An-Najah National University
Faculty of Graduate Studies

Modification of Biologically Active Compounds from Selected Medicinal Plants in Palestine

By
Ahmad Ibrahim Ahmad Husein

Supervisors

Prof. Mohammed Saleem Ali-Shtayeh

Dr. Nidal Zater **Dr. Waheed Jondi**

This thesis is Submitted in Partial Fulfillment of the Requirements for the Degree of Ph.D. of Science in Chemistry, Faculty of Graduate Studies, An-Najah National University, Nablus, Palestine.

2010

Modification of Biologically Active Compounds from Selected Medicinal Plants in Palestine

By
Ahmad Ibrahim Ahmad Husein

This Thesis was defended successfully on 28 / 9 /2010 and approved by:

Defense Committee Members		**Signature**
- Prof. Dr. Mohammed S. Ali-Shtayeh	**(Supervisor)**	MShtayeh
- Dr. Nidal Zatar	**(Co-supervisor)**	Nidal Zatar
- Dr. Waheed Jondi	**(Co-supervisor)**	
- Dr. Imad Odeh	**(External Examiner)**	
- Dr. Mohammed Al-Nuri	**(Internal Examiner)**	Mohammed Al-Nuri

Dedicated

This thesis is especially dedicated to all people who are interested in science.

Acknowledgements

This study was carried out at the Department of Chemistry and Department of Biology and Biotechnology, at An-Najah National University, Nablus, Palestine. I wish to express my deepest gratitude to my principal supervisor, Prof. Dr. Mohammed S. Ali-Shtayeh, for his encouragement to start and complete this work and for the opportunity to be a member of the inspiring research group. His endless support and constructive criticism has been precious during these years. I am greatly indebted to my other supervisors, Dr. Nidal Zatar and Dr. Waheed Jondi for their continuous support and valuable advice during my Ph.D. studies. I thank them for their professional experience, advice and patience during these years. Contributions of them all were vital to the success of the study. I owe my thanks to Dr. Shukri Khalaf for providing the facilities for my work in the department and for his support. I wish to thank Prof. Dr. Hikmat Hillal for his support and advice during my studies as a Ph.D. student. My thanks go to Dr. Mohamed Faraj and Mr. Shady Al-Refaay in the Department of Microbiology at Bir-Zeit University for providing the facilities to carry out anticancer tests in their laboratories. Special thanks to Dr. Malik Al-Zehluf in Jordan University for his assistance in carrying out anticancer tests.

I am grateful to all technicians in the chemistry laboratories for technical assistance in carrying out part or this study. My colleagues and friends in the Departments of Chemistry and Biology deserve warm thanks, for making my

work easier during these years, for giving hand in solving problems, and for providing a pleasant working atmosphere. Special thanks for Ahmad Othman for doing statistical analysis. My warm thanks go to my colleague Dr. Ahed Zyoud for inspiring discussions and sharing good moments during this program. Special thanks are also due to my colleagues in Tulkarm Directorate of Education for their patience and interest during the time of study. My brother, my uncle Musleh deserve special thanks for their friendship, advice and continuous encouragement during my academic study. Finally, my dearest thanks are addressed to my family, my wife for her love and endless support, and our wonderful and active sons for being the sunshine of my life.

إقـــــرار

أنا الموقع/ـــة أدناه ، مقدم/ ـــة الرسالة التي تحمل العنوان :

زيادة فعالية وتعديل على مركبات لها فعالية حيوية من نباتات مختارة في فلسطين.

اقر بأن ما اشتملت عليه هذه الرسالة انما هي نتاج جهدي الخاص، باستثناء ما تم الاشارة اليه حيثما ورد، وإن هذه الرسالة ككل، او اي جزء منها لم يقدم من قبل لنيل اية درجة او لقب علمي أو بحثي لدى أية مؤسسة تعليمية او بحثية اخرى.

Declaration

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

اسم الطالب: أحمد إبراهيم أحمد حسين Student's Name: Ahmad I. A. Husein

التوقيع: Signature :

التاريخ: Date :

Contents

List of Tables

List of Figures

List of Schemes

Modification of Biologically Active Compounds from Selected Medicinal Plants in Palestine

By
Ahmad Ibrahim Ahmad Husein

Supervisors

Prof. Mohammed Saleem Ali-Shtayeh

Dr. Nidal Zater **Dr. Waheed Jondi**

Abstract

Six plants used in traditional medicine in Palestine were tested for their biological activity. They were collected from Nablus region. These plants include four families namely Araceae (*Arum palaestinum),* Urticaceae *(Urtica pilulifera),* Labiatae *(Coridothymus capitatus, Majorana syriaca)* and Lamiaceae *(Teucrium creticum, Teucrium polium)*. The ethanolic extracts of those plants were tested for their antioxidant activity through DPPH assay and β-Carotene-linoleic acid assay. The concentrations of Phenolic compounds and Flavonoids were determined as μg/mg equivalent of quercetin and pyrocatechol. The extracts of the plants were tested also for their antifungal activity against four pathogenic fungi: *Trichophyton tonsurans, Microsporum canis, Trichophyton rubrum* and *Trichophyton violaceum,* compared with the activity of econazole. The extracts were also tested for their anticancer activity against prostate PC3 human carcinoma. All of the extracts showed different potential biological activities in all tests. Phytochemical screening indicated the presence of Flavonoids and phenolics in all extracts. *T. creticum* and *C. capitatus* were the most efficient antioxidants in DPPH assay, while *U. pilulifera* and *C. capitatus*

were the most efficient as antioxidants in β-Carotene-linoleic acid assay. *C. capitatus* and *M. syriaca* were the active against fungi between the six plants. *U. pilulifera* and *A. palaestinum* showed the highest potent antiproliferative activity. Some constituents were detected from plants extracts using GC-MS spectrophotometer and separated by flash chromatography. These constituents were tested for their biological activity. Many reactions were done to modify the activity of these constituents. Four phenolic acid esters were synthesized and tested for their biological activity in the same methodology used in testing the extracts. All of the synthesized compounds showed significant biological activity in all tests.

Chapter One
General Introduction

1.1 Traditional medicine

Thousands of years ago herbs and plant products were used in folk medicine in treating a wide spectrum of ailments and diseases. Folk remedies are prepared as powders, poultices, ointments, baths, decoctions, infusions and teas. The interest in studying the biological effects of traditional medicinal plants or isolating their active components for treatment of illness has been increasing all over the world and comprehensive screening programs have been established [1]. Floristic analysis showed that there are about 500,000 plant species on our planet. Out of these about 120,000 plant species can be used to create biologically active products, which are used in disease treatment [2]. Today a great number of different medicinal teas and other plant products are available on market including cosmetics and pharmaceuticals, which contain biologically active substances [3].

Recently some products of plant origin were shown to be effective sources of chemotherapeutic agents without undesirable side effects and with strong biological activity. This attracted the attention of many scientists and encouraged them to screen plants to study the biological activity of their constituents from chemical and pharmacological investigations to therapeutic aspects [4]. New systematic methods for separation, identification and determination of chemical constituents are applied, in addition to different biological activity tests carried out on plant extracts and their chemical constituents. Most of the new phytochemical studies follow similar methodologies such as plant collection, plant extraction, biological tests, chemical analysis and statistical analysis [5].

1.2 Modern Phytochemistry

Natural products have been a source of drugs and drug leads. It is estimated that 61 percent of the 877 small molecule new chemical entities introduced as drugs worldwide during 1981–2002 can be traced back to or were developed from natural products [6]. In some therapeutic areas, the contribution of natural products is even greater, e.g. about 78 percent of antibacterial and 74 percent of anticancer drug candidates are natural products or structural analogues of natural products. In 2000, approximately 60 percent of all drugs in clinical trials for the multiplicity of cancers were of natural origin. Modern drug discovery approaches applying full automation and robotics, hundreds of molecules can be screened using several assays within a short time, and with very small amounts of compounds. A number of associated techniques are used for identification and analysis of chemical constituents e.g. LC-PDA (liquid chromatography–photo-diode-array detector), LC-MS (liquid chromatography–mass spectrometry detector) and LC-NMR (liquid chromatography– nuclear magnetic resonance spectroscopy). While in the recent past it was extremely difficult, time consuming and labor intensive to build such a library from purified natural products, with the advent of newer and improved technologies related to separation, isolation and identification of natural products the situation has improved remarkably. Natural products libraries have been established to preserve crude extracts, chromatographic fractions or semi-purified compounds. However, the best result can be obtained from a fully identified pure natural product library as it provides scientists with the opportunity to handle the ‘lead’

rapidly for further developmental work, e.g. total or partial synthesis, dealing with formulation factors, in vivo assays and clinical trials [6].

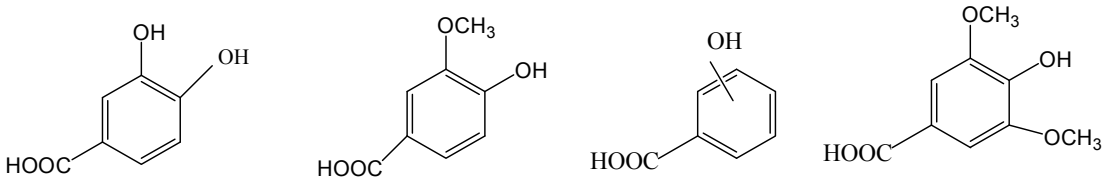
1.3 Main chemical constituents in medicinal plants

Plants have an almost limitless ability to synthesize chemical substances mainly secondary metabolites, of which at least 12000 have been isolated, a number estimated less than 10% of the total [7]. Chemical analysis of plant extracts showed the existence of many chemical compounds related to different classes such as alkaloids, quinines, sugar alcohols, terpenes, polyphenols, flavonoids, phenolics and many others. Flavonoids and phenolics and their derivatives are found in most plants. Such compounds are very important for both humans and plants [7]. They act as cell wall support materials [8] and as colorful attractants for birds and insects helping seed dispersal and pollination [9]. Flavonoids and phenolic acids also have antioxidative [10] and anticarcinogenic effects [11].

1.3.1 Phenolic compounds: Phenolic acids are large and heterogeneous group of biologically active non-nutrients. They are present in plants as hydroxylated derivatives of benzoic and cinnamic acids [12, 13]. Phenolic compounds are important in the defense mechanisms of plants under different environmental stress conditions such as wounding, infection, and excessive light or UV irradiation [14]. Phenolics are not only unsavory or poisonous, but also of possible pharmacological value [15]. The main two groups of phenolic compounds are:

1. Hydroxybenzoic acids: They have a general structure derived directly from benzoic acid and variations in the structures of individual hydroxybenzoic acids lie in the hydroxylations and methylations of the aromatic ring [16]. Four acids

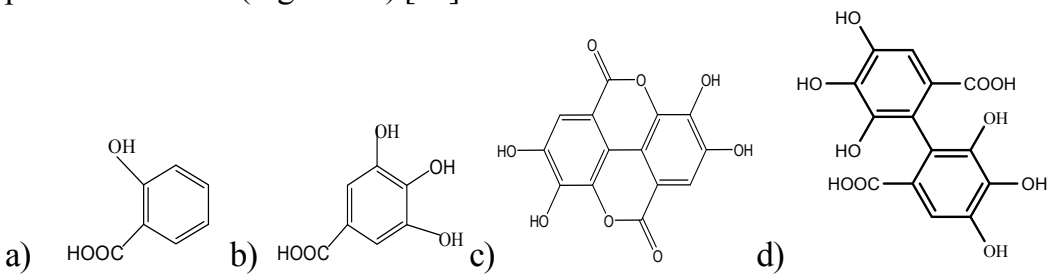
occur commonly: protocatechuic acid, vanillic acid, hydroxybenzoic acid and syringic acid, (Figure 1.1).



Protocatechuic acid Vanillic acid Hydroxy benzoic acid Syringic acid

(**Figure: 1.1) Chemical structures for common hydroxybenzoic acids.**

Other common hydroxybenzoic acids are also salicylic acid (2-hydroxybenzoic acid), and gallic acid (trihydroxyl derivative) which participate in the formation of hydrolysable gallotannins [17]. Its dimeric condensation product hexahydroxydiphenic acid related to dilactone, and ellagic acid are common plant metabolites (Figure 1.2) [18].

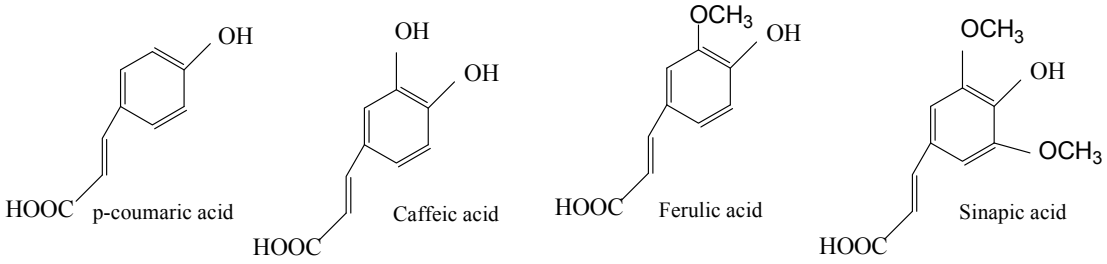


(Figure 1.2) Chemical structures of (a) salicylic acid, (b) gallic acid, (c) ellagic acid, (d) hexahydroxydiphenic acid

They may be present in soluble form or conjugated with sugars or organic acids as well as bound to cell wall fractions such as lignin [19].

2. Hydroxycinnamic acids: The four most widely distributed hydroxycinnamic acids in many plants are p-coumaric acid, caffeic acid, ferulic acid and sinapic

acids shown in (Figure 1.3) [16].



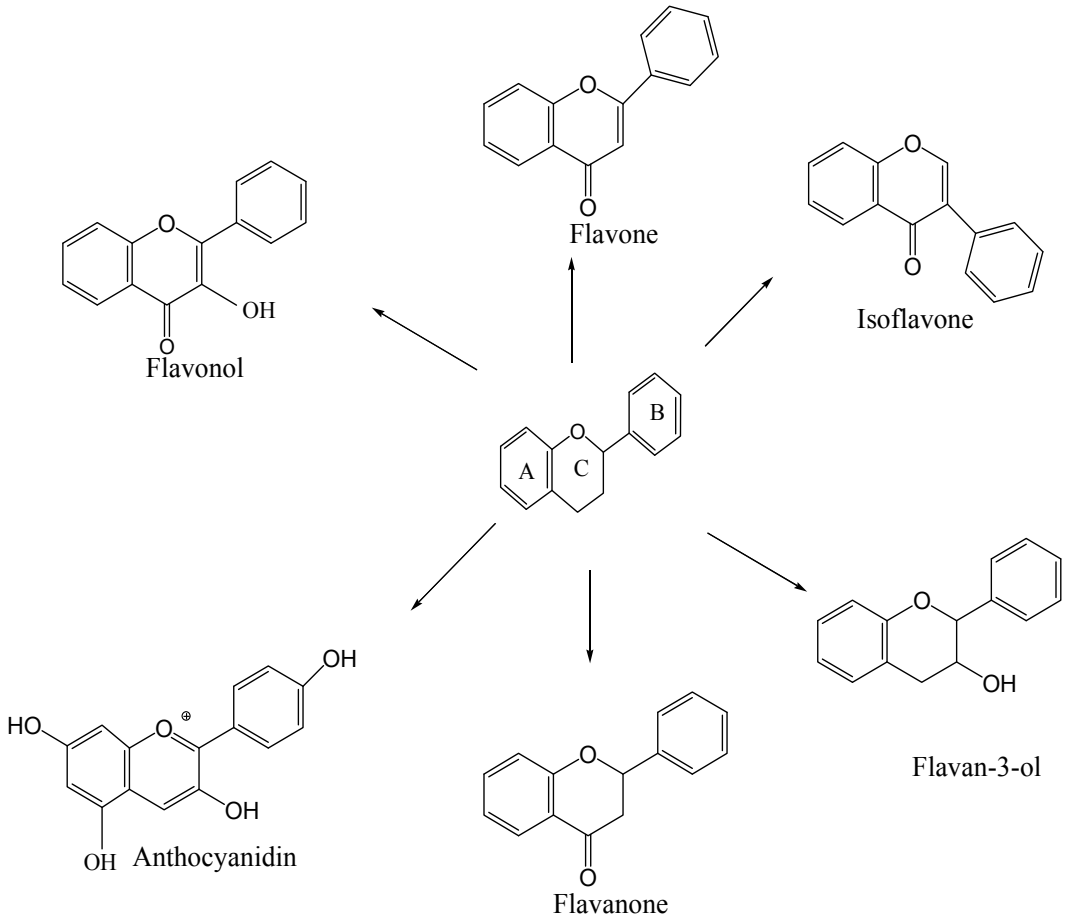
(**Figure: 1.3) Chemical structures of hydroxycinnamic acids**

Hydroxycinnamic acids usually occur in various conjugated forms, the free forms being artifacts from chemical or enzymatic hydrolysis during tissue extraction in the cis-forms. The conjugated forms are esters of hydroxy acids or sugar derivatives [20].

1.3.2 Flavonoids: Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings linked together with a pyrone ring in the case of flavones or a dihydropyrone ring in the case of flavanones [21] as shown in (Figure 1.4) and subgroups (Figure 1.5).

(Figure: 1.4) Chemical structures of (a) flavone skeleton, and (b) flavanone

Flavonoids are normal constituents of the human diet and are responsible for a variety of biological activities. Some of these act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties [22]. However, the molecular mechanisms explaining how flavonoids suppress the inflammatory response are not known in detail [23].



(Figure: 1.5) Chemical structures of the main flavonoid subgroups.

A few studies have shown that certain flavonoids down-regulate NO_2 mediators of inflammation production in response to inflammatory stimuli [24, 25] but no more precise mechanisms of action are known yet.

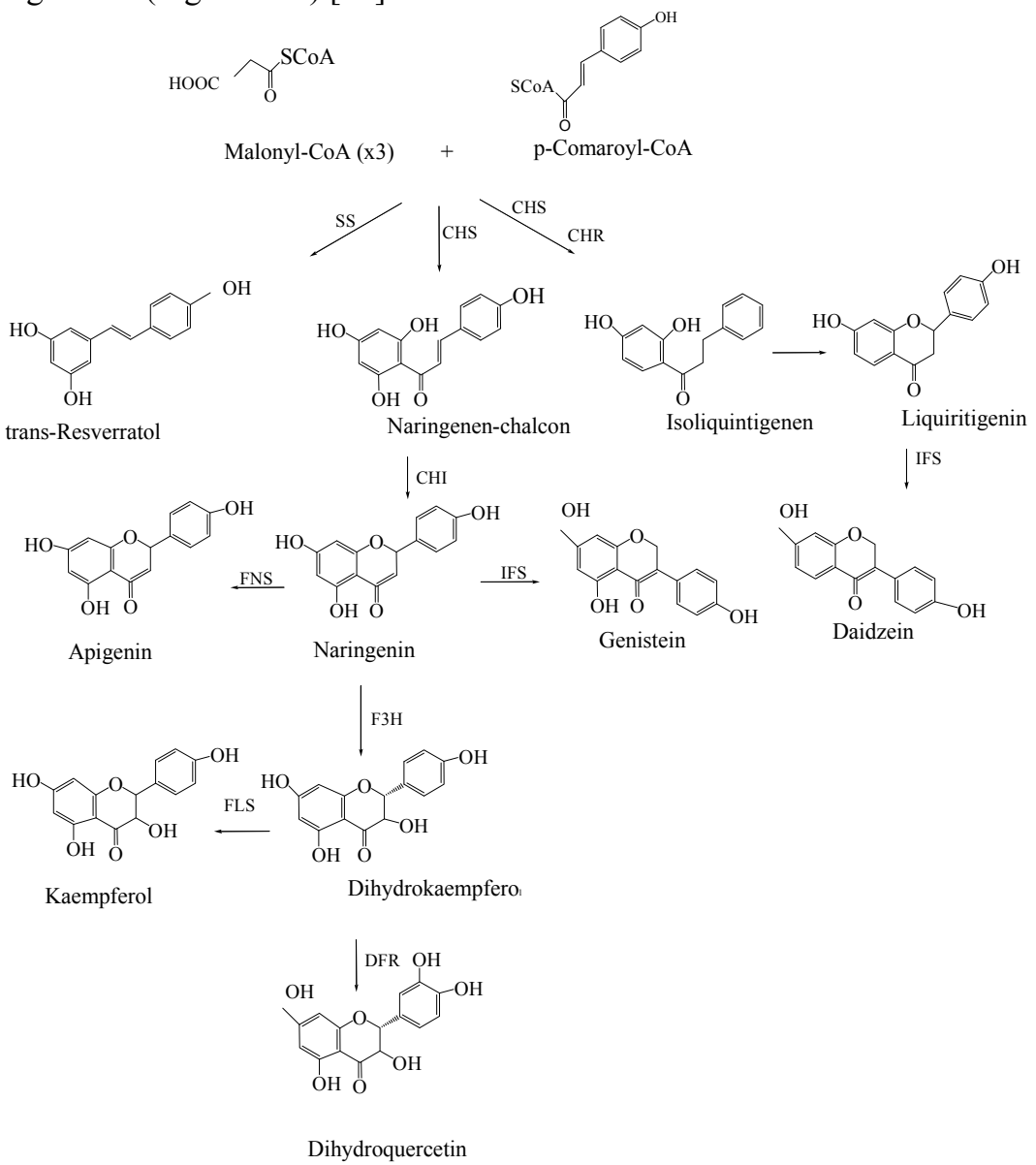
Flavonoids have long been recognized to possess antiallergenic, anti-inflammatory, antiviral, antiproliferative and antioxidative activities [26]. Epidemiological studies have shown inverse relationships between the intake of flavonoids specially flavonols and flavones and the risk of coronary heart disease [27], stroke, [28] lung cancer [29], and stomach cancer [30]. Other epidemiological studies have, on the other hand, found no association between

the intake of flavonoids and the risk of heart disease [31] or cancer [32]. Although the role of flavonoids and phenolic acids in the maintenance of health and prevention of diseases seems positive, the evidence is still limited and conflicting. Moreover, the bioavailability of flavonoids and phenolic acids from various foods, and the extent and mechanism of absorption in the human body are poorly known.

1.3.3 Overview of flavonoid and phenolic biosynthesis pathways

The biosynthesis of flavonoids, hydroxycinnamates, and phenolic acids involves a complex network of routes based principally on the Shikimate, phenylpropanoid, and flavonoid pathways. These biosynthetic pathways constitute a complex biological regulatory network that has evolved in vascular plants during their successful transition on land and that eventually is essential for their growth, development, and survival [33]. From the 1970s to the 1990s, there was a rapid and substantial progress in the research on the phenylpropanoid pathway, focusing mainly on a broad understanding of the metabolic pathway [34]. However, in more recent years, new information is also emerging regarding the regulation of the phenylpropanoid pathway. In the last few years, a great deal has been learned from studies in a variety of plant species, primarily about transcriptional regulation. A number of these studies were carried out using flavonoid mutants generated by activation tagging [35]. Characterization of flavonoid mutants in a variety of plant species has led to the identification of a number of novel regulatory proteins that are beginning to fill in the

void between signals that induce the pathway and well-known flavonoid regulators (Figures 1.6) [36].

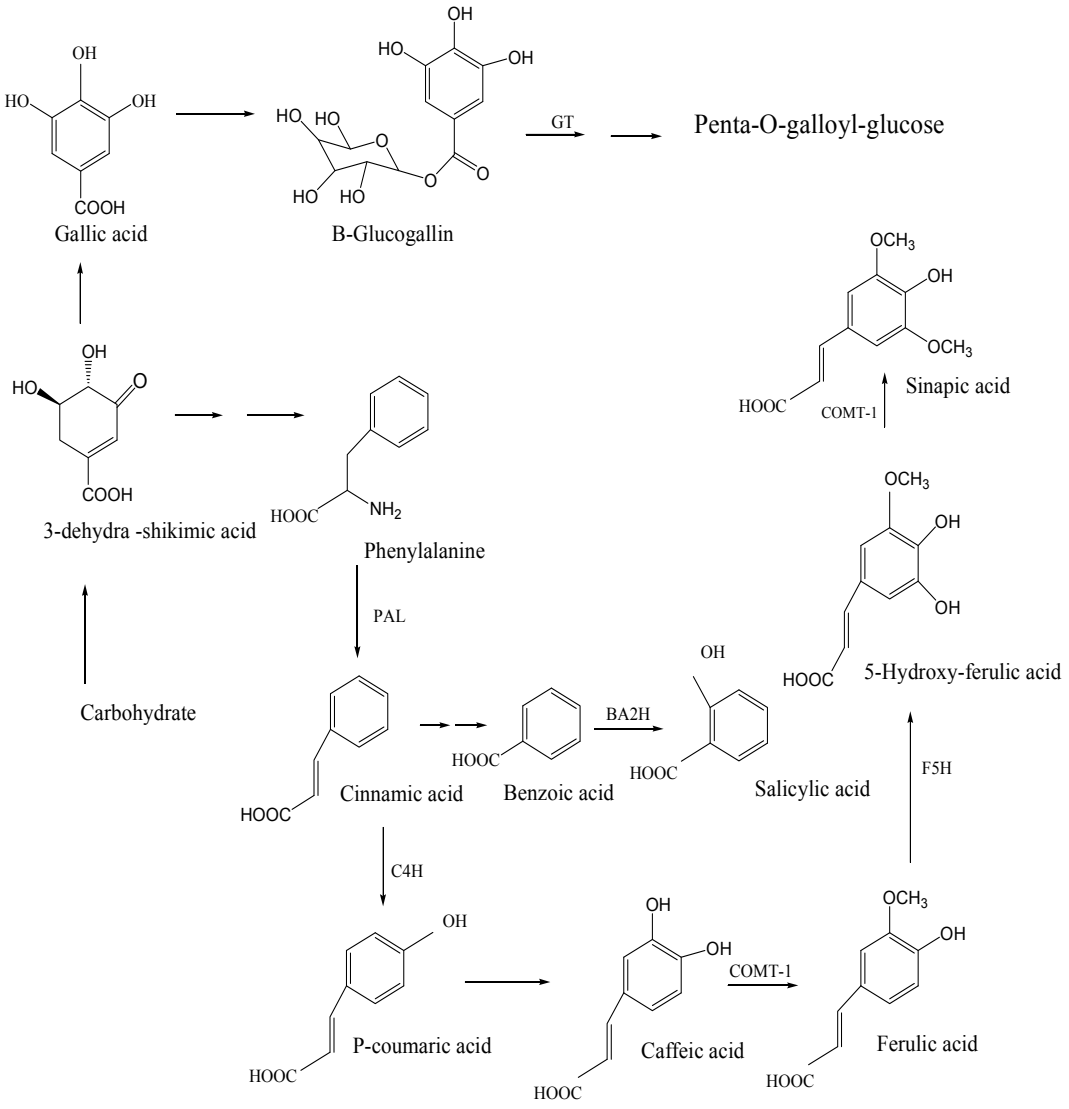


(Figure: 1.6) Schematic diagram of the stilbene and flavonoid biosynthetic pathway.

Enzyme abbreviations: SS, stilbene synthase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; FNS, flavone synthase; F3H,flavanone 3-hydroxylase; FLS, flavonol synthase; F3uH, flavonoid 3u-hydroxylase; DFR,dihydroflavonol 4-reductase; LAR, leucoanthocyanidin 4-reductase; LDOX, leucocyanidindeoxygenase; ANR, anthocyanidin reductase; EU, extension units; TU, terminal unit.

In addition, increasing evidence is being generated demonstrating that as well as inducing the phenylpropanoid pathway, these transcriptional regulators also influence the modification, transport, and deposition of metabolites in the vacuole [37]. In addition to the molecular techniques, technical advances both in chromatographic techniques and in identification tools, particularly the diverse forms of mass spectrometry, has allowed successful challenges to the separation and characterization of compounds of increasing complexity, poor stability, and low abundance [38]. Information generated utilizing these techniques has resulted in characterization of excess of complex secondary metabolites that, in conjunction with the characterization of the enzymatic steps, has permitted the complete or partial clarification of the flavonoid and the phenolic pathways present in many plants (Figure 1.7).

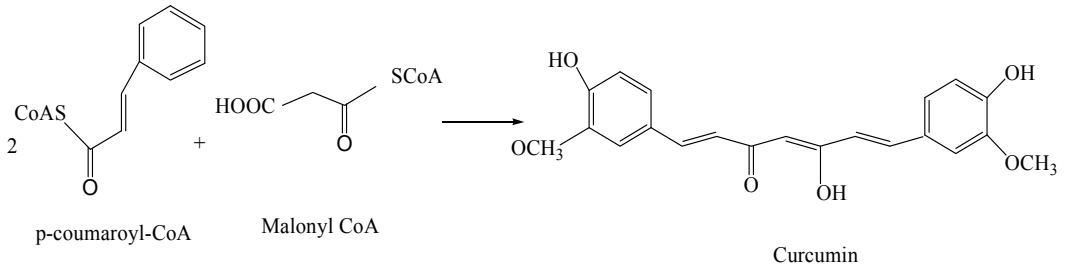
Nonetheless, the complete analysis of phenolic metabolic pathway is far from being complete. For example, recent reports underline those important questions still remain to be answered in the field of protoanthocyanidins and tannins [39], and that the exact nature of the biosynthetic pathway(s) leading to lignin monomers has not been fully elucidated [40]. An example of the phenolic pathway, which produces secondary metabolites that have health benefiting effects, is the biosynthesis of curcuminoids. The initial investigations into the biosynthesis of curcuminoids were carried over 25 years ago [41], although little has been done subsequently to elucidate fully the routes involved.



(Figure: 1.7) Schematic diagram of phenolic biosynthesis pathway accompined by the key enzymes involved. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid 2-hydroxylase; C4H, cinnamate 4-hydroxylase; COMT-1, caffeic/5-hydroxyferulic acid O-methyltransferase; 4CL, p-coumarate:CoA ligase; F5H, ferulate 5-hydroxylase; GT, galloyltransferase

The curcuminoids are thought to be formed from condensation of two molecules of p-coumaroyl-CoA with one molecule of malonyl-CoA via the action of possibly a polyketide synthase. The resulting product would then be transformed through demethoxycurcumin into curcumin via two

sequential rounds of hydroxylation followed by O-methylation (Figure 1.8).



(Figure: 1.8) Schematic diagram of curcumin formation

Alternatively, it is possible that the curcuminoid synthase enzyme may utilize the CoA esters of both p-coumaric acid and ferulic acid as substrates [42].

1.4 Methods of isolation and quantitative determination

1.4.1 Extraction

Extraction is the second step in the criteria of studying medicinal plants after plant collection. There are many methods used in extracting plant materials. Many factors may affect the extract such as, pH for the extracting medium, stability of the constituents, and hence biological activity of the chemical constituents of the plants. Type of solvents used in those methods may be organic or aqueous depending on the study needs. The following are the most popular methods used for extraction [43].

a. Decoction: It is one of the traditional extraction techniques where the extract is prepared by placing the plant in cold water, bringing it to boil for fifteen minutes, then the extract is decanted or filtered when required.

b. Infusion: It is carried out by pouring boiling water or organic solvent on a specific quantity of plant martial and allowing the mixture to stand for 10-15 minutes or more, then the extract is dried and kept for further studies.

c. Soaking: The plant martial is soaked in organic or aqueous solvent for 72 hours; the extract is decanted and dried, kept for further studies.

d. Soxhlet extraction: The plant martial is grounded and soaked in a solvent for 72 hours and then refluxed for 24 hours using soxhlet extractor.

e. Steam distillation: It is an ingenious method for isolation of slightly volatile, water insoluble compounds from plants. The plant material is grounded and boiled in water through distillation. The distillate is collected and separated by a separatory funnel.

1.4.2 Chromatographic types and techniques

Chromatography was discovered and named in 1906 by Michael Tswett, a Russian botanist while he was attempting to separate colored leaf pigments. Among all types of separation methods it has the unique position of being applicable to all types of problems in all areas of science. It is used for the separation of a mixture that contains a large number of organic compounds. Chromatography is a physical method that helps to avoid any reaction that may change the structure of the original compounds in the plant [44].

1- Liquid Chromatography (LC): In this method the mobile phase is a liquid whereas in gas Chromatography (GC) it is a gas. In Gas-liquid Chromatography (GLC) the stationary phase is a liquid spread over the surface of a solid support.

2- Thin layer Chromatography (TLC): In this method retention of solute, whether by partition or adsorption are described by their migration relative to that of the eluting agent. This linear flow in one direction, R_f is defined as

$$R_f = \frac{\text{distance travelled by solute}}{\text{distance travelled by mobile phase}}$$

Paper chromatographic methods were developed for flavonoids in the 1950s and 1960s [45]. These techniques were replaced by thin – layer chromatography (TLC) in the 1970s providing an inexpensive and useful technique for simultaneous analyses of several samples [46].

3- Column Chromatography processes: It is a useful technique for separation of large amounts of samples. The mobile phase may be liquid or gas. The separation may be due to adsorption that depends on the interactions between the solute and the adsorbent surface and the solvent, or may be due to partition, that depends on solute distribution between the mobile phase and stationary phase. A continuous passage of the solvent aids the setting of the packing particles. The active constituents are determined using spectrophotometric methods.

4- High-performance liquid chromatography (HPLC): It is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase. The differences in interaction with the column can help to separate different sample components from each other. High-performance liquid chromatography

(HPLC) has been the most widely employed chromatographic technique in flavonoid analysis during the past 20 years [47, 48]. It has been added a new dimension to the investigation of flavonoids in plant and food extracts. Particular advantages are the improved resolution of flavonoid mixtures compared to other chromatographic techniques, the ability to obtain both qualitative and accurate quantitative data in one operation, and the great speed of analysis [49, 50].

5- Gas Chromatography: The concept of GC was announced in 1941 by Martin and Synge. Chromatography is one of the most important and efficient methods of separation and identification of constituents of plants extracts. In this method the separation of constituents depend on the variety of their polarities through two phases, mobile phase and stationary phase. This is the role in all types of chromatography such as thin layer chromatography, column chromatography, flash chromatography and gas chromatography. Gas chromatography (GC) has only a limited applicability in the analysis of flavonoids and other phenolics due to their limited volatility, the main disadvantage is an extra step required to ensure the volatility of phenolics [36]. However, GC analysis with mass spectrometric (MS) detection has been applied for the analysis of flavonols in black tea [51] and cabbage [52]. Advantages of GC analysis include an improved separation of closely related isomers and simple coupling to MS detectors for identification through the fragmentation pattern [53, 54].

