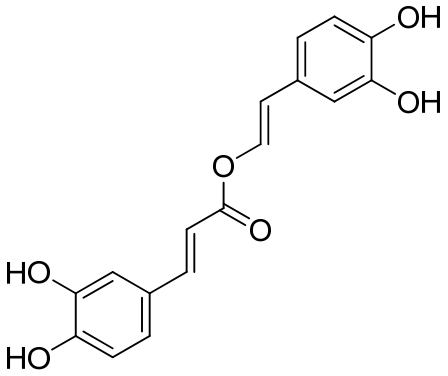
1.3.2 Alkaloids

They are groups of natural chemicals which mostly contain basic nitrogen atoms. Alkaloids are produced by plants, animals, fungi and bacteria. Many alkaloids can be purified from crude extracts by acid base extraction, most alkaloids are toxic to organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. For example, cocaine and morphine are used as local anesthetic and are also used as stimulant caffeine and nicotine, or the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste .[31]

Extracts from plants containing toxic alkaloids, such as aconitine and tubocurarine, were used since antiquity for poisoning arrows.[32]

1.4 Aromatic compounds

A large proportion of aromatic substances that are extracted from plants often show antifungal activity. [33] These include simple and alkylated phenols, phenolic acids, phenylpropanoids (**24**), coumarins, flavonoids, isoflavonoids , stillbenoids, quinones and xanthones.



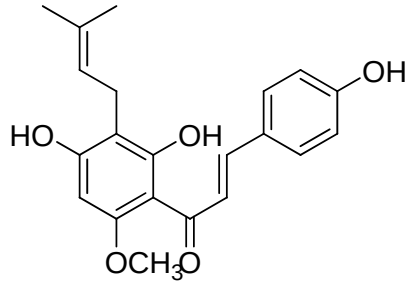
(E)-3,4-dihydroxystyryl 3-(3,4-dihydroxyphenyl)acrylate

24

Fig. (1.9): Example on aromatic compounds with antibacterial activity

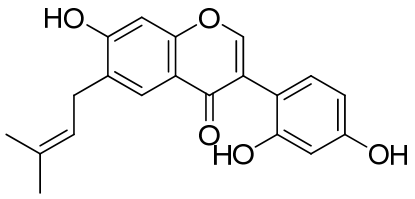
1.4.1: Flavonoids

Such as those shown in Figure **(1.10)**, and especially isoflavonoids Figure **(1.11)** were reported to have high antifungal activity against richophyton mentagrophytes and T. rubrum .[34-37]



(E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-enyl)phenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one

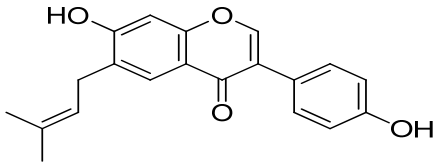
25



3-(2,4-dihydroxyphenyl)-7-hydroxy-6-(3-methylbut-2-enyl)-4H-chromen-4-one

26

Fig. (1.10): Example on flavonoids with antibacterial activities



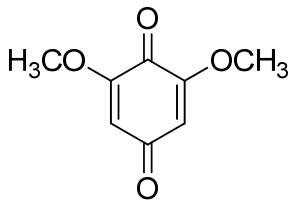
7-hydroxy-3-(4-hydroxyphenyl)-6-(3-methylbut-2-enyl)-4*H*-chromen-4-one

27

Fig. (1.11): Example on isoflavonoids with antibacterial activities

1.4.2 Quinones

Some quinones are also known to inhibit mycelial growth of fungi, examples are two naphthoquinonoid naphthaxirene derivatives and their glucosides from sesamum angolense pedaliaceae [38] and the benzoquinones juglone present in a number of plants, e.g pecan carya illinoesis, junglandaceae [39] and 2, 6- dimethoxybenzoquinone (**28**) from crotonlacciferus (euphorbiaceae).



2,6-dimethoxycyclohexa-2,5-diene-1,4-dione
28

The latter constituent displays antifungal activity against cladosporium cladosporiodes.[40]

1.5 Plants Used In Traditional Medicine in Palestine

More than 600 plant species have been used in Palestine in traditional medicine to treat various diseases .[41, 42] About fifty species of these plants are used to treat various skin diseases. The following paragraphs show a summary about some of these plants, the structure of the bioactive materials extracted form them and their applications.

1.5.1 Walnut Tree

The scientific name for this plant is Juglans Regial, it belongs to a family known as Juglandaceae .[43] Its name is derived from the Latin glans jovis, the corn of Jupiter. A very leafy tree with dark green leaves, the tree grows to a height of about 12-15 m. It has a straight, well-branched trunk with smooth, grayish-white bark, when young that develops deep, longitudinal furrows with age and becomes very rough.

The bioactive materials in this plant are available in leaves, bark, and fruit.

In Palestinian tradition, this plant is used to treat bacterial infection by isolating the juice from the green peels of the fruits and apply it on the infected skin.

Several bioactive materials have been isolated from walnut tree among these are inositol, tannin, gallic acid, junglandin, carotene, pyrogallic acid, monoterponids, sesquiterpenes and juglone. The structures of these extracted compounds are shown in Fig **(1-12)**.

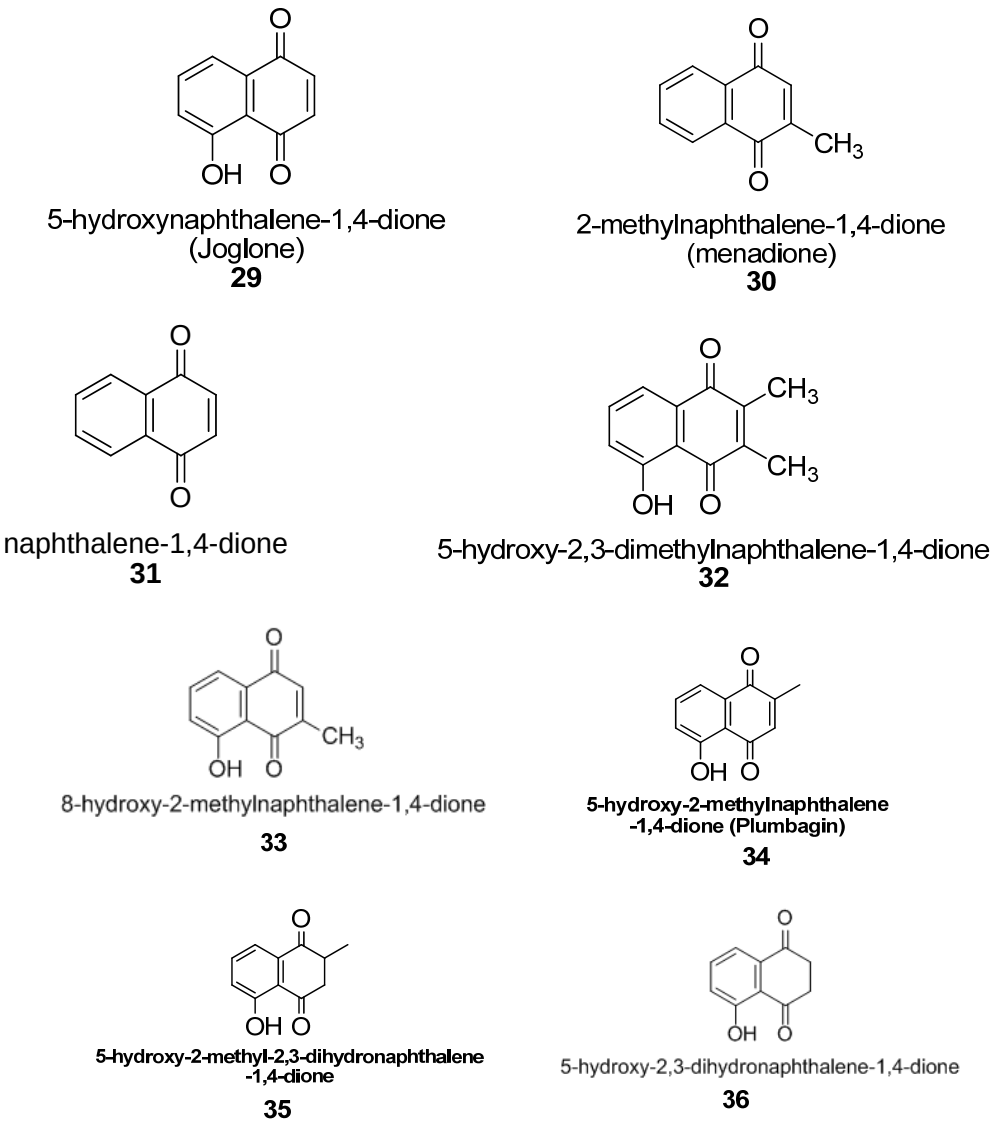


Fig. (1.12): Structures and name of materials extracted from walnut tree

The bioactive materials extracted from the walnut tree are used as: antihypoglycaemic, deputative, galactofuge, rubefacient, antiscrophutous and antidermatosic.

1.5.2 Plumbago

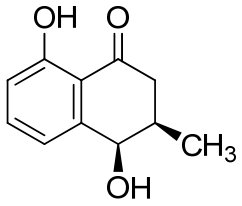
The scientific name for this plant is Plumbago europea L, it belongs to a family known as plumbaginaceae .[44] The Plumbago herb grows to height of 30-100 cm. It has a herbaceous stem which is ribbed, erect, branched, and leafy. The leaves are farinose, especially on the lower face, oblong elliptic obviate or oblanceolate, remotely denticulate. Lower most leaves are petiolate, whereas middle and upper leaves sessile and auriculate-clasping. Flowers of this plant are calyx 6-8 mm with corolla purple to lilac, lobes obovate, obtuse, mucronate. The medicinal parts are gathered in Summer.

Plumbago is native to South Africa, and is a popular ornamental in subtropical gardens in Florida and California, Plumbago may be found in gardens all over the world.

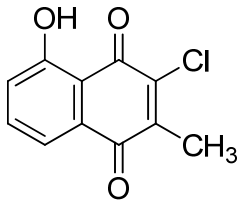
The bioactive materials in these plants are available in leaves, roots and whole plants.

In Palestinian tradition, green leaves are often macerated and applied on the infected skin. Also dry leaves are usually moistened and then macerated before being applied on infected skin parts.