1. 5 Importance of medicinal plants

Many people believe that medicinal plants are more natural and more accessible than manufactured drugs [55] .In addition to nutritional components, medicinal plants were used in treating a wide spectrum of ailments and diseases, and they have been screened for their potential uses as alternative remedies and the preservation of foods from toxic effects of oxidants [56]. A large number of plants have been found to contain ingredients that have antibacterial, antifungal, and anticancer activities. Other plants are used in traditional medicine due to their antioxidant properties [57, 58]. Recently some products of higher plant origin have been shown to be effective sources of chemotherapeutic agents without undesirable side effects and strong biological activity. Natural-product-like compounds are an attempt to enhance the productivity of synthetic chemical constituents used in preparing cosmetics and pharmaceuticals, which contain biologically active substances.

1. 6 Medicinal plants in Palestine

In Palestine, the screening of flora for pharmacological active compounds started in the late sixties [59]. The abundance of more than 2900 species condensed on a very small geographical area is a major advantage of studying the Palestinian flora. This richness is due to the diversity of the soil and climatic conditions [60]. Six of these plants used in Arab Tradition Palestinian Medicine were used in this study: *Arum palaestinu*m (Araceae), *Urtica pilulifera* (Urticaceae), *Coridothymus capitatus* (Labiatae), *Majorana syriaca* (Labiatae), *Teucrium creticum* (Lamiaceae) and *Teucrium polium,* (Lamiaceae) [43].

1- ***Arum palaestinum***: It is one of about 26 species of the arum genus which are flowering plants belonging to Araceae.This family includes many species distributed in different regions. Many species are used as traditional remedies or food. In Palestine the aerial parts of *A. Palaestinum* are considered edible after being soaked in salty water or drying. It is also used as animal fodder. The plant is also used in folk medicine to cure several chronic diseases such as stomach acidity, atherosclerosis, cancer, and diabetes and food toxicity [60]. Previous studies on the characteristics of secondary metabolites of the Araceae family showed that it has a simple profile of polyphenols and alkaloids with flavone c-glycosides, flavanols, flavones, proanthocyanidins and polyhydroxy alkaloids as main classes [61]. Another study was attempted to analyze the ethyl acetate fraction of the plant and led to the isolation and identification of a new polyhydroxy alkaloid compound $C_4H_5NO_4$ [62]. Few phytochemical and biological investigations have been reported on this plant [63]. One of them reported the inhibitory effect of an *A. Palaestinum* extract on the muscle contraction of rat and guinea-pig uteri [64]. And also, the isolation and structural elucidation of a novel pyrrole alkaloid, was investigated and showed anticancer activity [62]. Moreover, the ethyl acetate fraction of this plant was examined and found to possess antioxidant activity via evaluation of its scavenging of DPPH free radicals, in addition to its anti-cancer activity against hepatocarcinoma (Hep G2) ,breast carcinoma cells (MCF-7) and lymphoplastic leukemia (1301) using the 3-[4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) cytotoxicity assay [62].

2- *Urtica pilulifera*: It is commonly known in Roman as Nettle and in Palestine as (Qurraus), has been long used in many countries around the world as a traditional medicine for curing sore joints by mixing the plant juice with oil. The contents of the stinging hair provide a cure for rheumatism, hemorrhage. Decoction of the summits of the plant is diuretic, depurative. Seeds are used for renal stones and inflammation of the bladder, diuretic and aphrodisiac. It is used in the treatment of various diseases including Diabetes Mellitus and other ailments [65, 66]. Also the effect of methanol extract of the plant was studied on diabetes include male rat's reproductive structures and functions, such as sperm motility and count and testosterone secretion [67]. The oil of nettle showed a potent and completely inhibitory effect on the radial growth of a fungus called *A. alternaria* at 1500 ppm. [68].

3- *Coridothymus capitatus*: It is an aromatic plant found in Palestine and locally known under the common name (Zaa'tman). The essential oils of the plant were reported to have antimicrobial activities most of which are mediated by thymol and carvacrol [69]. Various species of thymus have been credited with a long list of pharmacological properties. The plant is also important as a source of perfume, cosmetics, flavoring and pharmaceutical industries [70]. In traditional food the plant has been used for its flavors as refreshing drink or in cooking. It's used in folk medicine against cold, influenza and throat infection [61], later on the plant was found to contain antiseptic and antimicrobial agents [71].

4- *Majorana syriaca*: It is one of the most popular herbs among Palestinian people The green leaves of the herb are rich in essential oil, which is responsible for its characteristics of flavor and fragrance. Oil of cultivated thyme is an

important commercial product and is obtained mainly by steam distillation of the fresh leaves. Plant extract is also found to have strong biological activity, and this may due to the presence of phenols, thymol and carvacrol as major constituents of thyme oil in the plant [72].

5- ***Teucrium creticum*** is found in Palestine and locally known under the common name Ja'adh. The plant is used traditionally to cure from diabetes in Nablus region. No phytochemical studies were found on this plant, and this may be attributed to its limited distribution in Palestine [61].

6- ***Teucrium polium*** is found in Palestine and locally known under the common name Ja'adhat al-sibian. The plant is well known for its diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive, anorexic, analgesic [73, 74], antibacterial [75] and antidiabetic effects [76]. Hot infusion of tender parts of plant is taken for stomach and intestinal troubles, plant used in a steam bath for colds and fevers, useful against smallpox and itch, stimulant, depurative, for feminine sterility, colds and tonic, astringent, vulnerary. Recently, it has been reported that the extract of T. polium reduces NADPH-initiated lipid peroxidation in rat liver microsomes in vitro [77]. The aqueous extract possesses a hypoglycaemic effect. Flowers and leaves of the plant possess conduolonic effect. It also stimulates the neuromotor centers for uterine and intestinal musculature. The plant shows antimicrobial activity against Bacillus subtilis and Staphylococcus aureus [78]. Plants belonging to the genus Teucrium have been shown to contain different classes of compounds such as fatty acid esters [79], terpenes [80], flavonoids and polyphenolics [81, 82].

1.7 Aims of study

The aims of our study were to:

1- Study the biological activity for six plants used in traditional medicine in Palestine: *Arum palaestinum*, *Urtica pilulifera*, *Coridothymus capitatus*, *Majorana syriaca*, *Teucrium creticum* and *Teucrium polium.*

   a- Antioxidant activity

   b- Antimicrobial activity

   c- Anticancer activity

2- Separation, identification and determination of active constituents from those plants.

3- Modify some of the separated constituents to improve their biological activity.

1.8 References

1- Azzam M. S. (1984). **Phytochemical investigation of certain plants used in Egyptian folk medicine as antidiabetic drugs**. Ph.D. thesis, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

2- Tivy J. (1995). **Biogeography, a study of plants in the ecosphere**. Essex, England: Longman House Burnt Mill, Halow, 12.

3- Nahrstedt A. (2000). ***Active constituents of traditional European medical plants-new developments. Natural products research in the new millennium,*** **Int. Congr. and Ann. Meeting of the Soc. for Med. Plant Res.** *(GA)*, September 3-7, 2000 Zurich, Switzerland.

4- Panizzi L., Flamini G., Cioni L.P., Moreli I. (1993). ***Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae.*** **J. Ethnopharm.** , **39**, 163-170.

5- Buchbauer G. (2000). ***The detailed analysis of essential oils leads to the understanding of their properties.*** **Perfumer & Flavorist**., **25**, 64-67.

6- Satyajit D. S., Lutfun N. (2007). **Chemistry for Pharmacy Students General, Organic and Natural Product Chemistry**, The Atrium, Southern Gate, Chichester, West Sussex, England : John Wiley & Sons Ltd,

7- Rafael C. D., Magda N. L., Nadia R. (2008). ***Quantification of phenolic constituents and antioxidant activity of pterodon emarginatys vogel seeds.*** **Int. J. Mol. Sci**., **9**, 606-614.

8-Wallace G., Fry S.C. (1994). ***Phenolic components of the plant cell wall.*** **Int. Rev. Cytol.**, **151**, 229–267.

9- Harborne J. B. (1994). **The Flavonoids: Advances in Research Since 1986**. London, UK: Chapman & Hall.

10- Robards K., Prenzler P. D., Tucker G., Swatsitang P., Glover W. (1999). ***Phenolic compounds and their role in oxidative processes in fruits*****. Food Chem.** ,**66**, 401–436.

11- Stavric B. (1994). ***Quercetin in our diet: from potent mutagen to probable anticarcinogen*****. Clin. Biochem.**, **27**, 245–248.

12- Shahidi F., Naczk M. (1995). ***Food Phenolics, Sources, Chemistry, Effects, Applications.*** **Lancaster**, USA: Technomic Publishing Company, Inc.

13- Havsteen B. (1983). **Flavonoids, a class of natural products of high pharmacological potency.** ***Biochem. Pharmacol.***, **32**, 1141–1148.

14- Dixon R. A., Paiva N. L. (1995). **Stress-induced phenylpropanoid metabolism.** ***Plant Cell.***, **7**, 1085–1097.

15- Strack D. (1997). **Phenolic metabolism**. In: Dey P. M., Harborne J. B., eds. ***Plant Biochemistry***. London, UK: Academic Press, 387–416.

16- Macheix J-J., Fleuriet A., Billot J. (1990) ***Fruit Phenolics***. Boca Raton, USA: CRC Press.

17- Haddock E., Gupta R., Al-Shafi S., Layden K., Haslam E., Magnolato D. (1982). **The metabolism of gallic acid and hexahydroxydiphenic acid in plants: biogenetic and molecular taxonomic considerations.** ***Phytochem.*** , **21**, 1049–1062.

18 - Maas J. L. , Galletta G. , Wang S. (1992). **Ellagic acid enhancement in strawberries**. In: Bills D., Kung S., eds. ***Biotechnology and Nutrition.*** Storeham, USA: Butteworth-Heinemann, 345–362.

19- Herrmann K. (1989). ***Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in food***. **Crit. Rev. Food Sci. Nutr., 28**, 315–347.

20- Schuster B., Herrmann K. (1985). ***Hydroxybenzoic and hydroxycinnamic acid derivatives in soft fruits***. **Phytochem., 24**, 2761–2764.

21- Giuseppe G., Barreca D., Gargiulli C., Leuzzi U., Caristi C. (2007). ***Flavonoids composition of citrus juice***. **Molecules, 12**, 1641-1673.

22- Middleton E., Kandaswami C., Theoharides T. (2000). ***The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer,*** **Pharmacological Reviews, 52**, 673–751.

23- Havsteen B. H. (2002). ***The biochemistry and medical significance of the flavonoids,*** **Pharmacology and Therapeutics, 96**, 67–202.

24- Liang Y. C, Huang Y. T., Tsai S. H., Lin-Shiau S. Y., Chen C. F., ,Lin J. K. (1999). ***Suppression of inducible cyclooxygenase and inducible nitric oxide synthesis by apigenin and related flavonoids in mouse macrophages*****, Carcinogenesis , 20**, 1945–1952.

25- Kim H., Kim Y. S., Kim S. Y., Suk K. (2001). ***The plant flavonoid wogonin suppresses death of activated C6 rat glial cells by inhibiting nitric oxide production*****, Neuroscience Letters, 309**, 67–71.

26- Robards K., Prenzler PD., Tucker G., Swatsitang P., Glover W. (1999). ***Phenolic compounds and their role in oxidative processes in fruits***. **Food Chem., 66**, 401–436.

27- Knekt P., Järvinen R., Reunanen A, Maatela J. (1996). ***Flavonoid intake and coronary mortality in Finland: a cohort study*****, Br. Med. J., 312**, 478–481.

28 - Keli S. O., Hertog M. G. L., Feskens E. J. M., Kromhout D. (1996). **Dietary flavonoids, antioxidant vitamins, and incidence of stroke: The Zutphen study, Arch. Int. Med., 156**, 637–642.

29 - Knekt P., Järvinen R., Seppänen R. (1997). ***Dietary flavonoids and the risk of lung cancer and other malignant neoplasms*****, Am. J. Epidemiol., 146**, 223–230.

30 - Garcia-Closas R., Gonzales C. A., Agudo A, Riboli E. (1999). ***Intake of specific carotenoids and flavonoids and the risk of gastric cancer in Spain.*** **Cancer Causes Cont., 10**, 71–75.

31 - Rimm E. B., Katan M. B., Ascherio A., Stampfer M. J., Willet W. C. (1996***). Relation between intake of flavonoids and risk for coronary heart disease in male health professionals*****, Ann. Intern. Med., 125,** 384–389.

32 - Hertog M. G. L., Sweetnam P. M., Fehily A. M., Elwood P. C., Kromhout D. (1997). ***Antioxidant flavonols and ischemic heart disease in a Welsh population of men: the Caerphilly Study*****, Am. J. Clin. Nutr. , 65,** 1489–1494.

33- Costa M. A., Collins R. E., Anterola A. M., Cochrane F. C., Davin L. B. (2003). ***An in silico assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in Arabidopsis thaliana and limitations thereof*, Phytochem., 64**, 1097–112

34- Heller W., Forkmann G. (1994). **Biosynthesis of flavonoids.** In: Harborne J. B, Ed. ***The Flavonoids.*** London: Chapman and Hall, 499–536.

35-Mathews H., Clendennen S. K., Caldwell C. G., Liu X. L., Connors K., Matheis N., Schuster D. K., Menasco D. J., Wagoner W., Lightner J., Wagner D. R. (2003). ***Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport*, Plant Cell., 15**, 1689–1703.

36- Winkel-Shirley B., (2001). ***Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology*, Plant Physiol., 126**, 485–493.

37- Broun P. (2004). ***Transcription factors as tools for metabolic engineering in plants,* Current Opinion Plant Biol.,7 ,** 202–209.

38- Whiting D. A., (2001). ***Natural phenolic compounds 1900–2000: A bird's eye view of a century's chemistry*, Nat. Prod. Rep., 18,** 583–606.

39- Xie D. Y., Dixon R. A. (2005). ***Proanthocyanidin biosynthesis still more questions than answers*, Phytochem., 66 ,** 2127–2144.

40- Boudet A. M. (2007). ***Evolution and current status of research in phenolic compounds,* Phytochem., 68**, 2722–2735.

41- Ramirez-Ahumada Mdel C., Timmermann B. N., Gang D. R. (2006). ***Biosynthesis of curcuminoids and gingerols in turmeric Curcuma longa and ginger Zingiber officinale: identification of curcuminoid synthase and hydroxycinnamoyl-CoA thioesterases***, **Phytochem. , 67,** 2017-29.

42- Harvey D. J. (1981). ***Gas chromatographic and mass spectrometric studies of ginger constituents : Identification of gingerdiones and new hexahydrocurcumin analogues*****, Journal of Chromatography, 212,** Issue **1** ,75-84.

43- Ali-Shatyeh M. S., Jamous R. M. (2008). **Traditional Arabic Palestinian Herbal Medicine.** (1st ed.), Nablus: Biodiversity and Environmental Research Center.

44- Skoog A., Hollar F., Nieman A., (1998). **Principles of instrumental analysis.** (5^{th} ed). USA: Thomson learning Inc.

45- Markham K. R. (1982). **Techniques of Flavonoid Identification.** New York, USA: Academic Press.

46- Robards K., Antolovich M. (1997). ***Analytical chemistry of fruit bioflavonoid: a review,*** **Analys., 122,** 11R– 34R.

47- Harborne JB. (1998). **Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis**. London, UK: Chapman & Hall.

48- Merken H. M., Beecher G. R. (2000). ***Measurement of food flavonoids by high-performance liquid chromatography: A review*****, J. Agric. Food Chem., 48,** 577–599.

49- Harborne J. B. (1988). **The flavonoids: recent advances.** In: Goodwin TW, ed. ***Plant Pigments***. London, England: Academic Press. 299–343.

50- Markham K. R. , Bloor S. J. (1998). **Analysis and identification of flavonoids in practice.** In: Rice-Evans CA, Packer L, eds. ***Flavonoids in Health and Disease.*** New York, USA: Marcel Dekker Inc., 1– 33.

51- Finger A., Engelhardt U. H., Wray V. (1991). ***Flavonol glycosides in tea - kaempferol and quercetin rhamnodiglucosides,*** **J. Sci. Food Agric., 55,** 313–321.

52- Nielsen J. K., Olsen C. E., Petersen M. K., (1993). ***Acylated flavonol glycosides from cabbage leaves*****, Phytochem., 34,** 539–544.

53- Mouly P., Gaydou E. M., Estienne J. (1993). **Column liquid chromatographic determination of flavanone in Citrus**, **J. Chromatogr., 634,** 129–134.

54- Schmidt T. J., Merfort I., Willuhn G. (1994). ***Gas chromatography-mass spectrometry of flavonoid aglycones II. Structure-retention relationships and a possibility of differentiation between isomeric 6- and 8-methoxyflavones,*** **J. Chromatogr. A., 669,** 236–240.

55-Jennifer K. (2000). Medicinal plants for livestock beneficial or toxic. http://WWW.ansci.cornell.edu/plants/inedicmal/index.html.

56- Huang G., Jiang J., Dai D. (2008). ***Antioxidative and antibacterial activity of the methanol extract of artemisia anomala S. Moore***, **African J. Biotech. , 7 (9),** 1335-1338

57- Schinella G. R., Tournier H. A., Prieto J. M., Mordujovich de Buschiazzo P., Ríos J. L. (2002). **Antioxidant activity of anti-inflammatory plant extracts, Life Sci., 70,** 1023-1033.

58- VanderJagt T. J., Ghattas R., VanderJagt D. J., Crossey M., Glew R. H. (2002). ***Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico,*** **Life Sci. ,70,** 1035-1040.

59- Silva, F., & Abraham, A. (1981). ***The potentiality of the Israeli flora for medicinal purposes*****, Fitoterapia , 52**, 195-200.

60- Ali-Shtayeh M. S., Yaghmour R. M., Faidi Y. R., Salem Kh., Al-Nuri M. A. (1998***). Antimicrobial activity of 20 plants used in Folkloric Medicine in Palestinian Area*****, Journal of Ethnopharmacol., 60,** 265-271.

61- Kite G. C., Sharp H. J., Hill P. S., Boyce P. C., (1997). ***Polyhydroxyalkaloids in the aroid tribes nephthytideae and aglaonemateae: Phytochemical support for an intertribal relationship.*** **Biochm. Syst. Ecol., 25,** 757- 766.

62- El-Desouky S. K., Kim K. H., Ryu S.Y., Eweas A. F., Gamal –Eldeen A. M., Kim Y. K. (2007). ***A new pyrrol alkaloide isolated from Arum palaestinum Boiss. and its biological activities*****, Arch. Pharm. Res., 30**, 927-931

63- Saad B., Azaizeh H., Said O. (2005). ***Tradition and Perspectives of Arab Herbal Medicine,*** **eCAM , 4 ,** 475–479.

64- Afifi F. U., Abdalla S. (1999). ***Effect of isoorientin isolated from Arum palaestinum on uterine smooth muscles of rats and guinea pigs,*** **J. Ethnopharmacol., 65,** 173-177.

65- Kavalali G., Tuncel H., Goksel S. , Hatemi H. H. (2003). ***Hypoglycemic activity of Urtica pilulifera in streptozotocin-diabetic rats,*** **J. Ethnopharmacol., 84,** 241-245.

66- Lopatkin, N. , Sivkov, A. Walther, C. Schlafke, S. Medvedev, A. Avdeichuk, J. Golubev, G. Melnik, K. Elenberger N. & Engelmann, U. (2005). ***Long-term efficacy and safety of a combination of sabal and Urtica pilulifera extract for lower urinary tract symptoms: A placebo-controlled, double-blind, multi-center trial. World,*** **J. Urol. , 12,** 742-749.

67- Irshaid F., Mansi K. (2009). ***Effects of leaf extract of Artica Pilulifera L. on male reproductive system of streptozotocin-diabetic rats***. **American J. of Pharmacol. and Toxicol., 2 (4),** 22-28.

68- Hadizadeh, I. ,Peivastegan B. , Hamzehzarghani H. (2009). ***Antifungal activity of essential oils from some medicinal plants of Iran against Alternaria alternate,*** **Am. J. of Appl. sci., 5(6),** 857-861.

69- Bhaskara M. V. Angers, P. Gosseline A., Arul, J. (1998). ***Characterization and use of essential oil from Thymus vulgaris against botrytis camera and rhizopus stolonnifer in strawberry fruits,*** **Phytochem., 47,** 1515- 1520.

70- Tabata M. , Honda G., Sezik E. (1988). **A report on traditional medicine and medicinal plants in Turkey,** Faculty of pharma. ceutical sciences, Kyoto University.

71- Bremnes L. H. (2002). **Eyewitness-Handbooks,** New York, USA: DK Publishing.

72- Abu-Lafi S., Odeh I. , Dewik Q., Imam A. ,Dembitsky M., Lumir O. (2007). ***Natural compounds Of Palestine flora. Comparison analysis by static headspace and steam distillation GC-MS of semi volatile secondary metabolites from leaves of cultivated Palestinian M. syriaca,*** **Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub, 1(151)**.21–29.

73- Suleiman M. S., Abdul-Ghani A. S., Al-Khali S., Amin R. (1998). ***Effect of Teucrium polium boiled leaf extract on intestinal motility and blood pressure,*** **J. Ethnopharmacol., 22,** 111–116

74- Tariq M., Ageel A. M., al-Yahya M. A. , al-Said M. S. (1989). ***Anti-inflammatory activity of Teucrium polium,*** **Int. J. Tissue React., 11**,185–188,

75- Mansouri S. (1999). ***Inhibition of Staphylococcus aureus mediated by extracts of Iranianplants,*** **Pharmac. Biol., 37,** 375–377.

76- Esmaeili M. A., Yazdanparast R. (2004). ***Hypoglycaemic effect of Teucrium polium: studies with rat pancreatic islets,*** **J. Ethnopharmacol., 95,** 27–30.

77- Panovska T. K. , Kulevanova S. (2005). ***Effect of some Teucrium species (Lamiaceae) on lipid peroxidation in rat liver microsomes,*** **Fresenius Environ. Bull., 14,** 957– 959

78- Lemordant D., Boukef K. , Bensalem M. (1997). ***Plantes utiles et toxiques de Tunisie,*** **Fitoterapia, 48,** 191-214.

79- Fonta G., Savona G., Redriguez, B. (1999). ***Usual 6'-fatty acid esters of (24S-24-ethyl- cholesta-5, 25- dien-3b-yl-b-D-Glucopyranoside) from Teucrium polium,*** **J. Ethnopharmacol. , 24,** 93–99

80- Mini D. A., Zhang P., Zhao X., Wang Chong S., Zheno Q. (1991). ***A neo chlordane deterrence from Teucrium japonicum,*** **Phytochem., 30,** 4175–4177.

81- Harborne J. B., Tomas B., Williams G. A., Gil. M. I. (1986). ***A chemotaxonomic study of flavonoids from European teucrium species,*** **Phytochem., 25,** 2811– 2816.

82- Rizk A. M., Hammouda F. M. , Rimpler H., Kamel A. (1986). ***Iridoids and flavonoids of Teucrium polium herb,*** **Planta. Medica., 2,** 87–88.

Chapter Two
Biological Activities of Selected Plants in Palestine

2.1 Antioxidants

2.1.1 Introduction

Antioxidants neutralize the toxic and 'volatile' free radicals by giving hydrogen atoms or scavenging them scheme 2.1 [1].

Unsaturated organic compounds → $RO^{\cdot}_2$ +ArOH → ROOH +$ArO^{\cdot}$

$RO^{\cdot}_2$ + $ArO^{\cdot}$ → non-radical products

(Scheme 2.1) Scavenging radicals by antioxidants

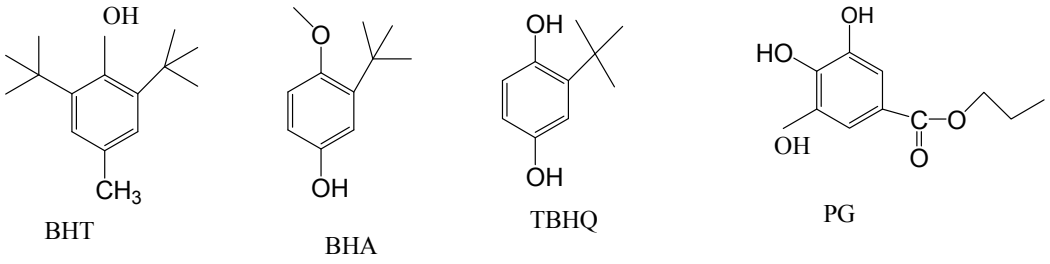
In biological systems free radicals and other reactive species like 'reactive oxygen species' (ROS), nitrogen (RNS) and chlorine (RCS), are normal by- products of metabolism and they are introduced into the body from outside sources of harmful chemicals in the environment, unhealthy foods, certain drugs, smoke and many other sources. In a normal healthy human, taking antioxidants neutralize the generation of those species and protect the body from cell damage [2]. However, when the humans get exposed to unfavorable physiochemical, environmental or pathological agents oxidative stress results from the imbalance between the extent of ROS formation and the oxidative defense mechanisms. Cellular damage induced by oxidative stress has been caught up in the etiology of a large number of human diseases (>100) such as pathology of cancer, atherosclerosis, malaria and rheumatoid arthritis as well as the process of ageing [3]. In recent years, there is a remarkable interest in the possible task of nutrition in prevention of disease. In this context, antioxidants especially derived from natural sources such as medicinal plants and herbal drugs derived from them require special attention. Antioxidants have many potential

applications, especially in relation to human health, both in terms of prevention of disease and therapy [4, 5]. Various antioxidants may prevent and/or improve diseased states [6]. These include the intracellular different levels of protection such as prevention, interception antioxidant enzymes and the dietary or oral supplements in the form of vitamin C, vitamin E, β-carotene, zinc and selenium [7, 8]. Antioxidants are also of particular importance because they might serve as leads for the development of novel drugs. Several plants used as neuroprotective, anti-inflammatory, digestive, antinecrotic, and hepatoprotective properties have recently been shown to have antioxidant and/or antiradical scavenging mechanism as part of their activity [9, 10].

A review of literature shows that there are many plants in Palestine showing antioxidant abilities at various levels of protection need to be examined for their potential therapeutic and beneficial properties [11]. More recent assays also should be included to study the antioxidant properties of medicinal plants or their chemical constituents. This will greatly help in identifying more potent compounds with potential applications in prevention.

There has been considerable interest in the field of antioxidants in recent years and these efforts have led to a better understanding of the mechanism involved and in the application areas of food and non-food commodities as well as in biological systems and as a dietary supplements. The early work by scientists was concentrated on synthetic antioxidants and currently butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), propyl

gallate (PG) and tert-butylhydroquinone (TBHQ) are most widely used in foods (Figure 2.1).



(Figure: 2.1) Structures of some antioxidants

Unfortunately some reports have indicated that certain synthetic antioxidants may possess weak carcinogenic effects in some animals at high levels. However natural sources of antioxidants such as tocopherols, ascorbic acid, erythrobic acid or their salts or derivatives have found extensive applications in the food industry [12]. There are many techniques and methodologies to evaluate the antioxidant activity; one should use several methods to provide complementary assessment that consider different mechanisms involved in rendering the effects. These included radical scavenging activity, metal chelation and reducing power among others [13].

Objective of study was to:

Evaluate the antioxidant activity of six medicinal plants: *Arum palaestinum*, *Urtica pilulifera*, *Coridothymus capitatus*, *Majorana syriaca, Teucrium creticum* and *T. polium*, using DPPH and β -carotene-linoleic acid assays.

2.1.2 Materials and methods

1- Chemicals

β-Carotene, linoleic acid, 1,1–Diphenyl-2-picrylhydrazyl (DPPH), buthylated hydroxytoluene (BHT), buthylated hydroxyanisol (BHA), and α-tocopherol were purchased from Sigma,(Sigma, Aldrich GmbH, Sternhheim, Germany). While pyrocatechol, quercetin, Tween-40, Folinciocalteus phenol reagent (FCR), sodium carbonate, ethanol, chloroform and other chemicals and reagents were purchased from Merck (Darmstat, Germany). All other chemicals and reagents were of analytical grade.

2- Plant Collection

The medicinal plant species screened in this study were collected in April – June 2008 from Nablus region and were identified by Prof. M. S. Ali-Shtayeh in the Department of Biological Sciences at An-Najah University. Voucher specimens are preserved in BERC /Til Village.

3- Extracts preparation

The aerial parts of the plants were dried in the shadow at 30°C and grinded using a Molenix (Mooele Depose type 241) for a minute. 50g sample from each dried plant was extracted by continuous stirring with 500 ml ethanol at 24°C for 72 h and filtering through Whatman No. 4 filter paper. The residue was then washed with additional 200 ml ethanol. The combined ethanolic extracts were dried using freeze drying and stored at -20 °C for future use.

4 - DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanolic solution of 1,1-diphenly-2-picrylhydrazyl-hydrate (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [14, 15]. One milliter of various concentrations of the extracts in ethanol was added to 4 ml of 0.004% methanol solution of DPPH. After 30 minutes, incubation period at room temperature, the absorbance was read against a blank at 517 nm. The percent Inhibition I (%) of free radical by DPPH was calculated as follows:

$I\ (\%) = ((A_{blank} - A_{sample})/ A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentrations providing 50% inhibition (I_C50) are calculated from the plot of inhibition (%) against extract concentration. Tests were carried out in triplicates.

5- β-Carotene-linoleic acid assay

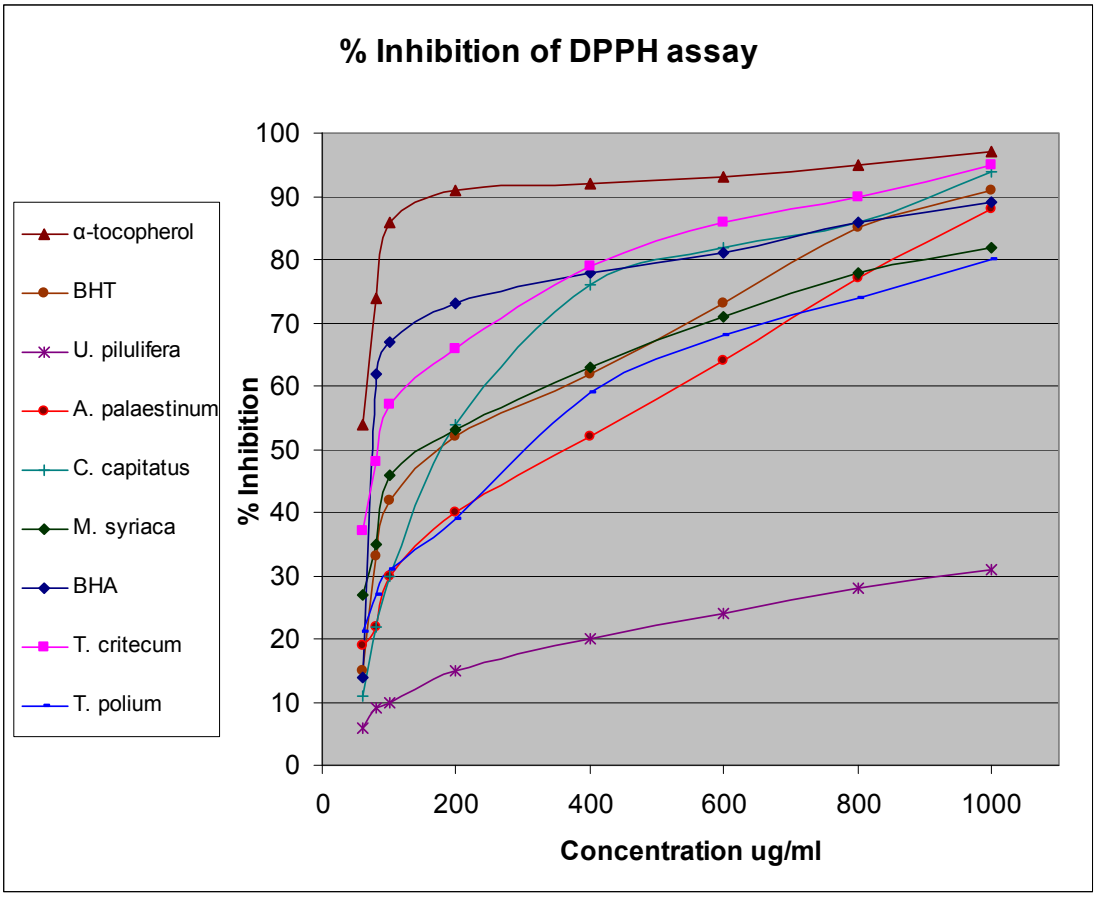
The antioxidant activity of the ethanol extracts, based on coupled oxidation of β-carotene and Linoleic acid emulsion, was evaluated following a modified method of Gazzani and Miller [16, 17]. Briefly, 1mg of β-carotene was dissolved in 2 ml chloroform and 20 mg of linoleic acid, 200 mg of Tween 40 were added. Chloroform was completely evaporated using a rotary evaporator under reduced pressure at low temperature (less than 30°C), and 200 ml of distilled water saturated with oxygen were added to the flask with vigorous shaking for 30 minutes. Aliquots (5ml) of the

prepared emulsion were transferred to a series of tubes each containing 0.1ml of extract or tocopherol (2mg/ml). A control sample was prepared exactly as before but without adding antioxidants. Each type of sample was prepared in triplicate. The test systems were placed in a water bath at 50°C for 2 hours. The absorbance of each sample was read spectrophotometrically at 470 nm, immediately after sample preparation and at 15-min intervals until the end (t = 120 min) of the experiment. Antioxidant activities in β-carotene-linoleic acid model were measured by the changes in the absorbance at 470 nm.

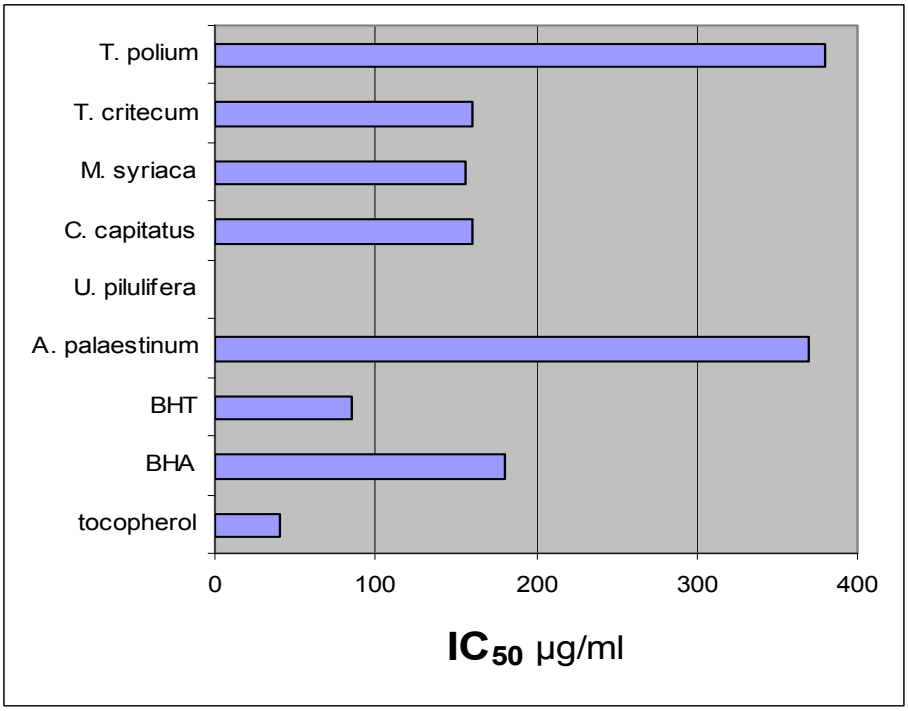
2.1.3 Results and discussion

In DPPH, a stable free radical scavenging assay with a characteristic absorption at 517 nm was used to study the radical scavenging effects of extracts. As antioxidants donate hydrogen atoms to these radicals, they lose their purple color which leads to decrease absorption. The decrease in absorption is taken as a measure of the extent of radical scavenging. All extracts showed significant free radical scavenging activity except *U. pilulifera* (Figure 2.2). It is found also that percent inhibition values increase with increasing concentrations. The 50% inhibition values for the extracts seem to be fairly significant at 400 µg/ml compared with commonly used synthetic antioxidants BHA (78%) and α-tocopherol (92%) at the same concentration (Figure 2.2). Free radical scavenging capacities (I_C50) values for *C. capitatus* (I_C50 =180µg/ml) and *T. creticum* (I_C50 =180 µg/ml) emphasize the high potential of those two plants when compared to commonly used synthetic antioxidant BHA (I_C50 =190 µg/ml) and α-

tocopherol (I_C50 =80 µg/ml) (Figure 2.3). The 50% inhibition values for the other plants are (*M. syriaca* =180 µg/ml), (*A. palaestinum* =380 µg/ml), (*T. polium* =390 µg/ml), showed a desirable antioxidant activities except (*U. pilulifera* =0.0µg/ml) that justify the usage of them in folk medicine.

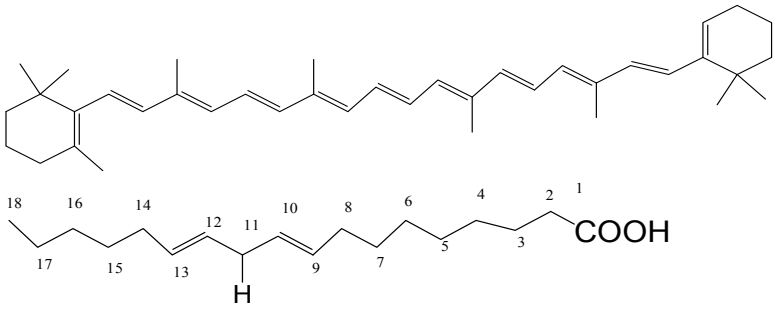


(Figure: 2.2) Percent inhibition of selected plants at different concentrations



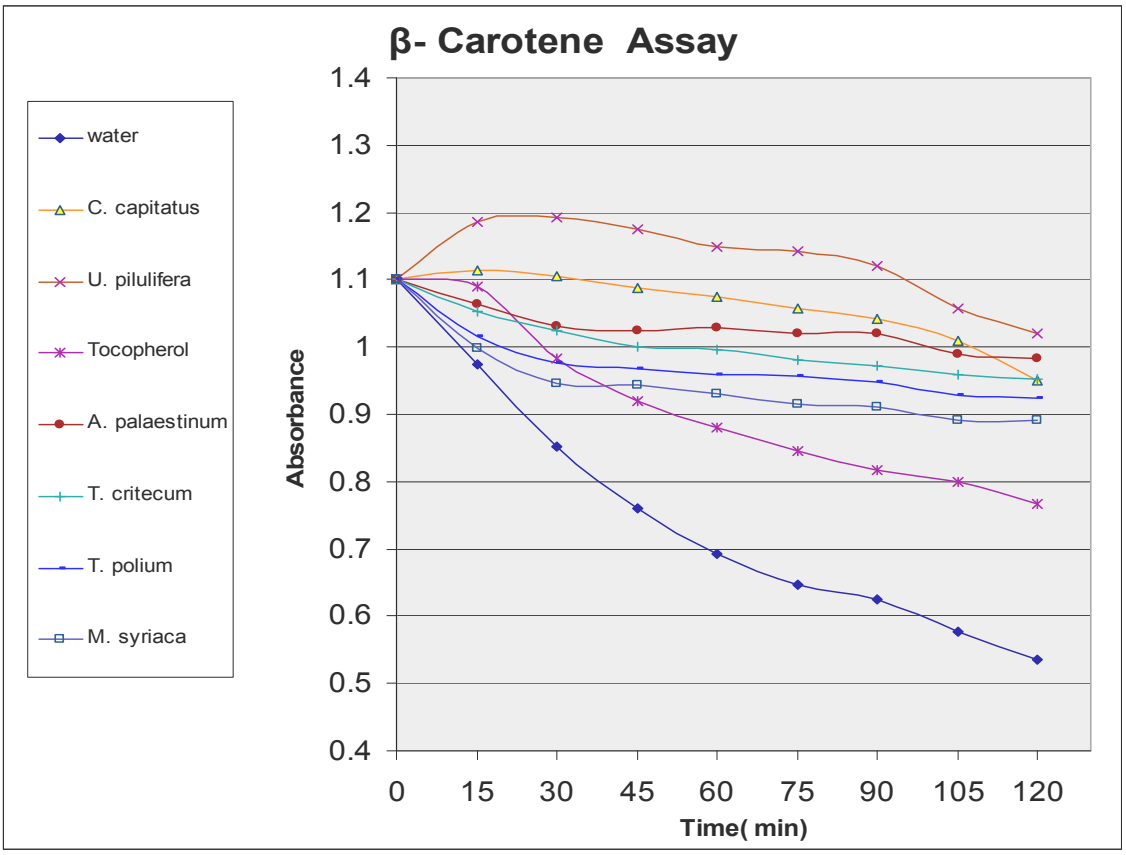
(Figure: 2.3) Free radical scavenging capacities I_C50of ethanolic extracts

Heat-induced oxidation of an aqueous emulsion system of β-carotene-linoleic acid was employed as another antioxidant test reaction. The test is based on the fact that β-carotene loses its color in the absence of antioxidant [17]. During oxidation, an atom of hydrogen is abstracted from the active methylene group of linoleic acid located on carbon-11 between two double bonds [18]. The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lose their conjugation, the carotenoids lose their characteristic orange color. This process can be monitored spectrophotometrically (Figure 2.4).



(Figure: 2.4) Structural formulas of β-carotene and linoleic acid

All ethanolic extracts of the selected plants revealed higher antioxidant efficiency compared with water (control) and the synthetic antioxidant α-tocopherol which gave the highest β-carotene color degradation (i.e. least antioxidant efficiency). *U. pilulifera* is the most efficient extract of them which is justified by the presence of 2-phenoxyethanol as one of the constituents of the plant. The compound gave the least β-carotene discoloration (i.e. highest antioxidant efficiency). The second extract is for *C. capitatus*, and the third is for *A. palaestinum* (Figure 2.5). The efficiency of those plants as antioxidants may be attributed to the presence of many different chemical compounds such as phenolics and flavonoids. The total phenolic amounts were calculated using pyrocatechol equivalent and total flavonoids were calculated using quercetin equivalent (see chapter 3). The presence of phenolic antioxidant can hinder the extent of β-carotene degradation by "neutralizing" the linoleate free radical and any other free radicals formed within the system. Hence, this forms the basis by which plant extracts can be screened for their antioxidant potential. In conclusion, the present study indicated that the use of these plants in Palestine either in traditional medicine or as edible plants is justified since they are promising sources of natural antioxidants.



(Figure: 2.5) Antioxidant activities of ethanolic extracts of the plants (*A. palaestinum*, *U. pilulifera*, *C. capitatus*, *M. syriaca*, *T. creticum*, *T. polium*), and α-tocopherol, as assessed by β-carotene-linoleic acid assay over 120 minute.

2.2 Antimicrobial activity of selected plants in Palestine

2.2.1 Introduction

The development of natural products used as medicines represents a way to rescue valuable aspects of traditional culture. Despite their unproven safety, some plant products are used as drugs or food supplements and they are sold without prescription. Natural phytochemicals derived from fruits, vegetables and herbs have been reported to posses a wide range of biological effects including antioxidant, antimicrobial and ant-inflammatory actions [19]. There are more than 35000 plant species being used in various cultures around the world for medicinal purpose [20, 21]. A wide range of medicinal plant parts are extracted and used as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant. While some of these raw drugs are collected in smaller quantities by the local communities and folk healers for local use, many other raw drugs are collected in larger quantities and traded in the market as the raw material for many herbal industries [22]. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of them have not been adequately evaluated [23]. Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, the present work is a systematic investigation was undertaken to screen six medicinal plants used in traditional medicine in Palestine.

The use of medicinal herbs in the treatment of skin diseases including mycotic infections is an old-age practice in many parts of the world. Fungal infections remain a therapeutic problem despite the availability of a number of treatments. Being largely synthetic and non-biodegradable, these agents used in treating

fungal infections can cause adverse effects and may have residual toxicity [24]. In Palestine, the screening of flora for pharmacological active compounds started in the late sixties [25]. The literature review and ethanobotanical surveys revealed that there is a list of 47 plant species used for treatment of skin diseases suggestive of dermatophyte infections [26]. Few studies of antifungal activity of those plants were performed. The oil of nettle showed a potent and completely inhibitory effect on the radial growth of *A. alternate* at 1500 ppm [27]. *C. capitatus* is an aromatic plant found in Palestine and locally known under the common name (zaatar). It is used in folk medicine against cold, influenza, throat infection [28] and also is important for the perfume, cosmetics, flavoring and pharmaceutical industries [29] .The essential oils of the plant were reported to have antimicrobial activities most of which are mediated by thymol and carvacrol [30]. Various species of thyme have been credited with a long list of pharmacological properties. They have been used in folk medicine as antiseptic and as antimicrobial agents [31]. There are no reported studies on antifungal activity of *C. capitatus* and *M. syriaca* in special case, but there are reports on the antifungal activities of the essential oils for other thymus species and their main components against soil-borne pathogens, food storage fungi, pathogens and opportunistic human pathogens [32]. *T. polium* is a medicinal plant which has been used for over 2000 years in traditional medicine due to its diuretic, diaphoretic, tonic, antipyretic and antispasmodic properties. Many bioinvestigations were done on the plant, but none of those on the antifungal activity of the plant [33]. Unlike bacterial diseases, fungal

diseases are more difficult to treat. Often topical and oral treatments are long term and may only be partially successful in controlling the fungus, if they work at all. Many infections will be chronic and if you are fortunate enough to rid the infection from your body, there is always the possibility of recurrence of the disease. This is why there is a difficulty in treating fungal diseases. Many serious bacterial diseases have been successfully treated and usually without side affects from the drugs used. This usually is not the case with treatment of fungal diseases.

Natural products including plants, animals and minerals have been the basis of treatment of human diseases. Traditional medical traditions can offer a more holistic approach to drug design and myriad possible targets for scientific analysis. For traditional medicines, newer guidelines of standardization, manufacture and quality control and scientifically rigorous research on the scientific basis for traditional treatments will be required. Numerous drugs have entered the international pharmacopoeia via the study of ethno pharmacology and traditional medicine. Even during the early part of this century, plants were a vital source of raw material for medicines.

2.2.2 Materials and methods

1. Chemicals: chloramphenicol, peptone, agar, dextrose, ethanol, Muller–Hinton agar, gentamicin, ampicilline and econazole, all chemicals and reagents were of analytical grade.

2. Extracts preparation

The aerial parts of the plants were dried in the shadow at 30°C and grinded using a Molenix (Mooele Depose type 241) for a minute. 50g sample from each dried plant was extracted by continuous stirring with 500 ml ethanol at 24°C for 72 h and filtering through Whatman No. 4 filter paper. The residue was then washed with additional 200 ml ethanol. The combined ethanolic extracts were dried using freeze drying and stored at -20 °C for further use.

3. Microorganisms

Bacteria:

The bacteria used included: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (JM109).

Fungi:

While the fungi used in this study were *T. tonsurans, M. canis, T. rubrum* and *T.* v*iolaceum.* The isolates have been maintained on SDA media at room temperature. Experimental cultures were kept on SDA media and subculture monthly (Murray et al., 1995; Yaghmour, 1997).

4. Antibacterial testing

The dried plant extracts were dissolved in ethanol to a final concentration of 100 mg/ml and sterilized by filtration through a 0.45 mm membrane filter. Antibacterial tests were then carried out by the disc diffusion method (Murray et al., 1995) using an inoculum containing 10^6 bacterial cells/ml spread on Muller–Hinton agar plates (1 ml inoculum/plate). The discs

(diameter= 6 mm) were impregnated with 2 ml of extract (10 mg/disc) at a concentration of 100 mg/ml and placed on the inoculated agar and incubated at 37°C for 24 h.

5. Antifungal testing:

The plants extracts were tested at different concentrations for their antifungal activity against the test pathogens using a modified poisoned food technique. Different amounts of each extract were incorporated in pre-sterilized SDA medium to prepare a series of concentrations of the extract (12.5, 25, 37.5, 50, 62.5 µg/ml). A mycelial agar disk of 5 mm diameter was cut out of 12 days old culture of the test fungus and inoculated on to the freshly prepared agar plates. In controls, sterile distilled water was used in place of the tested sample. Three replicate plates were used for each treatment (concentration).The inoculated plates were incubated in the dark at 24˚C and the observations were recorded after 10 days. Percentage of mycelial inhibition was calculated using the following formula:

% mycelial inhibition = (dc-ds/dc) x100%

dc: colony diameter of the control

ds: colony diameter of the sample

2.2.3 Results and discussion

a- Antibacterial activity

Six selected plants in Palestine used in folk medicine were tested in vitro against six bacterial species which are known to cause dermic and mucosal infections. Table 2.1 shows the effect of extracts on six kinds of bacteria at 200 µg/ml concentration. The best between the plants was *M. syriaca*

extract, and *C. capitatus*. They showed significant antibacterial activity against all kinds of tested bacteria. *E. coli* was the most type of bacteria that by affected by *C. capitatus* extract. The inhibition zone for was (37.8 mm) and *K. pneumoniae* was the most type affected by *M. syriaca*, inhibition zone (31.0 mm) compared to the control (6 mm) in diameter.

(Table: 2.1) Antimicrobial activity of plant extracts against bacteria

* Values of inhibition zone diameter in mm

No	Plant extracts (concentration)	Micro-organisms					
		P. vulgaris	*E. coli. JM109*	*E. coli*	*P. aerugino -sa*	Staphylo coccus aureus	*K. pneumon iae*
1	*A. palaestinum*	* 6.0	6.0	6.0	6.0	8.8± 0.7	6.0
2	*U. pilulifera*	6.0	6.0	6.0	6.0	6.0	6.0
3	*C. capitatus*	11.8± 0.8	13.0±0.6	19.4±1.5	20.8±0.8	22.0±0.8	31.0±0.6
4	*M. syriaca*	33.75±0.8	16.5±0.5	37.8±1.4	22.6±0.8	29.0±0.6	33.6±1.0
5	*T. creticum*	6.0	6.0	6.0	6.0	6.0	6.0
6	*T. polium*	6.0	6.0	6.0	6.0	6.0	10.2± 1.3
7	control	6.0	6.0	6.0	6.0	6.0	6.0

b- Antifungal activity

Only a few antifungal substances are known or available in the market as compared to antibacterial substances. Antimycotic substances are also relatively unsatisfactory in the control of dermatophytes. The discovery of active components exhibiting a broad spectrum antifungal activity may prove useful for the development of antifungal agents. Laboratory assessment showed the nature of fungi static activity encountered in plant extracts. Table 2.2 shows cross tabulation means of percent inhibition due to plant type and concentration level. The values of the means increase with increasing concentration of each plant.

(Table: 2.2) Cross tabulation means of percent inhibition due to plant type and concentration level.

Plant Type	Concentration	Mean	Std. Error	95% Confidence Interval

	Level			Lower Bound	Upper Bound
A. Palaestinum	12.5	28.000	4.980	18.106	37.894
	25.0	46.875	4.980	36.981	56.769
	37.5	66.000	4.980	56.106	75.894
	50.0	89.625	4.980	79.731	99.519
	62.5	100.000	4.980	90.106	109.894
U. Pilulifera	12.5	28.225	4.980	18.331	38.119
	25.0	66.875	4.980	56.981	76.769
	37.5	82.225	4.980	72.331	92.119
	50.0	95.475	4.980	85.581	105.369
	62.5	100.000	4.980	90.106	109.894
C. capitatus	12.5	71.550	4.980	61.656	81.444
	25.0	90.325	4.980	80.431	100.219
	37.5	100.000	4.980	90.106	109.894
	50.0	100.000	4.980	90.106	109.894
	62.5	100.000	4.980	90.106	109.894
M. syriaca	12.5	45.800	4.980	35.906	55.694
	25.0	72.550	4.980	62.656	82.444
	37.5	92.300	4.980	82.406	102.194
	50.0	100.000	4.980	90.106	109.894
	62.5	100.000	4.980	90.106	109.894
T. creticum	12.5	18.125	4.980	8.231	28.019
	25.0	43.625	4.980	33.731	53.519
	37.5	70.200	4.980	60.306	80.094
	50.0	87.125	4.980	77.231	97.019
	62.5	100.000	4.980	90.106	109.894
T. polium	12.5	48.525	4.980	38.631	58.419
	25.0	72.200	4.980	62.306	82.094
	37.5	90.300	4.980	80.406	100.194
	50.0	98.150	4.980	88.256	108.044
	62.5	100.000	4.980	90.106	109.894

All tested plants revealed antimycotic activity against all tested fungi, with *C. capitatus* extract showing the highest activity. Complete inhibition range for this plant was 12.5-25 µg/ml which is nearly 1/5 activity of econazole against *T. violaceum,* 1/6 and 1/7 of the activity for other tested fungi. *M. syriaca* extract have also showed considerable antimycotic activity against the same tested fungi (Table 2.3). This result is consistent with the value of those obtained by Sokovic, (2009) who also demonstrated significant antimicotic activity of two thymus spp. against *T. violaceum*. They showed complete inhibition range (0.25-1 µl/ml). Comparing the previous data with

the chemical composition of the oils, it becomes evident that there is a relationship between the high activities of the thyme species and the presence of phenol components, such as thymol and carvacrol. In addition to these plants *A. palaestinum* showed significant activity against *T. tonsurans*, complete inhibition range is (37.5 - 50 µg/ml). From those results we can suggest that plant extracts investigated here could find practical application in the introduction of highly active and safe fungi toxicants in addition to the limited commercial antifungal drugs.

(Table: 2.3) Complete inhibition range of the plant extracts

Concentration range of µg/ml				
plant	Fungi			
	T. tonsurans	*M. canis*	*T. rubrum*	*T. violaceum*
Arum palaestinum	37.5-50	50-62.5	50-62.5	50-62.5
Urtica pilulifera	50-62.5	50-62.5	50-62.5	37.5-50
Coridothymus capitatus	37.5-50	25-37.5	25-37.5	12.5-25
Majorana syriaca	37.5-50	37.5-50	37.5-50	25-37.5
Teucrium creticum	50-62.5	50-62.5	50-62.5	37.5-50
Teucrium polium	50-62.5	50-62.5	37.5-50	37.5-50
Econazole	2.5-5	2.5-5	2.5-5	2.5-5

Two ways ANOVA carried out on percent inhibition data showed a significant ($P \leq 0.05$) effect for plant type and concentration on the percent inhibition. Results of Duncan Post Hoc test (Tables 2.5a, 2.5b) carried out on the same data showed that there is no significant ($P \leq 0.05$) effect between the plants in the same subset.

(Table: 2.4) Two way ANOVA analysis of percent inhibition due to plant type and concentration level.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	**75281.837(a)**	**29**	**2595.925**	**26.164**	**.000**

Intercept	**707834.881**	**1**	**707834.881**	**7134.208**	**.000**
Plant Type	**11689.171**	**5**	**2337.834**	**23.563**	**.000**
Concentration	**57550.949**	**4**	**14387.737**	**145.013**	**.000**
Plant Type * Concentration	**6041.717**	**20**	**302.086**	**3.045**	**.000**
Error	**8929.533**	**90**	**99.217**		
Total	**792046.250**	**120**			
Corrected Total	**84211.369**	**119**			

a R Squared = .894 (Adjusted R Squared = .860)

(Table: 2.5a) Duncan Post Hoc tests of percent inhibition to determine the most homogeneous subsets of Plant Type in different concentration levels.

Plant Type	N	Subset			
		1	2	3	4
T. creticum	20	63.815			
A. palaestinum	20	66.100			
U. pilulifera	20		74.560		
T. polium	20			81.835	
M. syriaca	20			82.130	
C. capitatus	20				92.375
Sig.		.470	1.000	.926	1.000

Means for groups in homogeneous subsets are displayed. Based on Type III Sum of Squares The error term is Mean Square(Error) = 99.217.
a Uses Harmonic Mean Sample Size = 20.000. , b Alpha = .05.

(Table: 2.5 b) Duncan Post Hoc tests of percent inhibition to determine the most homogeneous subsets of Concentration Level

Concentration Level	N	Subset for alpha = .05			
		1	2	3	4
12.5	24	40.037			
25.0	24		65.408		
37.5	24			83.504	
50.0	24				95.063
62.5	24				100.000
Sig.		1.000	1.000	1.000	.264

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3 Anticancer activity of selected plants in Palestine

2.3.1 Introduction

Cancer is a major health problem in the world. In many countries, cancer is the second leading cause of death after heart disease. The estimated worldwide incidence of different carcinomas is about 10 millions, half of these in developed countries [34, 35]. Approximately five decades of systemic drug discovery and development have established a respectable accumulation of useful chemotherapeutic agents [36, 37], as well as a number of important successes in the treatment and management of human cancer [38]. Populations with a high level of natural herbal product use have a reduced incidence of gastric cancer [39, 40], as exemplified by the low incidence of colon cancer in Asian countries with a high consumption of soybean products [41]. In the scientific literature numerous citations reference epidemiological studies that support significant differences in the occurrence of carcinoma between oriental and occidental populations [42, 43]. Soybeans are the major dietary source of saponins, which have been suggested as possible anticarcinogens [44]. Increased consumption of vegetables reduces the risk of colorectal cancer mortality [45]. From its very beginnings almost half a century ago cancer chemotherapy has faced dramatic problems. Lack of selectivity of conventional anticancer agents which damage not only malignant but also normal cells, in particular blood cells has made scientists aware of the need for more specifically selective drugs [46]. Another drawback, which arose just after cancer chemotherapy was started, was the appearance of drug-resistant cancer cells [47]. This in turn justifies the interest in search of possible anticancer agents from the flora of different countries, which are found in the market as "natural

products" [48]. Since there are no efficient synthetic drugs available in the pharmacopeias. For this reason, there is a great interest in screening plants used in traditional medicine. Various medicinal plant extracts claimed to be effective as anticancer agents, have been used since ancient times. However, often objective and scientific efficacy has not been shown [49]. Recently, the ability of medicinal plant extracts to control the proliferation of prostate cancer cells was reported [50, 51]. The extract of *Cernilton pollen* was found to be highly active in inhibiting the growth of DU145 cells, a prostate cancer cell line. One of the active constituents of this plant was later identified using HPLC as cyclic hydroxamic acid [52]. Oenothein B was identified as the active compound of *Epilbium parvijlorum*, a plant used for the treatment of prostate disorders, inhibits 5-oc reductase , also the 5-α reductase which converts testosterone to the more potent androgen, dihydroxytestosteron (DHT) in the prostate, was identified as the molecular target for many plant extracts [53].

Aims of study

The current study reports a screening program of six selected medicinal plants in Palestine, used in traditional medicine .Some of them have anticancer activity against prostate cancer (PC3) human carcinoma and breast cancer MCF-7 human carcinoma. These plants include four families namely Araceae (*A. palaestinum*), Urticaceae (*U. pilulifera*), Labiatae (*C. capitatus*, *M. syriaca*) and Lamiaceae (*T. polium*, *T. creticum*).

2.3.2 Materials and methods

1- Chemicals: Trypsin, RPMI 1640 culture medium, fatal calf serum, glutamine, amphotricine B, Hank's balanced solution, Trypan blue solution, penicillin and gentamicin. All other reagents are of analytical grade.

2- Types of cancer cell lines

There many different types of cancer known by the position of infection or by the mechanism of action [57].

1- Breast cancer cell lines: MCF-7 human carcinoma .This test was done in Jordan University.

2- Prostate cell line: The cell line under investigation was Prostate cell line: resistant PC3 human carcinoma. The cell line was obtained from the laboratory cell culture of Bir-Zeit University.

3- Cell culture

The cells were cultured in RPMI 1640 medium supplement with 10% heated fetal bovine serum, 1% of 2 mM l-glutamine, 50 IU/ml penicillin, 50 μg/ml amphotricine B. After checking for the absence of mycoplasms and bacteria, cell grown at 35°C as monolayer confluent cells in RPMI 1640 medium supplemented with 10% calf serum. To avoid cell membrane sensitization, no antibiotics were used. For the assay, cells were washed three times with phosphate buffer saline (PBS). PBS was decanted, cells detached with 0.025% Trypsin – EDTA and RPMI 1640 medium was added to make up a volume of 10 ml. The cell suspension was centrifuged at 1000xg for 10 minutes and the pellet was re-suspended in 10 ml medium to make a single cell suspension. Viability of cells was determined by Trypan blue exclusion and it exceeds 96% as counted in a haemocytometer. Stock cultures were duplicate weekly after

inoculation. The cell line was cultured in 6-well tissue culture plates (9.8 cm^2) and incubated at 35 °C in a humidified atmosphere containing 5% CO_2. After 24 hours the cells were treated with extracts or pure compounds. 0.1 ml of each extract or pure compound was diluted to a serial dilutions (500, 250, 125, 62.5 µg/ml).

2.3.3 Results and discussion

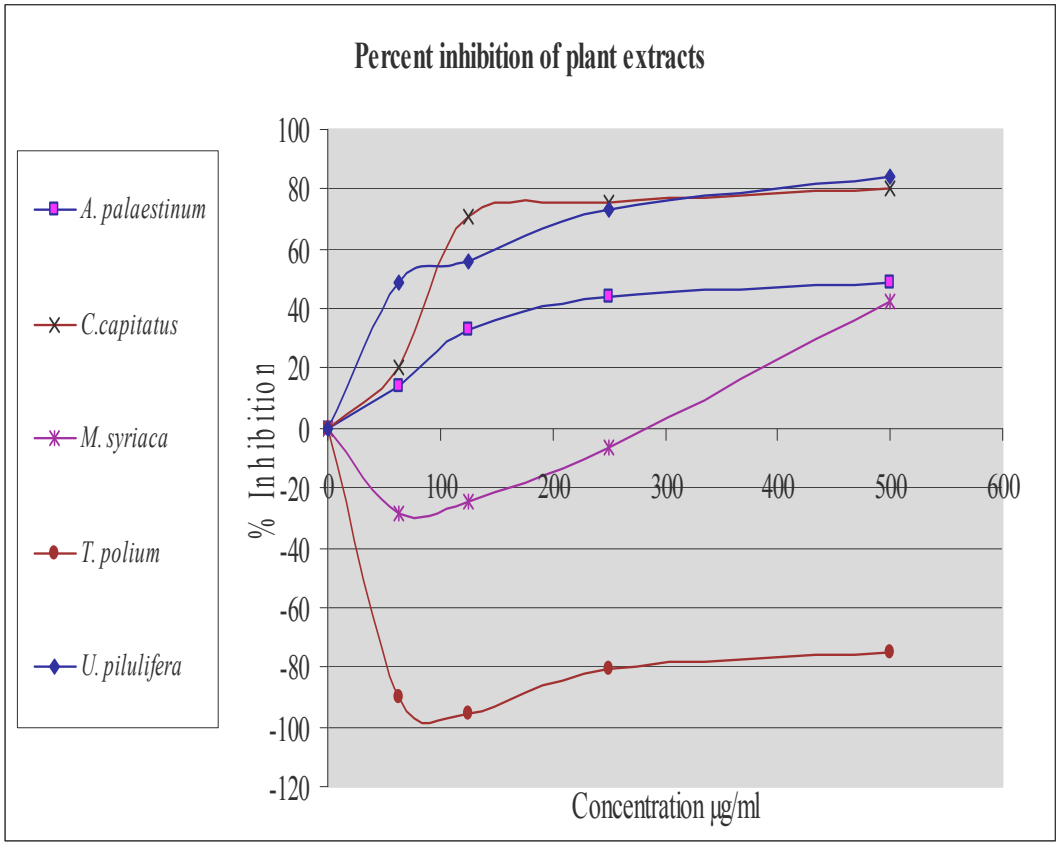
Laboratory experiments show the effect of the ethanolic extracts on breast cancer cells (MCF-7). Table 2.6 shows the percent inhibition and percent viability of each plant at different concentrations.

(**Table: 2.6)** Percent inhibition and percent viability of cancer cells for tested plants

No.	plant	Conc. *µg/ml*	*Absorbance*	*% inhibition*	*%viability*
1	*A. palaestinum*	62.5	0.52	-16	115
		125	0.42	7	93
		250	0.30	33	67
		500	0.25	44	56
2	*U. pilulifera*	62.5	0.23	49	51
		125	0.20	56	44
		250	0.12	73	27
		500	0.07	85	15
3	*C.capitatus*	62.5	0.36	20	80
		125	0.13	71	29
		250	0.11	76	24
		500	0.09	80	20
4	*M. syriaca*	62.5	0.58	-29	128
		125	0.56	-24	124
		250	0.48	-7	106
		500	0.26	42	58
5	*T. polium*	62.5	1.05	-80	200
		125	1.08	-85	190
		250	1.0	-71	180
		500	0.97	-65	175
control			0.45	100	

U. pilulifera showed the highest cytotoxicity plant against breast cancer, 85% of the cells are dead at the concentration of 500 µg/ml then *C.*

capitatus showed 80 % at the same concentration. From figure 2.6 IC_{50} for *U. pilulifera* was 63 µg/ ml and *C. capitatus was 100* µg/ ml. *A. palaestinum* and *M. syriaca showed* IC_{50} in the range between 500 µg/ ml and 600 µg/ ml, while *T. polium* did not exhibit any cytotoxicity against breast cancer as shown in (Figure 2.6).



(Figure: 2.6) Percent inhibition of plant extracts

2.4 References:

1- Vaidya D. B., Devasagayam P. A., (2007). ***Current status of herbal drugs in India: An overview*, J. Clin. Biochem. Nutr. , 41**, 1–11.

2- Ranjbar A., Khorami S., Safarabadi M., Shahmoradi A. , Malekirad A. Vakilian K., Mandegary A. , Abdolla M. (2006). ***Antioxidant activity of Iranian Echium amoenum Fisch & C. A. Mey flower decoction in humans: a cross sectional before / after clinical trial***, eCAM , **3(4)**, 469-473.

3- Ruiz-Terán F. , Medrano-Martínez A., Navarro-Ocaña A. (2008). ***Antioxidant and free radical scavenging activities of plant extracts used in traditional medicine***, **Mexico African Journal of Biotech. , 7 (12)**, 1886-1893.

4- Sies H., (1996). **Antioxidants in Disease, Mechanisms and Therapy**, New York: Academic Press.

5- Kelly S. A., Havrilla C. M., Brady T. C., Abramo K. H., Levin E. D. (1998). ***Oxidative stress in toxicology: established mammalian and emerging Piscine Model systems***, **Env. Hlth. Persp, 106**, 375–384.

6- Devasagayam T. P., Tilak J. C. , Boloor K. K., Sane K. S., Ghaskadbi S. S., Lele R. D. (2004). ***Free radicals and antioxidants in human health: Current status and future prospects***, **J. Assoc. Physicians India, 52**, 794–804.

7- Knight J. A. (2000). Review: ***Free radicals, antioxidants, and the immune system*****, Ann. Clin. Lab. Sci., 30**, 145–158.

8- De La Fuente M. , Victor V. M. (2007) . ***Antioxidants as modulators status of herbal drugs in India, J. Clin. Biochem. Nutr.*** , **41(1)**, 1–11.

9- Perry E. K., Pickering A. T., Wang W. W., Houghton P. J., Perru N. S. (1999). ***Medicinal plants and alzheimer's disease: from ethnobotany to phytotherapy*, J. Pharm. Pharmacol., 51**, 527-534.

10- Lin C. C., Huang P. C. (2002). **Antioxidant and hepatoprotective effects of acanthopanax senticosus, Phytother. Res., 14**, 489-494

11- Ali-Shatyeh M. S., Jamous R. M. (2008). **Traditional Arabic Palestinian Herbal Medicine.** Til, Nablus: Biodiversity &Environmental Research Center.

12- Shahidi F. (2008). ***Antioxidants: Extraction, identification, application and efficacy measurement*, EJEAF Che., 7(8)**, 3325-3330.

13- Shahidi F., Ho C.T. (2007). ***Antioxidants measurement and applications***, **American Chem. Soci., Symposium series ,956,** 2-7.

14- Burits M., Bucar F. (2000). ***Antioxidant activity of Nigella sativa essential oil.*** **Phytother. Res., 14,** 323–328.

15- Cuendet M., Hostettmann K., Potterat O. (1997). ***Iridoid glucosides with free radical scavenging properties from Fagraea blumei, Helv.*** **Chem. Ac., 80,** 1144-1152.

16- Gazzani G. A. ,Papetti G. M., Daglia M. (1998). ***Antioxidant and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment*, J. Agric. Food Chem., 46**, 4118-22.