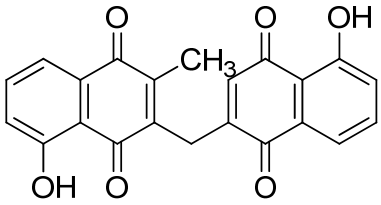
Chemical compounds that existing in Plumbago are shown in figure 1.13:



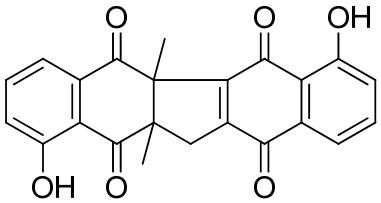
(3*R*,4*R*)-4,8-dihydroxy-3-methyl-3,4-dihydronaphthalen-1(2*H*)-one
(1,3-Isoshinanolone)
37



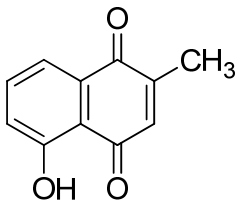
3-chloro-5-hydroxy-2-methylnaphthalene-1,4-dione
(Droserone)
38



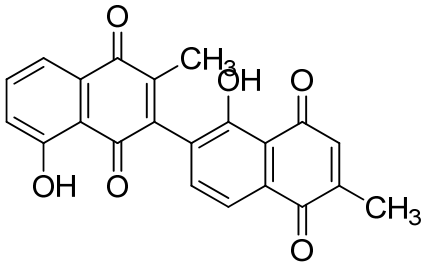
5-hydroxy-3-((5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)
methyl)-2-methylnaphthalene-1,4-dione
(Isozeyanone)
39



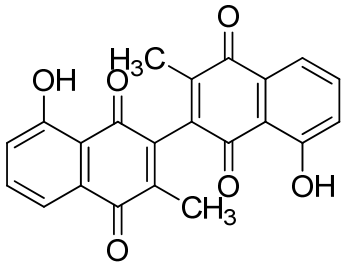
1,7-dihydroxy-5a,12a-dimethyl-12,12a-dihydro-5*H*-dibenzo
[*b*,*h*]fluorene-5,6,11,13(5a*H*)-tetraone
(Zeyalanone)
40



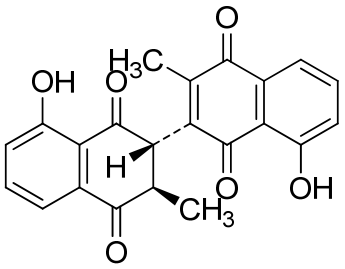
5-hydroxy-2-methylnaphthalene-1,4-dione
(Plumbagin)
41



1',8-dihydroxy-3,6'-dimethyl-2,2'-binaphthyl-1,4,5',8'-tetraone
(Chitranone)
42



8,8'-dihydroxy-3,3'-dimethyl-2,2'-binaphthyl-1,1',4,4'-tetraone
(3,3-Biplumbagin)
43



(2*S*,3*R*)-8,8'-dihydroxy-3,3'-dimethyl-2,3-dihydro-2,2'-binaphthyl-1,1',4,4'-tetraone
(1,2(3)-Tetrahydro-3,3-Biplumbagin)
44

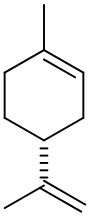
Fig. (1.13): Natural materials available in plumgbago europea L

1.5.3 Salvia L

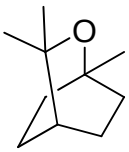
The scientific name for this plant is salvia fruticosa and it belongs to a family known as lamiaceae. Salvia L. is the largest genus of the family labaite, including over 900 species in the world. Since ancient times, species of salvia have been used in folk medicine for the treatment of diabetes and skin diseases such as psoriasis and eczema and are used with powder alum and other plants to treat mouth fungi.

Salvia due to has a wide range of biological activities such as anti bacterial activities [45-49]antitumor activities [50-57] and antifungal activities [51-56] salvia species also have some useful compounds to preserve raw and processed food [58]and some of them are used as a drink .[59]

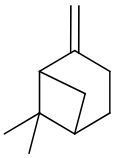
Salvia fruticosa is a very leafy plant which grows to height of about 60-120 cm; the light green gray leaves are used in traditional medicine in Palestine.



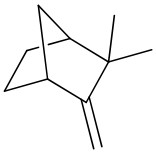
(*R*)-1-methyl-4-(prop-1-en-2-yl)
cyclohex-1-ene
(Limonene)
46



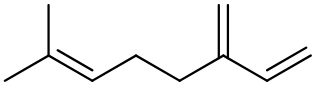
1,3,3-trimethyl-2-oxabicyclo
[2.2.2]octane
(1, 8-cineole)
45



6,6-dimethyl-2-methylenebicyclo
[3.1.1]heptane
(beta-Pinene)
48

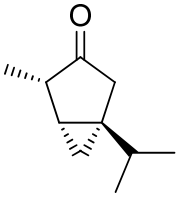


(1*S*,4*R*)-2,2-dimethyl-3-methylenebicyclo
[2.2.1]heptane
(Camphene)
47

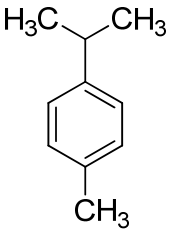


7-methyl-3-methyleneocta-
1,6-diene
(Myrcene)
50

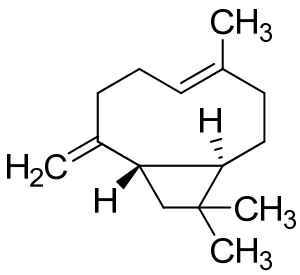
(1S,5S)-2,6,6-trimethylbicyclo
[3.1.1]hept-2-ene
(a-Pinene)
49



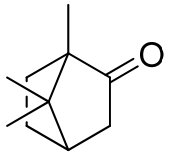
(1*S*,4*S*,5*R*)-1-isopropyl-4-methylbicyclo
[3.1.0]hexan-3-one
(a-Thujone)
52



p-cymene
51

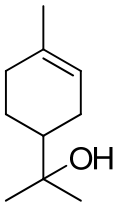


(1*R*,9*S*,*E*)-4,11,11-trimethyl-8-methylene bicyclo[7.2.0]undec-4-ene
(α-Caryophyllene)
54



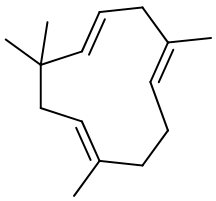
Camphor

53



2-(4-methylcyclohex-3-enyl) propan-2-ol

(a-Terpineol)
56



(1*E*,4*E*,8*E*)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene

(a-Humulene)
55

Fig. (1.14): Chemical compounds that are available in Salvia Fruticosa:

1.6 Aims and Scope of the Work

Scarce information on natural bioactive compounds and their properties in many plants in Palestine, as well as increasing demand for natural drugs were important motivations to start this study.

The general aims of this study can be placed into four folds:

1. Finding new natural compounds with bioactivities against bacteria in plants cultivated in Palestine.
2. Assessment of application possibilities of partially purified extracts containing these compounds.

3. Determination of their molecular structures, and

4. Determination of bioactivity.

Previous studies of aromatic and medicinal plants grown in Palestine have resulted in the discovery of new natural products with antibacterial activities [60] and the identification of new antioxidants .[61] These findings encouraged to initiate this work.

Inula viscosa (L.) is a perennial weed, native to the Mediterranean Basin known in the Palestinian folklore as Tayoon. It grows on hills tops, damp habitats, and roadsides. Extracts of the plant have been widely used in folk medicine to treat various diseases such as a diuretic, topical anti-inflammatic, and haemostatic[64]. Aqueous extracts of *I. viscosa* were also shown to exhibit antifungal activity *in vitro* [65, 66.] Cohen et al[67] provided evidence for the antifungal activity in plant of extracts made with organic solvents, including methanol, ethanol, ethyl acetate, acetone, chloroform, and n-hexane. Using thin-layer chromatography overlay assays, seven inhibitory zones against *Cladosporium cucumerinum* were observed in the extracts [67] In a recent study,[68] it was found that leaf extracts of *Inula viscosa* were highly effective in controlling downy mildew of grapevine, caused by *Plasmopara viticola*. Other biological activities of Inula viscosa include antiulcerogenic effects [69], prevent growth of pathogenic fungi [70], prevent zygote implantation in mammals [71], and have a strong anti oxidant activity [72].

There is also published evidence that *Inula viscosa* has also nematicidal/antihelm acologically active compounds [73, 74] including sesquiterpenes, sesquiterpenes acids [75], azulenes, lactones, flavonoids, and essential oils [76].

Currently, there is no published data on the cytotoxicity and genotoxicityof *I. viscosa* leaf extracts.

Inula viscosa was chosen for this study since it is an important plant in the Palestinian folklore and it has unlimited number of medical applications. Below are some examples on commercial medical applications of inula viscosa:

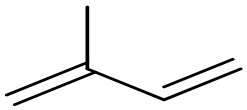
1- Inula head lice: Inula viscosa extract emulsified with olive oil or water is used in removing head lice and lice eggs.

2- Inula tea: Inula plant boiled in water is used to tract infection treatment, reduction of blood pressure, prevention of flu and cold, gum disorder treatment, and toothache treatment.

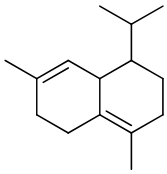
1.7 Chemical Compounds Extracted from *Inula viscosa*

1.7.1 Sesquiterpenes

Sesquiterpenes are a class of terpenes that consist of three isoprene units [77] and have the molecular formula $C_{15}H_{24}$. Like monoterpenes, sesquiterpenes could be acyclic or contain rings, including many unique combinations.



isoprene
57



1-isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene
58

1.7.2 Terpenes

Terpenes are hydrocarbons with the molecular formulas $(C_5H_8)_n$. Their building block is the hydrocarbon isoprene [77] and they are classified according to the number of isoprene units it composing them. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Many terpenes are hydrocarbons, but oxygen-containing terpenes such as alcohols, aldehydes or ketones (*terpenoids*) are also found.

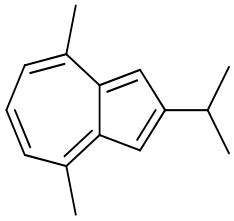
1.7.3 Lactones

Lactones are cyclic esters [78] which can be seen as the condensation product of an alcohol group -OH and a carboxylic acid group -COOH in the same molecule. It is characterized by a closed ring consisting of two or more carbon atoms and a single oxygen atom, with a ketone group C=O in one of the carbons adjacent to the other oxygen.