17- Miller H. E. (1971). ***A simplified method for the evaluation of antioxidants,*** **J. Am. Oil Chem. Soc. ,48 ,** 92-97.

18- Frankel E. N. (1998). **Hydroperoxide formation. In Lipid oxidation.** Dundee:The Oily Press. (23-41)

19- Hussin N. M. , Muse R., Ahmad S. , Ramli J., Mahmood M. (2009). ***Antifungal activity of extracts and phenolic compounds from Barringtonia racemmosa L. (Lecythidaceace)***, **African Journal of biotechnology, 8(12)**, 2835-2842.

20 –Koshy P., Nurestri A., Wirakaranain S., Sim K., Saravana K.(2009). ***Antimicrobial activity of some medicinal plants from Malaysia*, Am. J. Appl. Sci., 6 (8)**, 1613-1617.

21- Martinez-Valverde I., Periago M. J., Provan G., Chesson A. (2002). ***Phenolic compounds, lycopene and antioxidant activity in commercial varieties of tomato (Lycopersicum esculentum),* J. Sci. Food Agr.** , **82**, 323–330;

22- El-Desouky S. K., Kim Ki Hun , Ryu Shi Young, Eweas A. F., Gamal – Eldeen A. M. (2007) ***A new pyrrol alkaloide isolated from Arum Palaestinum Boiss and its biological activities*, Arch. Pharm. Res.** , **30**, 927-931.

23- Ali-Shtayeh M. S., Yaniv Z. , Mahajna J. (2000). ***Ethanobotinical survey in the Palestinian area: Aclassification of the healing potential of medicinal plants***, **J. Ethanopharmacol. ,73,** 221-232.

24- Soković D., Vukojević J., Marin P., Brkić D., Vajs V. (2009). ***Chemical composition of essential oils of thymus and mentha species and their antifungal activities,* Molecules, 14**, 238-249

25- Silva F., Abraham A. (1981). ***The potentiality of the Israeli flora for medicinal purposes,*** **Fitoterapia, 52**, 195-200.

26- Ali-Shtayeh M. S., Yaghmour R. M., Faidi Y. R., Salem Kh. , Al-Nuri M. A. (1998). ***Antimicrobial activity of twenty plants used in Folkloric Medicine in Palestinian area***, **Journal of Ethnopharma, 60,** 265-271.

27 - Irshaid F., Mansi K. (2009). ***Effects of leaf extract of Artica Pilulifera on male reproductive system of streptozotocin-diabetic rats,*** **American J. Pharma. Toxic., 4**, 22-28.

28- Hadizadeh I., Peivastegan B., Hamzehzarghani H. (2009). ***Antifungal activity of essential oils from some medicinal plants of Iran against Alternaria alternate, Am. J. Appl. sci.*****, 6**, 857-861.

29- Ali-Shtayeh M. S., Jamous R. M, Al-Shafie J. H., Elgharabah W. A. (2008) ***Traditional knowledge of wild edible plants used in Palestine (Northern West Bank): A comparative study,*** **J. Ethnobio. Ethnomed., 4**,13.

30- Bhaskara M. V., Angers P., Gosseline A., Arul J. (1998). ***Characterization and use of essential oil from Thymus vulgaris against Botrytis cinerea and Rhizopus stolonnifer in strawberry fruits,*** **Phytochem., 47,** 1515- 1520.

31- Tabata M., Honda G., Sezik E. (1988). **A report on traditional medicine and medicinal plants in Turkey.** Faculty of harmaceutical ciences, Kyoto University.

32- Derwich E., Benziane Z., Taouil R., Senhaji O. ,Touzani M. (2010). ***Comparative Essential oil Composition of Leaves of Mentha rotundifolia***

and Mentha pulegium, a Traditional Herbal Medicine in Morocco, **Amn-Euras. J. Sust. Agric., 4(1)**, 47-54.

33- Ljubuncic P., Dakwar S., Portnaya I., Cogan U., Azaizeh H., Bomazen A. (2006). ***Aqueous extract of Teucrium polium possess remarkable antioxidant activity in vetro*****, eCAM, 3(3),** 329-338.

34- Abu-Dahab R. , Afifi, F. (2007). ***Antiproliferative activity of selected medicinal plants of Jordan against breast adenocarcinoma cell line (MCF7), Scientia Pharmaceutica. (Sci. Pharm.) ,75,*** 121-136.

35- Figueroa-Hernández1 J. L., Sandoval González1 G., Jayme Ascencio V., Figueroa-Espitia J. L. , Fernández Saavedra G. (2005). ***Plant products with anti-cancer properties employed in the treatment of Bowel Cancer: Literature review 1985 and 2004,*** **Proc. West. Pharmacol. Soc., 48,** 77-83

36- Dennis R.A. , Darocha A. B. , Schwartsmann G. (2000). Anti-Cancer drug discovery and development in Brazil: ***Targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds***, **The Oncologist , 5,** 185-189

37- Chabner B. A. (1991). Anti-cancer drugs, In: DeVita VT Jr, Hellman S, Rosenberg AS,eds. **Cancer, Principles and Practice**, Philadelphia: Lippincott, 325-417.

38- Jessup J. M., McGinnis L. S., Winchester D. P. (1996). ***Clinical highlights from the National Cancer Database***, **CA Cancer J. Clin., 46**, 185-187.

39- Frantz D. J., Hughes B. G., Nelson D. R., Murria B. K. Christensen M. J. (2000). ***Cell cycle arrest and differential gene expression in HT-29 cells exposed to an aqueous garlic extract,*** **Nutr. Cancer, 38(2)**, 255.

40- Deenehy C. E. ,Tsourounis C. Botánicos ("hierbas medicinales") y suplementos nutrimentales, (ed) Katzung, BG. (2002). **Farmacología básica y clínica. México,** D.F.

41- Zhu Q., Meisinger J., Van Thiel D. H., Zhang Y. , Mobarhan S. (2002). ***Effects of soybean extract on morphology and survival of Caco-2, SW620, and HT-29 cells,*** **Nutr. Cancer ,42,** 131.

42- Hudson E. A., Dinh P. A., Kokubun T., Simmonds M. S., Gescher A. (2000). ***Cancer Epidemiol. ,*** **Biomarkers Prev., 9(11)** , 63.

43- Siergers C. P., Steffen B., Robke A., Pentz R. (1999). **Phytomedicine 6(1), 7.**

44- Oh Y. J. , Sung M. K. (2001). ***Soybean saponins inhibit cell proliferation by suppressing PKC activation and induce differentiation of HT-29 human colon adenocarcinoma cells,*** **Nutr. Cancer, 39(1),** 132

45- Rijken P. J., Timmer W.G., van de Kooij A. J., Van Benschop I. M., Wiseman S. A., Meijers M., Tijburg L.B. (1999). ***Carcinogenesis 20(12), 2267.***

46-Pisha E., Chai H., Lee I. S., Chagwedera T. E., Farnsworth N. R., (1995). ***Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis*****, Nat. Med., 1**, 1046-1051

47- Gottesman M. M. (1993). ***How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation award lecture***, **Cancer Res., 53**, 747-754.

48- Madhuri S., Pandey G.,(2009). ***Some anticancer medicinal plants of foreign origin,*** **Current Sci., 96 (6),** 779-783.

49- Bauer B. A. (2000). **Mayo Clin. Proc. ,75 (8)**, 835.

50- Hryb D. J., Khan M., Romas N., Rosner W. (1995). ***The effect of extracts of the roots of the stinging nettle (Jrtica dioicd) on the inter action of SHBG with its receptor on human prostatic membranes*****, Planta. Med., 61,** 32.

51- Hiremath S., Badami S., Swamy H., Patil S., Londonkar R. (1997). ***Anti-androgenic effect of Striga orobanchioides***. **Journal of Ethnopharma., 56,** 55-60.

52- Habib F., Ross M., Lewenstein A., Zhang X., Jaton J. (1995). ***Identification of a prostate inhibitory substance in pollen extract,*** **Prostate, 26,** 133-139.

53- Evans B., Griffiths K., Morton M. (1995). ***Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue bydietary lignans and isoflavonoids,*** **Journal of Ethnopharma., 747,** 295-302.

54-Medscape (2000). Prostate cancer. WWW.CBSHealthWatch.com.

55- Barinaga M. (1997). ***From bench to bedside,*** **Sci., 278**, 1036-1039.www.sciencemag.org.

56- Riss T., Moravec R. (1993). ***Comparison of MTT, XTT, and a novel tetrazolium compound MTS for in vitro proliferation and chemosensitivity assays,*** **Biomedical Products, 1 8,** 66-68.

57- Beljanski M. (2000). ***The anticancer agent PB-l00, selectively on malignant cells, inhibits multiplication of sixteen active malignant cell lines, even multidrug resistant,*** **Genetics and Molecular Biology, 23, 1**, 29-33.

Chapter Three
Phytochemistry

3.1 Introduction

Phenols and polyphenols, existing ubiquitously in nature, are commonly used as food additives and folk medicine in many countries. Interest in phenols and polyphenols has increased because many of them exhibit a broad spectrum of biological activities including anti-inflammatory, antiviral, antiatherogenic, antibacterial, as well as anticancer effects[1].These activities are associated, to a great extent, to their antioxidant properties, though different mechanisms may be involved. Derivatives of hydroxybenzoic acids such as salicylic acid and phenyl esters have been identified as one of the major active components of many plants. It has been shown that the antioxidative activity of phenolic compounds is determined by their molecular structure, and the position of hydroxyl group [2, 3] for instance, the ability of the delocalization of unpaired electrons to stabilize the formed radical after reaction with the initiator radical will be influenced [4]. Previous studies by many research groups demonstrated that the catechol moiety, with the 3, 4-dihydroxyl configuration, is important for the free radical scavenging activity for this type of phenolic compounds [5, 6]. Contrary to this, other studies suggest that the structure is not required for the activity [7]. Thus, it is worthy to compare the biological activities of four synthesized phenolic acid esters with different hydroxyl positions on the benzoic acid moiety beginning with the constituents separated from the selected medicinal plants as starting materials. Phenolic compounds absorb in the UV region and the most commonly used detector for HPLC is a variable-wavelength UV or

UV-vis detector [8, 9]. No single wavelength is ideal for monitoring all classes of phenolics since they display absorbance maxima at different wavelengths [10]. Most benzoic acid derivatives display their maxima at 246–262 nm, except for gallic acid and syringic acid which have absorption maxima at 271 and 275 nm, respectively [11]. Hydroxycinnamic acids absorb in two UV regions, one maximum being in the range of 225–235 nm and the other in the range of 290–330 nm [12]. At 320 nm, cinnamic acid derivatives can be detected without any interference from benzoic acid derivatives, which have a higher absorptivity at 254 nm. However, detection at 280 nm is the best alternative for the determination of both classes of phenolic compounds [13].

Objectives of study

1- Evaluate the amounts of phenolics and flavonoids in the ethanolic extracts of the selected plants as micrograms equivalent to quercetin and pyrocatechol.

2- Identify some of the constituents in plant's extracts using HPLC.

3- Modify some of the constituents separated from those plants.

4- Determine the biological activity of the modified compounds.

3.2 Materials and methods

3.2.1 Identification of some constituents from the selected plants.

Sampling:

Diluted samples (10 µl) of the ethanolic extracts of each plant were injected in the GC-MS spectrophotometer.

Apparatus:

The device "Perkin Elmer clarus 500 Gas chromatography" and "Perkin Elmer clarus 560D mass spectrometer" were used in the analysis.

Conditions:

He gas, temperature of the injector = 280 ºC, carrier was 1ml/ min and total flow was 60ml/min. Column was Elite – 5MS, 30 meter 0.25 µm df. Max projection at temperature 360 ºC, and minimum bleed at 320 ºC.

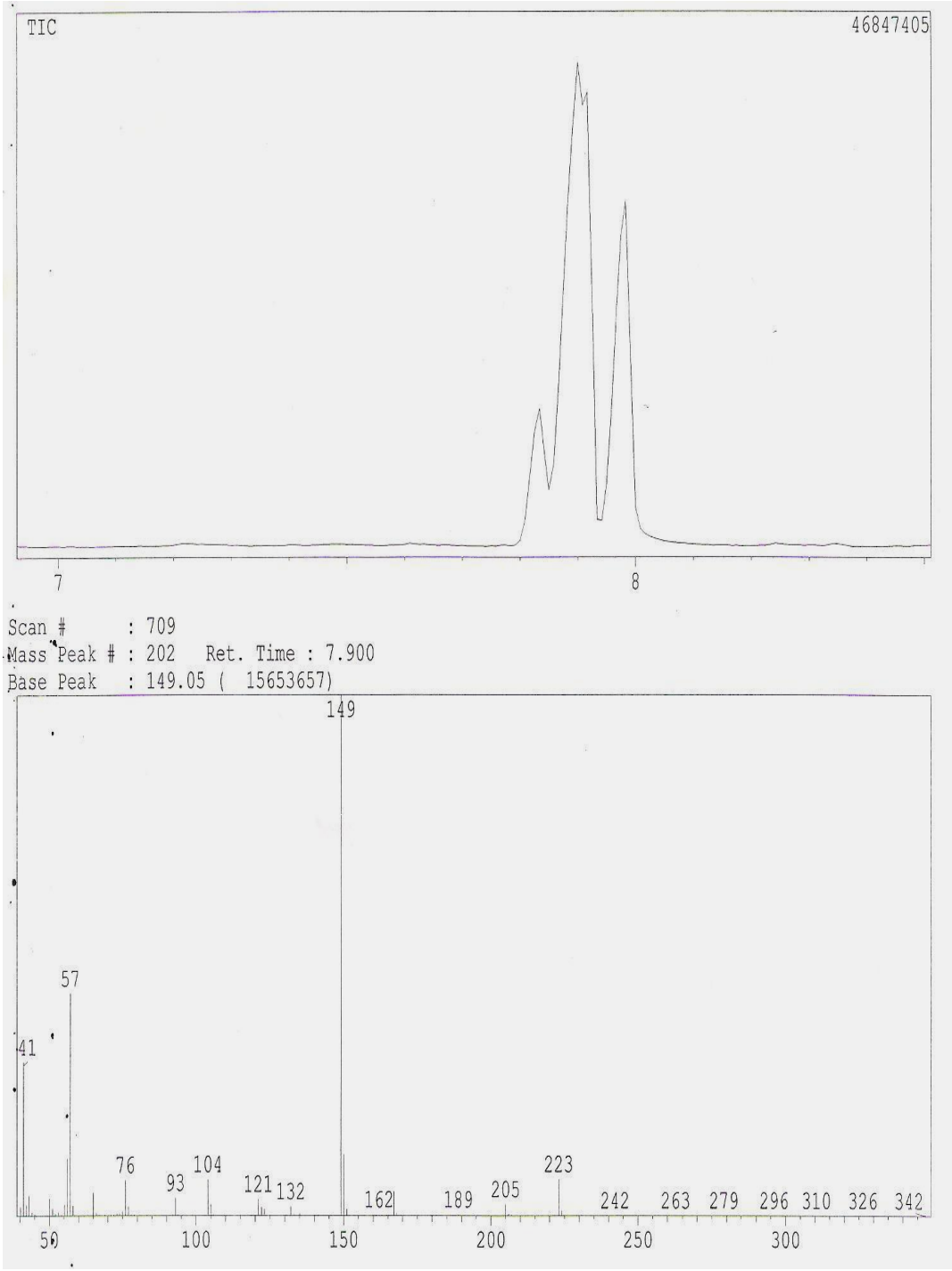
The experiment was done as follows:

Temperature (ºC)	Time (min.)	Rate (10/min.)
60 (beginning)	15	10
110	5	10
200	5	10
280	5	

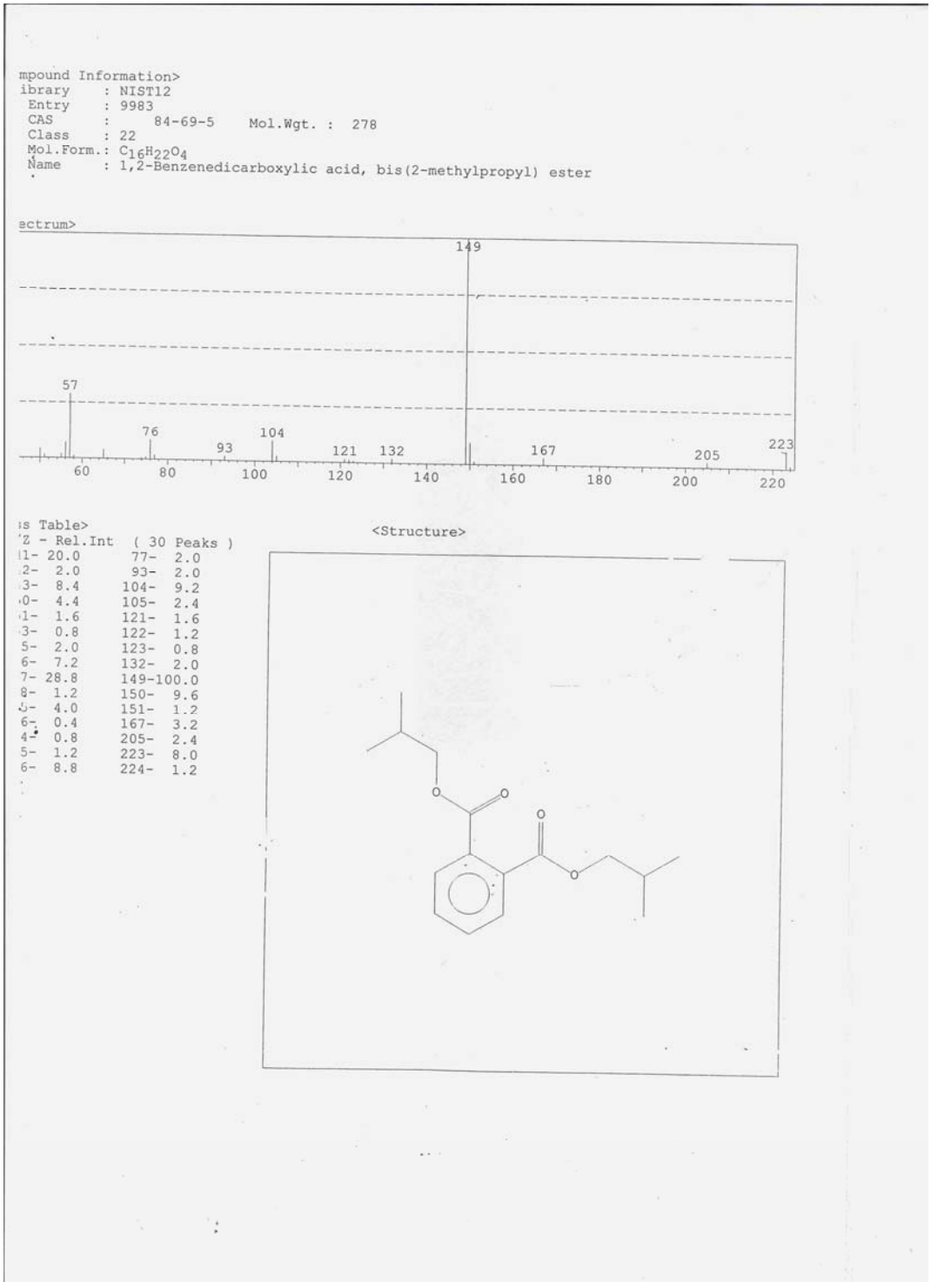
A few compounds were identified by GC-MS as shown in (Table 3.1), depending on the total ionic concentration values (TIC) and mass spectrum for each, in addition to many compounds were detected but not identified.

(Table: 3.1) Compounds identified from the selected plants by GC-MS

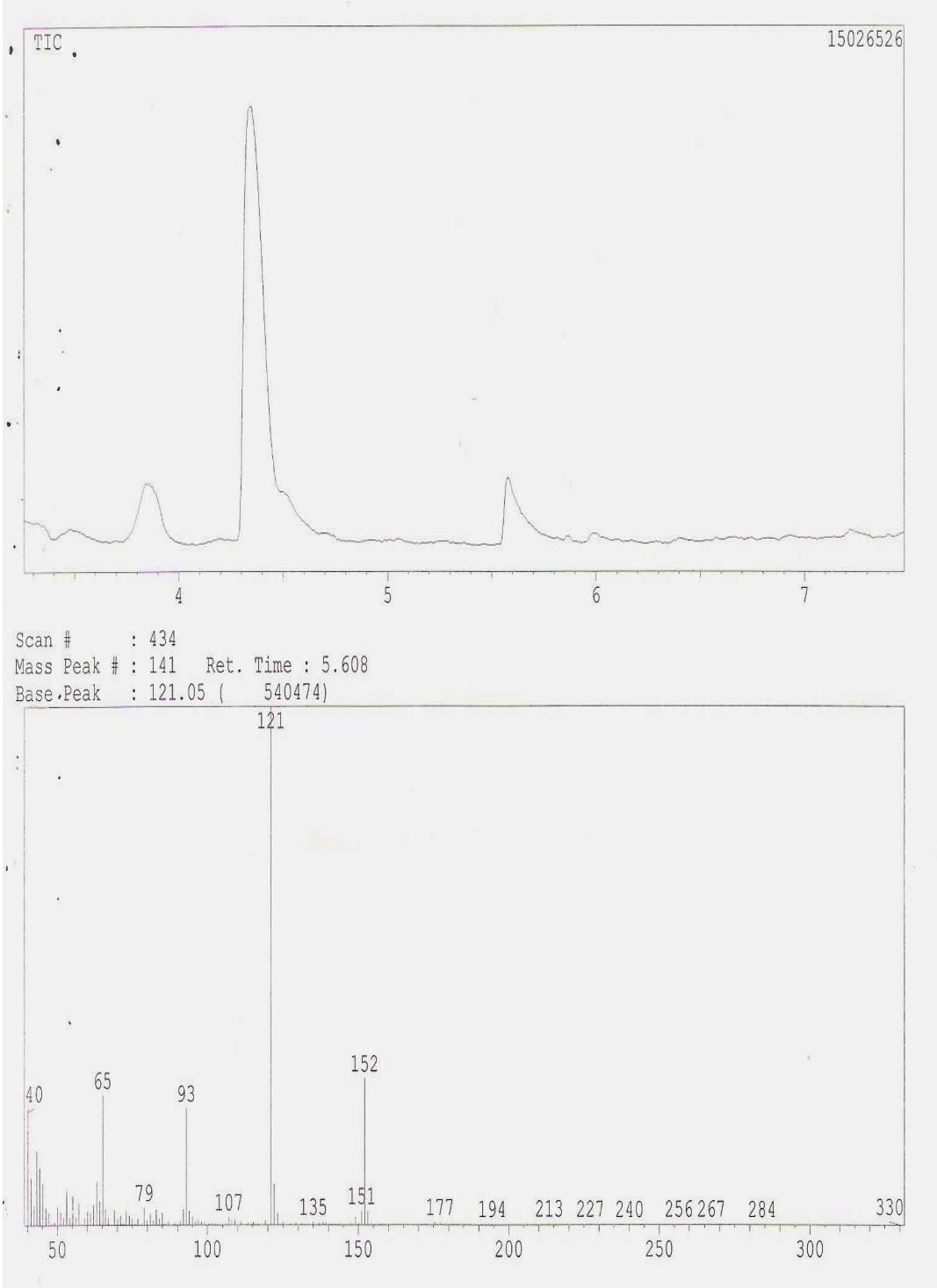
No	Plant name	Compound	Figure no.
1	*A. palaestinum*	diisobutyl phthalate	3.1a, 3.1b
2	*A. palaestinum*	Methyl 3-hydroxy benzoate	3.2a, 3.2b
3	*A. palaestinum*	Di-n-octyl phthalate	3.3a, 3.3b
4	*A. palaestinum*	4H-pyran-4-one 2,3 dihydro-3,5-dihydroxy -6-methyl	3.4a, 3.4b
5	*U. pilulifera*	2-phenoxy ethanol	3.5a, 3.5b
6	*C. capitatus*	Thymol	3.6a, 3.6b
7	*M. syriaca*	Thymol	3.6a, 3.6b



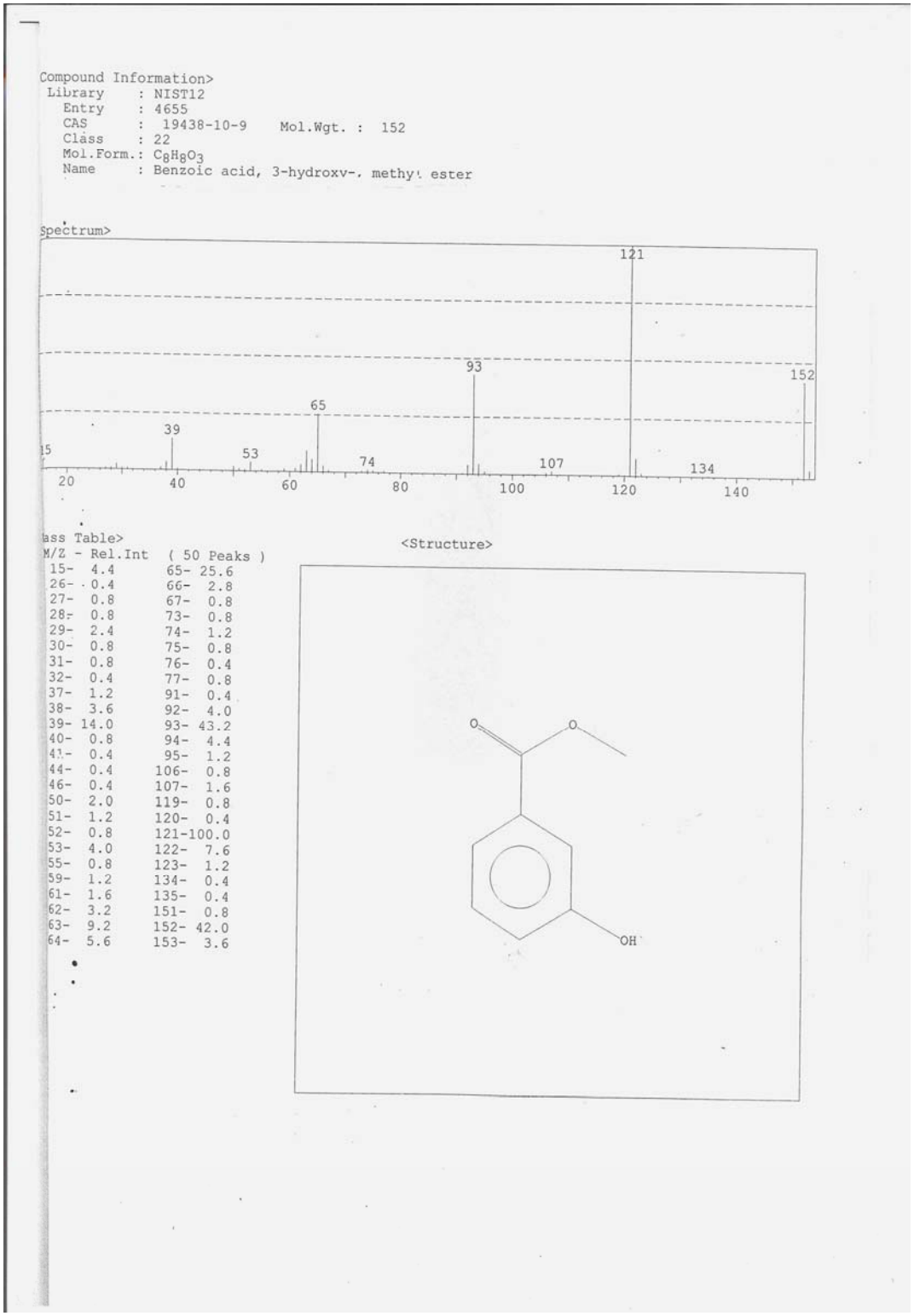
(Figure: 3.1a) TIC values for diisobutyl phthalate



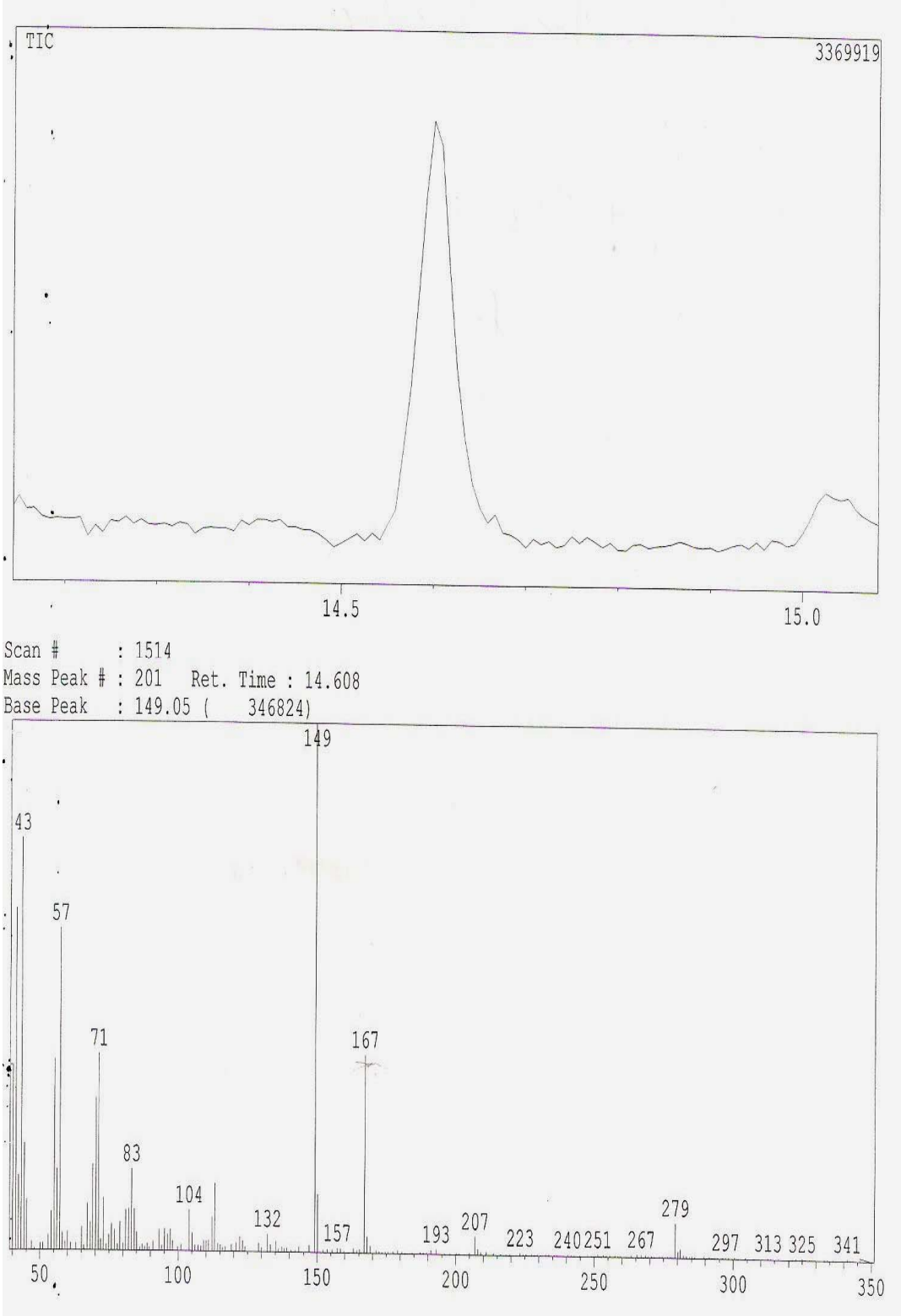
(Figure: 3.1 b) Mass spectrum of diisobutyl phthalate.