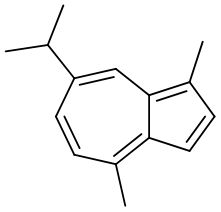
1.7.4 Azulene

Azulene is an isomer of naphthalene. Whereas naphthalene is colorless, azulene is dark blue. Its name is derived from the Spanish word azul, meaning "blue". Two azulenes, vetivazulene (4,8-dimethyl-2-

isopropylazulene) **59** and guaiazulene (1,4-dimethyl-7-isopropylazulene) **60**. , are found in nature as constituents of pigments in mushrooms, guaiac wood oil, and some marine invertebrates.



4,8-dimethyl-2-isopropylazulene
59



1,4-dimethyl-7-isopropylazulene
60

CHAPTER TWO
EXPERIMENTAL

All chemicals were purchased from Aldrich Chemical Company and used without any purification. Extracted and purified compounds from Inula viscosa were characterized by ^{1}H NMR, ^{13}C NMR, GC/MS and LC/MS. Nuclear Magnetic Resonance Spectra were recorded on Varian Gemini 2000, 300 MHz instrument, Gas Chromatography mass & spectrometry were recorded on Perkin Elmer 560D.

All ^{1}H NMR experiments were reported in unit of parts per million (ppm) downfield from tetramethylsilane.

All ^{13}C NMR spectra were reported in ppm relative to deuterchloroform (77.0 ppm).

All tests for antibacterial activity were reported in Al-Arabi hospital lab in Nablus, West Bank, Palestine.

Purification of extracted samples was performed by flash chromatography on silica gel (100-200) mesh.

2.1 General Procedure for extractions

2.1.1 Requirements of Efficient Extraction

Suitable solvent for extraction of organic compounds from plants must possess certain properties such as: water insoluble, low boiling point, medium polarity, and has higher affinity for plants extracts than water,

2.1.2 Plant Material

As mentioned earlier in the introduction chapter, the plant chosen for this study was Inula Viscosa known in Palestinian folklore as tayoon.

Plants were collected from hilly areas around Nablus city far from agricultural lands. It was collected in spring time, and dried in the shade.

2.1.3 Preparation of the Collected plants for Extraction

The dried material was ground and suspended in ethyl acetate. The produced suspension was mixed using mechanical mixer for about 48 h. Another method of extraction was also tried which is soxhlet extraction. In this method ground plant was extracted with ethyl acetate for about 24 h.

Produced solution was dried over magnesium sulfate and concentrated under reduced pressure at 50 ^{O}C using rotary evaporator.

2.2 Evaluation of Concentrate by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is an extremely useful technique for chemist in particular. TLC is an inexpensive, simple, rapid technique used to determining the number of components present in solution and helps in finding a suitable solvent for separating the components by flash chromatography as well as for monitoring reactions progress.

Several combinations of solvents of increasing polarity were evaluated as mobile phase in TLC to determine the number of compounds in Inula viscose extract. The solvents combinations were: hexane (100 %),

ethyl acetate/ n-hexane (3:7), ethyl acetate /n-hexane 1:1), ethyl acetate/n-hexane (7:3), ethyl acetate (100%), ethyl acetate/methanol (9:1). This study concludes that all of these solvent combinations are required to separate Inula viscosa extracts into pure components by flash chromatography.

2.3 Separation of Extract by Flash Chromatography

2.3.1 Flash Column Chromatography

Flash chromatography is very useful techniques for separating mixture of organic compounds into pure components.

It was performed by using a column with dimensions of 60 cm in length and 3.0 cm in diameter packed with silica gel 60 (230-400 mesh) purchased from Aldrich chemical company

The *Inula viscosa* extracts were loaded into the column as a solid mixture with some silica, which was prepared by suspending the mixture of about 5.0 g silica gel and 20 ml ethyl acetate, then ethyl acetate was removed under vacuum using rotary evaporator. The separation was started with pure hexane (low polarity) then the mobile phase polarity was increased gradually as follows: pure hexane, then hexane containing 20% ethyl acetate, then ethyl acetate was increased to 40%, then pure ethyl acetate was used and finally the column was flushed with ethyl acetate containing 10% methanol.

Table (2.1): A summary of the separated fractions from Inula viscose extract (about 10.0 g):

Fraction	Mobile Phase		Fraction weight (g)
	Hexane (%)	Ethyl acetate (%)	
1	80	20	2.0
2	60	40	2.2
3	0	100	2.5
4	0	90*	2.0

*** Ethyl acetate contains 10% methanol.**

All separated fraction were subjected to analysis by LC/MS. Fractions 1 to 3 were mixture of several components as shown in the results and the discussion part (chapter III) in addition they showed no bioactivity as shown in the next section (2.4). However, LC/MS results showed that, fraction 4 is a combination of two compounds that showed antibacterial activity. One of the two components of fraction 4 is also present in fraction 3. In order to determine the component that is responsible for the bioactivity, fraction 4 was subjected to fractionation by flash chromatography. The first eluent (Component 1) was eluted with ethyl acetate (minor) while the second eluent (component 2) was eluted with ethyl acetate containing 10% methanol. LC/MS results showed that component 1 is the one that is present in fraction 3. Since, fraction 3 is not bioactive; we believe the bioactivity of fraction 4 comes from component 2. So attention was turned to component 2 of fraction 4 and complete spectroscopic analysis was performed on it to determine its exact structure.

^{1}H NMR of component 2 of fraction 4; compound (61)

$CDCl_3$ δ (ppm): 3.8 (s, OCH_3), 3.83 (s, OCH_3), 6.18 (d, *J*, 2 Hz, C-5 H), 6.45 (d, *J*, 2 Hz C-7 H), 6.93 (d, *J*, 9 Hz, C-2' H) , 7.55 (dd, *J*, 2 and 9 Hz), 7.62 (d, *J*= 2Hz) doublet, 9.95 (bs, C4'-OH), 10.85 (bs, C6-OH) and 12.65 (bs, C4 OH).

^{13}C NMR of component 2 of fraction 4; compound (61)

$CDCl_3$ δ (ppm): 56.37, 60.41, 94.53, 99.53, 104.86, 116.32, 121.45, 138.4, 148.12, 150.13, 156.16, 157.03, 161.89, 164.84, 178.58.

2.4 Evaluation of fractions for Antibacterial Activity

2.4.1 Materials

Culture media: Mueller-Hinton, Tryptic Soy Broth (Hylabs ,Israel)

2.4.2 Microorganisms used

Bacteria strains used in the study were clinical isolates of Staphylococcus aureus, Escherechia coli, Proteus mirabilis, and Pseudomonas aerginosa, all of them were isolated from patients suffering from bacterial infections with the relevant bacteria.

2.4.3 Screening for Antimicrobial Activity

Fractions of Inula viscosa extracts were collected by flash chromatography and were screened for antimicrobial activity by using the agar well diffusion method reported in the literature by Perez et al.[62]

1. Three colonies of bacteria where transferred to sterile tubes each containing 5 ml of Tryptic Soy Broth.

2. Turbidity of the bacterial suspensions was adjusted to reach an optical density equivalent to a 0.5 McFarland standard to give a bacterial suspension of 10.cfu/ml. (cfu: colony forming unit).

3. Mueller-Hinton agar plates were inoculated by streaking bacterial swabs over the entire surface of the plates.

4. Plates were allowed to dry at room temperature.

5. Six millimeter wells were pushed into the plates.

6. Fifty microliters of the four fractions were added into duplicate wells.

7. Plates were allowed to stand at room temperature to let the tested derivative absorbed into the agar, and afterwards, they were incubated at 37 ^{o}C for 18 to 24 h.

8. Plates were examined for bacterial growth inhibition and zones of inhibition were measured in millimeters.

Table (2.2): Screening Results (Zone of Inhibition in mm):

Fraction (10 mg/ml)	***S.aureus***	***E.coli***	***P.mirabilis***	***P.aerginosa***
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	25	0	0	0

2.4.4 Determination of Minimum Inhibitory Concentration Method as Shown Below[63]

MIC was determined by broth dilution method as shown below:

1- Two-fold serial dilutions were prepared from fraction 4 in Tryptic Soy Broth.

2- Duplicate tubes of each dilution were inoculated with 5*10 of S.aureus.

3- All tubes were incubated at 37^{O}C for 18 to 24 hours.

4- The highest dilution of the drug that resulted in inhibition of bacterial growth was considered as the MIC.

Table (2.3): MIC results of fraction 4

Conc.(mg/ml)	Fraction 4
10	-ve
5	-ve
2.5	-ve
1.25	-ve
0.625	-ve
0.3125	-ve
0.15625	-ve
0.078125	-ve
0.039063	-ve
0.019531	+ve
Positive Control	+ve
Sterility Control	-ve

Bacterial growth (positive: +ve) or (negative: -ve)

Table (2.4): MBC results for S.aureus bacteria:

Fraction 4	MIC(mg/ml)	MBC (Mg\ml)
4	39	78

CHAPTER THREE
Results and Discussion

3.1 Analysis of the separated fractions

In this study, a comprehensive analysis was performed on tyoon extracts. Number of constituents and complete structure determination of at least one of the components was performed.

Tayoon was collected from areas in Nablus, Palestine, dried in the shade and grounded. Sample of dried and ground tayoon plant (about 500 g) was extracted with ethyl acetate. The extracts were concentrated in *vacuo* to afford about 30 g of extract. Another sample of tyoon was extracted with ethanol. Again the extracts were concentrated in *vacuo* to afford about 15 g of extract. The extracts of the two solvents were analyzed by HPLC and GC/MS. In the HPLC analysis, the mobile phase consists of acetic acid, and the results indicate the presence of phenolic compounds. Analysis by HPLC didn't give clear picture about the number of components present in tayoon. Analysis by GC/MS showed the presence of about 13 compounds as shown in Figure **a2** (Appendix).

The ethyl acetate extract (10.0 g) was fractionated by flash chromatography on silica gel 120 H using hexane–EtOAc solution of increasing polarity. Four fractions were separated. The first fraction was eluted with 20% EtOAc in hexane. Analysis of the first fraction by LC/MS showed the presence of multi components with very close retention time. The second fraction eluted with 40% EtOAc in hexane. Analysis of the

second fraction by LC/MS showed the presence of five components (Figure **a7**, page **58**, appendix), retention times and molar masses for the five components are summarized in Table **3.1**.

Table (3.1): LC/MS Analysis Results of Fraction 2

Component	Retention Time (min)	Molar Mass
1	0.55	392
2	1.27	253
3	1.49	294
4	1.74	409
5	2.40	584

3.1.1 Fraction Three

Analysis of Fraction 3 by LC/MS showed the presence of several components at low concentrations. No further work was performed on this fraction except for its biological activity against certain bacteria which was evaluated (see section below)

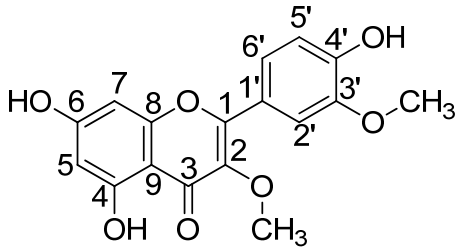
3.1.2 Fraction Four

Analysis of the fraction four by LC/MS showed the presence of two components (Figure 3a, Appendix), retention times and molar masses for the two components are summarized in Table **III.2**. Component 2 was the major product.