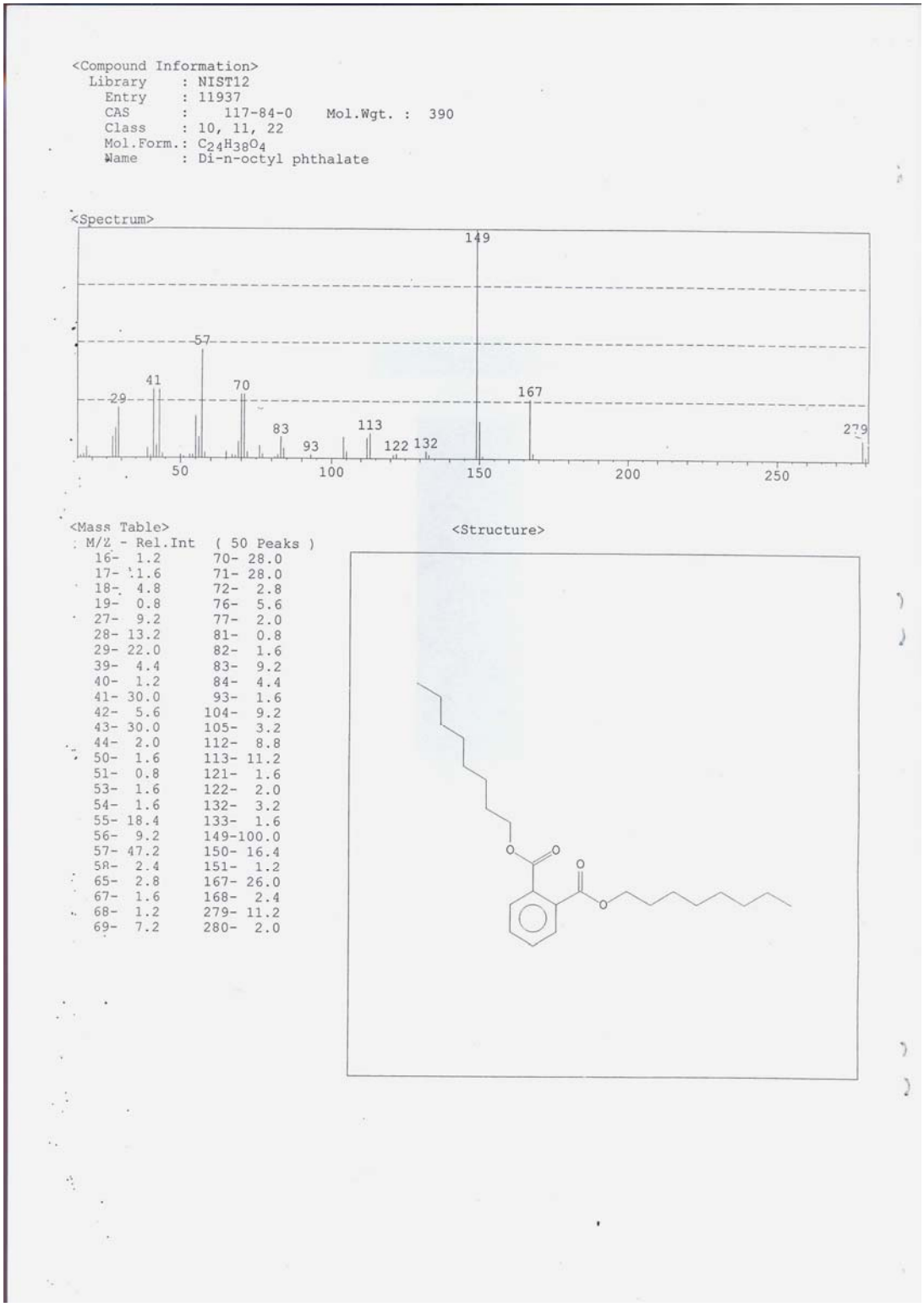
(Figure: 3.2 a) TIC values for Methyl 3-hydroxy benzoate



(Figure: 3.2 b) Mass spectrum of Methyl 3-hydroxy benzoate



(Figure: 3.3 a) TIC values for Di-n-octyl phthalate



<Compound Information>
Library : NIST12
Entry : 11937
CAS : 117-84-0 Mol.Wgt. : 390
Class : 10, 11, 22
Mol.Form.: $C_{24}H_{38}O_4$
Name : Di-n-octyl phthalate

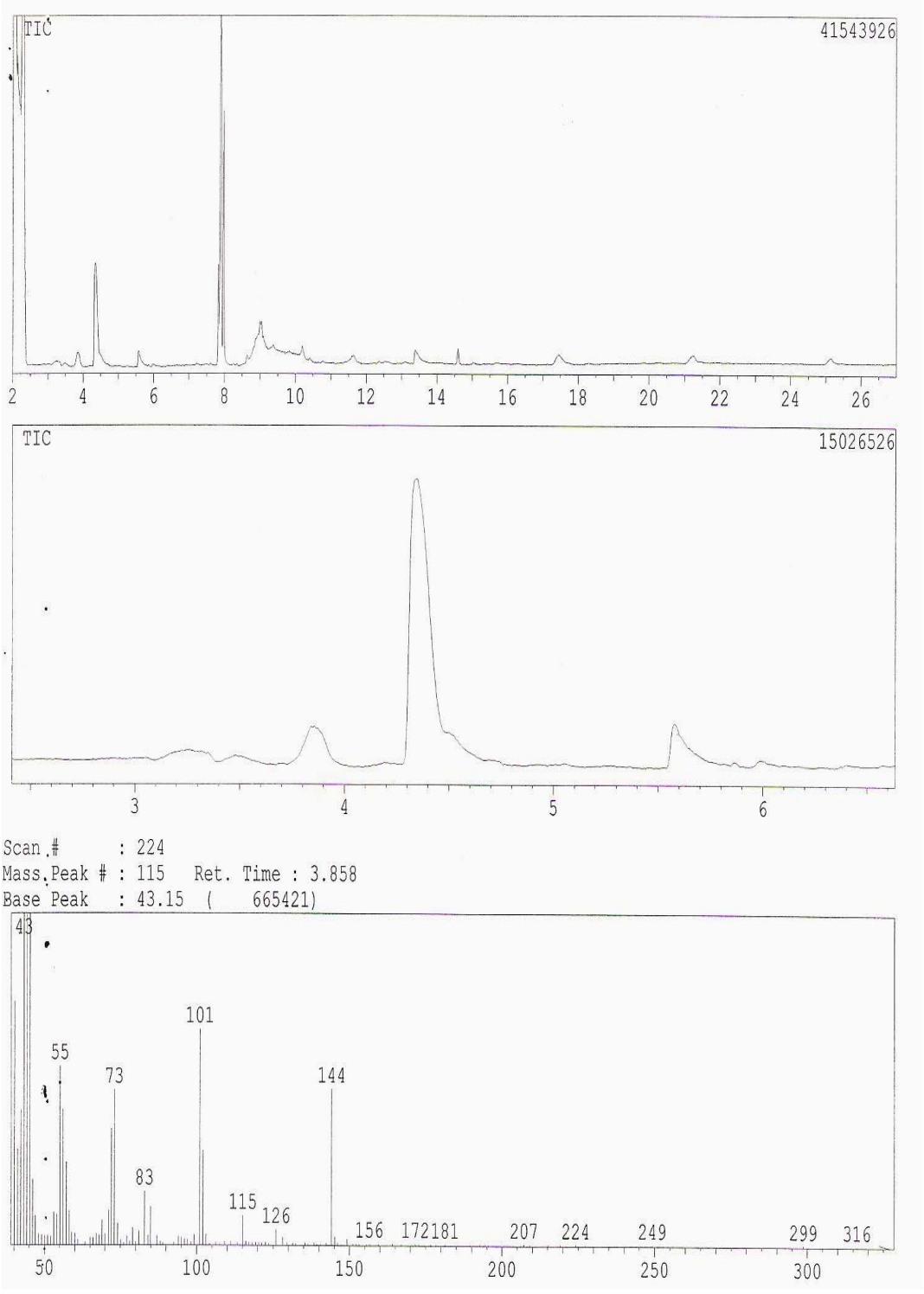
<Spectrum>

<Mass Table>

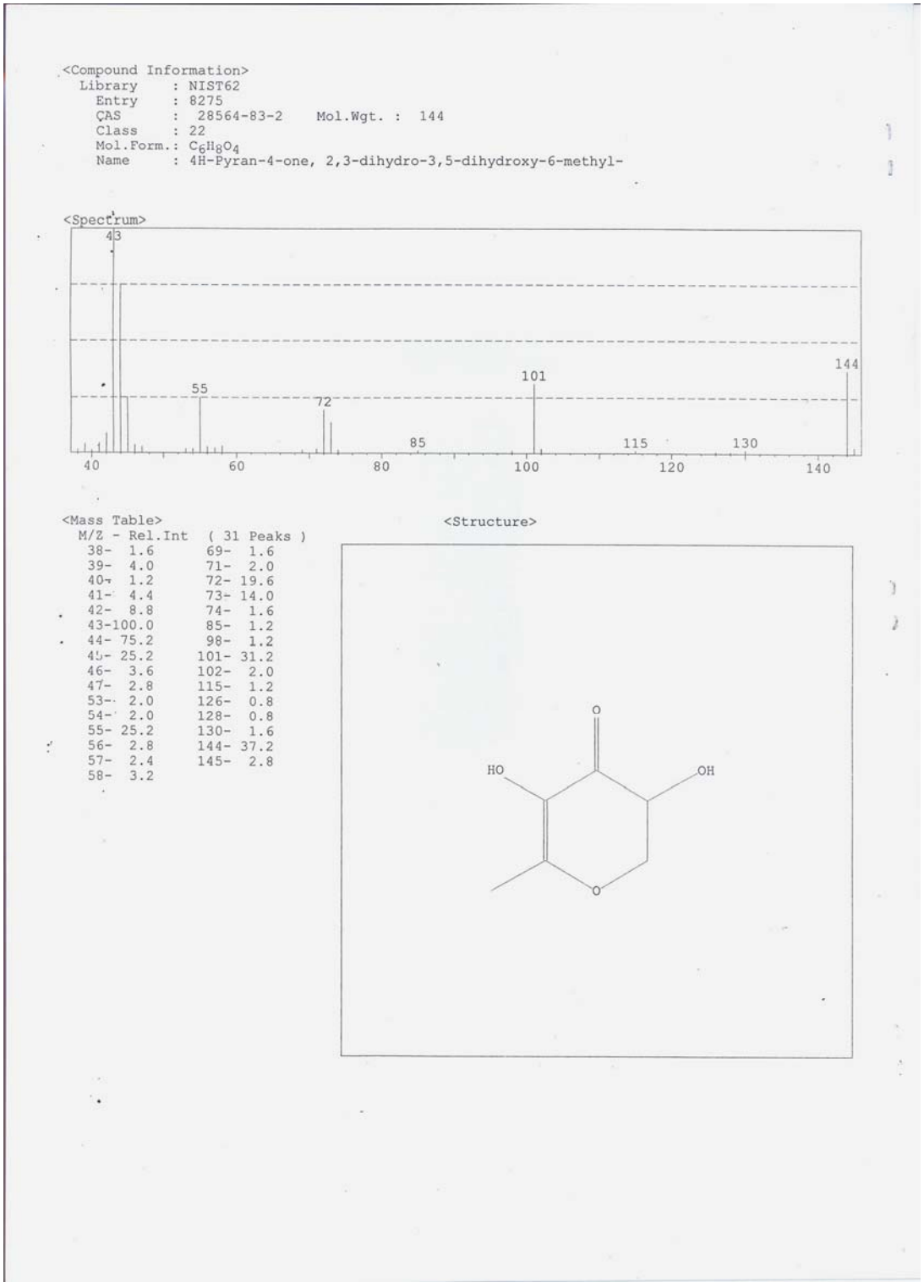
M/Z	Rel.Int	M/Z	Rel.Int
16-	1.2	70-	28.0
17-	1.6	71-	28.0
18-	4.8	72-	2.8
19-	0.8	76-	5.6
27-	9.2	77-	2.0
28-	13.2	81-	0.8
29-	22.0	82-	1.6
39-	4.4	83-	9.2
40-	1.2	84-	4.4
41-	30.0	93-	1.6
42-	5.6	104-	9.2
43-	30.0	105-	3.2
44-	2.0	112-	8.8
50-	1.6	113-	11.2
51-	0.8	121-	1.6
53-	1.6	122-	2.0
54-	1.6	132-	3.2
55-	18.4	133-	1.6
56-	9.2	149-	100.0
57-	47.2	150-	16.4
58-	2.4	151-	1.2
65-	2.8	167-	26.0
67-	1.6	168-	2.4
68-	1.2	279-	11.2
69-	7.2	280-	2.0

<Structure>

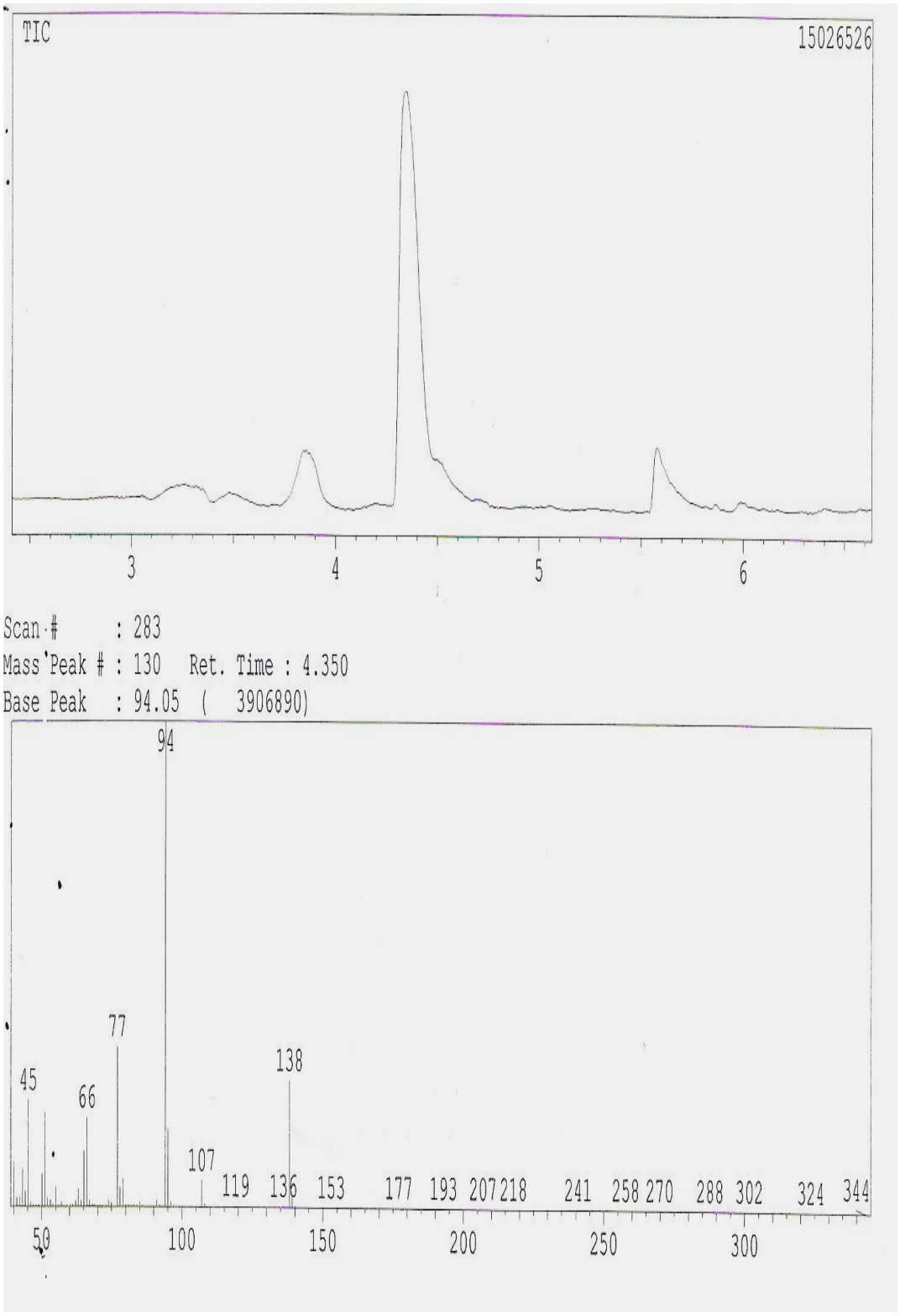
(Figure: 3.3 b) Mass spectrum of Di-n-octyl phthalate



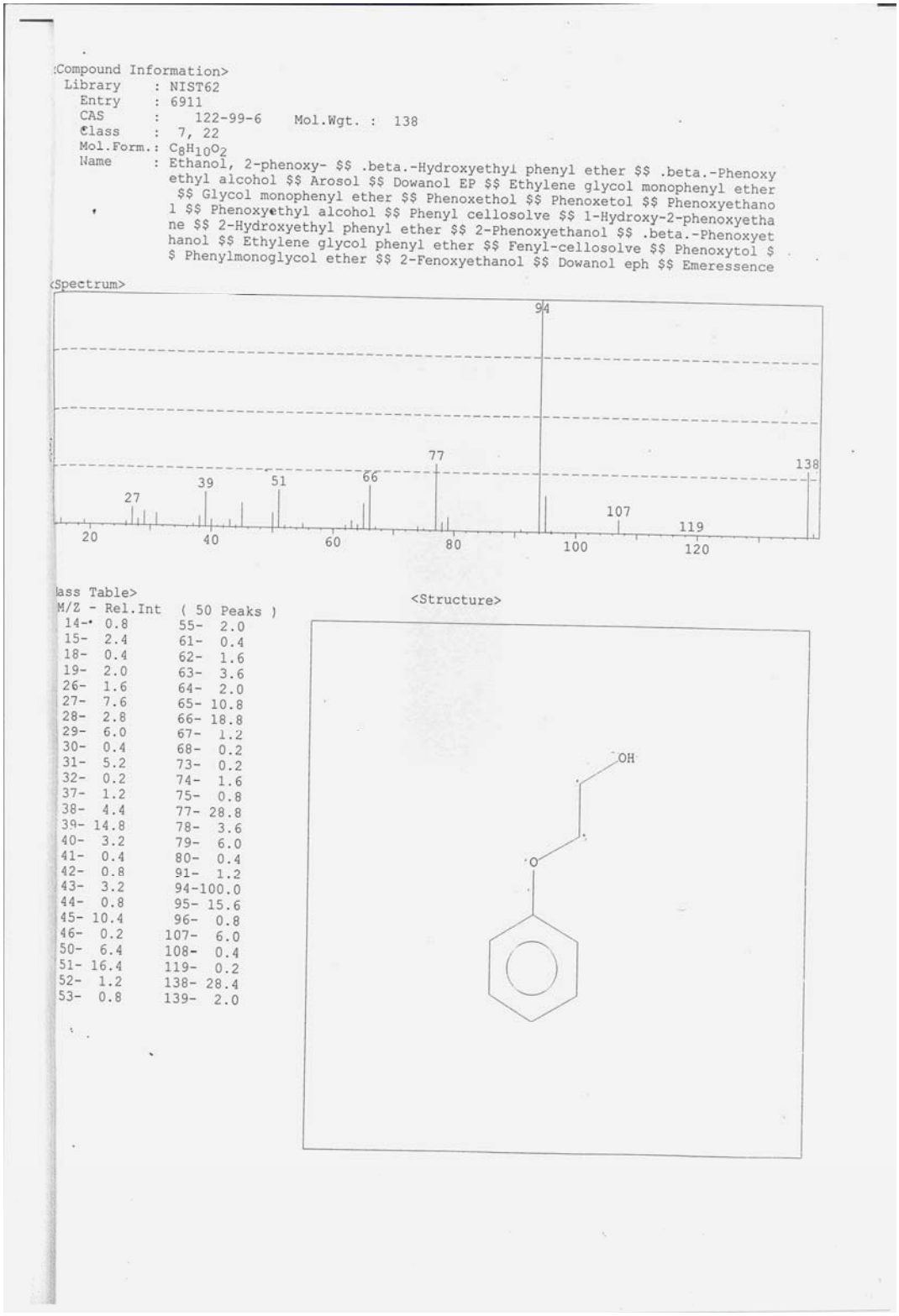
(Figure: 3.4 a) TIC values for 4H-pyran-4-one 2, 3 dihydro-3, 5-dihydroxy -6-methyl



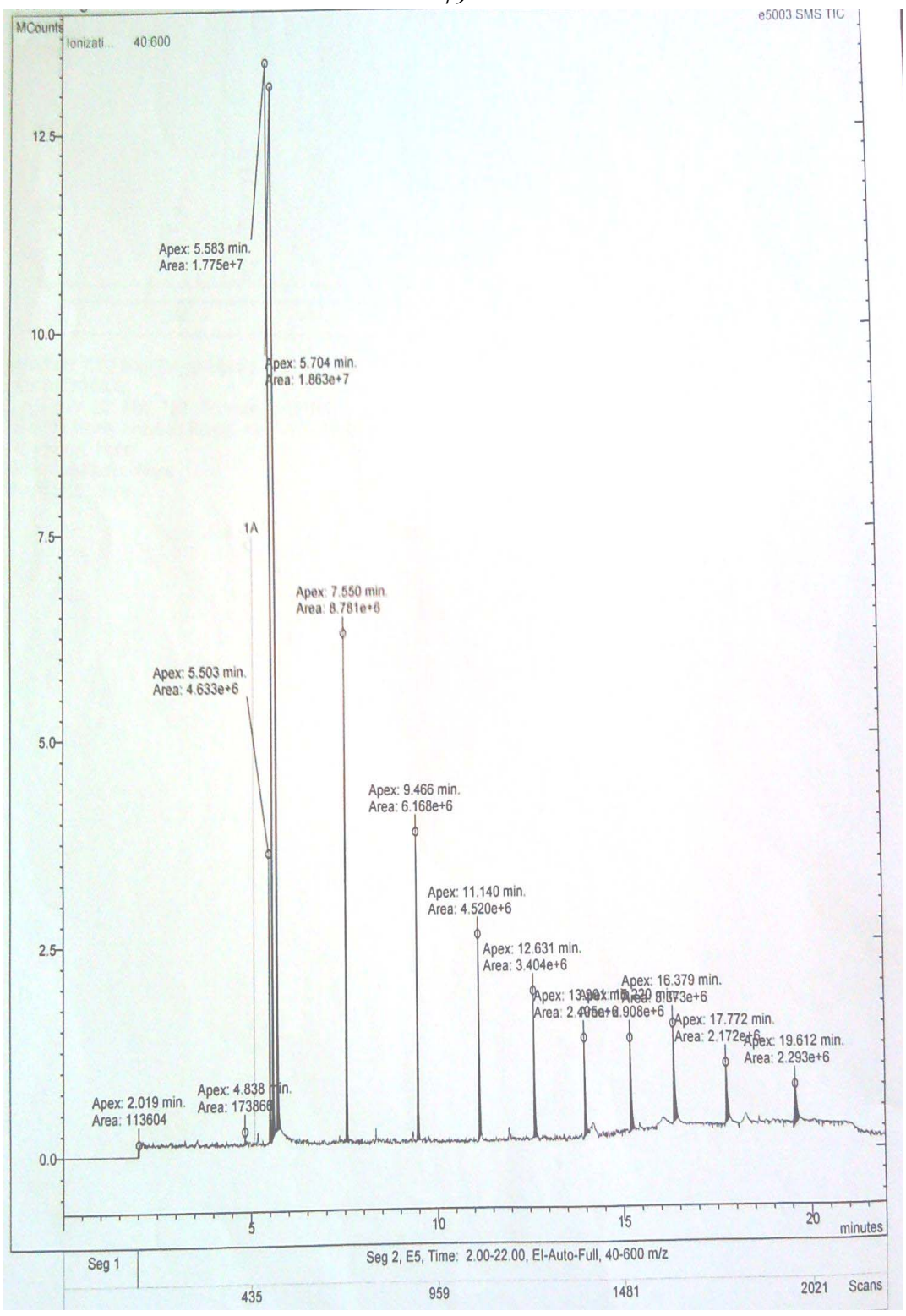
(Figure: 3.4 b) Mass spectrum of 4H-pyran-4-one 2, 3 dihydro-3, 5-dihydroxy -6-methyl



(Figure: 3.5 a) TIC values for 2-Phenoxyethanol.



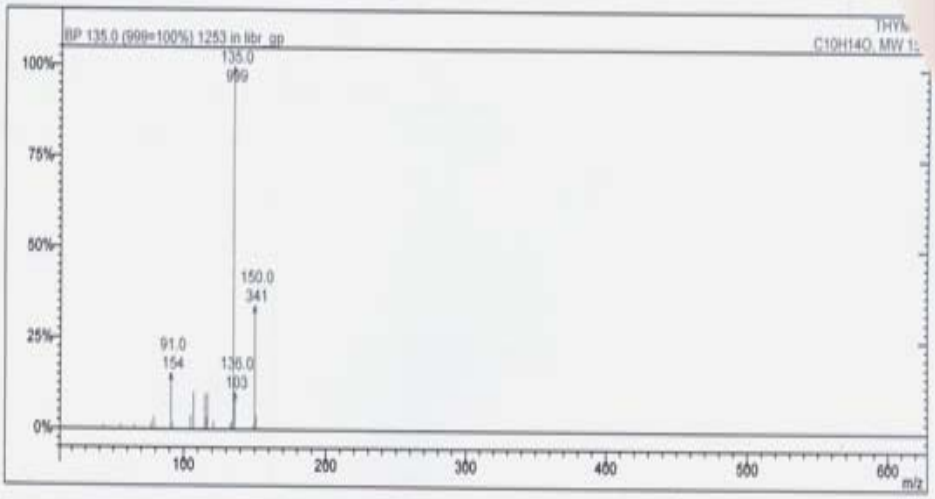
(Figure: 3.5 b) Mass spectrum of 2-Phenoxyethanol.



(Figure: 3.6 a) TIC values for Thymol

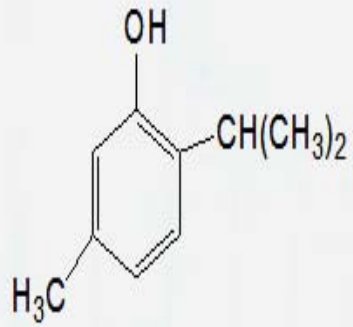
Print Date: 11 Mar 2009 11:38:23

Entry 1253 from libr_gp NIST Library



Spectrum 1253 from libr_gp Library
Name: THYMOL
Pair Count: 20 MW: 150 Formula: C10H14O
CAS No: None Acquired Range: 43.0 - 151.0 m/z
Synonyms: None
Other Databases: None
Replicates: None

Ion	Int	Norm	Ion	Int	Norm	Ion	Int	Norm
43.0	10	10	105.0	37	37	134.0	20	20
55.0	11	11	107.0	101	101	135.0	999	999
65.0	12	12	115.0	94	94	136.0	103	103
77.0	18	18	116.0	30	30	149.0	12	12
79.0	34	34	117.0	100	100	150.0	341	341
91.0	154	154	121.0	22	22	151.0	36	36
92.0	19	19	133.0	15	15			

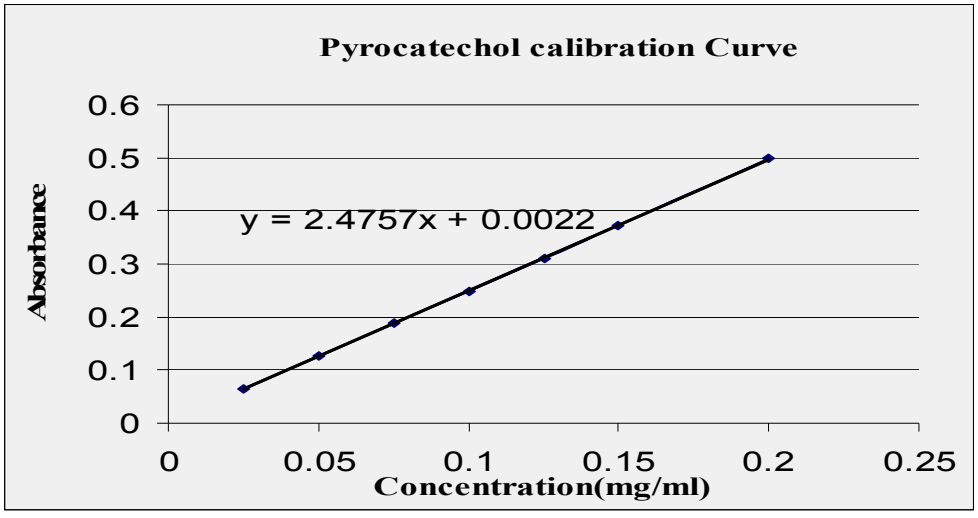


(Figure 3.6 b) Mass spectrum of Thymol

3.2.2 Determination of total phenolic compounds

Total soluble phenolics in the ethanolic extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard using pyrocatechol as a standard [14, 15]. Briefly, 1 ml from extract solution (2 mg/ml) was transferred into a volumetric flask of 50 ml, and made up to 46 ml with distilled water. Folin-Ciocalteu reagent (1 ml) was added and the contents of flask were mixed thoroughly. After 3 min, 3 ml of 2% aqueous solution of sodium carbonate (Na_2CO_3) was added, then the mixture was allowed to stand for 2h with intermittent shaking. The absorbance was measured at 760 nm (Figure 3.7). The concentration of total phenolic compounds in the ethanolic extracts was determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol calibration curve is given as:

Absorbance = 0.00 4757 µg pyrocatechol + 0.0022 (R^2: 0.9997)

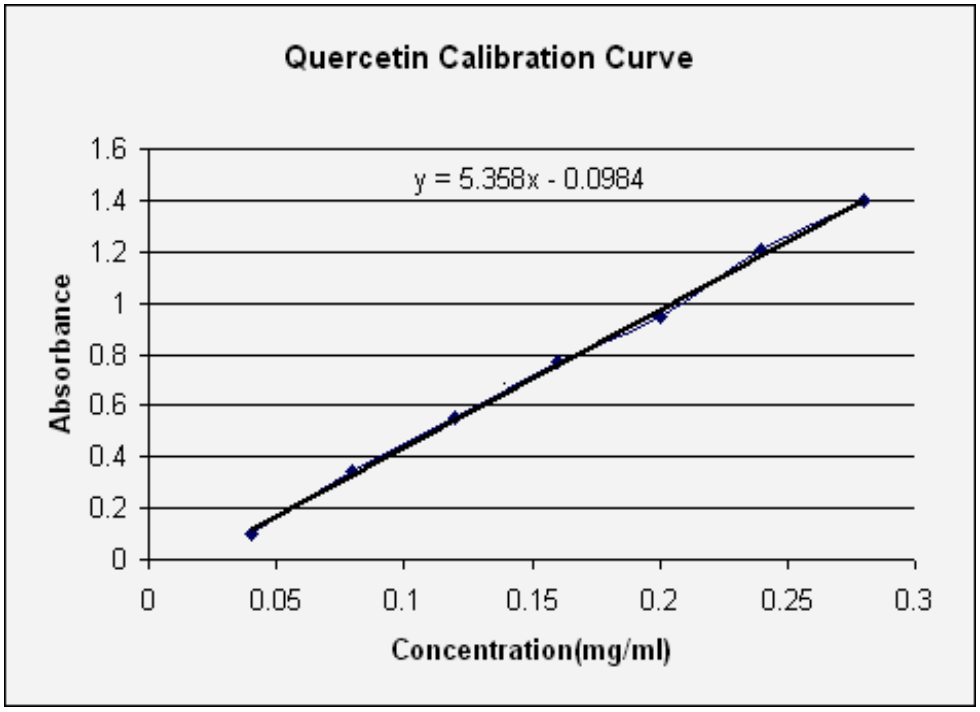


(Figure: 3.7) standard pyrocatechol calibration graph

3.2.3 Determination of total flavonoid compounds

Flavonoid concentration was determined as follows: From each ethanolic extract solution (1 milliter) was diluted with 4.3 ml of 80% aqueous ethanol and to the test tubes were added 0.1 ml of 10% aluminum nitrate $Al(NO_3)_3$ and 0.1 ml of 1 M aqueous potassium acetate (CH_3COOK). After 40 min at room temperature, the absorbance was determined spectrophotometerically at 415 nm (Figure 3.8) .Total flavonoid concentration was calculated using quercetin as standard [16].

Absorbance = 0.005358 µg quercetin – 0.0984 (R^2: 0.9994)



(Figure: 3.8) Standard quercetin graph.

(Table: 3.2) Total flavonoid and phenolic compounds in the ethanolic extracts determined as µg/mg of quercetin and pyrocatechol equivalents, subsequently.

No	Plant name	Total flavonoids (concentration µg/mg)	Total phenolics (concentration µg/mg)
1	A. palaestinum	270	3.9
2	U. pilulifera	213	120.6
3	C. capitatus	160.2	103.5
4	M. syriaca	146.4	37.3
5	T. creticum	154.2	35.2
6	T. polium	203.5	30.8

3.2.4 Determination of total phenolics and flavonoids using HPLC

HPLC methodologies represent, to date, the most widely used approach to the analysis of phenolics [17]. In most cases, HPLC techniques allow flavonoid profiles in plant extracts to be obtained without the need for preliminary derivatization and sample preparation. Reversed-phase chromatography has been extensively employed for the separation of flavonoids on C8 or C18 columns [18, 19, 20] with polar mobile phases, such as methanol, acetonitrile, tetrahydrofuran or acid solutions [21]. Gradient elution has often been used to obtain the profile of separated flavonoids [17, 22]. Under normal reversed-phase conditions, the more polar compounds are generally eluted first. The classes of flavonoids that characterize citrus species (flavanones, flavones, and, to a lesser extent flavonols/flavanols) have their maximum absorption at specific wavelength ranges: flavanones (280- 290 nm), flavones (304-350 nm) and flavonols (352-385 nm). Moreover, at 325 nm flavones show an absorption peak, which is similar to the corresponding peak at 280 nm.

3.2.5 Identification of phenolic compounds and flavonoid compounds using HPLC analysis

Plant extracts used in this study were tested for the presence of phenolic compounds and flavonoids according to the retention time of calibration curve standards (Table 3.3).

(Table:3.3) Phenolic compounds and flavonoids detected by HPLC according to the Retention time

No		Ret. Time (min)	Plants					
			A. palaestinum	*U. pilulifera*	*C. capitatus*	*M. syriaca*	*T. creticum*	*T. polium*
1	Benzoic acid	10.5	present					
2	Salicylic acid	2.9	present		present	Present	present	
3	Cinnamic acid	18.8	present					
4	Quercetin	22.8						
5	Pyrocatechol	4.4		Present				present
6	Diisobutyl phthalate	3.5	present					
7	Ascorbic acid	*N.D						
8	Resorcinol	3.1						
9	Thymol	9.8			present	Present		
10	Phthalic acid	3.3	present				present	
11	Sorbic acid	1.8					present	
12	2-Phenoxy ethanol	21.1		present				
13	Methyl 3-hydroxy benzoate	19						

*Not determined

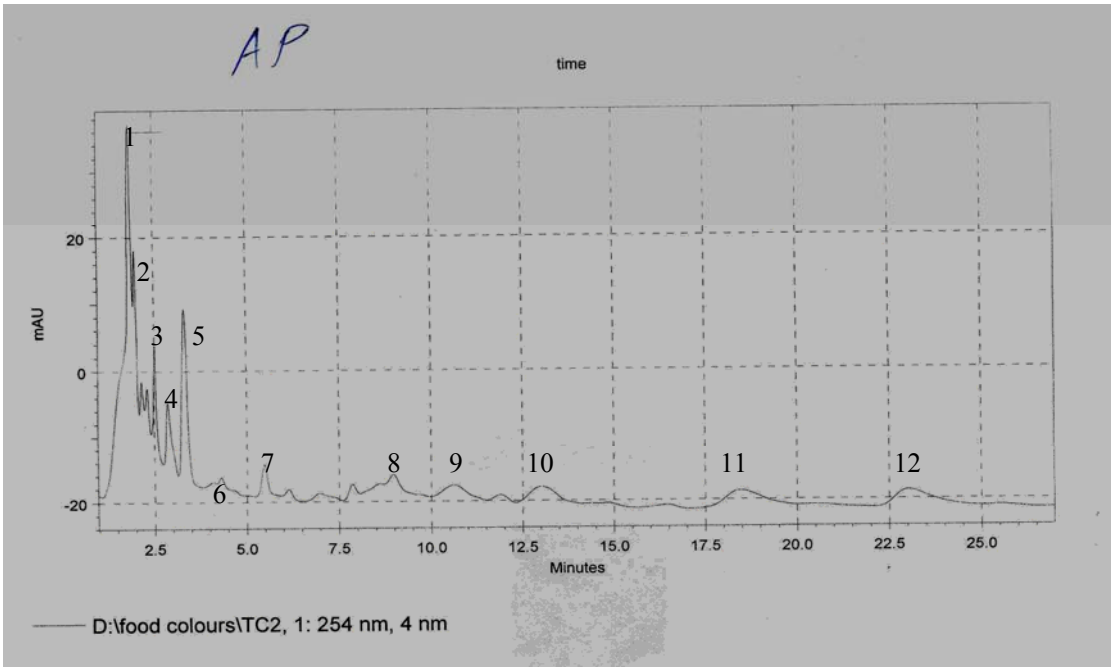
Apparatus and reagents

a- Liquid chromatography: Equipped with suitable injection device (Shimadzu SPD-M 10AVP HPLC), solvent delivery system, UV detector, electronic integrator and μBondapak C_{18} column, 300x3.9 (id) mm waters/Millipore. Flow rate 2mL/min, injection volume 10 μL, detector wavelength 254 nm, temperature ambient.* Mobile phase: 20% aqueous CH_3COOH (volume/ volume) buffered to pH 3.0 with saturated sodium acetate solution.

b- Standard solutions: Fifty mg of each standard was dissolved in 5ml ethanol and diluted to 100 ppm. The final solution was filtered through a 0.45 membrane filter.

c- Sample preparation: Fifty mg of each extract was dissolved in 5ml ethanol and diluted to 100 ppm. The final solution was filtered through a 0.45 membrane filter.