Table (3.2): LC/MS Analysis Results of Fraction 4

Component	Retention time	Molar Mass
1	1.22	331.9
2	1.29	347.9

Fraction 4 was subjected to purification by Flash chromatography; component with relatively high concentration was collected and analyzed by ^{1}H NMR and ^{13}C NMR. The results are consistent with the structure shown in Figure **(3.1)**. The proposed structure was confirmed with that reported in the literature.[79, 80] The compound was identified to be 3,3'-di-*O*-methylquercetin **(61)**.



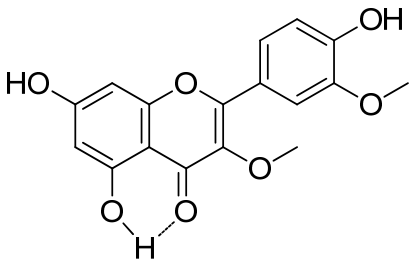
61

3,3'-di-O-methylquercetin

(Fig. 3.1)

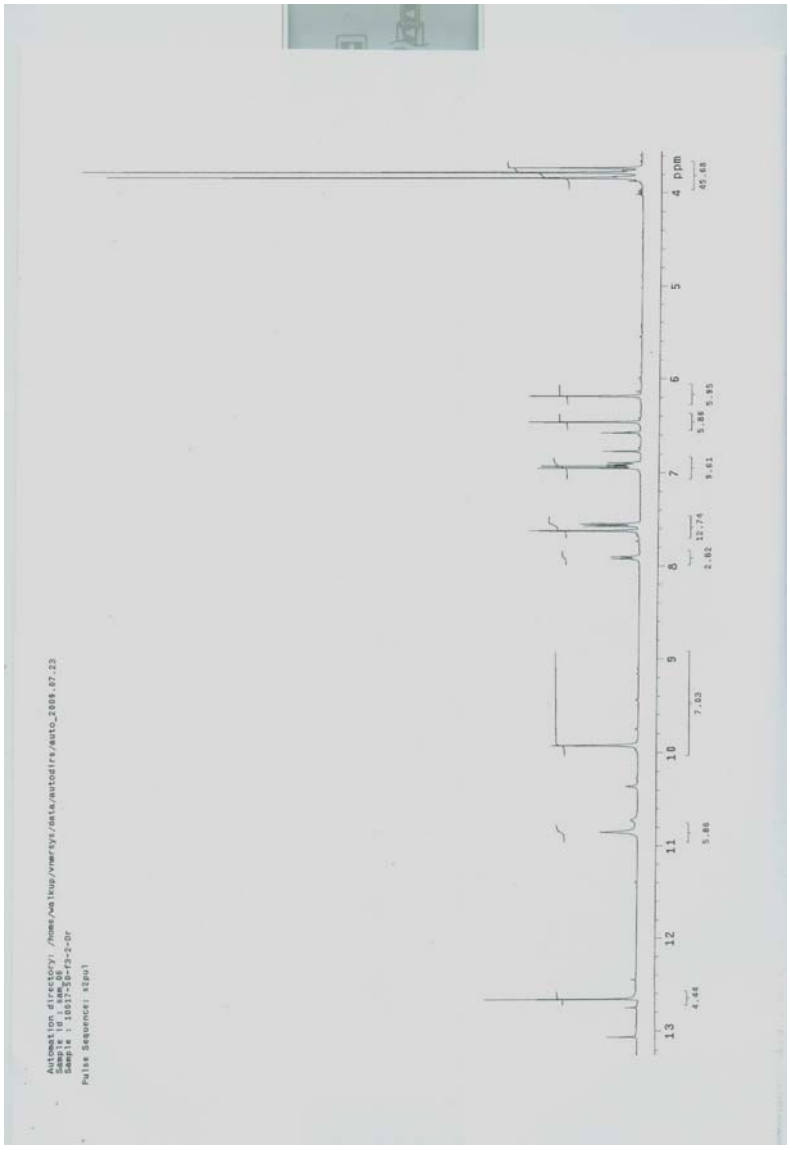
The ^{1}H NMR of the major component of fraction four exhibits the following signals (Figure **III.2**): two methoxy singlet C-2 and C-3' at δ 3.8 ppm and 3.83 ppm respectively, doublet C-5 H at 6.18 with coupling constant of $J_{5,7}$= 2 Hz, doublet C-7 H at 6.45 with coupling constant of $J_{5,7}$=2 Hz, doublet C-2' at 6.93 with a coupling constant of$J_{5,'6}$= 9Hz, doublet of doublet at 7.55 with coupling constants of $J_{6',2'}$=2 and $J_{6',5}$=9 Hz, doublet 7.62 with a coupling constant of 2Hz, broad singlet C4'-OH at 9.95, broad singlet C6-OH at 10.85, and broad singlet C4 OH at 12.65. The high desheilding of C4-OH could be attributed to intra H-bonding between β-hydroxyl group (C4-OH) and the carbonyl group as shown in Figure II.2.

Fig. **(3.2)**: The following figure shows the intramolecular H-bonding between β-hydroxyl group (C4-OH) and the carbonyl group



61

(Fig. 3.2): ^{1}H NMR of major component of fraction 4 of *I. Viscosa:*



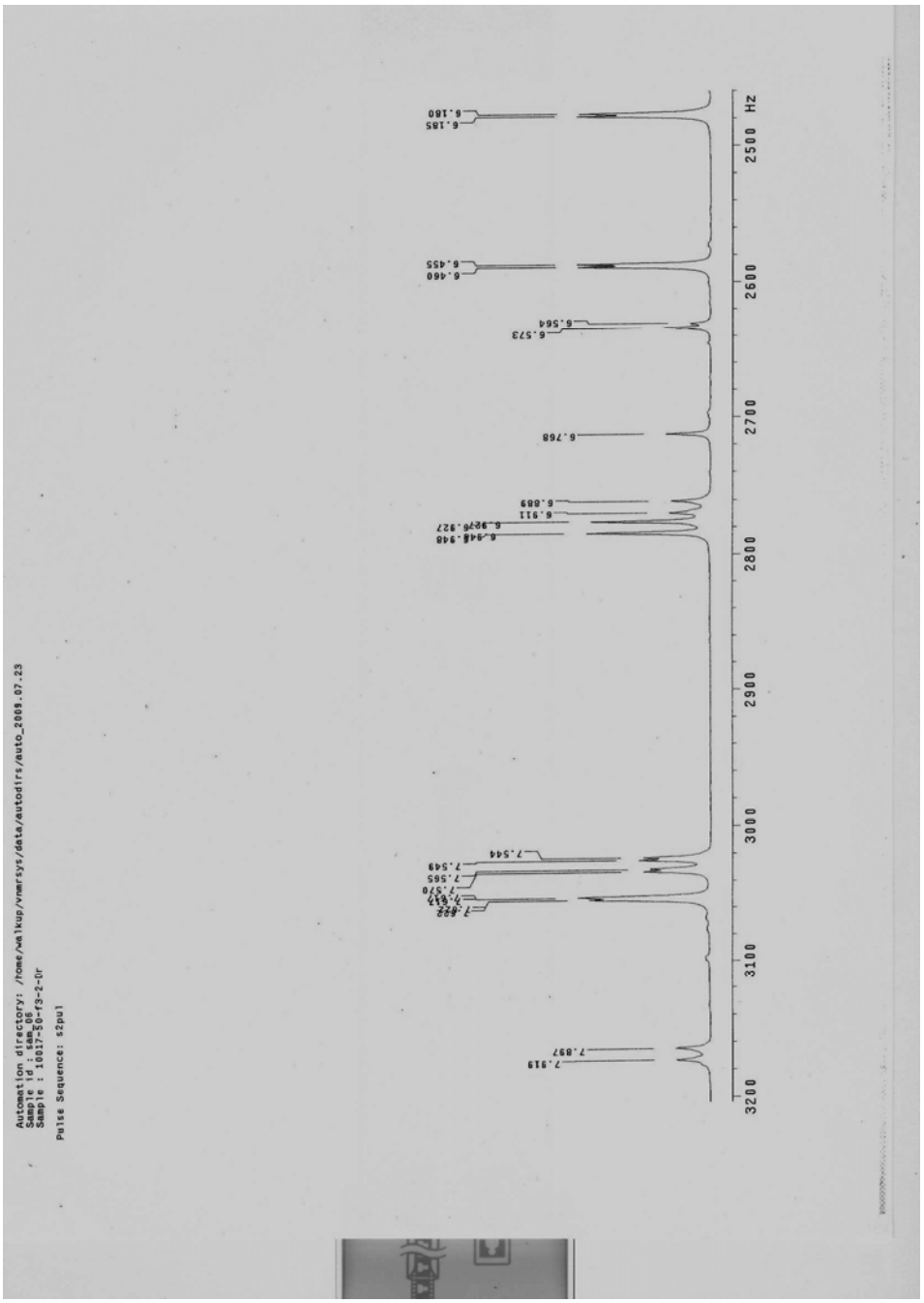


Fig. (3.3): ^{1}H NMR of major component of fraction 4 of *I. Viscosa*, Showing the coupling constants between major peaks:

Analysis of 3,3'-di-*O*-methylquercetin (61) by ^{13}C NMR showed the presence of 15 peaks (figure III.4), they are summarized in table II.3

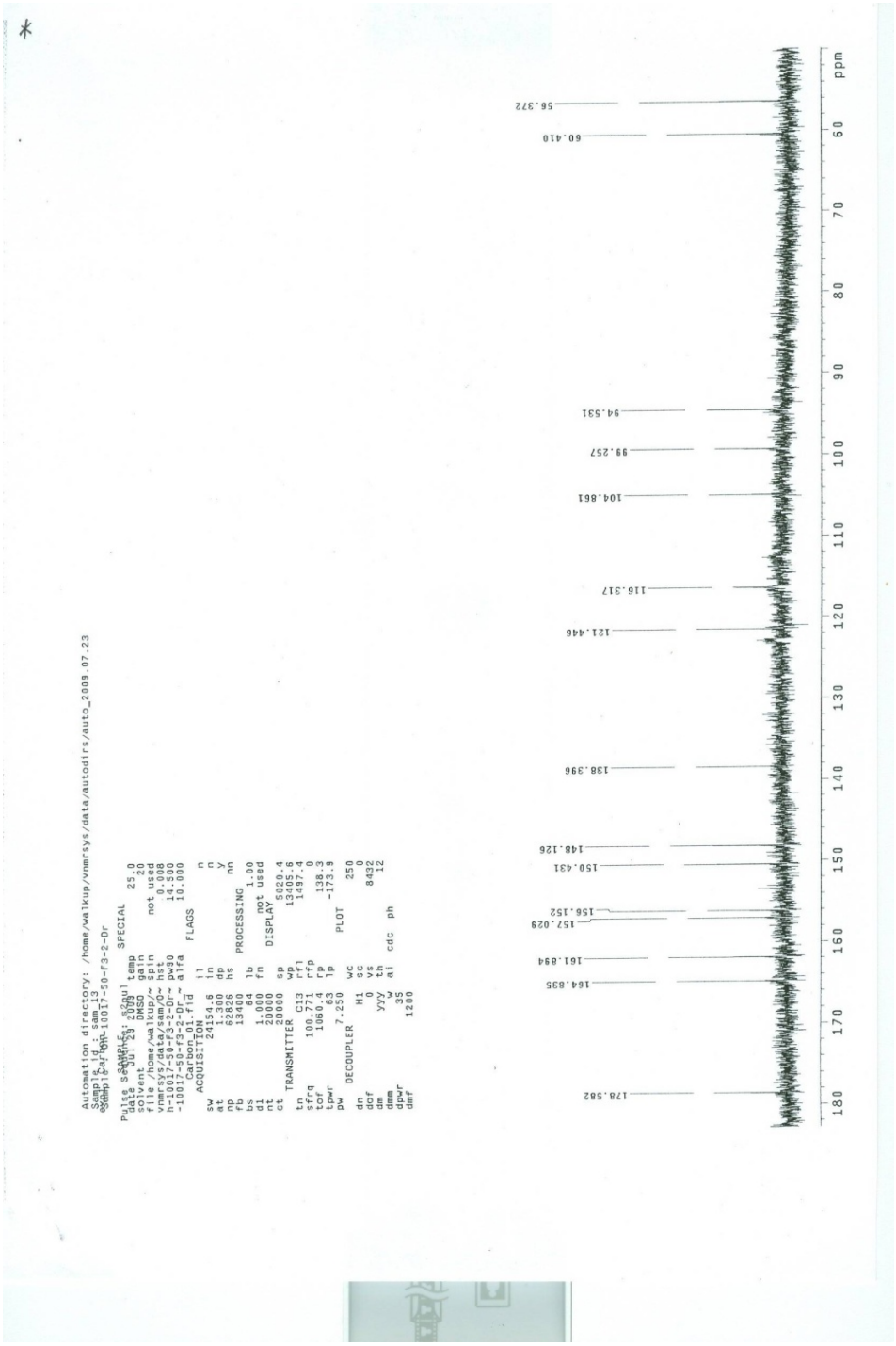


Fig. (3.4): ^{13}C NMR of major component of fraction 3 of *I. Viscosa*

Table (3.3): Summary of the ^{13}C chemical shifts of 3,3'-di-*O*-methylquercetin (61):

Chemical Shift δ (ppm)	
56.37	C-3' OMe
60.41	C-2 OMe
94.53	C-7
99.53	C-9
104.86	C-2'
116.32	C-5'
121.45	C-1'
138.40	C-4'
148.12	C-3'
150.13	C-2
156.16	C-1
157.03	C-8
161.89	C-4
164.84	C-6
178.58	C-3

3.2Antibacterial Activity

As mentioned earlier, four fractions were separated from I. Viscosa using flash chromatography technique. Fractions of components were evaluated for their antimicrobial activity against four types of bacteria: *S. aureus*, *E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.These bacterial strains were clinical isolates; all of the strains were isolated from patients suffering from bacterial infections with the relevant bacteria.

3.2.1 Screening Results

Fractions, separated from Inula Viscosa extracts, were screened for antimicrobial activity using the well known diffusion method reported in the literature by Perez et al[62], the efficiency of the drug was measured by

the zone of inhibition in millimeters of the bacteria cultured on the Mueller-Hinton agar plate. The results are summarized in Table **III.5**. As can be seen in Table **5**, all four fractions were inactive (zones of inhibition were zero), for *E. Coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. However, fraction four showed antimicrobial activities against *S. aureus*, others fractions 1, 2 and 3 showed no activities as shown in table 4, zone of inhibition for compounds in these fractions were 0 mm. The zone of inhibition for fraction four was 25 mm.

Table (3.4): Screening Results (Zone of Inhibition in mm):

Fraction (10 mg/ml)	***S.aureus***	***E.coli***	***P.mirabilis***	***P.aerginosa***
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	25	0	0	0

All fractions, except fraction four, were dropped for further evaluation because they showed zero activities against the four different types of bacteria studied.

3.2.2 Determination of Minimum Inhibitory Concentration (MIC)

The aim of this test is to determine the minimum concentration of bioactive material that inhibits the growth of bacteria. Results were compared to that of triclosan. Both fraction four and triclosan were tested on *clincal isolate S. aureus*.[63] MIC was determined by broth dilution method. Two solutions were used to obtain correct results; one is positive control solution (contains bacteria alone) where bacterial growth always

positive, another is sterility control solution (contains curcumin alone) where bacterial growth always negative.

The results obtained from this test are summarized in table III.5. As shown in Table **6**, MIC of fraction four was about 0.04 mg/mL, which is almost equal to that of tricolsan (0.025-0.1 mg/mL).[81]

Table (3.5): A Summary of MIC Results of Fraction 4

Conc.(mg/ml)	Fraction 4
10	-ve
5	-ve
2.5	-ve
1.25	-ve
0.625	-ve
0.3125	-ve
0.15625	-ve
0.078125	-ve
0.039063	-ve
0.019531	+ve
Positive Control	+ve
Sterility Control	-ve

3.2.3 Determination of Minimal Bactericidal Concentration (MBC)

The concentration of bioactive material that results in a total inhibition of bacterial growth is known as Minimal Bacterial Concentration (MBC). This method was applied on fraction four, the results are summarized in Table **6**.

Table (3.6): MBC and MIC results of fraction four against *S. aureus* bacteria:

Fraction 4	MIC(mg/ml)	MBC (Mg\ml)
4	**39**	**78**

As shown before, the major component of fraction four was identified by H^1 and ^{13}C NMR to be 3,3'-di-*O*-methylquercetin (61). As mentioned earlier in the experimental part, the antibacterial activity of fraction 4 could be attributed to 3,3'-di-*O*-methylquercetin (61). Since fraction 4 contains two components one on them (minor) is also present in fraction 3 as shown by LC/MS, however, fraction 3 showed no sign bioactivities which suggested that this minor component is not the active one, hence, no further evaluation was done to it.

References

1- Phillipson, J. D. Phytochemistry and Medicinal Plants. *Phytochemistry***,** 56, 237−243, **2001**.

2- Kong, J. M.; Goh, N. K.; Chia, L. S.; Chia, T. F. Recent advances in traditional plant drugs and orchids. *Acta Pharmacologica Sinica***,** 24, 7−21, **2003**.

3- Traditional medicine strategy launched. (WHO News). 80, 610, **2002**.

4- Benowitz, S. As war on cancer hits 25-year mark, scientists see progress, challenges. Scientist, 10, 1−7, **1996**.

5- Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70, 461−477, **2007**.

6- Newman, D. J.; Cragg, G. M.; Snader, K. M. Natural products as sources of new drugs over the period **1981-2002**. J. Nat. Prod. 66, 1022−1037, **2003**.

7- Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D.; Guo, Z., Global importance of medicinal plants. In *The Conservation of Medicinal Plants*, **1991**.

8- Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D.; Guo, Z., Global importance of medicinal plants. In *The Conservation of Medicinal Plants*, **1991**.

9- Kaplan, W. Codylamata Acuminata. New Orleans Med. Surg. J. 94, 388, **1942**.

10- Wilson, L. B., Jr.; Mizel, SB.; Grisham I. M.; Creswell, KM.,Session V- Networks and Shared Facilities- Rapporteurs Summary. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 33, 158, **1974**.

11- *Catharanthus Roseus*. http://www.nybg.org/bsci/belize/gallery.html as accessed on 22/07/10.

12- Noble, R. L. The discovery of the vinca alkaloids - chemotherapeutic agents against cancer. Biochem. Cell Biol. 68, 1344−1351, **1990**.

13- Ingram,J. http://www.daveingram.ca/knowingnature/C1534673850/E20070119212411/index.html as accessed on 22/07/10.

14- Kinghorn, A. D.; Seo, E. K. Plants as sources of drugs. Agricultural Materials as Renewable Resources, 647, 179−193, **1996**.

15- Oberlies, N. H.; Kroll, D. J. Camptothecin and taxol: Historic achievements in natural products research. J. Nat. Prod. 67, 129−135, **2004**.

16- Spande, T. F.; Garraffo, H. M.; Edwards, M. W.; Yeh, H. J. C.; Pannell, L.; Daly, J. W. Epibatidine- A novel (chloropyridyl) Azabicycloheptane with Potent Analgesic Activity from an Ecuadorian Poison Frog. J. Am. Chem. Soc. 114, 3475−3478, **1992**.

17- Blood Pressure. http://www.medicinenet.com/captopril/article.htm.

18- Chin, Y. W.; Balunas, M. J.; Chai, H. B.; Kinghorn, A. D. Drug discovery from natural sources. Aaps Journal, 8, E239−E253, **2006**.

19- Haefner, B. Drugs from the deep: marine natural products as drug candidates. Drug Discovery Today, 8, 536−544, **2003**.

20- Huang, G. S.; Lopez-Barcons, L.; Freeze, B. S.; Smith, A. B.; Goldberg, G. L.; Horwitz, S. B.; McDaid, H. M. Potentiation of taxol efficacy and by discodermolide in ovarian carcinoma xenograft-bearing mice. Clin. Can. Res. 12, 298−304, **2006**.

21- Davis J. Inactivation of antibiotic and the dissemination of resistance genes. Science, 264, 375- 382, **1914**.

22- Robin EH, Anril W, Alexander M, Loeto M, Keith K (**1998**) Nasopharyngeal carriage and antimicrobial resistance in isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae* Type b in children under 5 years of age in Botswana. International Journal of Infectious Diseases, 3(1), 18-25, **1998**.