1-A. palaestinum:

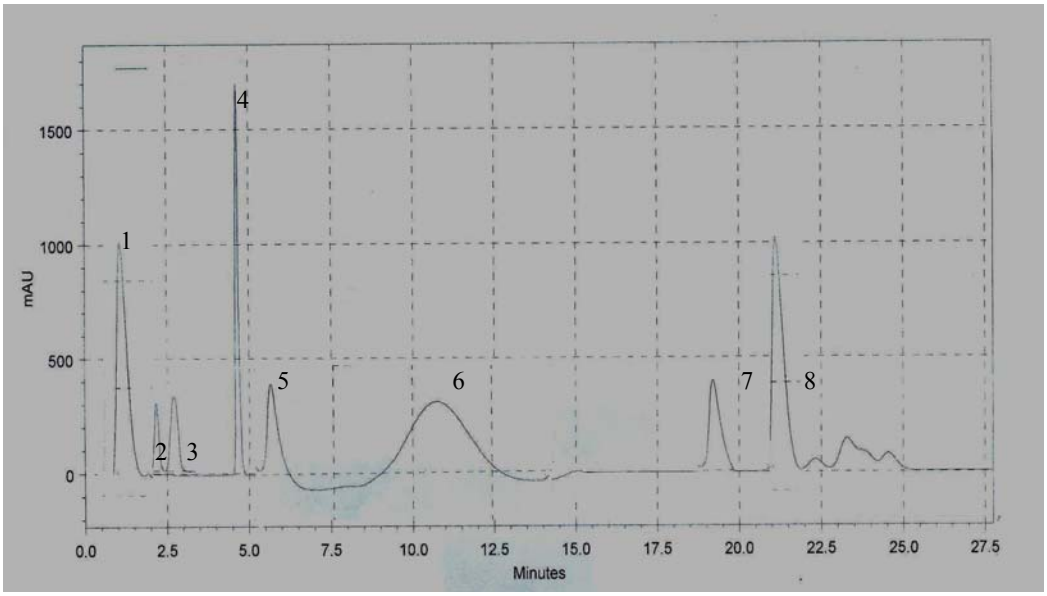


(Figure: 3.9) Chromatogram of *A. palaestinum*

(Table: 3.4) Retention time of separated compounds from *A. palaestinum*

Peak No.	Retention time (minute)	Compounds
Unknown	2.1	Unknown
2	2.3	Unknown
3	2.5	Unknown
4	2.9	Salicylic acid
5	3.3	Phthalic acid
6	3.5	Diisobutyl phthalate
7	5.5	Unknown
8	9	Unknown
9	10.6	Benzoic acid
10	13.3	Unknown
11	18.8	Cinnamic acid
12	23.4	Unknown

2- *U. pilulifera*

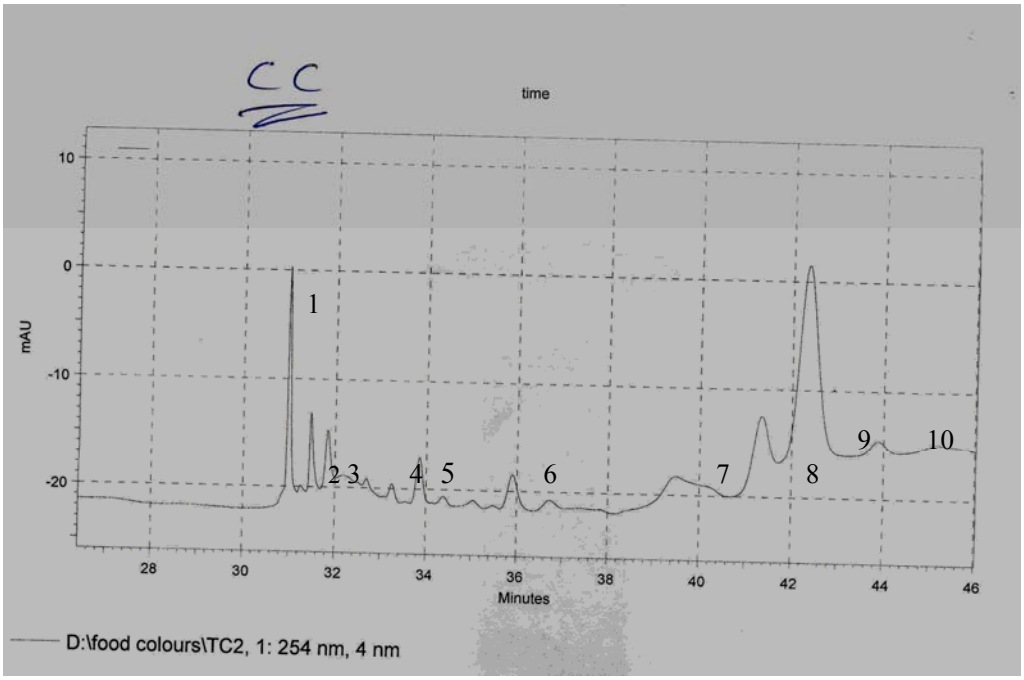


(Figure: 3.10) Chromatogram of *U. pilulifera*

(Table: 3.5) Retention time of separated compounds from *U. pilulifera*

Peak No	Retention time (minute)	Compounds
1	2.1	Unknown
2	2.4	Unknown
3	2.6	Unknown
4	4.5	Pyrocatechol
5	5.5	Unknown
6	11	Unknown
7	19	Methyl 3-hydroxy benzoate
8	21	2-phenoxy ethanol

3- *C. capitatus*

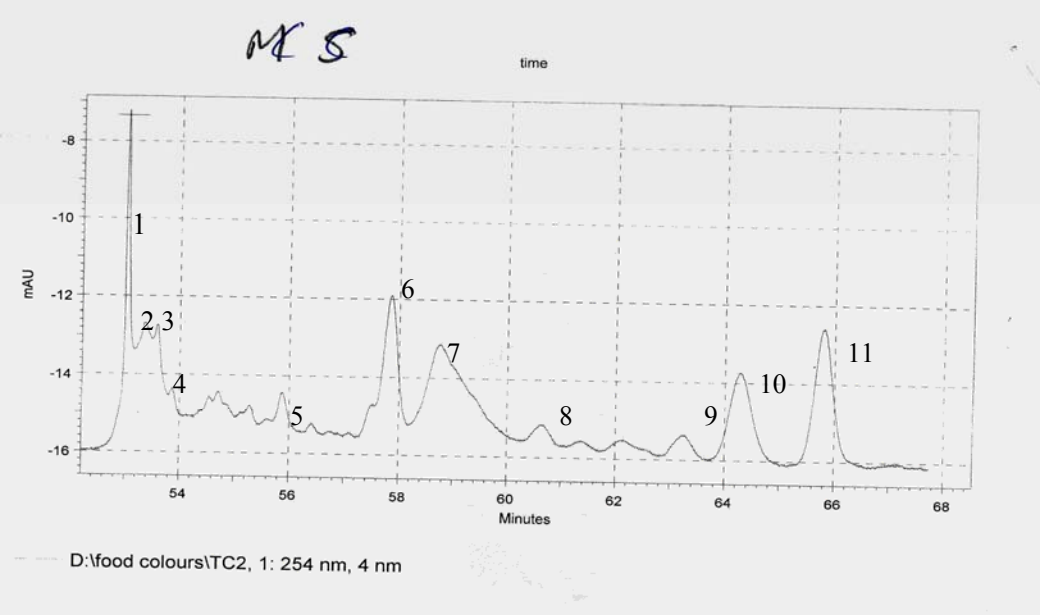


(Figure: 3.11) Chromatogram of *C. capitatus*

(Table: 3.6) Retention time of separated compounds from *C. capitatus*

Peak No	Retention time (minute)	Compounds
1	1.9	Unknown
2	2.4	Unknown
3	2.8	Salicylic acid
4	4.9	Unknown
5	6.9	Unknown
6	7.7	Unknown
7	9.8	Thymol
8	11.4	Unknown
9	12.3	Unknown
10	13.9	Unknown

4- M. syriaca

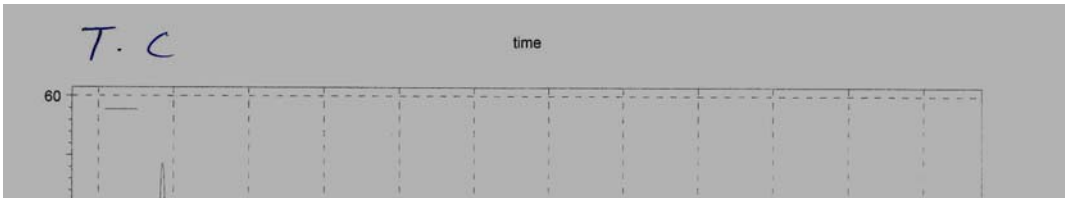


(Figure: 3.12) Chromatogram of *M. syriaca*

(Table: 3.7) Retention time of separated compounds from *M. syriaca*

Peak No.	Retention time (minute)	Compounds
1	2	Unknown
2	2.3	Unknown
3	2.6	Unknown
4	2.8	Salicylic acid
5	4.8	Unknown
6	6.8	Unknown
7	7.7	Unknown
8	9.8	Thymol
9	12.2	Unknown
10	13.3	Unknown
11	14.3	Unknown

5- T. creticum



1

2

6

3 4

5

7

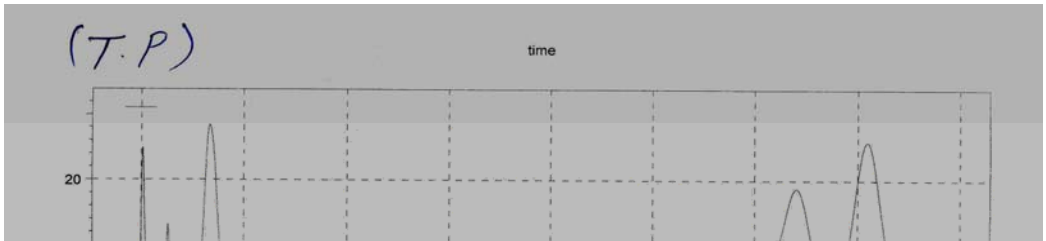
8 9

(Figure: 3.13) Chromatogram of *T. creticum*

(Table: 3.8) Retention time of separated compounds from *T. creticum*

Peak No.	Retention time (minute)	Compounds
1	1.9	Sorbic acid
2	2.1	Unknown
3	2.3	Unknown
4	2.5	Unknown
5	2.9	Salicylic acid
6	3.3	Phthalic acid
7	5.3	Unknown
8	8	Unknown
9	9.3	Unknown

6-T. polium



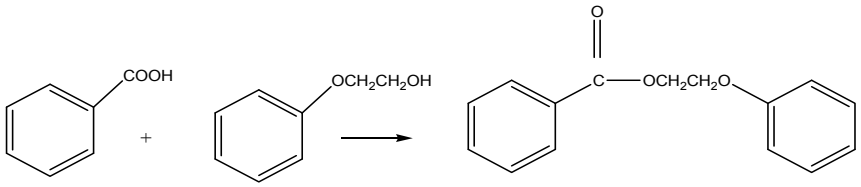
(Figure: 3.14) Chromatogram of *T. polium*

(Table: 3.9) Retention time of separated compounds from *T. polium*

Peak No.	Retention time (minute)	Compounds
1	2	Unknown
2	2.5	Unknown
3	4.3	Pyrocatechol
4	5.4	Unknown
5	6.9	Unknown
6	12.4	Unknown
7	14.8	Unknown
8	16.2	Unknown

3.2.6 Modification of some biologically active compounds

1- Preparation of 2-phenoxyethyl benzoate*:* Benzoic acid (10.0g, 0.08 mol) was mixed with 2-phenoxy ethanol (11.0g, 0.08 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 24 hours. The product was collected as solid crystals. The crystals were purified by flash chromatography using (n-hexane /ethyl acetate 3:1) as shown in Scheme 3.1.



(Scheme 3.1) Preparation of 2-phenoxyethyl benzoate

TLC spotting for starting materials and product emphasize a new product (three spots with different R_f). Melting point of pure crystals was found to be (112-114). ºC and percentage yield was 52%.

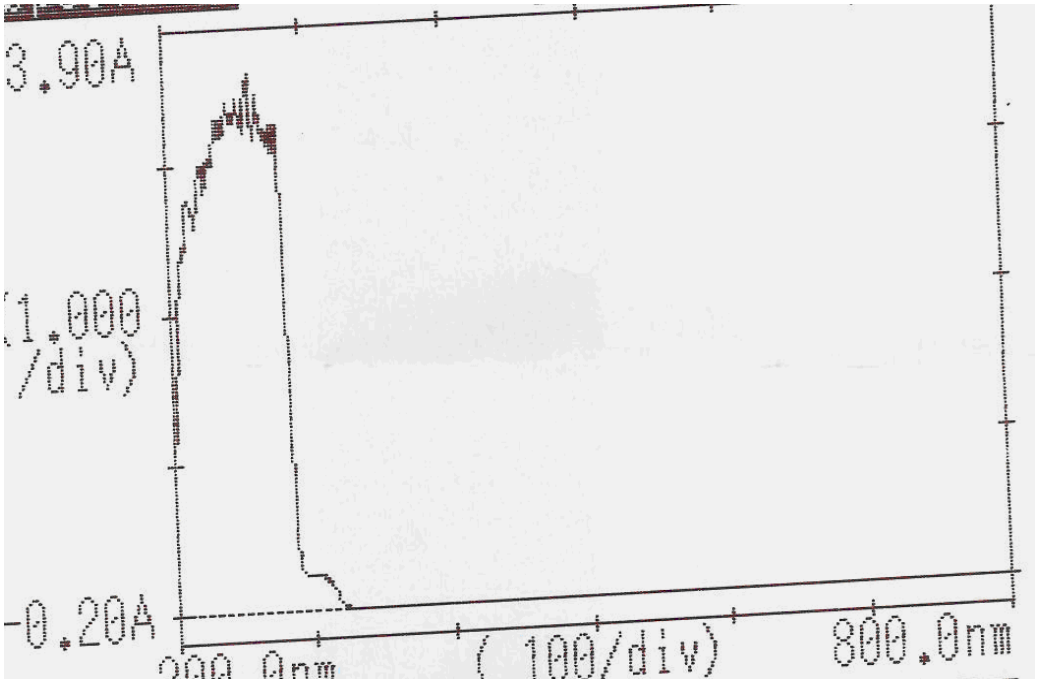
UV λmax 240-270 (Figure 3.15).

IR spectroscopic data *ν*max (cm-1) (Figure 3.16).

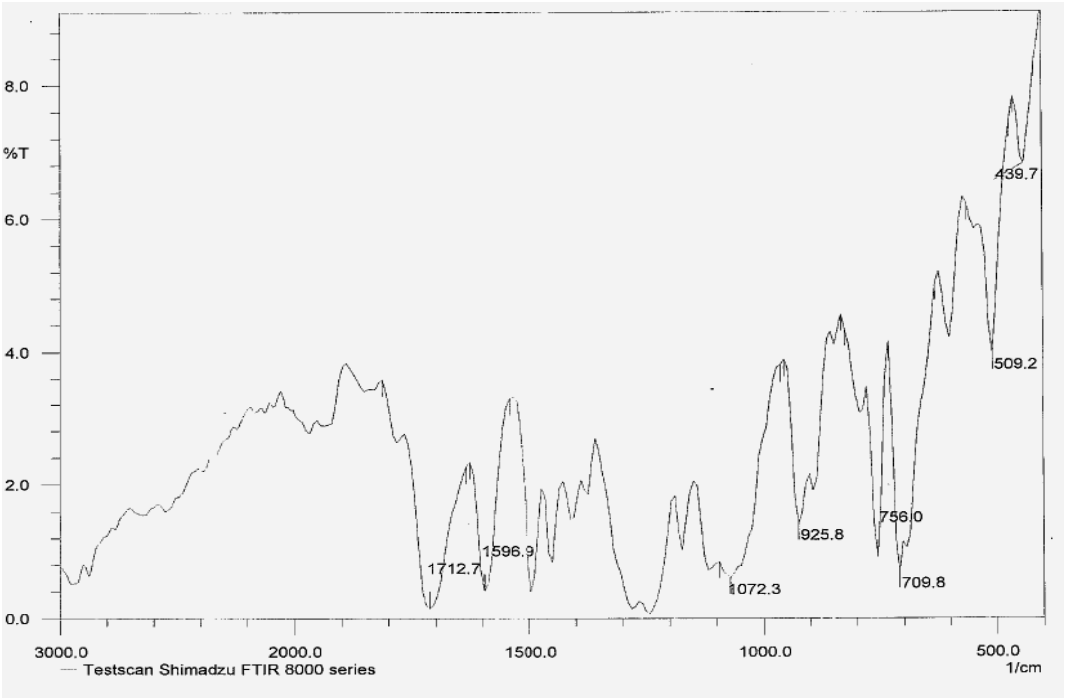
1697	C=O
1596	Aromatic ring
1242	-COO
1064	-O-R

There is no identity card for the compound in GC-MS library

From the above data we can conclude that the product is 2-phenoxyethyl benzoate.



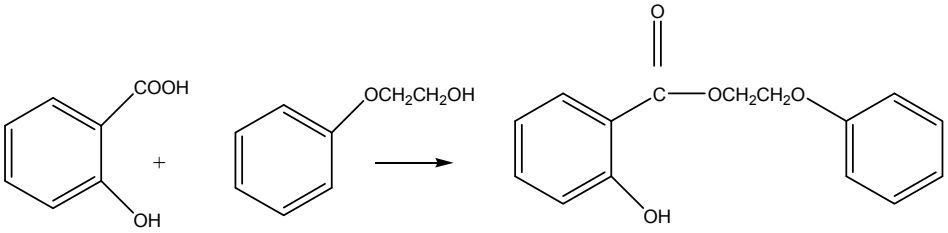
(Figure: 3.15) U.V spectrum of 2-phenoxyethyl benzoate



(Figure: 3.16) IR spectrum of 2-phenoxyethyl benzoate

2- Preparation of 2-phenoxyethyl 2-hydroxy benzoate

2-Hydroxy benzoic acid (10.0g, 0.07 mol) was mixed with 2-phenoxy ethanol (11.0g, 0.08 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 24 hours. The product was collected as solid crystals. The crystals were purified by flash chromatography using (n-hexane /ethyl acetate 3:7) as shown in Scheme 3.2.



(Scheme 3.2) Preparation of 2-phenoxyethyl 2-hydroxy benzoate

TLC spotting for starting materials and product emphasize a new product (three spots with different R_f). The product was purified as white crystals with melting point (104-106 ºC) and percentage yield was 71%.

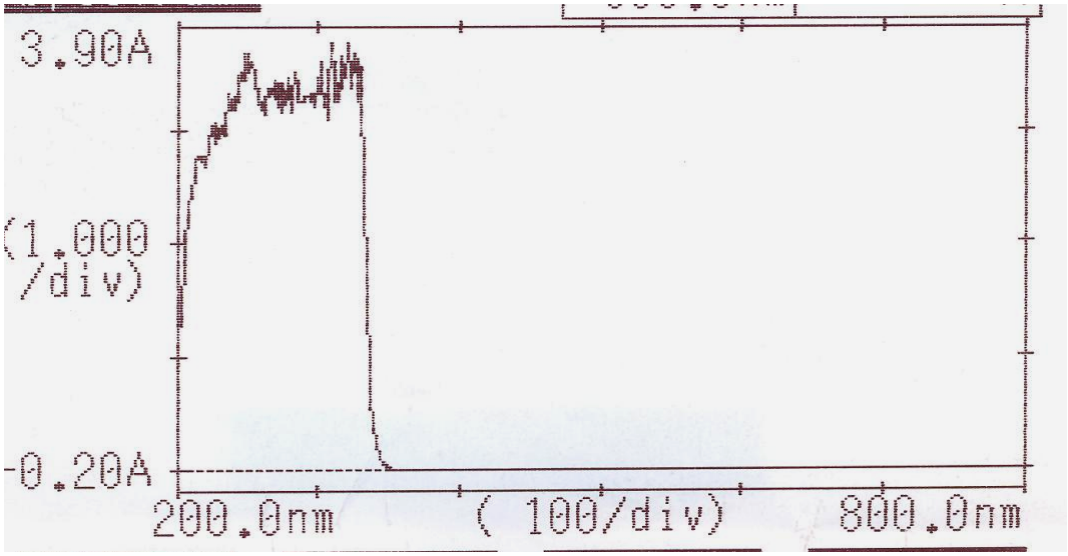
UV λmax 240-270 (Figure 3.17)

IR spectroscopic data *v*max (cm-1) (Figure 3.18)

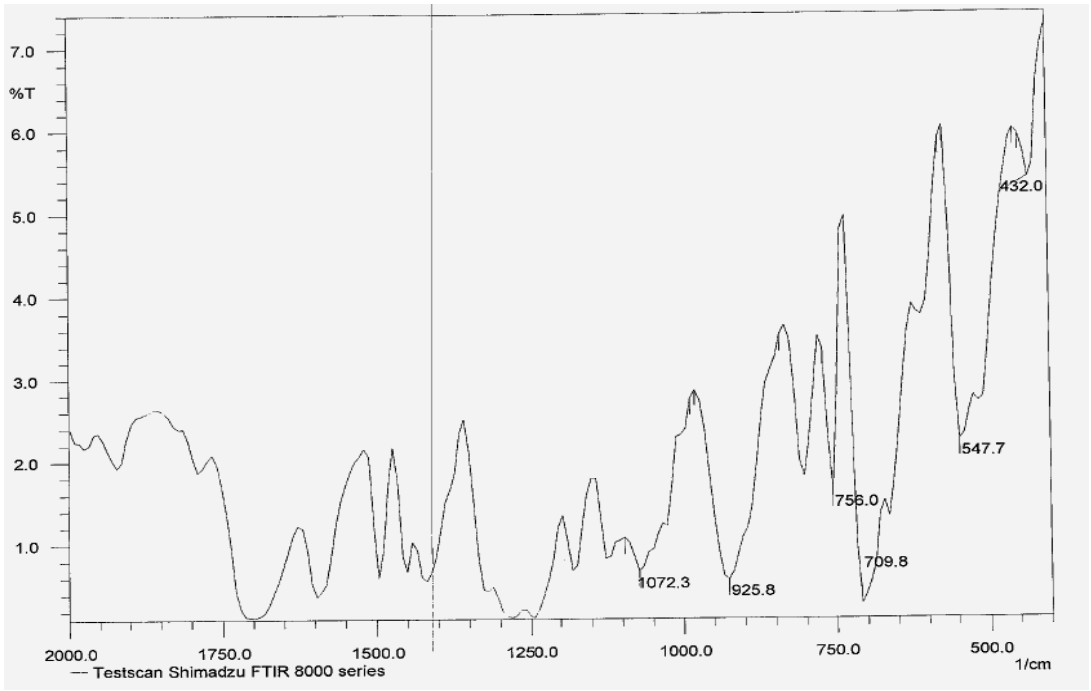
3309	OH group
1712	C=O
1596	Aromatic ring
1242	-COO
1072	-O-R

There is no identity card for the compound in GC-MS library

From the above data we can conclude that the product is 2-phenoxyethyl 2-hydroxybenzoate.



(Figure: 3.17) UV spectrum of 2-phenoxyethyl 2-hydroxybenzoate

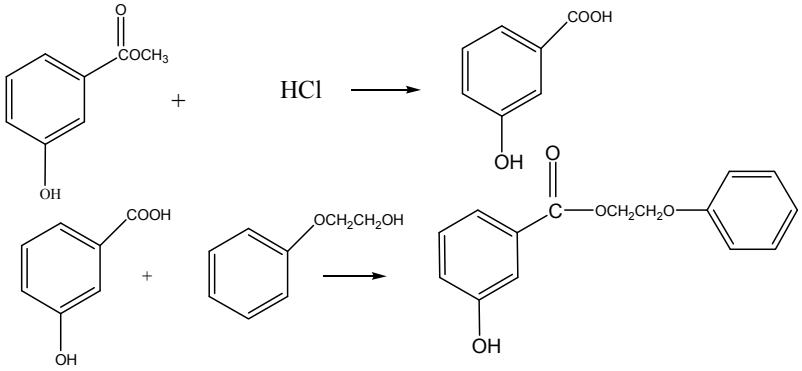


(Figure: 3.18) IR spectrum of 2-phenoxyethyl 2-hydroxybenzoate

3- Preparation of 2-phenoxy ethyl 3-hydroxy benzoate

Methyl 3-hydroxy benzoate (10.0g, 0.07 mol) was boiled with 5ml 6 M HCl for 10 minutes. The product was mixed with 2-phenoxy ethanol (11.0g, 0.08 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 24 hours. The product was collected as solid crystals. The crystals were purified by flash chromatography using (n-hexane /ethyl acetate 3:7) as shown in Scheme 3.3. Melting point was measured to be (138 -141

^{o} C). The percentage yield of the reaction was 31%. The product was confirmed by UV and IR spectroscopic data (Fig 3.19, Fig 3.20).



(Scheme 3.3) Preparation of 2-phenoxyethyl 3-hydroxy benzoate

TLC spotting for starting materials and product emphasize a new product (three spots with different R_f)

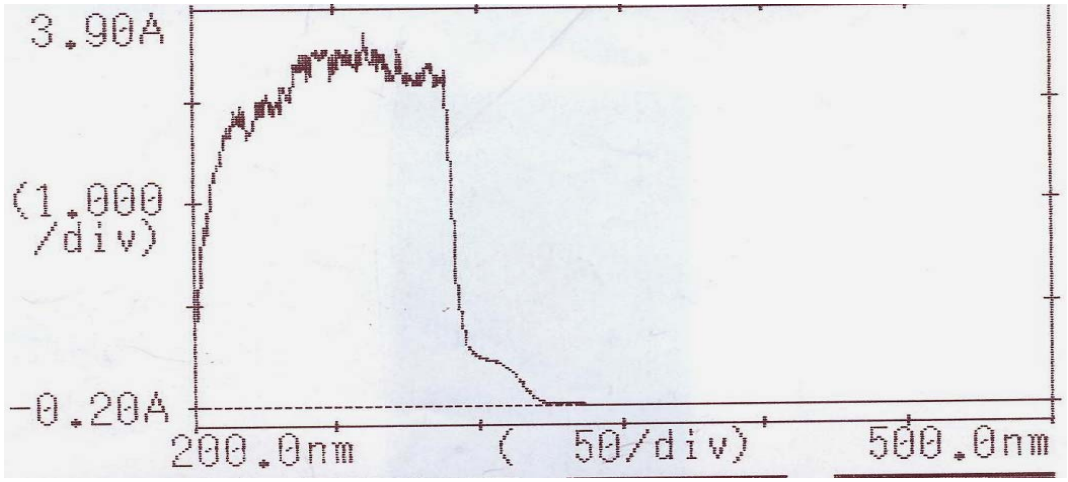
UV λmax 240-270 (Figure 3.19)

IR spectroscopic data *ν*max (cm-1) (Figure 3.20)

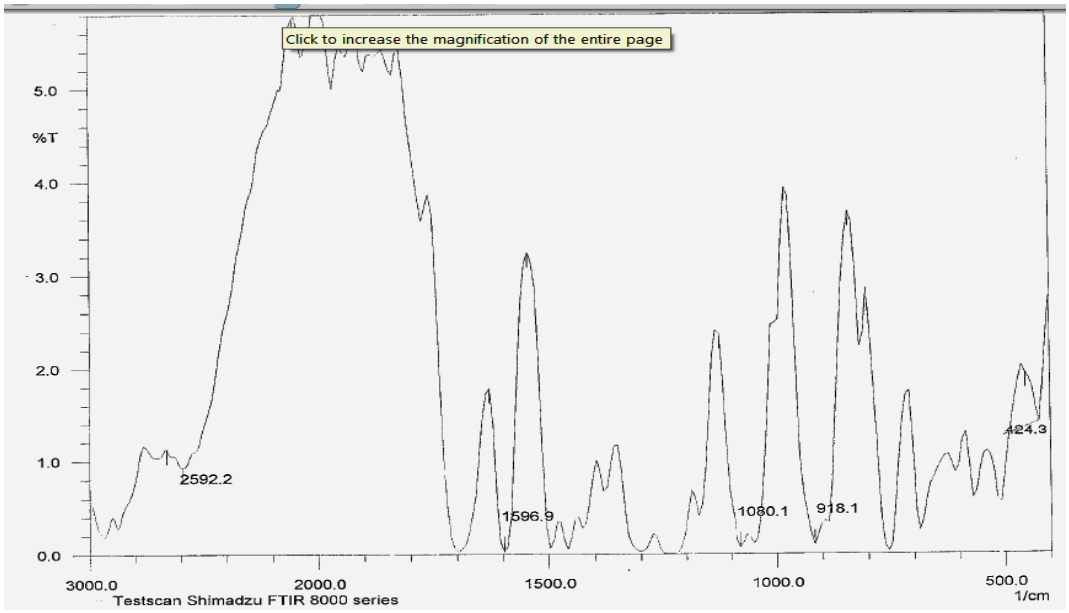
3309	OH group
1689	C=O
1596, 1496	Aromatic ring
1242	-COO
1087	Para OH
673,694	mono substituted

There is no identity card for the compound in GC-MS library

From the above data we can conclude that the product is 2-phenoxyethyl 3-hydroxybenzoate



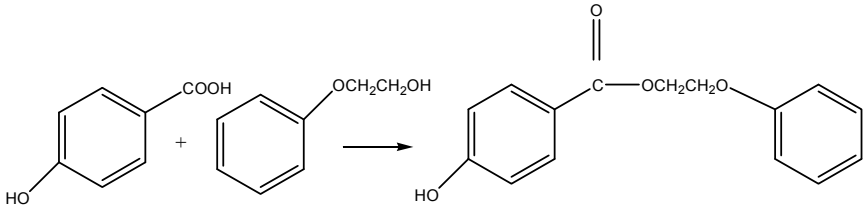
(Figure: 3.19) UV spectrum for 2-phenoxy ethyl 3-hydroxybenzoate



(Figure: 3.20) IR spectrum of 2-phenoxyethyl 3-hydroxybenzoate

4- Preparation of 2-phenoxyethyl 4-hydroxybenzoate

4-Hydroxy benzoic acid (10.0g, 0.07 mol) was mixed with 2-phenoxy ethanol (11.0g 0.08 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 24 hours. The product was collected as solid crystals. The crystals were purified by flash chromatography using (n-hexane /ethyl acetate 3:7) as shown in Scheme 3.4.



(Scheme 3.4) Preparation of 2-phenoxyethyl 4-hydroxybenzoate

TLC spotting for starting materials and product emphasize a new product (three spots with different Rf). Melting point was found to be (146-148 ºC). The percentage yield was 57%.

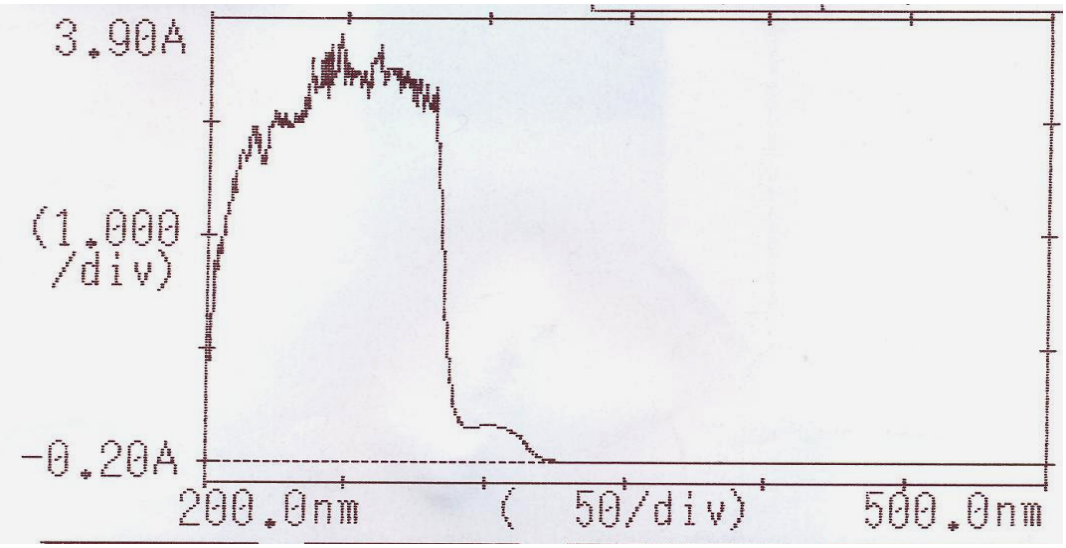
UV λmax 240-270 (Figure 3.21).

IR spectroscopic data *ν*max (cm-1) (Figure 3.22).