23- Harborne J. B. and baxter H.(eds) , Phytochemical Dictionary, Taylor and Francis, London **(1993)**.

24- Picman A. K. Biochem. Syst. Ecol. 12, 13 (1984).

25- Franich, R. A. , Gadgil. P. D. and Shahin, L. physiol Plant Path. 23, 183 (**1983**).

26- Marston A. , Gafner F. , Dossagi S. F. and Hostettmann K. , Phytochemistry 27, 1325 (**1988**).

27- Ohtani K. , Mavi S. and Hostettmann K. , Phystochemistry 33,83 (**1993**).

28- Nagata T., Tsushida T., Hamaya E., Enoki N., Manabe S. and Nishno C., Agric. Biol. Chem. 49, 1181 (**1985**).

29- Renee J. G. and Jeffry B. H. Phystochemistry, Vol. 37, #1, pp 19-42 (**1994**).

30- Takechi M. and Tanaka Y. Phytochemistry 29. 451 (**1990**).

31- Rhoades, David F (1979). "**Evolution of Plant Chemical Defense against Herbivores**". In Rosenthal, Gerald A., and Janzen, Daniel H. *Herbivores: Their Interaction with Secondary Plant Metabolites*. New York: Academic Press. p. 41. ISBN 0-12-597180-X.

32- Aniszewski, p.182.

33- Renee J. G. and Jeffry B. H. Phystochemistry, Vol. 37, #1, pp 19-42 (**1994**).

34- Ingham J. L., Tahra S. and Harborne J. B. Z. Naturforsch 38c, 194 (**1983**).

35- Tahra S. , Ingham J. L. , Nakahara S. , Mizutani J. and Hrborne J. B. Phytochemistry 23, 1889 (**1984**).

36- Geibel M. , Geiger H. and Treutter D. Phytochemistry 29, 1351 (**1991**).

37- Mizobouchi S. and Sato Y. , Agric. Biol. Chem. 48, 2771 (**1884**).

38- Pottert O. , Stoecki-Evans H. , Msonthi J. D. and Hostermann K. , Helv. Chim. Acta 70, 155 (**1987**).

39- Henry H. B. , Gary D. C. and James E. O. Instrumental Analysis (**1978**).

40- Snyder L. R. and Kirkland J. J. Modern Liquid Chromatography New York: Wily-Interscience (**1974**).

41- Palevitch Pros. D. , Yaniv Z., Medicinal Plants of the Hollyland.

42- Ali-Shtayeh M. S. , Medicinal Plants of the West Bank, Department of Biological Sciences, An-Najah Univ., Nablus, Unpublished work.

43- Brinkman, K.A. (**1974**) "*Juglans* L. - Walnut", in: Schopmeyer, C.S. (ed.), ***Seeds of woody plants in the United States***, Agriculture Handbook **450**, Washington, D.C.: U.S. Department of Agriculture, Forest Service, p. 454–459, (rev. ed.: 1992), ISBN 0-931146-21-6.

44- "*Plumbago* L.". *Germplasm Resources Information Network*. United States Department of Agriculture. 2002-01-02. http://www.ars-grin.gov/cgi-bin/npgs/html/genus.pl?9608. Retrieved **2010-01-29**.

45- Tzakou O, Pitarokili D, Chinou IB. Composition and antimicrobial activity of the essential oils of *Salvia ringens*. *Planta Med* 67: 81-83, **2001**.

46- Tepe B, Daferera D, Sokmen A et al. Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). Food Chem 90: 333-340, **2005**.

47- Dobrynin VN, Kolosov MN, Chernov BK et al. Antimicrobial substances of *Salvia officinalis*. Khim Prir Soedin 5: 686-686, **1976**.

48- Albayrak S, Aksoy M, Hamzaoglu E. Determination of antimicrobial and antioxidant activities of Turkish endemic *Salvia halophila* Hedge, Turk J Biol 32: 265-270, **2008**.

49- Ogutcu H, Sokmen A, Sokmen M et al. Bioactivities of the various extracts and essential oils of *Salvia limbata* C.A.Mey. and Salvia sclarea L., Turk J Biol 32: 181-192, **2008**.

50- Kurkcuoglu M, Baser KHC, Duman H. Composition of essential oils from two varieties of *Salvia aucheri* Bentham growing in Turkey. J Essent Oil Res, 14: 241-242, **2002**.

51- Honda G, Koezuka Y, Tabata M. Isolation of an antidermatophytic substance from the root of *Salvia miltirrhiza*. Chem Pharm Bull 36: 408-411, **1988**.

52- Soliman KM, Badeea RI. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food Chem Toxicol 40: 1669-1675, **2002**.

53- Daferera DJ, Ziogas BN, Polission MG. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. J Agric Food Chem 48: 2576-2581, **2000**.

54- Pitarokili D, Tzakou O, Loukis A et al. Volatile metabolites from *Salvia fruticosa* as antifungal agents in soilborne pathogens. J Agric Food Chem 51: 3294-3301, **2003**.

55- Viuda M, Ruiz-Navajas Y, Fernandez-Lopez J et al. Chemical composition and antifungal activity of the essential oils of *Salvia* (*Salvia officinalis* L.) and rosemary (*Rosemarinus officinalis* L.). Alimentaria 4: 101-105, **2006**.

56- Gao YG, Song YM, Yang YY et al. Pharmacology of tanshinone. Yao Hsueh Pao 14: 75-82, **1979**.

57- Chen XG, Li Y, Yan CH et al. Cancer chemopreventive activities of S-3-1, a synthetic derivative of danshione. J Asian Nat Prod Res 3: 63-75, **2001**.

58- Rota C, Carraminana JJ, Burillo J et al. In vitro antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. J Food Prot 67: 1252-1256, **2004**.

59- Lima CF, Andrade PB, Seabra RM et al. The drinking of a *Salvia officinalis* infusion improves liver antioxidant status in mice and rats. J Ethnopharmacol 2: 383-389, **2005**.

60- Yu, B.-W.; Chen, J.-Y.; Wang, Y.-P.; Cheng, K.-F.; Li, X.-Y.; Qin, G.-W.; *Phytochemistry*, *61*, 439, **2002**.

61- Zygmunt, B.; Namiesnik, J.; *J. Chromatogr. Sci*. *41*, 109, **2003**.

62- Perez C, Pauli M, Bazevque P. An Antibiotic Assay by the Agar Well Diffusion Method. Acta Biologiae et Medicine Experimentalis. **15:113-115, 1990.**

63- NCCLS-National Committee for Clinical Laboratory Standards: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobiacally. Approved Standards M7-A5, Wayne, PA, USA, **2000**.

64- A. Shtacher, G.; Kashman, Y. *J. Med. Chem.* **1970**, *13*, 1221-1223. b. Azoulay, P.; Reynier, J. P.; Balansard, G.; Gasquet, M.; Timon-avid, P. *Pinharm. Acta Helv. 61*, 345-352, **1986**.

65- Maoz, M., and Neeman, I. Antimicrobial effects of aqueous plant extracts on the fungi *Microsporum canis* and *Trichophyton rubrum* and on three bacterial species. Lett. Appl. Microbiol. 26:61-63, **1998**.

66- Qasem, J. R., Al-Abed, A. S., and Abu-Blan, M. A. activity of clammy inula (*Inula viscosa*) on *Helminthrosporium sativum* and *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathol. Mediterr. 34:7-14, **1995**.

67- Cohen, Y., Baider, A., Ben-Daniel, B. H., and Ben-Daniel, Y. Fungicidal preparations from *Inula viscosa*. Plant Prot. Sci. 38:629-630, **2002**.

68- Wang, W. Q., Ben-Daniel, B. H., and Cohen, Y. Extracts of *Inula viscosa* control downy mildew caused by *Plasmopara viticola* in grapevines. (Abstr.) Phytoparasitica 32:208, **2004**.

69- A. Alkofahi and A. H. Atta, "Pharmacological screening of the anti-ulcerogenic effects of some Jordanian medicinal plants in rats," *Journal of Ethnopharmacology*, vol. 67, no. 3, pp. 341–345, **1999**.

70- M. Maoz and I. Neeman, "Effect of *Inula viscosa* extracton chitin synthesis in dermatophytes and *Candida albicans*,"*Journal of Ethnopharmacology*, vol. 71, no. 3, pp. 479–482, **2000**.

71- N. M. Al-Dissi, A. S. Salhab, and H. A. Al-Hajj, "Effects of *Inula viscosa* leaf extracts on abortion and implantation in rats," *Journal of Ethnopharmacology*, vol. 77, no. 1, pp. 117–121, **2001**.

72- G. R. Schinella, H. A. Tournier, J. M. Prieto, P. Mordujovich, and J. L. Rios, "Antioxidant activity of anti-inflammatory plant extracts," *Life Sciences*, vol. 70, no. 9, pp. 1023–1033, **2002**.

73- A. Ulubelen, S. O¨ ksu¨z, and N. Go¨ren, "Sesquiterpene acids from *Inula viscosa*," *Phytochemistry*, vol. 26, no. 4, pp. 1223– 1224, **1987**.

74- E. Wollenweber, K. Mayer, and J. N. Roitman, "Exudate flavonoids of *Inula viscosa*," *Phytochemistry*, vol. 30, no. 7, pp. 2445–2446, **1991**.

75- B. Marongiu, A. Piras, F. Pani, S. Porcedda, and M. Ballero, "Extraction, separation and isolation of essential oils from natural matrices by supercritical CO2," *Flavour and Fragrance Journal*, vol. 18, no. 6, pp. 505–509, **2003**.

76- L. Lauro and C. Rolih, "Observations and research on an extract of *Inula viscosa*," *Bollettino della Societa Italiana di Biologia Sperimentale*, vol. 66, no. 9, pp. 829–834, **1990**.

77- http://en.wikipedia.org/wiki/sesquiterpene.