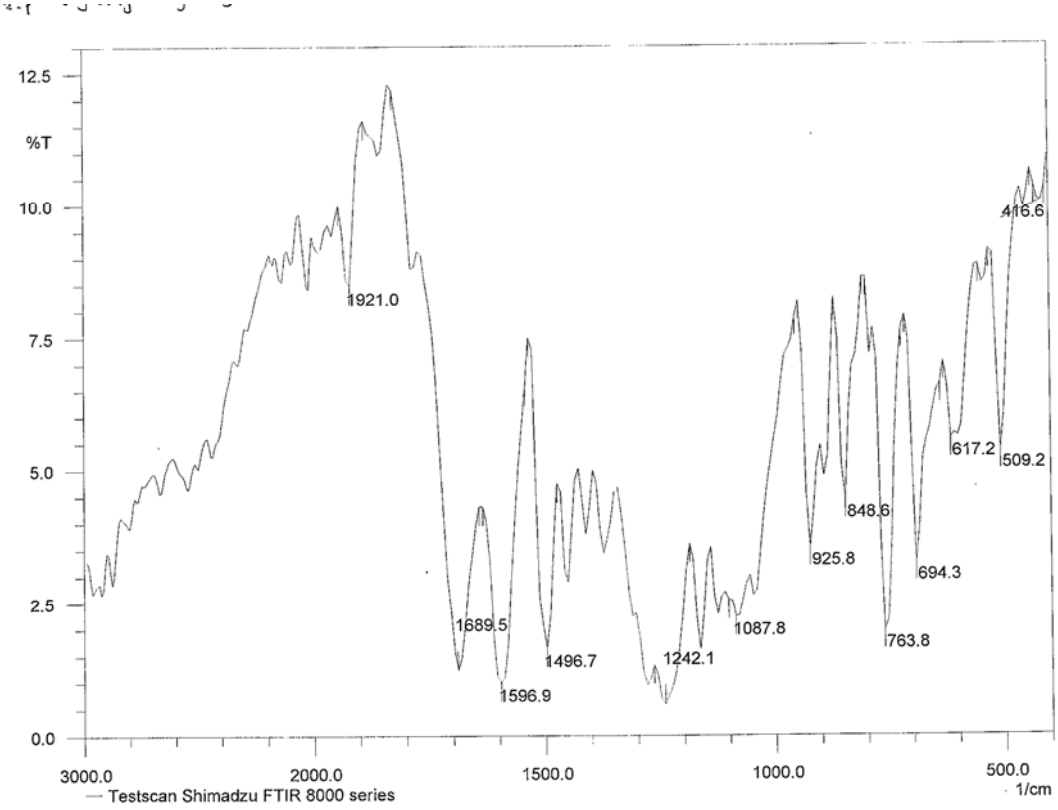
3309	OH group
1689	C=O
1596, 1496	Aromatic ring
1242	-COO
1087	-O-R
484	Para OH
673,694	mono substituted

There is no identity card for the compound in GC-MS library

From the above data we can conclude that the product is 2-phenoxyethyl 4-hydroxybenzoate.



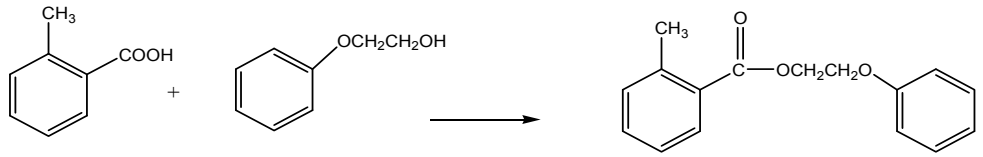
(Figure: 3.21) UV spectrum of 2-phenoxyethyl 4-hydroxybenzoate



(Figure: 3.22) IR spectrum of 2-phenoxyethyl 4-hydroxybenzoate

5- Preparation of 2-phenoxyethyl toluate

2-Methyl benzoic acid (13.6g, 0.1 mol) was mixed with 2-phenoxyethanol (13.8g, 0.1 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 24 hours. The product was collected as solid crystals. The crystals were purified by flash chromatography using (n-hexane /ethyl acetate 3:7) as shown in Scheme 3.5. Melting point was found to be (134-136ºC). TLC spotting for starting materials and product emphasize a new product (three spots with different R_f).The percentage yield of the product was 73%.

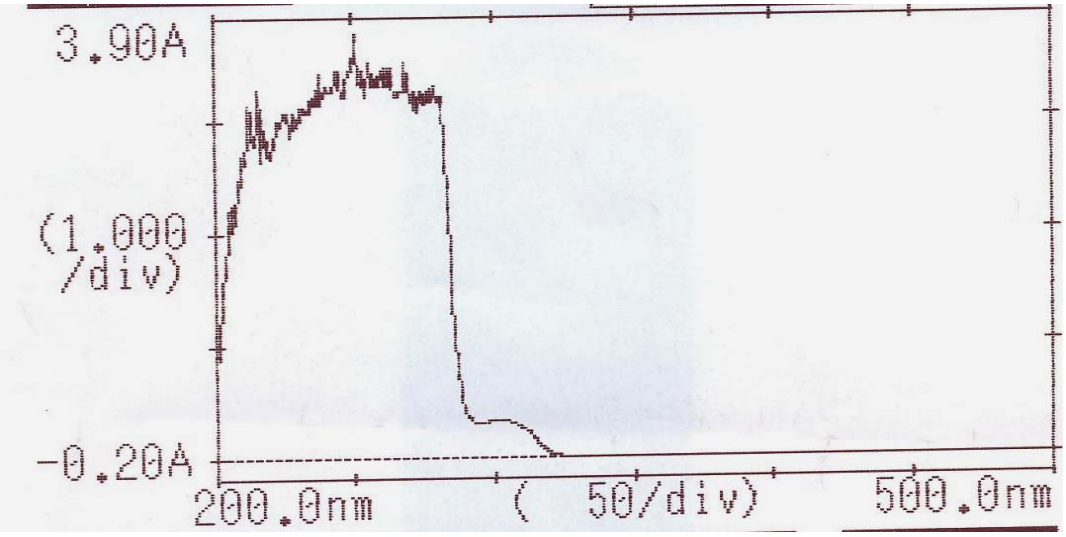


(Scheme 3.5) Preparation of 2-phenoxyethyl toluate.

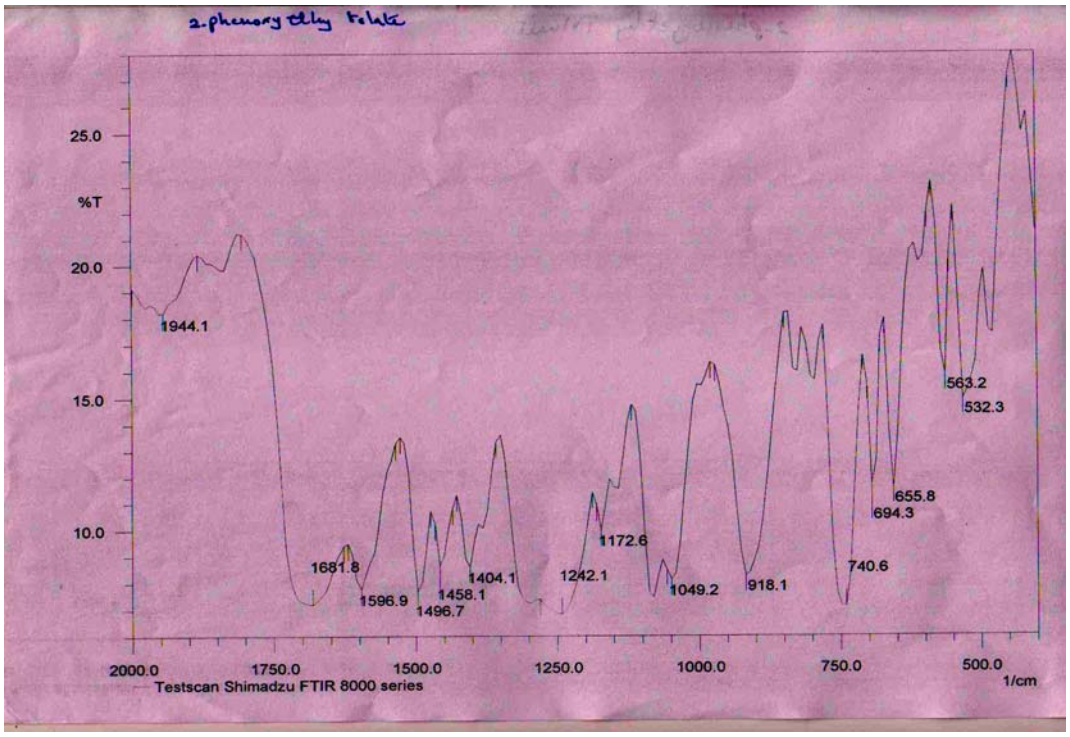
UV λ_{max} 240-270 (Figure 3.23).

IR spectroscopic data ν_{max} (cm^{-1}) (Figure 3.24).

1689	C=O
1596, 1496	Aromatic ring
1242	-COO
1087	-O-R
673,694	mono substituted



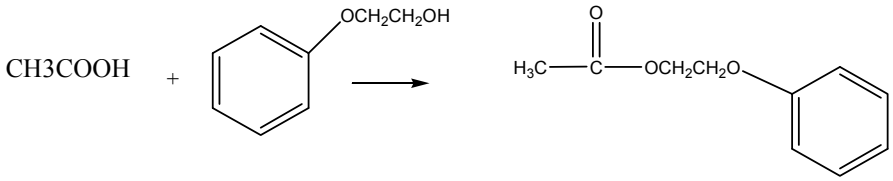
(Figure: 3.23) UV spectrum of 2-phenoxyethyl toluate



(Figure: 3.24) IR spectrum of 2-phenoxyethyl toluate

6- Preparation of 2-phenoxyethyl acetate

Glacial acetic acid (6.0g, 0.1 mol) was mixed with 2-phenoxyethanol (13.8g, 0.1 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 3 hours as shown in Scheme 3.6. The product was a clear liquid. The percentage yield of the product was 68%. The product was confirmed by UV and IR spectroscopic data (Fig 3.25, Fig 3.26).

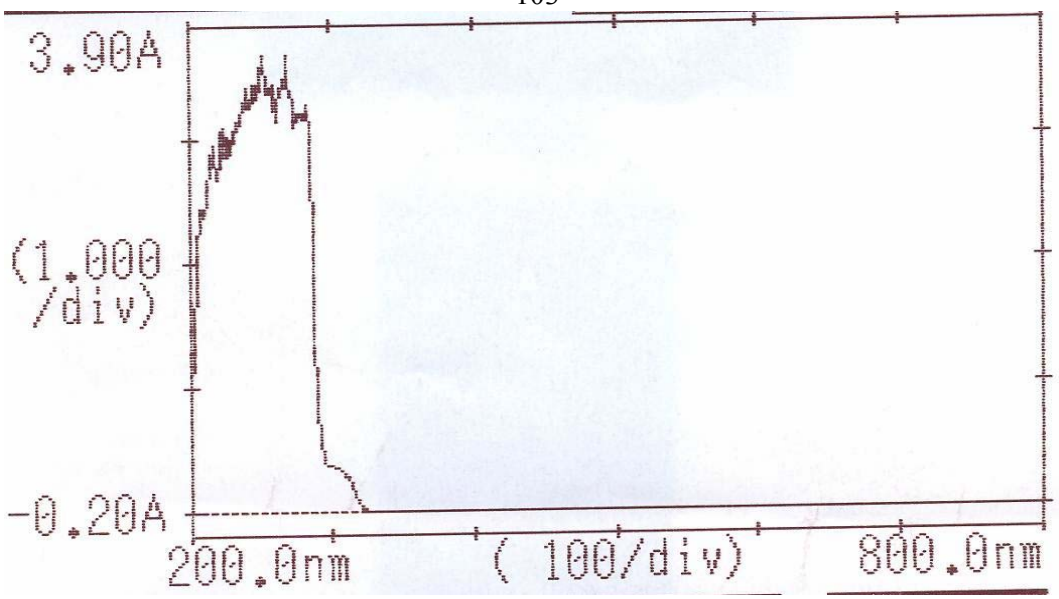


(Scheme: 3.6) Preparation of 2- phenoxyethyl acetate

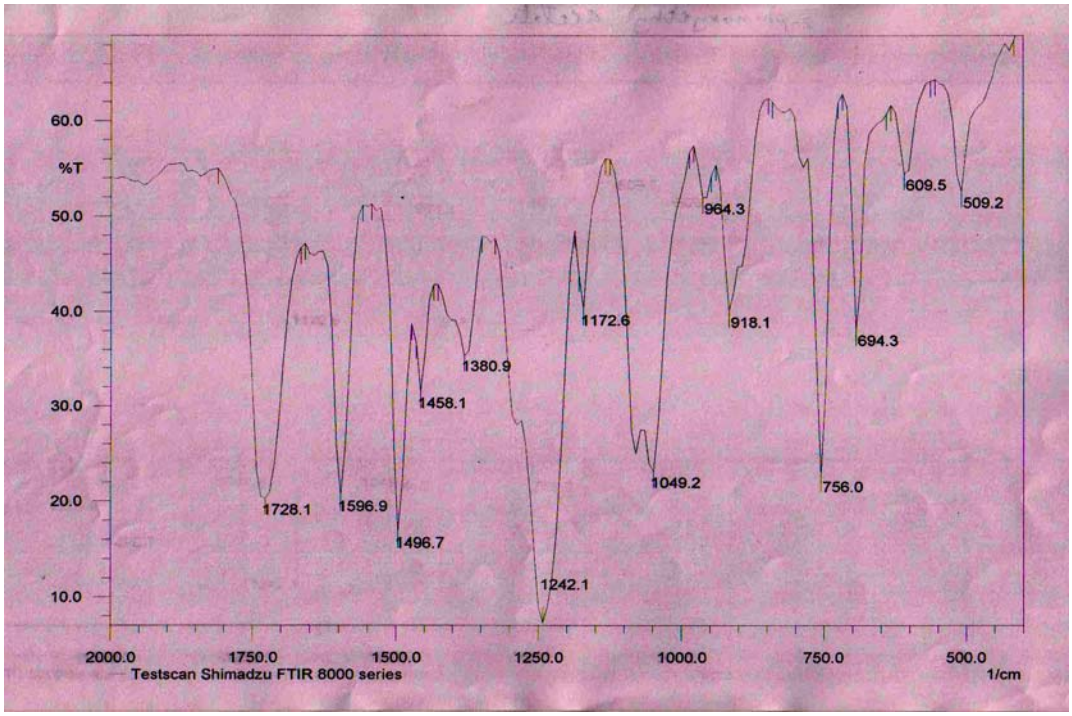
UV λ_{max} 220-245 (Figure 3.25).

IR spectroscopic data ν_{max} (cm^{-1}) (Figure 3.24).

1689	C=O
1596, 1496	Aromatic ring
1242	-COO
1087	-O-R



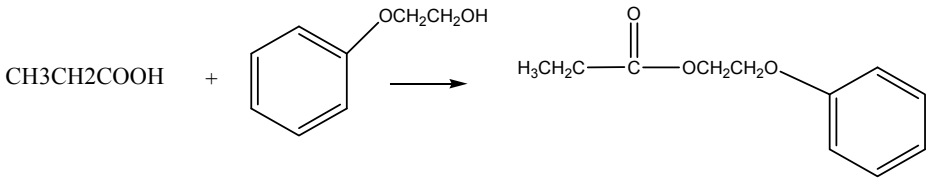
(Figure: 3.25) UV spectrum of 2-phenoxyethyl acetate



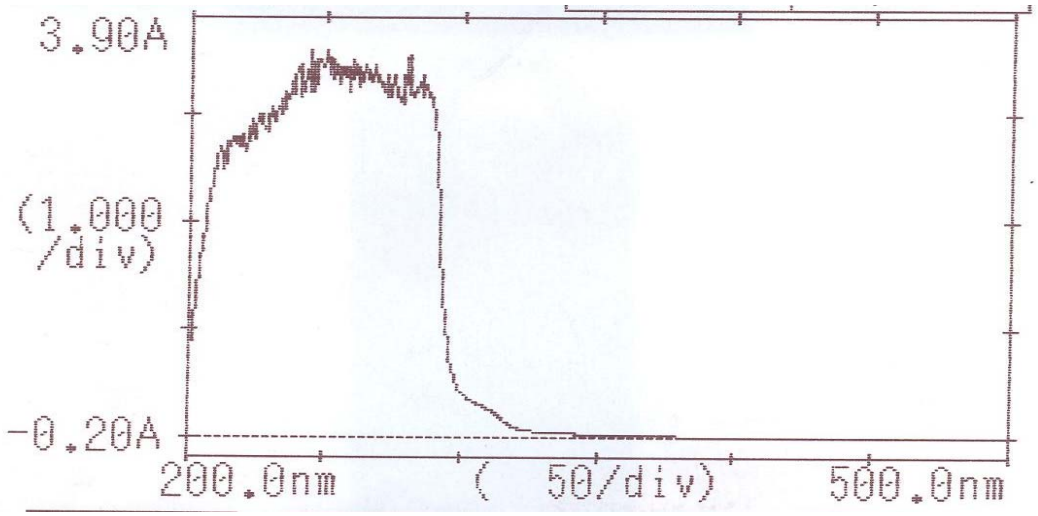
(Figure: 3.26) IR spectrum of 2-phenoxyethyl acetate

7- Preparation of 2-phenoxyethyl propionate

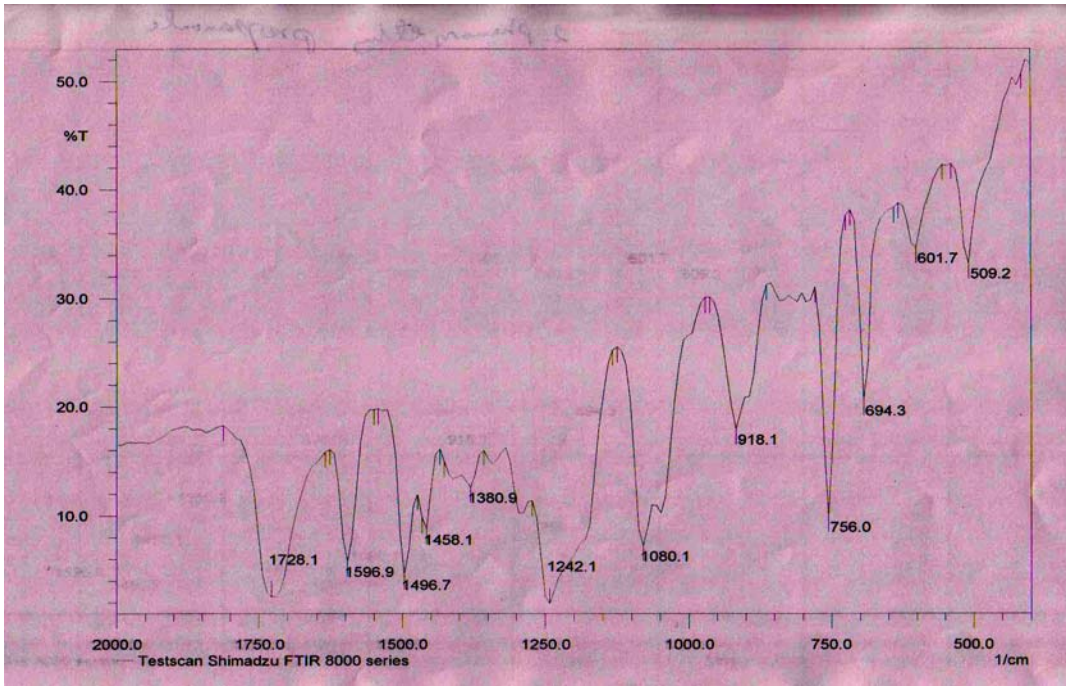
Propanoic acid (7.4g, 0.1 mol) was mixed with 2-phenoxyethanol (13.8g, 0.1 mol) and reflux for 3 hours. The reaction was cooled and left at room temperature for 2 hours as shown in Scheme 3.7. The product was a clear liquid. The percentage yield of the product was 61%. TLC spotting for starting materials and product emphasize a new product (three spots with different R_f).The product was confirmed by UV and IR spectroscopic data (Fig 3.27, Fig 3.28).



(Scheme: 3.7) Preparation of 2-phenoxyethyl propionate



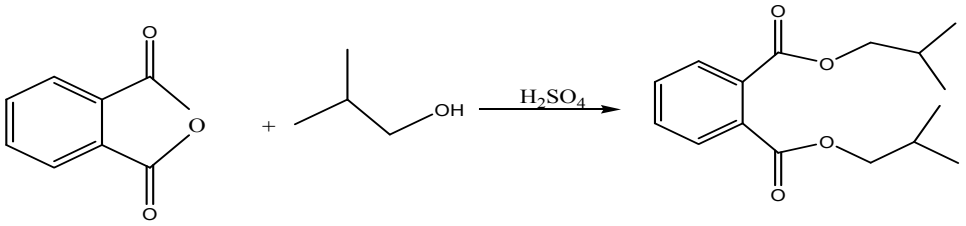
(Figure: 3.27) UV spectrum of 2-phenoxyethyl propionate



(Figure: 3.28) IR spectrum of 2-phenoxyethyl propionate

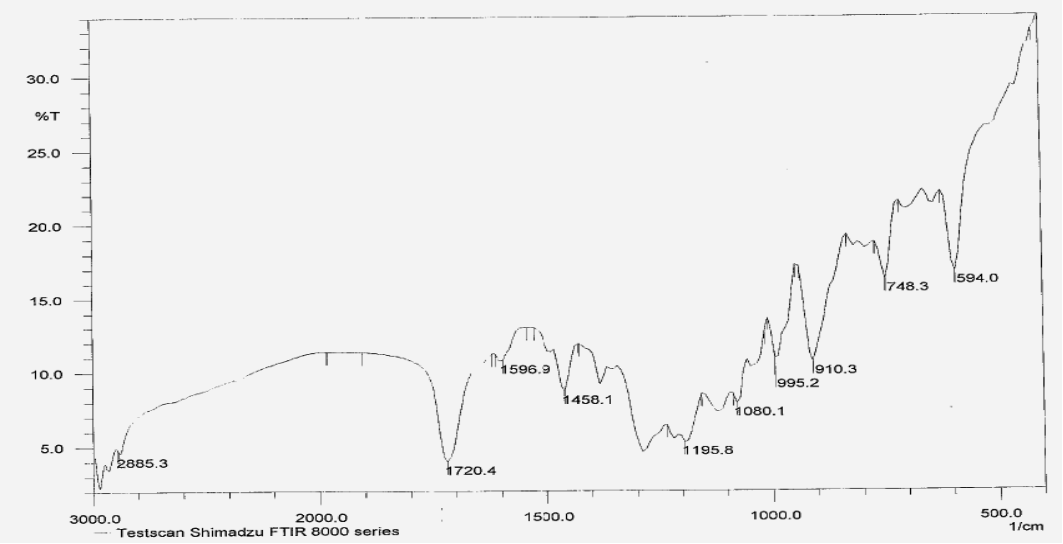
8- Preparation of diisobutyl phthalate

Isobutyl alcohol (80.0g, 1.08 mol) was mixed with phthalic anhydride (10.0g, 0.07 mol). The mixture was catalyzed with 2ml concentrated sulfuric acid and reflux for 3 hours according to the following scheme:



(Scheme 3.8) Preparation of diisobutyl phthalate

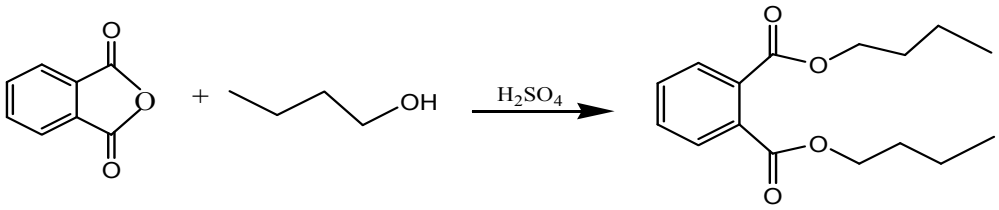
The product was a yellow liquid with boiling point 325-328 ^{o}C. It was confirmed by comparing it with an authentic sample in addition to IR analysis (Figure: 3.29).



(Figure: 3.29) IR spectrum of diisobutyl phthalate

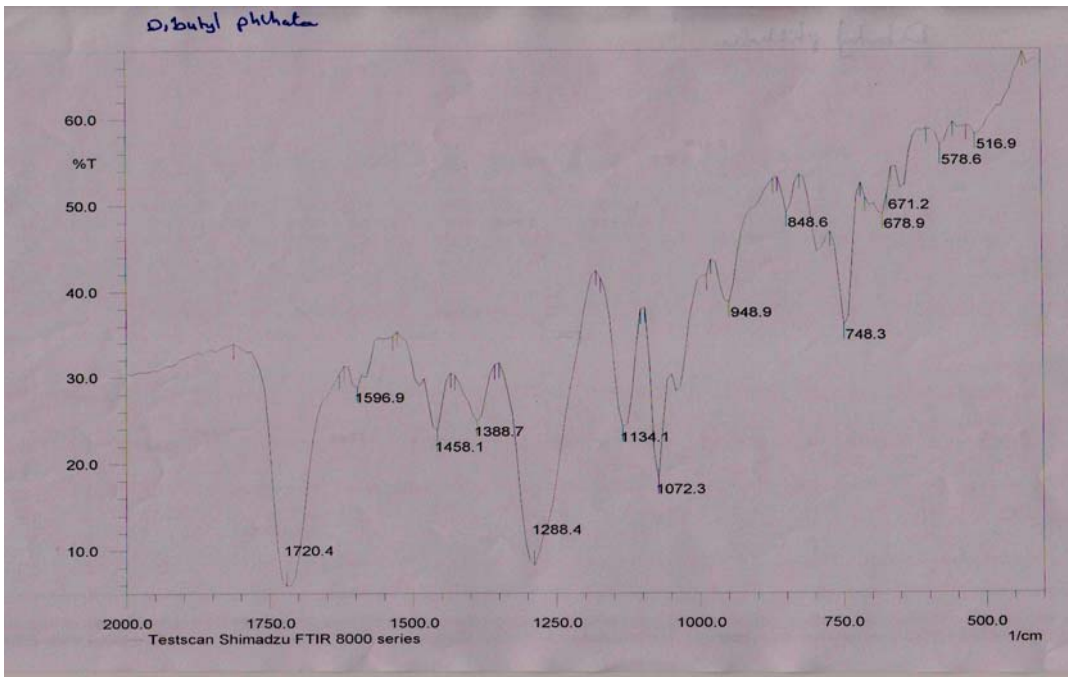
9- Preparation of dibutyl phthalate

Butyl alcohol (80.0g, 1.08 mol) was mixed with phthalic anhydride (10.0g 0.07 mol). The mixture was catalyzed with 2ml concentrated sulfuric acid and reflux for 3 hours according to the following Scheme:



(Scheme 3.9) Preparation of dibutyl phthalate

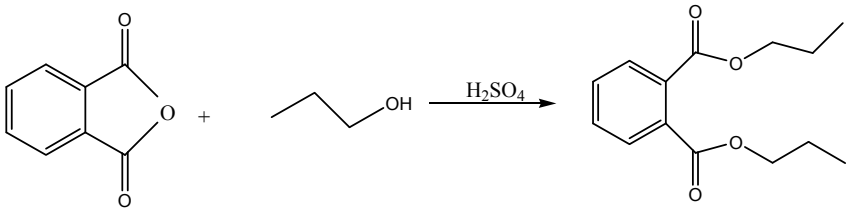
The product was a clear liquid with a boiling point of 336-341 ºC. Spectrophotometric analysis IR Figure 3.30 emphasizes the production of dibutyl phthalate.



(Figure: 3.30) IR spectrum of dibutyl phthalate

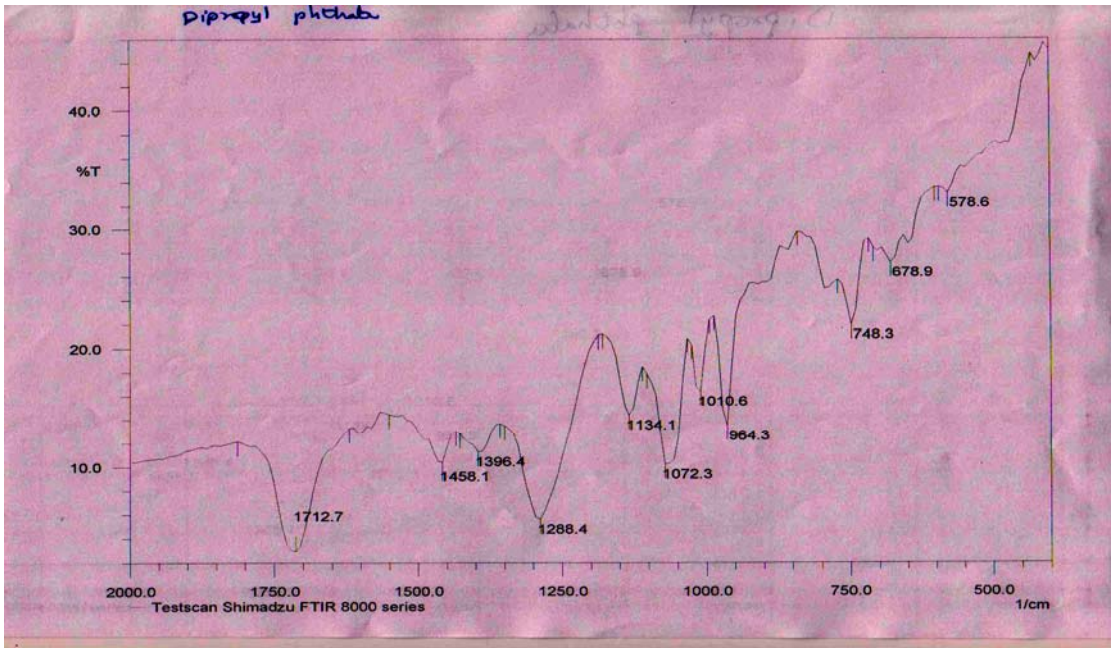
10- Preparation of dipropyl phthalate

Propyl alcohol (80.0g, 1.6 mol) was mixed with phthalic anhydride (10.0g 0.07 mol). The mixture was catalyzed with 2ml concentrated sulfuric acid and reflux for 3 hours according to the following Scheme:



(Scheme 3.10) Preparation of dipropyl phthalate

The product was a clear liquid with a boiling point of 315-318 ºC. The mass of the product was 1.2g (percentage yield of the product was 68 %). The product was confirmed by IR pectroscopic data (Fig 3.31).



(Figure: 3.31) IR spectrum of dipropyl phthalate

3.2.7 Biological activities of the modified compounds

Starting and modified compounds were tested for their biological activities using the same procedures used for testing the activity of plant extracts (chapter two).

3.3 Results and discussion

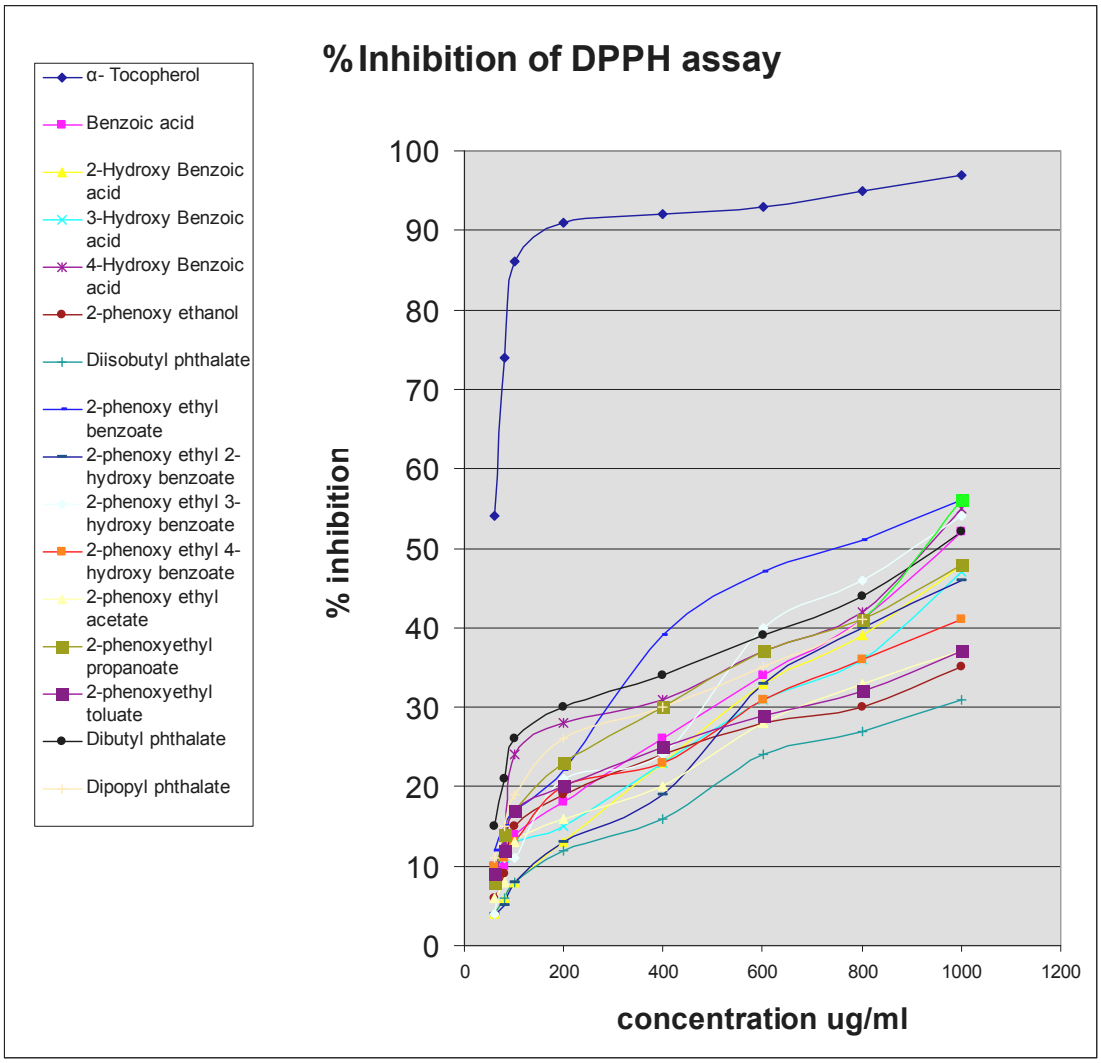
1- Antioxidants

Ethanolic solutions (10 mg/ml) of each compound were prepared to study their antioxidant activities. DPPH is one of the methods used to evaluate the antioxidative activity of antioxidants. As shown in (Table 3.10) and (Figure 3.32) these phenolic acid esters gave various degrees of free radical scavenging, but the values were lower than that of α-tocopherol. The concentrations of the tested compounds needed to reduce DPPH absorption by 50 % at 517 nm were nearly in the range of 900- 1100 µg/ml. While for α- tocopherol was 40 µg /ml. These results show that there is a little difference in the antioxidant activity between phenolic acid ester. It has been shown that the antioxidant activities of the phenolic compounds are slightly affected by the molecular structure and the position of hydroxyl group on the site of ortho, meta and para on the benzoic moiety. This is consistent with studies done on polyphenolic compounds and showed that the structure is not required for the antioxidant activity [23, 24].

(Table: 3.10) Percent inhibition of phenolic acid esters at different concentration

No.	Concentration µg/ml	% inhibition							
		60	80	100	200	400	600	800	1000
1	α- Tocopherol	54	74	86	91	92	93	95	97
2	Benzoic acid	10	10	14	18	26	34	41	52
3	2-Hydroxy Benzoic acid	4	6	8	13	23	33	39	48
4	3-Hydroxy Benzoic acid	10	11	13	15	23	31	36	47
5	4-Hydroxy Benzoic acid	10	13	24	28	31	37	42	55
6	2-phenoxy ethanol	6	9	15	19	24	28	30	35
7	Diisobutyl phthalate	4	6	8	12	16	24	27	31
8	2-phenoxy ethyl benzoate *	12	15	17	22	39	47	51	56
9	2-phenoxy ethyl 2-hydroxy benzoate *	4	5	8	13	19	33	40	46
10	2-phenoxy ethyl 3-hydroxy benzoate *	4	11	11	21	24	40	46	54
11	2-phenoxy ethyl 4-hydroxy benzoate*	10	11	13	20	23	31	36	41
12	2-phenoxy ethyl acetate *	6	8	13	16	20	28	33	37
13	2-phenoxyethyl propanoate *	8	14	17	23	30	37	41	48
14	2-phenoxyethyl toluate *	9	12	17	20	25	29	32	37
15	Dibutyl phthalate *	15	21	26	30	34	39	44	52
16	Dipopyl phthalate *	11	15	19	26	30	35	41	56

* Modified compounds



(Figure: 3.32) Percent inhibition of phenolic acid esters at different concentrations

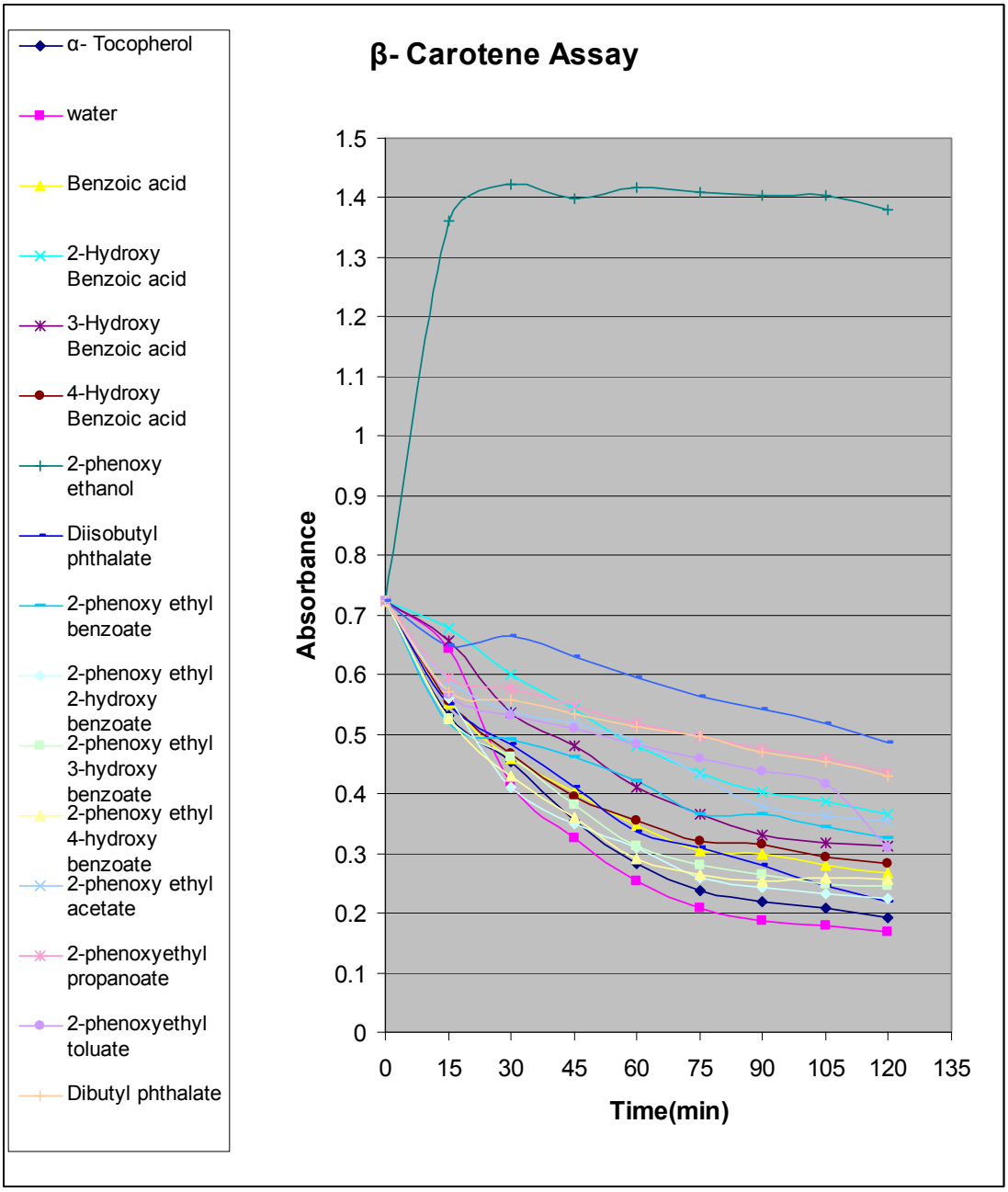
β-Carotene-linoleic acid is another antioxidant test used to measure antioxidant activity of synthetic and modified compounds (Table 3.11).

The same compounds were tested for their antioxidant activity using emulsion system of β-carotene linoleic acid depending on the fact that β-carotene loses its color in the absence of antioxidants (see chapter two). Table 3.11 and figure 3.33 show the nature of activity of those compounds. All of them showed higher antioxidant efficiency compared with water (control) and the synthetic antioxidant α-tocopherol which gave the highest β-carotene degradation (i.e. least antioxidant efficiency). 2-Phenoxy ethanol was the least β-carotene discoloration (i.e. the highest antioxidant efficiency. Dipropyl phthalate, dibutyl phthalate and 2-phenoxy ethyl propionate were the second group that gave significant activity.

(Table: 3.11) Absorbance of phenolic acid esters using *β-carot*ene assay

No	Compounds	Time (minute)								
		0	15	30	45	60	75	90	105	120
1	α- Tocopherol	0.722	0.532	0.453	0.355	0.282	0.237	0.218	0.209	0.191
2	water	0.722	0.643	0.416	0.325	0.254	0.208	0.186	0.178	0.169
3	Benzoic acid	0.722	0.553	0.458	0.402	0.346	0.304	0.299	0.281	0.266
4	2-Hydroxy Benzoic acid	0.722	0.678	0.601	0.543	0.48	0.435	0.402	0.386	0.365
5	3-Hydroxy Benzoic acid	0.722	0.656	0.536	0.481	0.411	0.365	0.332	0.318	0.311
6	4-Hydroxy Benzoic acid	0.722	0.556	0.467	0.396	0.355	0.321	0.315	0.294	0.283
7	2-phenoxy ethanol	0.722	1.36	1.422	1.398	1.416	1.409	1.405	1.405	1.38
8	Diisobutyl phthalate	0.722	0.551	0.483	0.412	0.335	0.31	0.279	0.246	0.218
9	2-phenoxy ethyl benzoate *	0.722	0.517	0.49	0.461	0.423	0.366	0.365	0.343	0.326
10	2-phenoxy ethyl 2-hydroxy benzoate *	0.722	0.561	0.411	0.35	0.309	0.258	0.242	0.231	0.223
11	2-phenoxy ethyl 3-hydroxybenzoate *	0.722	0.526	0.463	0.382	0.312	0.279	0.263	0.248	0.246
12	2-phenoxy ethyl 4-hydroxybenzoate *	0.722	0.525	0.43	0.361	0.292	0.265	0.253	0.26	0.256
13	2-phenoxy ethyl acetate*	0.722	0.589	0.54	0.518	0.488	0.43	0.38	0.363	0.354
14	2-phenoxyethyl propanoate *	0.722	0.596	0.576	0.548	0.519	0.497	0.476	0.458	0.435
15	2-phenoxyethyl toluate *	0.722	0.566	0.531	0.509	0.482	0.46	0.438	0.417	0.309
16	Dibutyl phthalate *	0.722	0.57	0.558	0.534	0.513	0.496	0.47	0.454	0.431
17	Dipopyl phthalate *	0.722	0.648	0.664	0.629	0.596	0.563	0.541	0.517	0.486

* Modified compounds



(Figure: 3.33) Antioxidant activities of phenolic acid esters as assessed by β-carotene-linoleic acid assay over 120 minute.

2- Antibacterial activity

The entire compounds (Table 3.12) were tested in vitro against six bacterial species which are known to cause dermic and micosal infections [25] beside other infections in human. All compounds studied in this work showed antibacterial activity except 2-phenoxyethanol which showed no such activity. 2-phenoxy ethyl propionate and 2-phenoxyethyl acetate were the most active modified compounds also 2-phenoxyethyl benzoate showed significant activity. It is noteworthy to take attention for those modified compounds although they possess less activity than synthetic antibiotics. It is important to prepare new therapeutics because some kinds of bacteria become resistant to certain drugs after a period of time.

(**Table: 3.12)** Antibacterial activity of modified compounds at concentration 200µg/ml

No.	Compound	Inhibition zone diameter (mm)[a] Micro-organisms

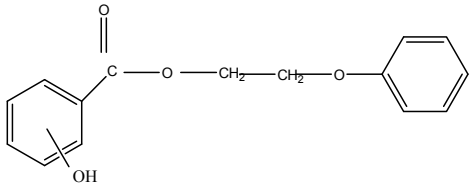
		P .vulgaris	*E . coli. JM109*	*E. coli*	*P. aeruginosa*	*S. aureus*	*K. pneumoniae*
1	Gentamicin	15.8±0.8	26±0.7	30.8±0.8	31.3±1.3	33.8±0.8	34.8±0.8
2	Streptomycin	14±0.71	17±0.7	26.3±0.8	30.8±0.8	29±1.2	37.8±1.1
3	Benzoic acid	7.3±3.8	8.3±0.8	9.8±1.2	7.5±1.1	10.5±1.1	10.5±0.5
4	2-Hydroxy Benzoic acid	7.5±0.5	9.0±1.2	8.5±1.1	9.5±1.1	8.5±0.5	8.0±0.7
5	3-Hydroxy Benzoic acid	7.5±0.5	8.4±1.2	7.5±1.1	7.8±1.3	8.5±1.1	8.0±0.7
6	4-Hydroxy Benzoic acid	9.0±0.8	7.0±0.7	7.3±0.5	7.0±0.7	7.8±0.8	8.5±1.1
7	2-phenoxy ethanol	6.0	6.0	6.0	6.0	6.0	6.0
8	Diisobutyl phthalate	8.5±1.1	8.0±0.7	9.5±1.1	8.0±0.71	9.5±0.5	7.8±1.3
9	2-phenoxyethyl benzoate	6.5±0.5	7±0.7	7±0.7	6.0	6.0	10.8±0.8
10	2-phenoxyethyl 2-hydroxy benzoate	7.8±0.4	11.5±0.9	8.5±0.5	10±0.8	10.8±0.8	23.3±0.8
11	2-phenoxyethyl 3-hydroxy benzoate	6.0	11.5±0.9	6.0	9.5±0.5	6.0	6.0
12	2-phenoxyethyl 4-hydroxy benzoate	6.0	7.0±0.0	8.5±1.1	8.0±0.7	7.5±0.5	8.0±0.0
13	2-phenoxyethyl acetate	20.3±0.8	30.5±0.9	23±0.0	24±0.7	24±0.5	25.1±0.9
14	2-phenoxyethyl propanoate	17±1.2	24±0.5	24±0.5	22±0.0	24±0.0	22.8±0.8
15	2-phenoxyethyl toluate	12.8±0.8	14.8±0.8	6.0	6.0	13.3±.8	6.0
16	Dibutyl phthalate	7.5±0.5	15.8±0.4	9±0.8	10.8±0.8	10.8±0.8	10.8±0.8
17	Dipopyl phthalate	14±0.7	14.8±0.8	6.0	10±0.8	6.0	6.0

[a]: Disc diameter, 6mm

3-Antifungal activity

Four compounds were tested in vitro for their antifungal activity against two types of dermatophytes *M. canis* and *T. rubrum* (Tables 3.13, 3.14).

The most active compound is 2-phenoxyethyl 4-hydroxy benzoate. It showed complete inhibition at a concentration less than 37.5 µg/ml against *M. canis* and less than 50 µg/ml against *T. rubrum*. The other two didn't show significant activity. The tested compounds have the same molecular weights and formulas, but different in structural formula especially in the position of hydroxyl group as shown below. These results emphasize the importance of functional group position in the activity of the compound.



(Figure: 3.34) General formulas of acid esters

(Table: 3.13) Antifungal activity of modified compounds on *M. canis*

No	Compound	Concentration µg/ml				
		12.5	25	37.5	50	62.5
1	2-phenoxy ethyl benzoate	11.0 ± 2.9	19.2 ± 3.6	28.7 ± 4.2	39 ± 2.7	46 ± 3.4
2	2-phenoxy ethyl 2-hydroxy benzoate	6 ± 3.9	15 ± 6.7	20 ± 4.2	36 ± 2.6	48 ± 5.6
3	2-phenoxy ethyl 3-hydroxy benzoate	13.8 ± 2.4	23.7 ± 1.9	29.3 ± 3.3	33.5 ± 1.7	42.5 ± 4.1
4	2-phenoxy ethyl 4-hydroxy benzoate	76 ± 4.3	88 ± 3.4	100	100	100
5	Econazole *	100	100	100	100	100

* Econazole showed complete inhibition at concentration 2.5 µg/ml

(Table: 3.14) Antifungal activity of modified compounds on *T. rubrum*

		% Inhibition				
No	Compound	Concentration µg/ml				
		12.5	25	37.5	50	62.5
1	2-phenoxy ethyl	21.5 ± 5.2	26 ± 1.4	27.8 ± 2.2	39 ± 1.6	40.3 ± 3.0

	benzoate					
2	2-phenoxy ethyl 2-hydroxy benzoate	12.3 ± 5.3	17.3 ± 2.1	20.3 ± 3.9	28 ± 3.9	41.3 ± 2.1
3	2-phenoxy ethyl 3-hydroxy benzoate	10.8 ± 5.6	28.3 ± 5.6	34.3 ± 6.1	36.5 ± 2.9	46.8 ± 5.5
4	2-phenoxy ethyl 4-hydroxy benzoate	48.8 ± 4.6	70 ± 5.7	98.6 ± 1.1	100	100
5	Econazole *	100	100	100	100	100

* Econazole showed complete inhibition at concentration 2.5 µg/ml

4- Anticancer activity

Two compounds were tested in vitro against breast cancer using the same test and procedure in Chapter Two. 2-Phenoxyethyl 4-hydroxy benzoate showed considerable activity against MCF-7 (Table 3.15). It showed 36 % viability at 62.5 µg/ml and 11% at 500 µg/ml, while 2-phenoxy ethyl 2-hydroxybenzoate showed 52% at 500 µg/ml. This means that IC_{50} for 2-Phenoxyethyl 4-hydroxy benzoate is less than 62.5 µg/ml.

(Table: 3.15) Percent viability of cancer cells

No	Compound	Concentration µg/ml				
		62.5	125	250	500	1000
1	2-phenoxy ethyl benzoate	-	-	-	-	-
2	2-phenoxy ethyl 2-hydroxybenzoate	94	89	72	52	-
3	2-phenoxy ethyl 3-hydroxybenzoate	-	-	-	-	-
4	2-phenoxy ethyl 4-hydroxybenzoate	36	21	14	11	-

3.4 References

1- Cos P., Calomme M., Pieters M., Vietinck A. J., Vanden Berghe D., (2000). ***Studies in chemistry of natural products by Atta-Ur-Rahman,*** Amsterdam. **Elsevier Science, 22**, 307-341.

2- Middleton E., Kandaswami Jr. C., Theoharides T. C. (2000). ***The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer,*** **Pharm. Rev., 52**, 673-751

3- Chen J. H., Ho C-T. (1997). ***Antioxidant activities of caffeic acid and its to hydroxy cinnamic acid compounds*****, J. Agric. Food Chem., 45**, 2374-2378

4- Silva F. , Borges F., Gulmaraes C., Lima J. , Matos C., Reis S. (2000). ***Phenolic acids and derivatives: Studies on the relationship among structure, radical scavenging activity, and physicochemical parameters,*** **J. Agric. Food Chem., 48,** 2122-2126

5- Son S., Lewis B. A., (2002). ***Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: Structureactivity relationship*****, J. Agric. Food Chem., 50,** 468- 472.

6-Nardini M., A'Aquino M., Tomassi G., Gentili V. C., Di Felice M., Scaccini *C.,* (1995). **Free Radic. Biol. Med., 19,** 541- 552.

7-Moon J. H., Terao J., (1998). ***Antioxidant activity of caffeic acid and dihydrocaffeic acid in lard and human low-density lipoprotein,*** **J. Agric. Food Chem. ,46**, 5062- 5065.

8-Lee H. S., Widmer B. W. (1996). **Phenolic compounds.** In: Nollet LML, ed. Handbook of Food Analysis. ***Physical Characterization and Nutriet Analysis***. New York, USA: Marcel Dekker, Inc., 821–894.

9-Robards K., Antolovich M. (1997). ***Analytical chemistry of fruit bioflavonoids, a review*****, Anal , 122,** 11R 34R.

10-Delage E. , Baron G. B. , Drilleau J-F., (1991***). High-performance liquid chromatography of the phenolic compounds in the juice of some French cider apple varieties,*** **J. Chromatogr., 555,** 125–136.

11-Torres A. M., Mau-Lastovicka T., Rezaaiyan R. (1987). ***Total phenolics and high-performance liquid chromatography of phenolic acids of avocado, J*****. Agric. Food Chem., 35,** 921–925.

12- Justesen U., Knuthsen P., Leth T.,(1998). ***Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection,*** **J Chromatogr** , **A; 799**,101–110.

13-Pussayanawin V., Wetzel D. L. (1987). ***High-performance liquid chromatographic determination of ferulic acid in wheat milling fractions as a measure of bran contamination,*** **J. Chromatogr., 391**, 243–255.

14- Taga M. S., Miller E. E. , Pratt D. E. (1984). ***Chia Seeds as a Source of Natural Lipid Oxidant,*** **J. Am. Oil Chem. Soc. ,61,** 928-31.

15- Slinkard K., Singleton V.L. (1977). ***Total phenol analyses: automation and comparison with manual methods.*** **American J. Enolo. Viticul., 28,** 49-55.

16- Gazzani G., Papetti A., Massolini G., Daglia M., (1998). ***Antioxidant and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment,*** **J. Agric. Food Chem. ,46 ,** 4118-41121.

17- Martinez-Valverde I., Periago M. J., Provan G., Chesson A., (2002). ***Phenolic compounds, lycopene and antioxidant activity in commercial***

***varieties of tomato (Lycopersicum esculentum*), J. Sci. Food Agr., 82,** 323–330.

18- Conde E., Cadahia E., Garcia-Vallejo M. C. ,(1997). ***Low molecular weight polyphenols in leaves of Eucalyptus camaldulensis, E-globulus and E-rudis,* Phytochem. Anal., 8,** 186–193.

19- Molyneux R. J., Mahoney N., Bayman P., Wong R. Y., Meyer K. Irelan N., (2002). ***Eutypa dieback in grapevines: differential production of acetylenic phenol metabolites by strains of Eutypa lata,* J. Agric. Food. Chem., 50,** 1393–1399;

20- Penazzi G., Caboni M. F., Zunin P., Evangelisti F., Tiscornia E., Toschi T. G., Lercker G., (1995). ***Routine high-performance liquid chromatographic determination of free 7-ketocholesterol in some foods by two different analytical methods,* J. Am. Oil Chem. Soc., 72,** 1523–1527.

21- Merken H. M., Beecher G. R. (2000). ***Measurement of food flavonoids by high-performance liquid chromatography: a review,* J. Agric. Food. Chem., 48**, 577–599.

22-Khokhar S., Magnusdottir S. G. M. (2002). ***Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom,* J. Agric. Food. Chem., 50,** 565–570.

23- Hiremath S., Badami S., Swamy H., Patil S., Londonkar R. (1997). ***Anti-androgenic effect of Striga orobanchioides,* Journal of Ethnopharm., 56,** 55-60.

24- Vitalone A., Guizzetti M., Lucio G. , Tita B. (2003). ***Extracts of various species of Epilobium inhibit proliferation of human prostate cells,* J. P. P., 55,** 683–690.

25- Evans B., Griffiths K., Morton M. (1995). ***Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids,* Journal of Ethnopharm. , 747,** 295-302.

Chapter Four

General Discussion and Conclusions

4.1 General discussion

Plants are important source of useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* biological activity assays [1]. Many reports are available on the antioxidant, antibacterial, antifungal, anticancer properties of plants [2, 3]. Some of these reports have helped in identifying the active principle responsible for such activities and in the developing of drugs for therapeutic use in human beings. Medicinal plants represent a rich source

of antimicrobial agents [4]. A wide range of medicinal plant parts is used for extracts as raw drugs and they possess varied medicinal properties [5, 6]. The present work was therefore aimed at investigating the biological activity (antioxidant, antibacterial, antifungal and anticancer) of six plants used in Traditional Arab Palestinian Herbal Medicine, TAPHM, to treat several ailments and health problems in humans. The work was also aimed at separating and identifying biologically active constituents in these plants, and thereafter modifying some of these compounds in order to promote their biological activities.

All the ethanolic extracts of the tested plants in this study revealed varying levels of antioxidant radical scavenging activity, with *M. syriaca* showing the highest radical scavenging activity using the DPPH assay, and *U. pilulifera* showing the highest antioxidant activity in β-carotene assay. Recently, methanol extract of different parts of *U. pilulifera* extracts were found to exhibit powerful antioxidant activity against various oxidative systems *in vivo* [7]. The antioxidant activity of these extracts has been attributed to the reduction of lipid peroxidation and elevation of antioxidant enzyme activities.

However, some of the species in the present work have showed low radical scavenging activity compared to positive controls but high antioxidant activity against linoleic acid peroxidation or vice versa. This result is in agreement with that of Lee (2003) [8]. Different antioxidant and radical scavenging activity may be partly, due to wide variety of antioxidant constituents such as phenolics, flavonoids and other types of

free radical inhibitors, initiates oxidation and inhibits free radical chain propagation reactions. Different mechanisms of action and kinetics of the inhibitory effect of these antioxidants using different procedures may result in the discrepancy of these findings [9]. Owing to the complexity of the antioxidant materials and their mechanism of actions, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied samples and a combination of different methods is necessary. Despite such limitations, DPPH or linoleic acid peroxidation methods can be helpful for primary screening and finding of novel antioxidants [10].Taken together, our results combined with those of recent findings allow us to suggest that the extract of *U. pilulifera* contains bioactive compounds with antioxidant activities.

In the present study ethanolic extracts of the six plants used in traditional medicine in Palestine were tested for their ability to inhibit the proliferation of breast cancer cells (MCF-7) and Prostate cancer cells (PC3) using the MTT reduction assay. The extract of *U. pilulifera* showed the highest cytotoxicity against breast cancer, and this may be attributed to high concentrations of phenolics and flavonoids present in the plant, as was revealed in our work. This result is in agreement with that of Gülçin (2004) [11] who also found that the antioxidant properties of *Urtica dioica* L. have lead to anticancer activities. The results of the present study and of other comparable studies emphasize the effect of polyphenolic compounds on mutagenesis and carcinogenesis in humans by stabilizing lipid peroxidation and protecting the cells from oxidative damage [12].

Furthermore, *M. syriaca and C. capitatus* have also showed considerable antimicrobial activity against all different types of tested bacteria and dermatophytic fungi. This result is in agreement with those of Darwish (2010) [13] and Ahmet (2003) [14] who also found both plants to posses antibacterial activity against many types of bacteria. Chemical analysis of these plants in this work has revealed that they are rich in phenols, thymol and carvacrol, which may therefore be responsible for their characteristic flavor, fragrance and antimicrobial activity [15].

In the present work, chemical analysis of the test plants, the isolation of some of their constituents, and investigating their biological activities (antioxidant and radical scavenging, antimicrobial, and anticancer activities), have revealed some compounds with significant activity. One of the most promising of these constituents was 2-phenoxyethanol which was isolated from *U. pilulifera*. The compound showed the highest antioxidant activity among all bioactive constituents isolated and identified from the six plants. To promote its activity, the compound was treated with a set of carboxylic acids to form phenolic acid esters. These acid esters were tested for the first time for their biological activities. The most active ester products were 2-phenoxyethyl 2-hydroxybenzoate and 2-phenoxyethyl 4-hydroxybenzoate, with the latter showing remarkable antifungal and anticancer activities. Such activity may be attributed to the molecular structure, and the position of hydroxyl group. Several previous studies have demonstrated that the catechol moiety, with the 3, 4-dihydroxyl configuration, is important for the free radical scavenging activity for this

type of phenolic compounds. On the other hand, other studies suggested that the structure was not required for the activity [5, 6]. Thus, it is worthy to compare the biological activities of synthesized phenolic acid esters with different hydroxyl positions on the moiety beginning with the constituents separated from the selected medicinal plants as starting materials.

Another interesting result from the present study was the separation and identification of phthalate compounds such as diisobutyl phthalate and di-n-octyl phthalate for the first time from the extract of *A. palaestinum*, using HPLC and GCMS techniques. Synthetic phthalate esters are used commonly in paints and polymer products as plasticizers [16], and widely detected in the environments, such as sediments, natural waters, soils, plants, and aquatic organisms [17]. Although phthalate derivatives are harmful chemicals which are regarded as environmental health hazards due to their toxicity, carcinogenicity [18].Other studies showed that phthalate ester (PE) has been detected in water samples from various aquatic environments [19], as well as in bacteria, plants and the fatty acid fractions of certain species of marine macro-algae [20]. However, there are reports that phthalate esters are naturally produced extracellularly by microorganisms such as bacteria, fungi and yeasts [21]. The isolation of these compounds from *Arum palaestinum*, a food and medicinal plant, justifies for further studies on the plant, especially with regard to safety and potential biological activity.

4.2 References:

1- Belpaire F. M., Bogaert M. G., (2003). **The fate of xenobiotics in living organisms, Practical Medicinal Chemistry,** 501-515, Academic press.

2- Koshy P., Sri N. A., Wirkarian S., Sim K. S., Saravana K., Hong S. L., Lee G. S., Syarifah N. S. A. Rahman. (2009). ***Antimicrobial activity of some medicinal plants from Malaysia,*** **American J. Appl. sci., 6 (8),** 1613- 1617.

3- Fyhrquist Pia. (2007). **Traditional medicinal uses and biological activities of some plant extracts of African *Combretum* Loefl., *Terminalia* L.and *Pteleopsis* Engl. species (Combretaceae),** Academic dissertation, Faculty of Biosciences of the University of Helsinki.

4- Abu-Dahab R., Afifi F., (2007). ***Antiproliferative activity of selected medicinal plants of Jordan against a breast adenocarcinoma cell line,*** **Scientia Pharmaceutica, 75,** 121- 136.

5- Charles M. (2005). **Elevating Antioxidant Levels in Food through Organic Farming and Food Processing.** An Organic Center State of Science Review.

6- Riss T., Moravec R., (1993). ***Comparison of MTT, XTT, and a novel tetrazolium compound MTS for in vitro proliferation and chemosensitivity assays,*** **Biomedical Products , 1 8 ,** 66-68.

7- Irshaid F., Mansi K. (2009). ***Effects of leaf extract of Urtica pilulifera L. on male reproductive system of streptozotocin-diabetic rats,*** **American J. Pharmacol. Toxicol. , 4 (2)**, 22-28.

8- Lee S. E, Hwang H. J, Ha J. S, Jeong H.S., Kim J. H. (2003). ***Screening of medicinal plants for antioxidant activity,*** **Life Sci. ,73 ,** 167-179

9- Kaur C., Kapoor H. C., (2002). ***Antioxidant activity and total phenolic content of some Asian vegetables,*** **Int. J. Food Sci. Tech., 37,** 153-162.

10- Koleva I, Van B. T. A., Linssen J. P. H., De G. A., Evstatieva L. N. (2002). ***Screening of plant extracts for antioxidant activity: a comparative study on three testing methods,*** **Phytochem. Anal., 13,** 8-17.

11- ¨Ilhami G., Ö. ¨Irfan K., Münir O. , Mehmet E. B., (2004). ***Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (Urtica dioica L.),*** **J. Ethnopharm., 90,** 205-215.

12- Tanaka M., Kuei C.W., Nagashima Y., Taguchi T., (1998). ***Application of antioxidative maillrad reaction products from histidine and glucose to sardine products,*** **Nippon Suisan Gakkaishi, 54 ,** 1409–1414.

13- Ahmet C. G., Gokhan B., Mine B. , Huseyin D., Esin K. E., (2003). ***Analysis of essential oil of Coridothymus capitatus (L.) and its antibacterial and antifungal activity, Z.*** **Naturforsch., 58c,** 687- 690

14- Darwish R. M., Aburjai T. A., (2010). ***Effect of ethnomedicinal plants used in folklore medicine in Jordan as antibiotic resistant inhibitors on Escherichia coli,*** **BMC Complementary and Alternative Medicine,** 1- 8.

15- Ravi K. U., Pratibha D., Shoeb A. (2010). ***Screening of antibacterial activity of six plant essential oils against pathogenic bacterial strains,*** **Asian J. Med. Sci., 2(3),** 152-158.

16- Ljungvall K., Veeramachaneni D. N. R., Hou M., Hulten F., Magnusson U., (2008). **Theriogenology, 70,** 984.

17- Sultan M. Z., Moon S. S., Park K., (2010). ***Natural phthalate derivatives from the bacterium burkholderia cepacia K87,*** **J. Sci. Res., 2 (1)**, 191-195.

18- Heudorf U., Mersch-Sundermann V., Angerer, (2007). **J. Int. J. Hyg. Environ. Health, 210** , 623.

19- Hashizume K. , Nanya J. , Toda C. , Yasui T. , Nagano H. , Kojima N. (2002). ***Phthalate esters in various water samples and biodegradation of the phthalates by microbes isolated from river water,*** **Biol. Pharm. Bull., 25,** 209–14.

20- Namikoshi M., Fujiwara T., Nishikawa T., Ukai K. (2006). ***Natural abundance 14C content of dibutyl phthalate (DBP) from three marine algae,*** **Mar. Drugs, 4 ,** 290–7.

21- Mahmoud A. H., Motawa H. M., Wahba H. E., Ebrahim A. Y., (2006). ***Study of some antioxidant parameters in mice livers affected with Urtica pilulifera extracts*****, Trends Med. Res., 1,** 67-74.

4.3 Suggestions for further studies

1- Separation identification and determination of chemical constituents for *Teucrium creticum* using GC-MS analysis.

2- Check the activity of *A. palaestinum, U. pilulifera and C. capitatus* plant extracts on different types of cancer.

3- Modify new compounds depending on the other active ingredients in the selected plants.

4- Identify unknown constituents which are detected by HPLC from the extracts of the plants.

جامعة النجاح الوطنية
كلية الدراسات العليا

زيادة فعالية وتعديل على مركبات لها فعالية حيوية من نباتات مختارة في فلسطين.

اعداد
أحمد إبراهيم أحمد حسين

اشراف
أ. د. محمد سليم علي اشتية
د. نضال زعتر **د. وحيد جندي**

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

2010م

زيادة فعالية وتعديل على مركبات لها فعالية حيوية من نباتات مختارة في فلسطين

اعداد
أحمد ابراهيم أحمد حسين

اشراف
أ.د. محمد سليم علي اشتية
د. نضال زعتر د. وحيد جندي

الملخص

يهدف هذا البحث إلى فحص مستخلصات كحولية لست نباتات مختارة تستخدم في الطب الشعبي في فلسطين لعلاج أمراض مختلفة واجراء بعض الفحوصات الحيوية عليها مثل مدى منع أو وقف عمليات التأكسد، أثر المستخلصات على أنواع مختلفه من البكتيريا والفطريات التي تصيب الإنسان وكذلك أثرها على الخلايا السرطانية التي تصيب الثدي وغدة البروستاتا من خلال اختبار خاص بقياس حساسية هذه الخلايا يسمى (MTT assay).

أما النباتات المختارة فهي اللوف ، القريص ، الزعتر الفارسي ، الزعتر البري ، جعدة زيته ، جعدة الصبيان.

وقد أظهرت النتائج المخبرية أن جميع المستخلصات لها أثر حيوي في بعض الفحوصات التي تمت. أما عملية فصل المستخلصات فقد اعتمد فيها طرق الاستخلاص و الفصل بأنواع مختلفة من الكروموتوغرافيا منها ، كروموتوغرافيا الورقة و غروموتوغرافيا الغاز (GC) و غروموتوغرافيا الضغط المرتفع (HPLC) ، كما تم التعرف على بعض المركبات الفعالة المستخلصة من النباتات قيد الدراسة باستخدام أجهزة طيف الكتلة (MS) ، وأجهزة الأشعة فوق البنفسجية (UV) و أجهزة طيف الأشعة تحت الحمراء (IR) . ومن اهم المركبات التي فصلت مركب 2- فينوكسي ايثانول الذي تم مفاعلته مع بعض الحموض الكربوكسيلية لانتاج مركبات جديدة ،اظهرت اثرا كبيرا على خلايا السرطان وبعض انواع الفطريات .

كما تم التعامل مع المركبات الفعالة المستخلصة من النباتات الطبية كيماويا لزيادة فعاليتها عن طريق التغيير في التركيب الكيماوي لها بتغيير موقع بعض المجموعات الوظيفية عليها، أو تحضير بعض المركبات المشابهة لها كيماويا و التي يمكن أن يكون لها فعالية في علاج بعض

الأمراض التي تصيب الإنسان. ومن نتائج هذه الدراسة ايضا فصل بعض مركبات الفيثاليت لأول مرة من نبتة اللوف.