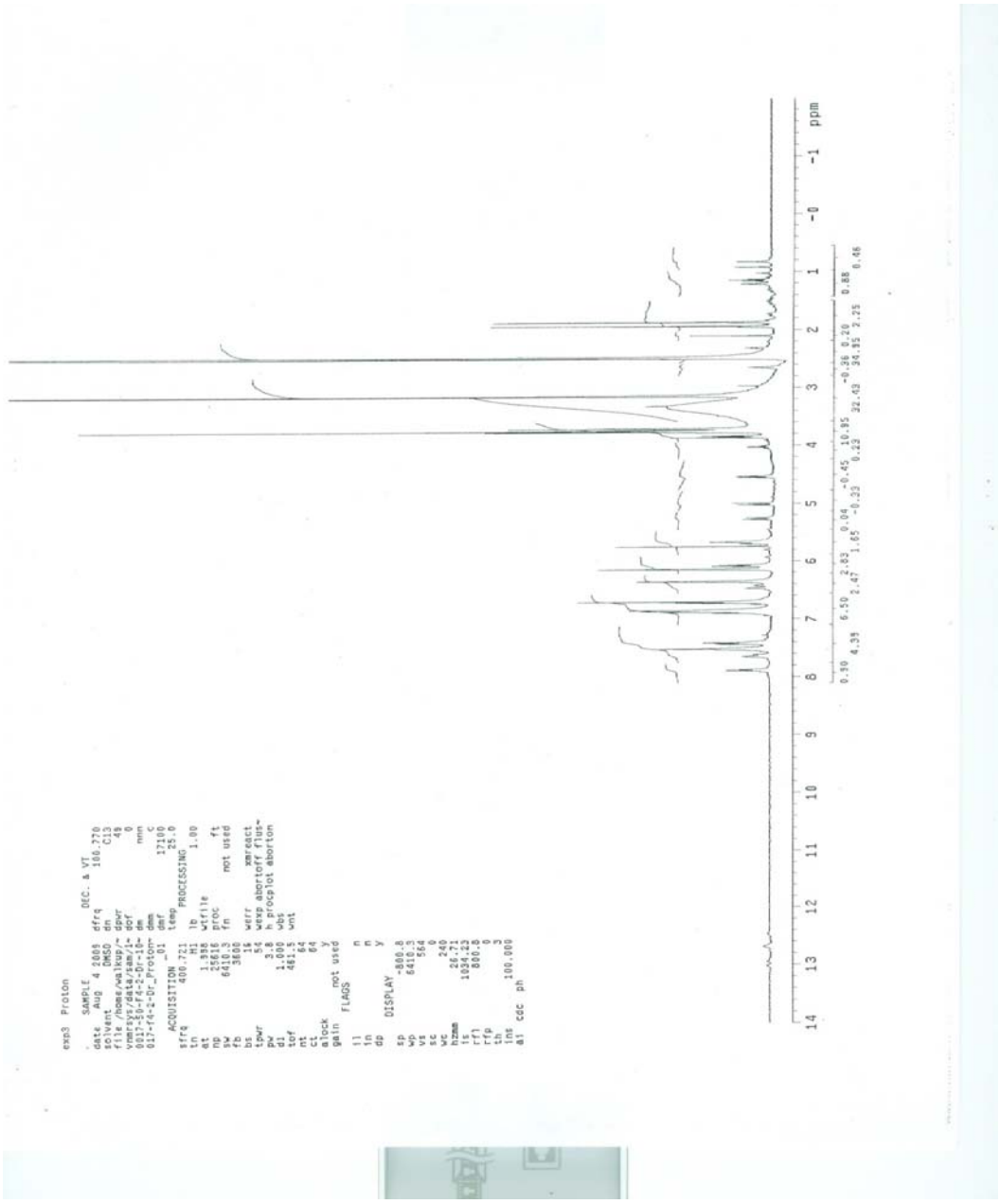
78- *March's* ***Advanced Organic Chemisty****: Reactions, Mechanisms, and Structure* Michael B. Smith, Jerry March Wiley-Interscience, 5th edition, **2001**, ISBN 0-471-58589-0.

79- Chiappini, I.; Fardella, G.; Menghini, A.; Rossi, C. *Planta Med. 44*, 159-161, **1982**.

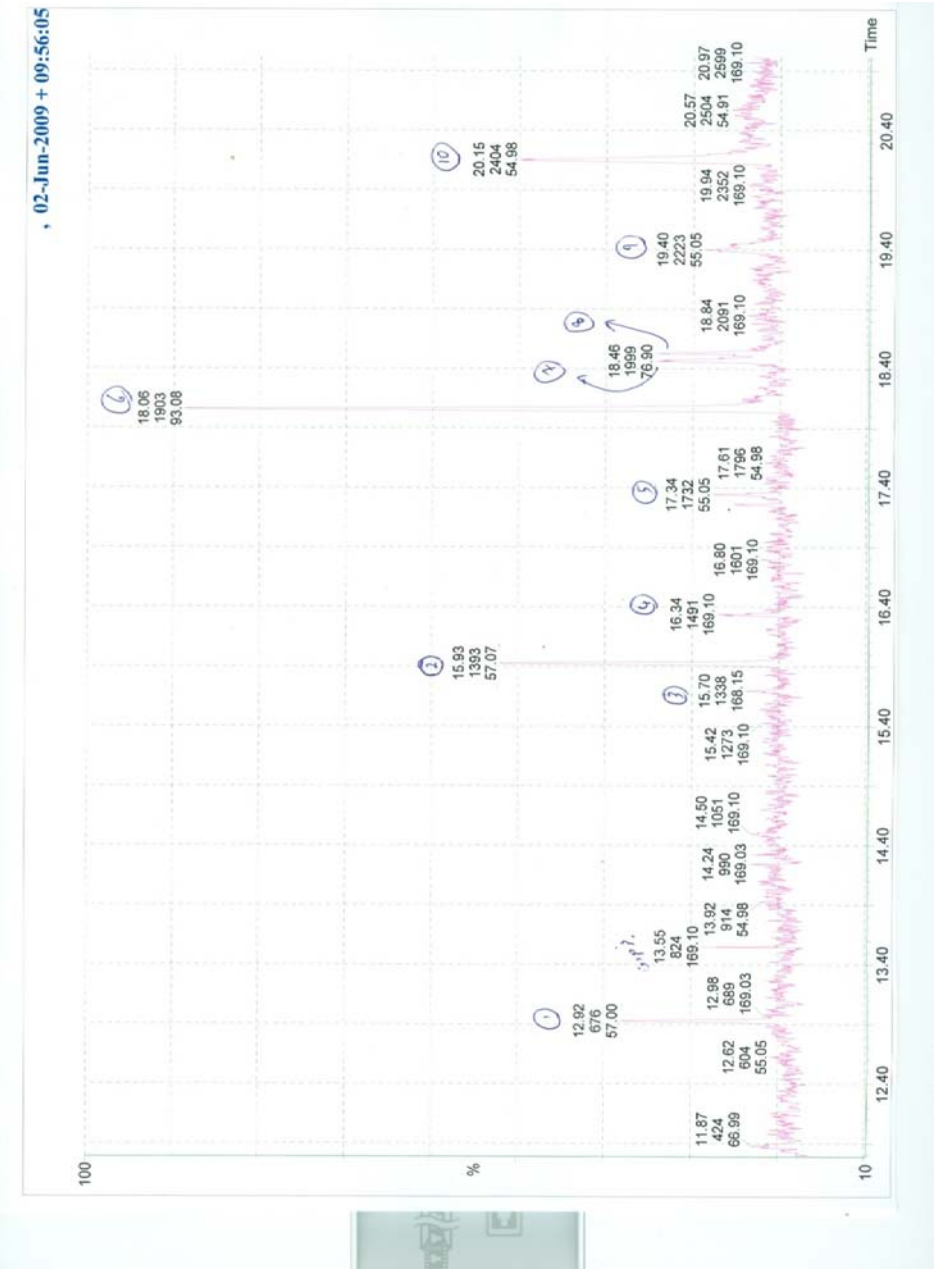
80- Grande, M.; Piera, F.; Guenca, A.; Torres, P.; Bellido, I. S. *Planta Med. 51*, 414- 419, **1985**.

81- Suller, M. T. E.; Russell, A. D. " Triclosan and antibiotic resistance in *Staphylococcus aureus*" Journal of Antimicrobial Chemotherapy 46, (1), Pp. 11-18, **1999.**

Appendix



(Fig a1): ^{1}H NMR of fraction 3 of *I. Viscosa*, showing the presence of a mixture of two components



(Fig a2): GC/MS of crude of *I. Viscosa*

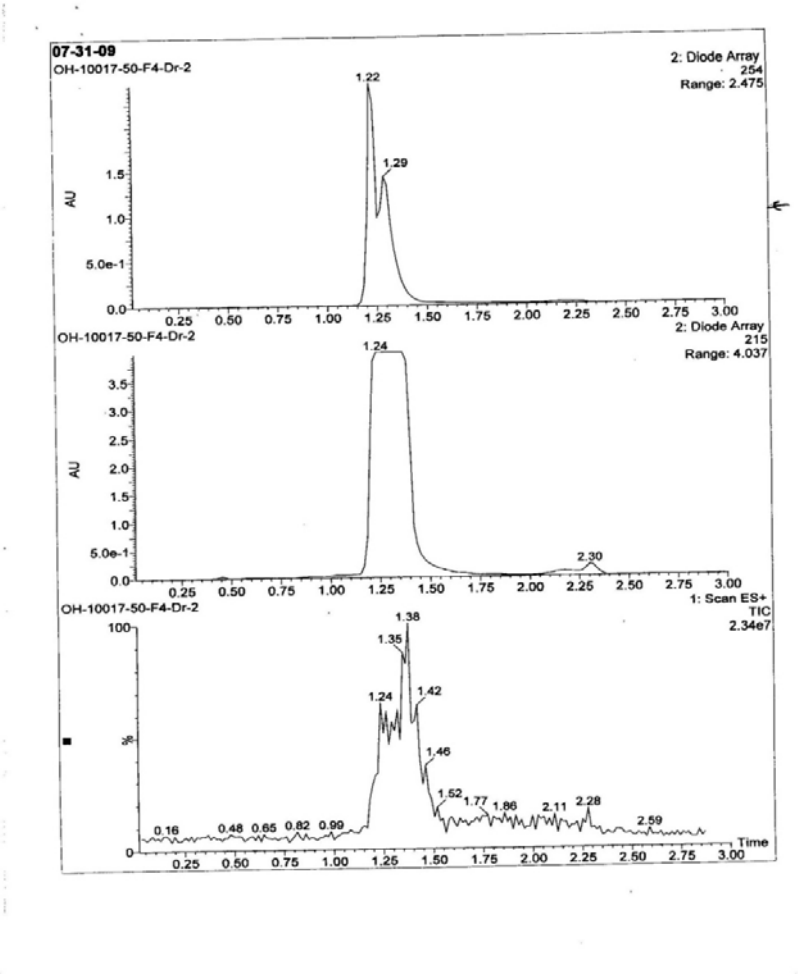


Figure a3: LC/MS of fraction 4 of *I. Viscosa*, showing the presence of a mixture of two components

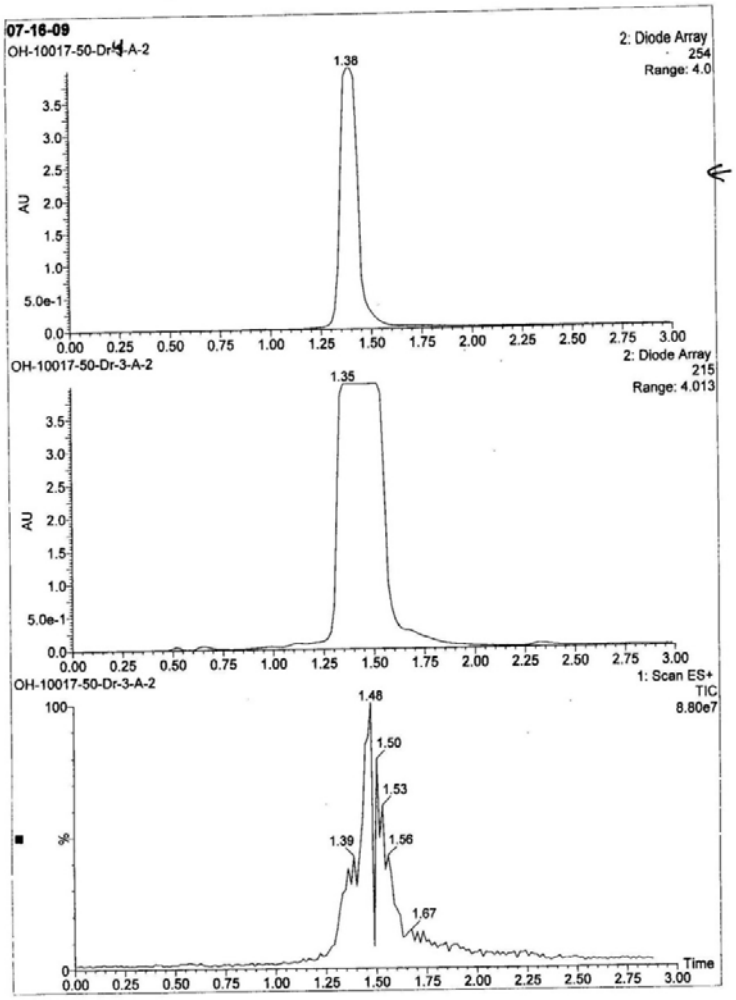


Fig: a4: LC/MS of component 1 of fraction 4 after purification

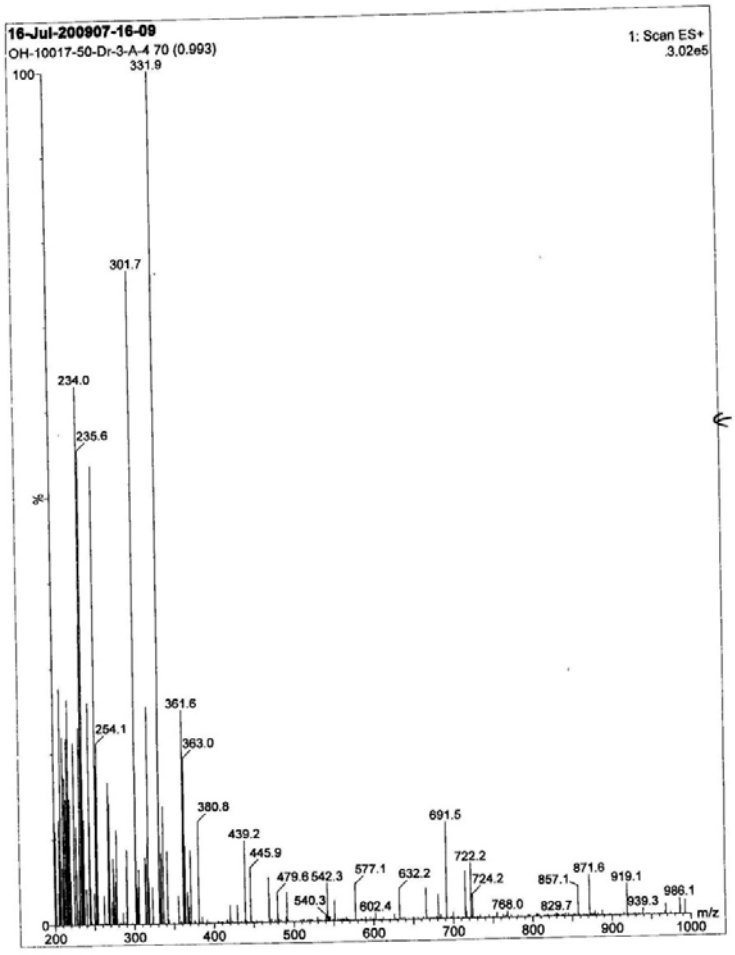


Fig: a5: MS of component 1 of fraction 4 after purification

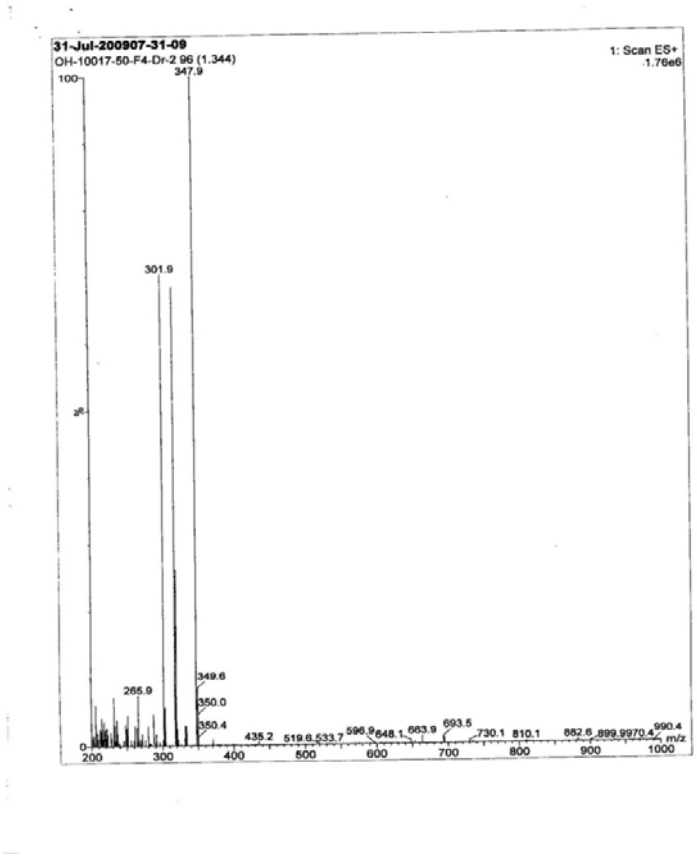


Fig: a6: MS of component 2 of fraction 4 after purification

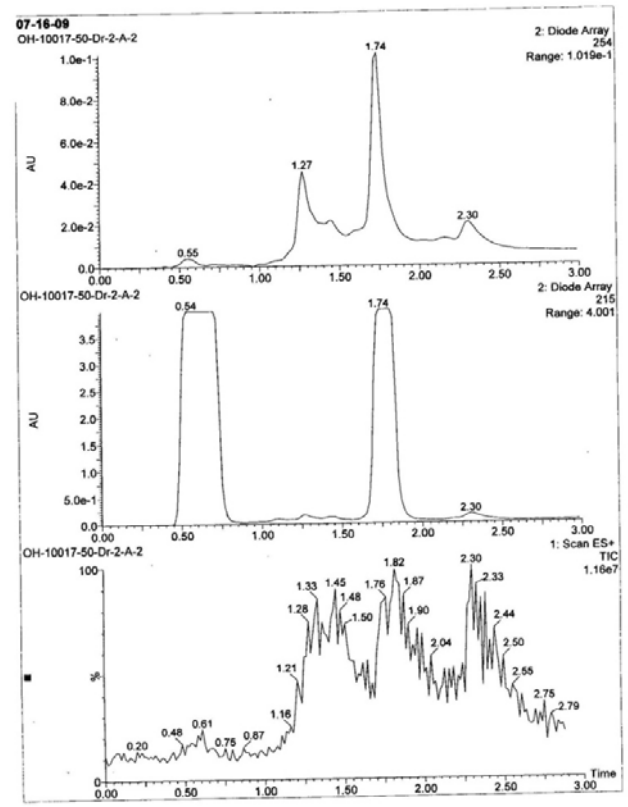


Fig: a7: LC/MS of fraction 2 of *I. Viscosa*, showing the presence of a mixture of five components

جامعة النجاح الوطنية
كلية الدراسات العليا

دراسة عزل وتشخيص مواد نشطة طبياً من أصول نباتات طبية

إعداد
ضرار عيسى "محمد حسن" صمادي

إشراف
د. عثمان حامد

قدمت هذه الأطروحة استكمالا لمتطلبات درجة الماجستير في الكيمياء بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

2011م

دراسة عزل وتشخيص مواد نشطة طبياً من أصول نباتات طبية

إعداد

ضرار عيسى "محمد حسن" صمادي

إشراف

د. عثمان حامد

الملخص

يستخدم أكثر من 600 نوع من النباتات في الطب الشعبي الفلسطيني, ويستخدم نحو خمسين نوع من هذه النباتات لعلاج الإمراض الجلدية المختلفة. وعليه جاء هذا العمل استمرارا لإيجاد أدوية جديدة تستخرج من نباتات تنمو في فلسطين. وقد تم اختيار نبات الطيون هنا لهذا الغرض، حيث اختير هذا النبات لما يحمله من أهمية في الطب الشعبي الفلسطينيني، اذ ان له عددا غير محدود من التطبيقات الطبية.

في هذه الدراسة تم اتباع ثلاث مراحل أساسية في فصل وتحديد محتويات الطيون. ففي المرحلة الأولى تم نقع الطيون في اسيتات الايثيل (ethyl acetate) ، وفي المرحلة الثانية تم فصل محتويات الطيون بطريقة flash chromatography الى اربعة اجزاء. أما في المرحلة الثالثة فقد تم تخصيص الجزء الرابع من النبات في عملية الفصل ودراسته كمضاد لنوع من أنواع البكتيريا هي (S.aureus) .

وبسبب تفرد الجزء الرابع بالتاثيرات الطبية المذكورة، فقد تم فصله مرة أخرى من أجل الحصول على نقاوة مرتفعة حيث أعطى مركبين، فرعيا بنسبة ضئيلة ورئيسا بنسبة عالية. وتم تشخيص المركب الرئيس حيث كان تركيبه هو (5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-methoxy-4*H*-chromen-4-one) ويظهر شكل المركب كما هو مبين أدناه: