An-Najah National University

Faculty of Graduate Studies

Biological and phytochemical screening of *Erodium laciniatum* and *Lactuca orientalis*

By

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This thesis was defended successfully on 12/01/2017 and approved by:

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Dedication

This thesis is dedicated to:

My own little heroes Eyas, Emad and Maymoona.

My great parents Dr Ghazi Othman and Mrs Amina Ghanim.

My husband Moathe Deek.

My brothers, sisters and friends.

All truth researchers and pharmacists in Palestine and world.

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الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان

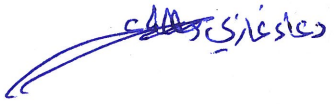
Biological and phytochemical screening of Erodium laciniatum and Lactuca orientalis

أقر بأن ما شملت عليه الرسالة هو نتاج جهدي الخاص, باستثناء ما تمت الإشارة إليه حيثما ورد, وأن هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحثي لدى أي مؤسسة علمية أو بحثية

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 degrees or qualifications.

Student's Name: **اسم الطالب: دعاء غازي عثمان**

Signature **التوقيع:** 

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List of Abbreviations

λ_{max}	Maximum Wave Length of Absorption
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ATCC	American Type Culture Collection
CA	Catechin
CFU	Colony-Forming Unit
CLSI	Clinical Laboratory Standard Institute
CUPRAC	Cupric Ion Reducing Antioxidant Capacity
DMPD	N,N-dimethyl-p-phenylene diamine dihydrochloride
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
FRAP	Ferric Reducing-Antioxidant Power
FTC	Ferric Thiocyanate
GA	Gallic acid
IC_{50}	Half Maximal Inhibitory Concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MOPS	3-[N-morpholino] propanesulfonic acid
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
ORAC	Oxygen Radical Absorbance Capacity
QU	Quercetin
ROS	Reactive Oxygen Species
RP	Reducing Power
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SOD	Superoxide Radical
TBA	Thiobarbituric Acid
TEAC	Trolox Equivalent Antioxidant Capacity
TRAP	Total Radical-Trapping Antioxidant Parameter
UV	Ultraviolet

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Abstract

From ancient times, various herbal remedies and various medical agents containing flavonoids, tannins and phenols have been utilized for prevention and treatment of different diseases and disorders. Currently, such groups of bioactive compounds have become the subject of many antimicrobials researches, with possessed antiviral, antifungal and antibacterial activities. Moreover, many of high quality studies assessed the relationship between flavonoid's, tannin's and phenol's chemical structures and their antibacterial activities, and also studied their mechanisms of action on the microbial growth. These investigations approved that such classes of natural compounds can possess many therapeutic properties, including oestrogenic, antiinflammatory, enzyme inhibition and antimicrobial activities.

In this study we aimed to screen *Erodium laciniatum* and *Lactuca orientalis*

phytoconstituents and to evaluate their total flavonoids, tannins and phenols contents. An additional aim is to evaluate their antioxidant and antimicrobial activities.

Phyto-constituents, total flavonoids, phenols and tannins were screened and evaluated by using standard analytical and phytochemical methods.

Antioxidant activity was evaluated by using DPPH method; meanwhile antibacterial activities were examined by using several reference bacterial strains obtained from the American Type Culture Collection (ATCC) and multidrug resistant clinical isolates. The tested strains included *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (MRSA Positive), *Enterococcus faecium* (ATCC 700221), *Shigella sonnei* (ATCC 25931), *Enterobacter cloacae* (clinical) and *Klebsiella pneumoniae* (clinical).

In addition to that anti-fungal activities of these extracts were also examined against two reference strains *Candida albicans* (ATCC 90028) and *Epidermatophyton floccosum* (ATCC 52066). Antibacterial and antifungal activity of all prepared *Erodium laciniatum* and *Lactuca orientalis* organic and aqueous extracts were determined by using several methods including agar diffusion well-variant method, agar diffusion disc-variant method and broth micro-dilution method. The results of the phytochemical screening showed that *L. orientalis* and *E. laciniatum* contain phenols, tannins, flavonoids, saponins, monosaccharides, reducing sugars, carbohydrates, cardiac glycosides, and steroids, while alkaloids were not detected in both of the studied species. However, proteins, starch and terpenoids were not detected in *L. orientalis* and detected in *E. laciniatum* which means that *E. laciniatum* had more phyto-constituents than *L. orientalis*. Moreover, total flavonoid, tannins and phenols content in *E. laciniatum* extract were higher than the *L. orientalis* extracts. The IC_{50}

values of the antioxidant activity were almost the same in *L. orientalis* and *E. laciniatum* extracts. Additionally the lowest value of MIC was for organic extract of *E. laciniatum* against *Staphylococcus aureus*. The same organic extract also showed to have the largest diameter of inhibition against *Enterococcus faecium.* However, *L. orientalis* aqueous extract showed to have the largest diameter of inhibition against *Staphylococcus aureus.* Whereas, the antifungal activity which was tested by using agar dilution method against *Epidermatophyton floccosum* showed that the organic extract of *E. laciniatum* had the lowest MIC which was 0.391mg/ml. In conclusion, both of the studied species have a mixture of phytochemicals and rich in flavonoids, phenols and tannins and have potential antioxidant and antimicrobial activities which can be used as therapeutic agents or can be used in cosmeceutical and pharmaceutical industries.

Chapter One

1. Introduction:

1.1. Background

The majority of the world populations especially in the underdeveloped and developing countries depend mainly on herbals and herbal extracts for treatment of their health problems [1]. Recently, there is a continuous increase of the world populations, lack of enough supply of medications, side effects of chemical drugs, high cost of treatments and expansion of drug resistance to the most of the antibiotics that used in the treatment infectious diseases. These factors have led the pharmacologists, pharmacognosists, microbiologists and other scientific specialties to increase their attention on the natural sources specially from plants as a source of medicaments for treatment of a wide variety of human ailments [2, 3].

In the recent time, wild and cultivated medicinal plants represent an alternative biological source of therapeutic agents instead of the chemical synthetic agents as they are safe for both human and environment. These natural agents have been clinically tested on humans from ancient time as they were used for treatment of various infectious and noninfectious diseases throughout folkloric complementary and alternative medicine on humans and domestic animals [4, 5]. In addition to their therapeutic values, plants had been utilized for other purposes throughout the human history such as flavoring agents for foods and drugs, food natural preservatives, coloring agent and cosmetics [6, 7]. Medicinal plants are

still widely being used in crude forms or as extracts and different parts of these plants such as seeds, fruits, flowers, leaves, barks and roots are used for preparing of these therapeutic forms [8].

Plant kingdom has been a major source of medicaments for thousands of years, and phyto-products continue to play an essential role in medicine which is considered an endless source of therapeutic agents which provided and still provide therapeutically active compounds and substances for curing several diseases such as atropine from Belladonna, vincristine from Catharanthus, morphine from Poppy capsule, reserpine from Rauwolfia, eugenol from Clove, cineole from Eucalyptus and many others [9, 10].

However, it must be taken into consideration that the use of medicinal plants with their all therapeutic and non-therapeutic benefits must be balanced with their possible risks and safety precautions [11, 12].

1.2. Antioxidant

In the human body, the lack of balance between formation of free radicals and their neutralization can cause oxidative stress which may cause many types of diseases. Oxidative stress disorders can cause damage in macromolecules of the living cells, DNA, lipids and proteins [13] .The major source of oxidative stress is endogenous oxidants which resulted from normal cellular metabolism. Many common diseases are caused by these conditions such as cancer, hypertension, diabetes, atherosclerosis, Alzheimer and Parkinson [14].

In fact, there are several mechanisms of protection from these free radical formations in the human cell, this system may be disrupted due to pathogenic conditions. Some synthetic antioxidants like NSAIDs are available in the markets and widely used but they have many adverse reactions, drug interactions, contraindications and dangerous side effects. Since natural compounds have lower side effects and better desirable effects in most cases, the use of antioxidant compounds from medicinal plants and other natural sources is recently increased [15, 16].

Antioxidant compounds from natural sources can be used to maintain human health and protect from oxidative stress so they protect from many diseases and improve a healthy lifestyle [17]. Recent researches have reported that antioxidant compounds found in medicinal plants not only useful in neutralization of free radicals, but also have several pharmacological effects like antimicrobial effect, antimutagenic and anti-inflammatory effects. For these reasons, antioxidant compounds became a promising title for researchers to work on worldwide [18, 19].

To estimate and evaluate the antioxidant activities of plant extracts, there are many available methods to be used in vitro [20]. These methods include 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method, hydrogen peroxide scavenging (H_2O_2) assay, trolox equivalent antioxidant capacity (TEAC) method, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolorization assay (ABTS), nitric oxide scavenging activity, peroxynitrite radical scavenging activity, total radical-trapping antioxidant parameter (TRAP)

method, superoxide radical scavenging activity (SOD), ferric reducing-antioxidant power (FRAP) assay, hydroxyl radical scavenging activity, phosphomolybdenum, oxygen radical absorbance capacity (ORAC), reducing power (RP), ferric thiocyanate (FTC), thiobarbituric acid (TBA), β-carotene linoleic acid method (conjugated diene assay), xanthene oxidase, cupric ion reducing antioxidant capacity (CUPRAC), metal chelating activity and N,N-dimethyl-p-phenylene diamine dihydrochloride (DMPD) methods [21].

The DPPH method uses the molecule 1, 1-diphenyl-2-picrylhydrazyl (DPPH) to determine the antioxidant activity of plant extracts in a UV-visible Spectrophotometric method [22]. The DPPH molecule is a free radical molecule which is known to have stable formula with spare electron that delocalizes over a molecule as a whole for that no dimerization may occurred. This delocalization of spare electron provides the deep violet color which is characterized by an absorption band of UV radiation of wavelength 517nm. When a solution of antioxidant agent is mixed with another which contains DPPH, the antioxidant will scavenge the free radical and reduce the DPPH molecule to DPPH-H molecule and the deep violet color will be lost. Due to these characteristics antioxidant activity of any substances including plant extracts can be examined by measuring the decrease of absorption of the UV radiation at 517nm for mixed solutions compared to DPPH solution at the same concentration. A reference standard is used usually in this method to compare with the tested extract like 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic

acid compound which is commonly known as Trolox. Trolox is a vitamin E analogue which is water soluble and has very high antioxidant activity [23].

1.3. Phenols and total phenols

Phenolic compounds are a very important type of antioxidants that are found in many medicinal plants and have multiple functions against free radicals. They have one or more aromatic rings in their molecular skeleton in addition of one or more hydroxyl groups. Phenolic compounds have reducing, hydrogen donating, and singlet oxygen quenching activities as well as they have chelating properties for metals [24].

These compounds are found widely in the nature and separated into many classes that range from simple phenols to highly complex polymeric compounds like tannins [25].

Total phenol content can be measured by using Folin Ciocalteau's method which depended on the use of Folin Ciocalteau's reagent with the extract solution in alkaline media where the reduction of the reagent by phenols turns the color of solution from yellow to blue. By using the spectrophotometer the resulting solution is tested for absorption on the wave length of 765nm. The intensity of light absorption at 765nm is proportional to phenol concentration in the sample. Gallic acid is a standard that may be used in this method for comparative purposes [26].

1.4. Tannins

Tannins are phenolic compounds that give rise to the astringency and bitterness in plants. They are known to have highly complex structured

types with high molecular weight called true tannins which are subdivided into hydrolysable tannins and non-hydrolysable tannins. Moreover, there are simple tannins with relatively low molecular weights that called pseudo-tannins [27].

Hydrolysable tannins are formed by esterification of sugars with simple phenolic acids and these compounds may be hydrolyzed by acids or enzymes such as tannase enzyme. Non-hydrolysable tannins are condensed tannins which occur due to condensation reactions between flavanols [28].

1.5. Flavonoids and flavonoids glycosides

Flavonoids are the largest group of phenolic compounds in the nature that are found in free state and as glycosides. They give rise to colors which are found in several parts of plants like flowers and fruits and some of them affect the taste and fragrance of them. They are known to have a strong antioxidant activity because of their ability to scavenge damaging oxidizing species [29].

Total flavonoids content can be determined using aluminum chloride colorimetric method. The plants samples are mixed with aluminum chloride and potassium acetate solutions. Using a spectrophotometer, the intensity of light absorption at 510 nm is measured for solutions. Quercetin is a standard material which is commonly used to establish a standard calibration curve to determine flavonoids concentrations in plant extracts [30].

Flavonoids can reduce the activity of reactive oxygen species by several means. They suppress singlet oxygen, inhibit enzymes that generate Reactive Oxygen Species (ROS), chelate ions of transition metals that may catalyze the production of ROS, quench cascades of free-radical reactions in lipid peroxidation and recycle other antioxidants [31, 32] .

Flavonoids can be classified into classes according to their chemical structure and these classes include flavonols, isoflavones, flavones, flavanone, aurones, dihydrochalcones, leucoanthocyanidins, anthocyanins and proanthocyanidins. It was found that flavonoids play an important role in plant resistance against pathogenic microorganisms [33].

1.6. Antibacterial and antifungal activities

Currently used and available antibiotics have many side effects on the human body such as hypersensitivity, allergic reactions, immune suppression as well as many other side effects. In addition to that, the increase of resistant of bacteria against these antibiotics is a challenging issue nowadays [34]. High number of illnesses and deaths resulted from resistance bacteria worldwide and this requires introducing new developed antibiotic drugs. However, the development of such drugs has decreased in recent years although currently used antibiotics are getting less potent. For that, there is an urgent need to search about novel antimicrobial drugs from natural sources that have high potency and relatively low side effects. Many species of medicinal herbs were found to have antibacterial and antifungal activities and can be used as an

efficient alternative in place of currently available antibiotics with fewer side effects [35].

Antibacterial activity of a plant extracts can be determined by several methods [36]. Agar diffusion well-variant is a method which requires using sterile Petri dishes filled with Mueller-Hinton agar and the bacterial inoculum will be uniformly spread with a sterile swab. Wells are to be applied in agar and solution of agar is added to these wells. After incubation, inhibition of bacterial growth is measured in mm [37]. Another method for testing of antibacterial activity is agar diffusion disc-variant which the bacterial inoculums are prepared and applied to Mueller-Hinton agar as the previously mentioned. Discs of filter loaded with plant extract are placed on separated points in agar. After incubation, the inhibition of bacterial growth is measured in mm [38]. A micro-dilution method is used for determination of minimum inhibitory concentration (MIC). Mueller-Hinton broth is usually used as media added to 96-well plates. Plant extract solution is then added and serially diluted. Certain volume of bacterial inoculum is added and the results are taken after 24 hours incubation at 35^{o}C. The minimal inhibitory concentration (MIC) is the lowest plant extract concentration that is required to inhibit the visible bacterial growth completely [37]. All previously mentioned methods need preparation of bacterial inoculums in certain concentrations to achieve accuracy and consistency and this can be done by comparison of turbidity of the bacterial suspension with a turbidity of a standard solution (McFarland standard). Standardized

suspensions of bacteria, which have turbidity that match turbidity of McFarland standard represent starting point of dilutions to reach the final wanted bacterial concentration [38].

For evaluation of antifungal activity the same methods can be applied on candida but with some modifications [39].

1.7. *Lactuca orientalis* and *Erodium laciniatum* members of Palestinian flora

Palestine, the Holy Land, has ethnic high rooted inconsistency (Christians, Druze, Muslims, Jews from West and East and Samaritans) [40]. Such context has enriched its culture, especially folkloric, medicine, herbal food and cosmetic. In fact, Palestine is a unique country, in its bio-diversity due to its geographical location and due to that, various climatic, phyto-geographic and zoogeographic zones covered Palestine, creating huge biological multi-diversity [41]. In the flora of Palestine, there is about 2700 plant species distributed in all its regions but few of them have been sufficiently and scientifically studied [42].

In this study we focused on *Lactuca orientalis* and *Erodium laciniatum*, which are two of unstudied plant species in the Palestinian flora.

1.7.1. *Lactuca orientalis*

Lactuca orientalis (Boiss.) Boiss. is one species of Lactuca genus which belongs to the Asteraceae (*Compositae*) family. Its common English name is split-leaf lettuce which grows wildly in several regions of the world including the rocky Mediterranean, Irano-Turanian, Northern

African, Western Asian, Caucasian, Middle Asian and Indian Sub-continental regions. It is found in Palestine in the Southern regions in the deserts and rocky mountains. *Lactuca orientalis* grows as dwarf-shrup (chamaephyte) with length ranges between 20 and 50 centimeters. It looks to have wooly-floccose appearance in early stages of growth then becomes glabrescent later. Its stems and branches are white colored and intricate. Branchlets of it are rigid, short and spreading and in late stages they become spinescent at tips. The leaves of *L. orientalis* -as shown in Figure 1- are pinnatifid into few triangular to oblong lobes, and they wither soon. Its radical leaves are tapering to a petiole and its cauline leaves, which are growing immediately on caulis which are long-decurrent with adnate linear appendages. Its heads are solitary and they are sessile and have 5-flower each. It has involucres with length about 8 to 14 millimeters in fruit. The color of its flowers is yellow. The produced achenes (naked seeds) are 6 to 8 millimeters long, there color is tawny or dirty purple, there shape is linear to narrowly elliptic, they are sub-compressed, they are 7 to 9-striate on each side, they are tapering above and nearly beakless. Each of them has pappus which its color is white and it is about as long as achene. These pappi are easily deciduous. The flowering time of *L. orientalis* is often between August and September.

Figure 1: *Lactuca orientalis* plant

The phytochemical constituents of *L. orientalis* are saponins, flavonoids and tannins [43]. According to the best of our knowledge there were no previous studies on *L. orientalis* about its antioxidant activity, total phenol, tannin and flavonoids content, and possible antibacterial and antifungal activities.

1.7.2. *Erodium laciniatum*

Erodium laciniatum (Cav.) Willd. is one species from genus *Erodium* and it belongs to the family of *Geraniaceae.* Its common known names are Cutleaf Heron's-Bill, Qarnawah, and Ibrat Al Rahib. It grows wildly in Mediterranean region and other regions worldwide. It is found in Palestine among shrubs and in grassy places and it grows also in sandy soil.

E. laciniatum is an annual herbaceous plant and appears to be covered in crisp soft downy hairs which are dense to sparse and these hairs are retrorse or antrorse and sometimes it seems to be sparingly glandular and hairy as shown in Figure 2. It may be glabrescent but this is rare. It is 20 to 50 centimeters long. Stems of *E. laciniatum* are grooved and they are

procumbent or prostrate and rarely found to be erected. *E. laciniatum* leaves are 1 to 7 centimeters long and it has also stipules which are membranous, ovate and 5 to 8 millimeters long. Blades of lower leaves of the plant are mostly cordate-ovate and obscurely 3-lobed. Blades of the other leave are 3-lobed or 1 to 2-pinnatepartite or pinnatescent into short, narrow and acutish lobes. Rarely some of its leaves are mostly undivided. The flowers of *E. laciniatum* grow as flower clusters on very long peduncles, these clusters are called umbels. Every umbel contains three to eight flowers. *E. laciniatum* has bracts which are two or more with length between 0.25 to 0.8 centimeters. They are broad, membranous, more or less ovate and their color is brown or white. Pedicels of flowers are twice as long as the calyx, they are often hispidulous to glandular-hairy, but they rarely found to be glabrous. Sepals are about 8 millimeters long. They are mucronate and usually patulous or appressed-hairy. Petals are longer than sepals, their color is purple and they are oblong. Filaments of flowers are toothless or maybe 1 to 2-ciliate-dentate. Mericarps are 4 to 8 millimeters long and they have no furrows beneath their pits. They have beaks which are slender and 4 to 8 centimeters long. The flowering time is between March and April.

Figure 2: *Erodium laciniatum* plant

According to the limits of our knowledge, there are no previous studies about *E. laciniatum* species that give details about its antioxidant activity, total phenol, tannin and flavonoids contents, and possible antibacterial and antifungal activities.

1.8. Objectives and significance of the study

1.8.1. Objectives of the study:

This study aimed to investigate phytochemical constituents, evaluate total phenols, tannin and flavonoids contents and to estimate antioxidant and antimicrobial activity of *Lactuca orientalis* and *Erodium laciniatum* which are two species from Palestinian flora that have not been studied previously.

1.8.2. Significance of the study:

To the best of our knowledge there are no previous studies about phytochemical constituents, total phenols, tannin and flavonoids contents as well as antioxidant and antimicrobial activity of *L. orientalis* and *E. laciniatum* plants. Investigating the phytochemical active constituents of these species therefore would be of great value.

Chapter Two

2. Methodology:

2.1 Materials:

All the chemicals and reagents used in this study were from the Pharmacy Department at An-Najah National University and were purchased form reliable resources as shown in Table 1, 2 and 3.

Table 1: Chemicals and reagents used for phytochemical screening

Chemicals and reagents	Manufacturer	Country
Millon's reagent	Gadot	Israel
Acetic acid	Frutarom LTD	Israel
Benedict's reagent	Gadot	Israel
$FeCl_3$	Riedeldehan	Germany
NaOH	Gadot	Israel
Ninhydrain solution	Alfa Aesar	England
Magnesium ribbon	SDFCL	India
Molish's reagent	Alfa Aesar	England
H_2SO_4	Alfa Aesar	England
Iodine solution	Alfa Aesar	England
Chloroform	Sigma-Aldrich	Germany
HCl	SDFCL	India
Wagner's reagent	Sigma-Aldrich	Germany

Table 2: Chemicals and reagents used for antioxidant evaluation

Chemicals and reagents	Manufacturer	Country
Methanol	Lobachemie	India
N-hexane	Frutarum LTD	Israel
(DPPH) 2, 2-Diphenyl-1-picrylhydrazyl	Sigma-Aldrich	Germany
Trolox (6-hydroxy- 2, 5, 7, 8 -tetramethychroman-2 carboxylic acid)	Sigma-Aldrich	Denmark

Table 3: Chemical reagents used for estimation of total phenols, tannin and flavonoid contents

Chemicals and reagents	Manufacturer	Country
Folin-Ciocalteu's	Sigma Aldrich	Denmark
quercetin	Sigma Aldrich	Denmark
AlCl3	Sigma Aldrich	Germany
Na_2CO_3	Sigma Aldrich	Germany
Catechin	Sigma Aldrich	Israel
vanillin	Sigma Aldrich	Denmark

While Mueller-Hinton broth (Becton, Dickinson and copany, USA), Mueller-Hinton Agar (Becton and Dickinson company, USA), Sabouraud Dextrose Agar (Becton, Dickinson and company, France), RPMI-1640 media (Sigma Aldrich, UK) and Dimethyl sulfoxide (DMSO) (Riedeldehan, Germany) were used for antimicrobial screening experiments.

2.2. Instrumentation

All the used instrumentations in this study were from the Pharmacy Department at An-Najah National University except microbiological tests instrumentations which were conducted at Faculty of Science, Microbiology Department, An-Najah National University and the all used instrumentations presented in Table4.

Table 4: instrumentations used for biological and chemical screening of plants extracts

Instrumentations	Manufacturer	Country
Rotary evaporator	Heidolph OB2000, VV2000	Germany
Freeze dryer	Mill rock technology, model BT85, Danfoss	China
Grinder	Moulinex model, Uno.	China
Balance	Radwag, AS 220/c/2	Poland
Micropipettes	Finnpipette	Finland
Incubator	Nuve	Turkey
Syringe filter 0.45 *µm* pore size	Microlab	China
Micro-broth plate	Greiner bio-one	North America
Vortex	VELP Scientifica	Europe
Shaker	Memmert Shaking Incubator	Germany
Hot plate stirrer	Daihan Labtech	India
Multi 12-Channel Micropipette	Eppendorf	Germany
Spectrophotometer	Jenway 7135	England

The used glasswares were volumetric flasks (50ml, 150ml, 100ml, 25ml), glass rod, graduated cylinders (1000ml, 100ml, 50ml), separatory funnels and simple funnels.

2.3. Collection and preparing plant materials:

The entire *L. orientalis* plant was collected during the flowering time in June, 2015 from Jerusalem area of Palestine while *E. laciniatum* plant was collected from Nablus area during the same time. Botanical identification was carried out at Pharmacognosy and Herbal Products Laboratory at An-Najah National University. Botanical identification was based on live-herbal specimens and photographs. Voucher specimen code

was Pharm-PCT-1317 for *L. orientalis* and Pharm-PCT-942 for *E. laciniatum*.

Collected aerial parts were washed, completely dried in shade at room temperature and the dried leaves were grounded into a fine powder by using a mechanical blender. Generated powders were stored in proper storage conditions separate special containers till use.

2.4. Preparation of plant extracts

A total of 25g of powder of each plant species was exhaustively extracted by adding 50ml of n-hexane and 125 ml of 50% ethanol in triple-distilled water. Mixtures were then shacked for 72 hour at room temperature using a shaker that was set at 200 rpm. After that, each mixture was filtered using suction flask and Buchner funnel filtration. The obtained liquid filtrates were separated individually by separatory funnel into 2 phases; a lower aqueous phase representing the first aqueous extract and the upper organic phase representing the organic extract. The remaining solid materials were re-extracted separately, by 125 ml of 50% ethanol in triple distilled-water and the re-extraction process was carried out as described earlier. The extracts were then filtered individually to obtain the second aqueous extract. Both first and second aqueous extracts were pooled together and dried using a freeze dryer for 12 h. Meanwhile, organic extracts were placed in rotary evaporator for 1 h. at 35ºC to evaporate leftover organic solvents and completely dried organic extracts were then stored at 2-8ºC till use [44]

2.5. Antioxidant activity

2.5.1. Trolox standard and plant working solutions:

A stock solution of a concentration of 1 mg/ml in methanol was initially prepared for each of the plants aqueous extracts and Trolox standard. Stock solutions were used to prepare working solutions with the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 70, 80, 100µg/ml) by using serial dilution in methanol.

2.5.2. Spectrophotometric measurements :

Solution of DPPH was freshly prepared at a concentration of 0.002% w/v. Then, it was mixed with methanol as well as with each of the working concentration in a ratio of 1:1:1. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentrations was DPPH with methanol only. The solutions were incubated at room temperature in a dark cabinet for about 30 minute. Then, their optical densities were determined by using the spectrophotometer at a wave length of 517 nm. The change of the color of solutions from purple to clear solution indicated the antioxidant activity.

2.5.3. Percentage of inhibition of DPPH activity :

The percentage of antioxidant activity of each of the obtained plants extracts and the Trolox standard were calculated using the following formula :

DPPH inhibition activity (%) = (A-B)/A ×100%

Where A is optical density of the blank and B is optical density of the sample .

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant extracts as well as the standard deviation were both calculated using GraphPad Prism 6 Program.

2.6. Qualitative phytochemical analysis :

Preliminary qualitative phytochemical screening of primary and secondary metabolic compounds such as proteins, starch, phenols, cardiac glycosides, saponin glycosides, flavonoids, alkaloids, steroids, volatile oils, and tannins were carried out according to the standard common phytochemical methods described by Trease and Evans, 1983 [45] and Harborne, 1998 [25] for aqueous extracts of entire *L. orientalis* and *E. laciniatum* plants.

2.6.1. Qualitative tests for proteins

Ninhydrin test: boiled 2 ml of 0.2% Ninhydrin solution with the plants crude extracts, the violet color that appeared indicate the presence of proteins and amino acids.

2.6.2. Tests for carbohydrates

Fehling's solutions test: Fehling solutions A and B were mixed in equal volumes, boiled and then this mixture was added to both crude plants extracts. A red color precipitate indicated the presence of reducing sugars.

Benedict's reagent test: Boiled 2 ml of Benedict's reagent with crude extracts; a reddish brown color indicated the presence of the carbohydrates.

Molisch's solution test: crude plants extracts were shaken with 2 ml of Molisch's solution and 2 ml of H_2SO_4 concentrated was added and poured carefully along the side of the test tube. A violet ring appeared at the inter phase of the test tube indicated the presence of carbohydrate.

Iodine test: 2 ml of iodine solution was mixed with both crude plants extracts. Purple or dark blue colors indicated the presence of the carbohydrate.

2.6.3. Test for phenols and tannins

2 ml of 2% solution of $FeCl_3$ was mixed with crude extracts. Black or blue-green color indicated the presence of tannins and phenols.

2.6.4. Tests for flavonoids

Alkaline reagent test: 2ml of 2% NaOH solution was mixed with plants crude extracts, intensive yellow color was formed, which turned into colorless when 2 drops of diluted acid were added to solution. This result indicated the presence of flavonoids.

2.6.5. Test for saponins

About 5 ml of distilled water was added to crude plants extracts in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.

2.6.6. Keller-kilani test

A mixture of Acetic acid glacial (2ml) with 2 drops of 2% $FeCl_3$ solution was added to the plants extracts and conc. H_2SO_4. A brown ring, which was produced between the layers, indicated the entity of cardiac steroidal glycosides.

2.6.7. Test for steroid

In a beaker, every plant crude extract was mixed with 2 ml of chloroform and conc. H_2SO_4. In the lower chloroform layer the produced red color indicated the presence of steroids. Another test was performed by mixing 2ml of each of acetic acid with conc. H_2SO_4 and crude extracts with 2ml of chloroform. Green color indicated the presence of steroids.

2.6.8. Test for terpenoids

About 2 ml of chloroform was mixed with the plants extracts and evaporated on the water path then boiled with 2 ml of conc. H_2SO_4. A grey color which was produced indicated the presence of terpenoids.

2.6.9. Test for alkaloids (Wagner's test)

Few drops of Wagner's reagent were added carefully in a test tube to about 2 ml of each plant extract. A reddish brown precipitate indicated the presence of alkaloids.

2.7. Determination of total Tannin content:

Total tannin content of aqueous extract for the two studied species was determined according to Vanillin colorimetric method with some

modifications [46]. Serial dilutions were prepared from 0.1mg/ml plants extracts and catechin stock aqueous solutions for the construction of the calibration curve. The reaction mixture was prepared by mixing 0.4 ml of each solution with 3 ml of 4% Vanillin solution (dissolved in methanol) and 1.5 ml of concentrated HCL solution. The samples were incubated in a thermostat at 25ºC for 15 min. Then, the absorbance of each sample was determined by using spectrophotometer at wave length of 500nm. Samples were prepared in triplicates and the mean value of absorbance was calculated for each sample. The concentration was expressed in terms of catechin equivalents (mg of CA/g) for both extracts of each species.

2.8. Determination of total phenol content:

Total phenols content in the plants aqueous extracts were determined using spectrophotometric method with some modifications [47]. Stock aqueous solutions for aqueous plants extracts with a concentration of 0.1mg/ml were prepared. The reaction mixture was prepared for the two species included in this study. Each mixture was prepared by mixing 0.5ml of both plants extracts solutions, 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml of 7.5% of $NaHCO_3$ aqueous solution. The samples were incubated in a thermostat at 45°C for 45min. After that, the absorbance of each mixture was determined using spectrophotometer at wave length of 765nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was calculated for each sample. The same procedure was repeated for the

standard solution of Gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of Gallic acid equivalent expressed in terms of (mg of GA/g of extract).

2.9. Determination of total flavonoids content:

Total flavonoid content was determined according to the procedure of Chang et al.,2002 [48]which was validated by Nugroho, 2011 [49], with some modifications using quercetin as reference standard. Quercetin 10 mg was dissolved in distilled water up to 100 ml. Plants aqueous extracts stock solutions were prepared at the same concentrations. Subsequently, every stock solution was used to provide a series of diluted concentrations. From each solution, 0.5 ml was mixed with 3 ml of methanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M potassium acetate and 5 ml distilled water, and then incubated at room temperature for 30 min. The absorbance of each dilution was then measured at 510nm wavelength, and distilled water with methanol, 10% $AlCl_3$ and potassium acetate was used as a blank. The total flavonoid content was determined from the calibration curve of Quercetin and expressed as milligram of Quercetin Equivalent per gram of extract (mg QU/g extract)[50].

2.10. Statistical analyses:

Determination of antioxidant activity, total flavonoids, total phenols and total tannins contents of both plants species was carried out in triplicate for each sample. The obtained results were presented as means ± standard deviation (SD) [51].

2.11. Antimicrobial tests :

Both aqueous and organic extracts of each of the tested plants species included in this study were investigated for their anti-bacterial and anti-fungal activities. Antibacterial activities of the extracts were examined against five referenced bacterial strains obtained from the American Type Culture Collection (ATCC) and against multidrug resistant clinical isolates. The tested strains included *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (MRSA Positive), *Enterococcus faecium* (ATCC 700221), *Shigella sonnei* (ATCC 25931), *Enterobacter cloacae* (clinical) and *Klebsiella pneumoniae* (clinical).

As well as the anti-fungal activities of these extracts were examined against two referenced strains of *Candida albicans* (ATCC 90028) and *Epidermatophyton floccosum* (ATCC 52066).

Antibacterial and antifungal activity of all prepared organic and aqueous extracts was determined by using several methods including agar diffusion well-variant method, agar diffusion disc-variant method and broth micro-dilution method. Agar dilution method was applied for *Epidermatophyton floccosum* [52-55].

2.11.1. Plants extracts solutions, media and inoculums preparations

Each of the prepared organic extracts was dissolved in 100% Dimethyl sulfoxide (DMSO) achieving a concentration of 25 mg/ml. Aqueous extracts were dissolved in distilled water achieving a concentration of 50

mg/ml. Syringe filters with 0.45µm pore size were used to sterilize the solutions.

Mueller-Hinton broth was prepared by addition of 22g of broth powder to 1L distilled water, heated and gently stirred until it became clear, added to test tubes in 2, 5 and 10ml volumes, covered with tight cotton sealing and then sterilized using an autoclave. It was used for inoculums preparations and dilutions of bacteria and *Candida* as well as in Minimum Inhibitory Concentration method for bacteria.

Mueller-Hinton agar was prepared by addition of 38g of broth powder to 1L distilled water, heated and gently stirred until boiling, covered with tight cotton sealing, sterilized using an autoclave and poured in sterile Petri dishes in 20ml volume. It was used in agar diffusion well-variant method and agar diffusion disc-variant method.

RPMI-1640 broth was prepared by addition of 10.4 g RPMI and 34.53 g MOPS (3-[N-morpholino] propanesulfonic acid) buffer in 900 mL distilled water, pH was adjusted to 7 using 1 mol/L sodium hydroxide, then water was added to get a final volume of 1 Liter. Sterilization was carried out by 0.45 µm filter syringes. It was used for determination of minimum inhibitory concentration method for *Candida albicans* [56].

Sabouraud dextrose agar was prepared by addition of 65g of broth powder to 1L distilled water, heated and gently stirred until boiling; 1ml was added to each test tube, covered with tight cotton sealing and sterilized using an autoclave. It was used for agar dilution method to

determine minimum inhibitory concentration of *Epidermatophyton floccosum*.

Freshly cultivated bacterial isolates were used to prepare inoculums for testing purposes. It was taken using sterile swap and suspended in sterile MH broth until turbidity matched the 0.5 McFarland standard ($1.5x\ 10^8$ CFU/ml), then dilutions were applied until final standard bacterial concentration of ($5x10^5$ CFU/ml) was achieved. The same procedure was applied for *Candida albicans* but the final standard concentration was 0.5×10^3 to 2.5×10^3 CFU/ml [54].

Epidermatophyton floccosum inoculum was prepared by suspending its spores in sterilized distilled water with 0.05% Tween until turbidity matched a McFarland standard.

2.11.2. Agar diffusion well-variant method:

Using a sterile cotton swap the bacterial inoculum was uniformly spread on the MH agar (20 ml). Holes with diameters of 6 mm and distances more than 20 mm were applied in every MH plate. Solutions of plants extracts with concentrations of 25 mg/ml and 50mg/ml for organic and aqueous extracts respectively and 100% DMSO were added to the wells in 90µl volume. The Petri dishes were incubated for 24 hours for all reference strains of bacteria and for 48 hours for *Candida albicans* which was tested using the same method. After incubation, inhibition of bacterial growth was measured in mm. Tests were performed in duplicate.

2.11.3. Agar diffusion disc-variant method: Using a sterile cotton swap the bacterial inoculum was uniformly spread on the MH agar(20 ml). Sterile filter papers with diameters of 6mm were placed in several points on every MH plate with distances more than 20 mm . Solutions of plants extracts with concentrations of 25 mg/ml and 50mg/ml for organic and aqueous extracts respectively and 100% DMSO were added to the filter papers in 10µl volume. Inoculated plates were incubated for 24 hours for all reference strains of bacteria and for 48 hours for *Candida albicans* which was tested using the same method. After incubation, inhibition of bacterial growth was measured in mm. This test was applied using bacterial isolates *Enterobacter cloacae* and *Klebsiella pneumonia*. In addition reference commercial discs of antibiotics were used for comparison. All the conducted tests were performed in duplicate.

2.11.4. Minimum Inhibitory Concentration (MIC)

The wells of 96- well plates were filled with sterile Mueller-Hinton broth. For each plant extract solution, 11 dilutions were applied in the plates by using two-fold serial dilution method. For each of these serial dilutions, well number 11 was considered as the negative control for microbial growth, while well number 12, which contained Mueller-Hinton broth only, was used as positive control for microbial growth. The bacterial inoculums were added to all wells except well number 11.The final bacterial concentration in each of the inoculated wells (except negative control) was adjusted to 5×10^5 CFU/ml (CFU: colony-forming unit). For *Candida albicans* testing, the previously mentioned

method was applied using RPMI-1640 broth instead of Mueller-Hinton broth . The *Candida* concentration in each of the inoculated wells was adjusted to 0.5×10^3 to 2.5×10^3 CFU/ml .

During the anti-bacterial and anti-fungal activity tests, the achieved 10 concentrations of both the aqueous and organic extracts of each plant were from 0.049 to 25 mg/ml and 0.024 to 12.5 mg/ml, respectively. After inoculation of microorganisms into their assigned wells, the plates were covered and incubated at 35□C. The incubation period was 24 hours for bacterial strains and 48 hours for *Candida albicans*. The anti-microbial activity of each extract was examined in duplicate. The lowest concentration of each of the plant extracts that did not allow any visible microbial growth in the inoculated wells was considered to represent the minimal inhibitory concentration (MIC) of that extract.

2.11.5. *Epidermatophyton floccosum* inhibition test

SDA was prepared by addition of 65g of broth powder to 1L distilled water, heated and gently stirred until boiling; 1 ml was added to every test tube, covered with cotton and sterilized using an autoclave. Test tubes with SDA were placed in 40^{o}C water path to provide solidification of agar during serial dilution applications. All test tubes were labeled from 1 to 8 numbers for every tested solution . Solutions of plants extracts with concentrations of 25 mg/ml and 50mg/ml for organic and aqueous extracts, respectively and 100% DMSO were added to the number 1 test tubes in 1ml volume . Serial dilutions were applied and concentrations were 0.195 to 25 mg/ml for aqueous extracts , 0.098 to 12.5 mg/ml for

organic extracts and 0.391 to 50% for DMSO tubes. Tubes were left at room temperature to solidify then 20µl of *Epidermatophyton floccosum* freshly prepared inoculum was added to all tubes. Tubes with SDA only were used as control. Results was taken after 7 and 10 days of leaving at room temperature . Minimum inhibitory concentration was the lowest concentration that completely inhibit the growth of *Epidermatophyton floccosum* .

Chapter Three

3. Results and Discussions

Natural phenolic and polyphenolic compounds including flavonoid, tannins and simple phenols are well known scientifically for their antioxidants properties [57, 58].

Many conducted scientific studies stated that the risk of various degenerative diseases can be prevented by consuming large quantities of natural products containing antioxidants [59-61].

Recently, it has been proven clinically that oxidative stresses and free radicals in the living organisms are the major causes of many disorders including cancer, chronic renal failure, atherosclerosis, aging, diabetes mellitus and autoimmune disease [62-68].

Meanwhile, antibiotic resistance considered a main global crisis and this harmful resistance reached lethal point in many clinics around the world. For that reason, there is an urgent need to investigate new antimicrobial agents which can inhibit this bacterial resistance without toxic effects and with minimum side effects [69, 70].

Flavonoids are a group of plant derived from natural compounds with potentially credulous properties, including potential antimicrobial activity [71]. Moreover, since ancient times, various pharmaceutical formulations containing flavonoids have been utilized for prevention and treatment of various diseases and disorders [72]. In addition to that, this group of bioactive compounds becoming the subject of many of the antimicrobial research, which possessed antiviral, antifungal and antibacterial activities

[73-75]. Moreover, many of high quality studies have assessed the relationship between flavonoids chemical structures and their antimicrobial activities and also they studied their mechanisms of action on the microbial growth [71, 76-80].

Many of the medical and pharmaceutical investigations have been approved that flavonoids can possess many therapeutic properties, including oestrogenic, anti-inflammatory, enzyme inhibition and antimicrobial activities [81-83].

Ideally, this group of natural compounds should be in the form of new agents of antimiocrobial drugs.

3.1. Qualitative phytoconstituents

Phytochemical screening tests for *L. orientalis* plants aqueous extracts showed the presence of monosaccharide, reducing sugar, carbohydrates, phenol, tannins, flavonoids, saponins, cardiac glycosides, steroid while protein, starch, terpenoids and alkaloids were absent. At the same time phytochemical screening tests for *E. laciniatum* showed the presence of Protein, monosaccharide, reducing sugar, starch, phenol, tannins, flavonoids, saponins, cardiac glycosides, steroid and terpenoids while alkaloids was absent in this extract as shown in Table 5.

Table 5: Phytochemical screening tests results for the aqueous extracts for *L. orientalis* and *E. laciniatum* plants

Chemical constituent	*L. orientalis*	*E. laciniatum*
Protein	-	+
Monosacharrides	+	+
Reducing sugar	+	+
Carbohydrates	+	+
Starch	-	+
Phenol	+	+
Tannins	+	+
Flavonoids	+	+
Saponins	+	+
Cardiac glycosides	+	+
Steroid	+	+
Terpenoids	-	+
Alkaloids	-	-

3.2. Total flavonoids, phenols and tannins contents results

Standard calibration curves used for the determination of total flavonoids, phenols and tannins contents which were prepared by using different concentrations of Quercetin equivalent (mg of QU/g), gallic acid equivalent (mg of GA/g) and catechin (mg of CA/g) and their respective optical density as shown in Figures 3, 4 and 5 respectively. The absorption values of several concentrations of the standard quercetin are listed in Table 6.

Table 6: Absorption values of several concentrations of the standard quercetin

Concentration of quercetin (µg/ml)	Absorption at λ_{max} =510nm
0	0
10	0.007
30	0.013
50	0.022
70	0.032
100	0.043

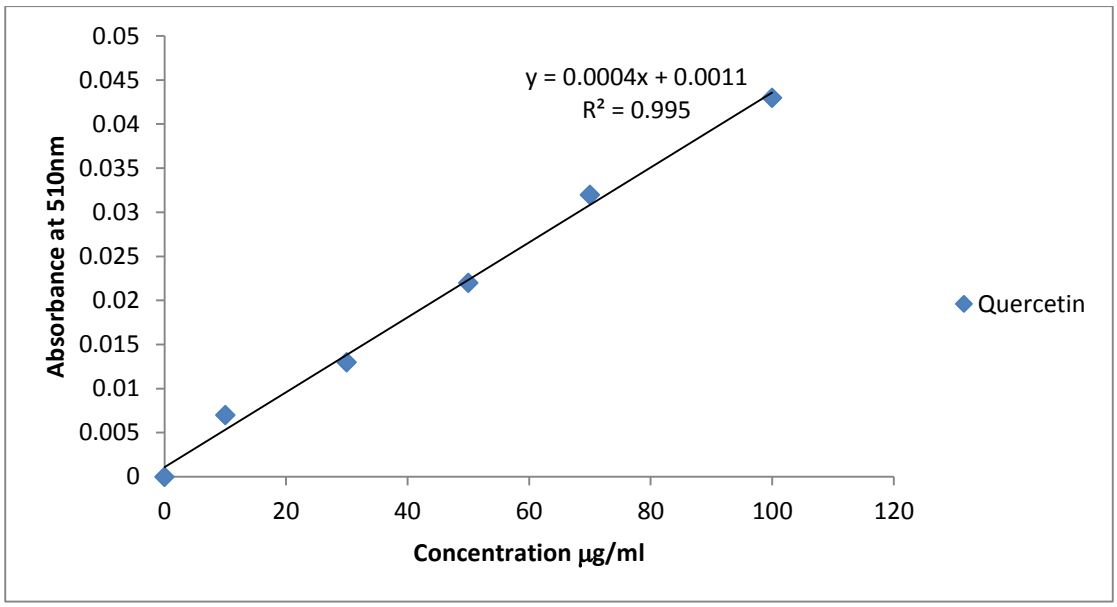

Figure 3: Standard calibration curve of quercetin(Table 6)

The total flavonoid was expressed as mg/g quercetin equivalent using the standard curve equation: Y = 0.0004*X + 0.0011, R2=0.995, where:

Y- Absorbance at 510 nm

X- Total flavonoid in the extracts

From this equation the total flavonoid content in *L. orientalis* extract was 418.6±28.1mg/g equivalent of quercetin. In the same time the total flavonoid content in *E. laciniatum* extract was 455.7±35.8mg/g equivalent of quercetin.

For evaluation of total phenol content, the absorption values of several concentrations of the standard gallic acid were listed in Table 7.

Table 7: Absorption values of several concentrations of the standard gallic acid

Concentration of gallic acid (µg/ml)	Absorption at λ_{max} =765nm
0	0.000
10	0.142
40	0.496
50	0.557
70	0.798

The total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: Y = 0.0112*X + 0.0176, R^2= 0.9956, Where:

Y- Absorbance at 765 nm

X- Total phenols in the extracts.

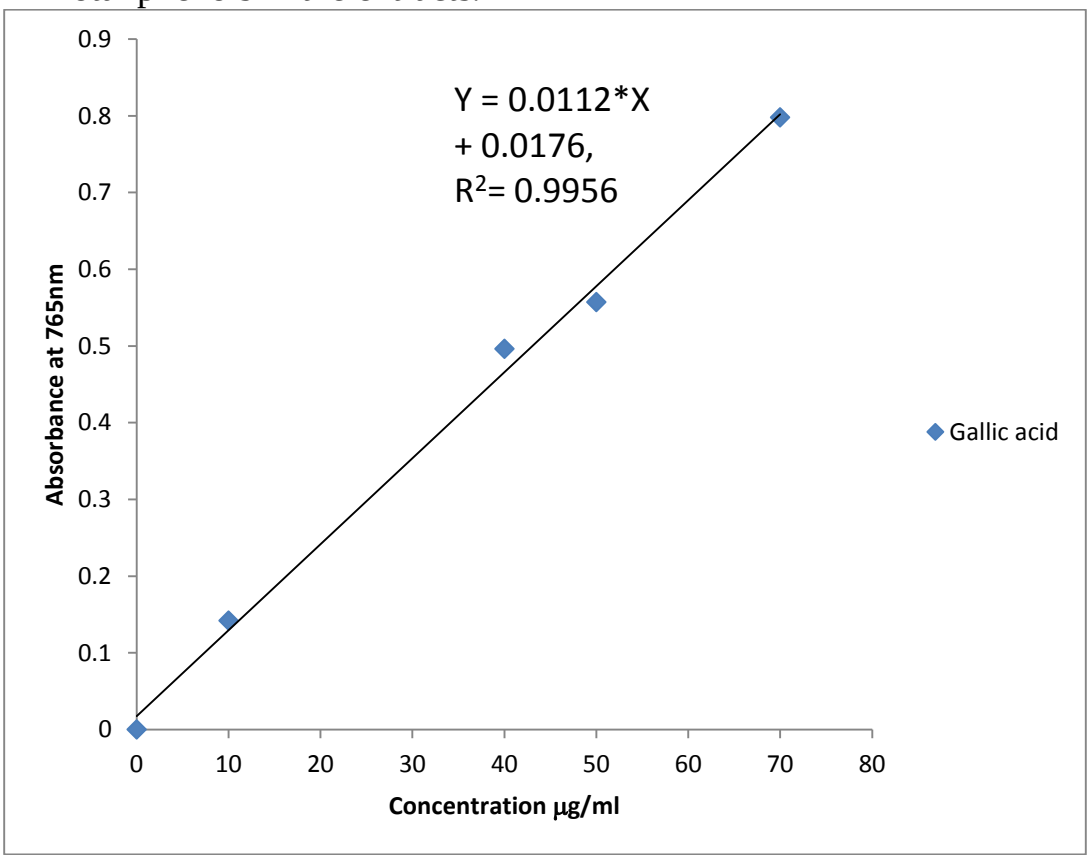

Figure 4: Standard calibration curve of gallic acid(Table 7)

Total phenols content was 5.2 mg/g gallic acid in *L. orientalis* and 10.3 mg/g gallic acid in *E. laciniatum.*

For evaluation of total tannin content, the absorption values of several concentrations of the standard catechin was performed as listed in Table 8.

Table 8: Absorption values of several concentrations of the standard catechin

Concentration of catechin (µg/ml)	Absorption at λ_{max} =276nm
0.	0.000
0.010	0.028
0.030	0.041
0.050	0.056
0.070	0.077
0.100	0.095

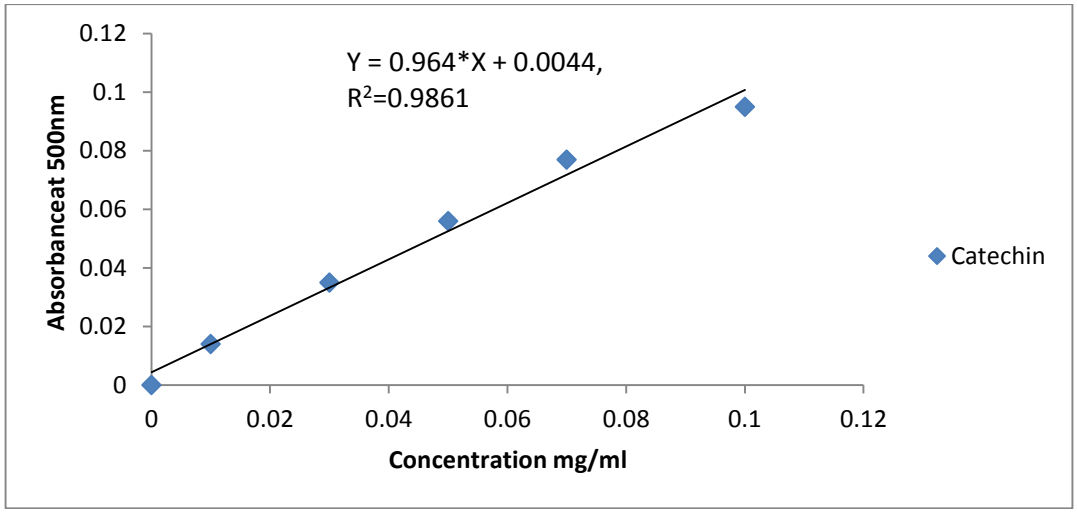

Figure 5: Standard calibration curve for catechin (Table 8)

The total tannins were expressed as mg/g catechin equivalent using the standard curve equation: Y = 0.964*X + 0.0044, R^2=0.9861.

Where:

Y- Absorbance at 500 nm

X- Total tannin in the extracts

According to the catechin standard calibration curve the total tannin content was 62.33±5.68 mg/g catechin in *L. orientalis* and 123.33±11.5 mg/g catechin total tannin in *E. laciniatum.*

3.3. Antioxidant activity

The free radical scavenging activity of the plant extracts of *L. orientalis* and *E. laciniatum* was tested using DPPH radical method and Trolox was used as a reference standard. Percentage of inhibition was calculated using the formula:

Percentage of inhibition of DPPH activity (%) = (A-B)/A ×100%.

Where:

A = UV absorption of the blank,

B = UV absorption of the sample all at λ_{max} = 517nm.

Table 9: Percentage inhibition activity for Trolox , *L. orientalis* and *E. laciniatum* extract.

Concentration (µg/ml)	% of inhibition		
	Trolox	*L. orientalis*	*E. laciniatum*
1	43.54	33.86	32.84
2	52.32	34.39	33.94
3	61.82	37.04	36.51
5	80.20	39.68	39.27
7	94.75	44.44	42.94
10	97.17	48.94	46.97
20	97.17	62.70	61.28
30	97.17	73.81	73.39
40	97.17	83.07	83.49
50	97.27	89.68	90.09
70	97.27	92.86	93.21
80	97.27	92.86	93.21
100	97.27	93.39	93.21
IC_{50}	1.210	28.94	30.49

IC_{50} was calculated using GraphPad prism program .

The IC_{50} of antioxidant activities of *L. orientalis* and *E. laciniatum* are shown in Figure 6 and 7 respectively.

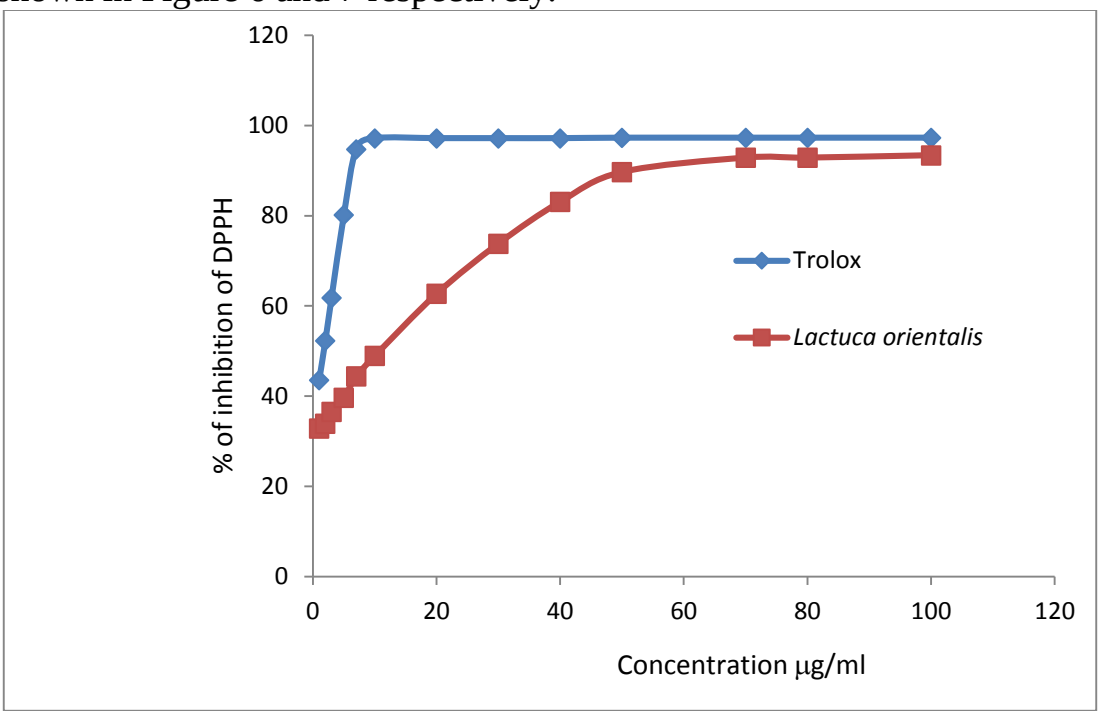

Figure 6: Antioxidant inhibition activity of Trolox standard and *L. orientalis* (Table9)

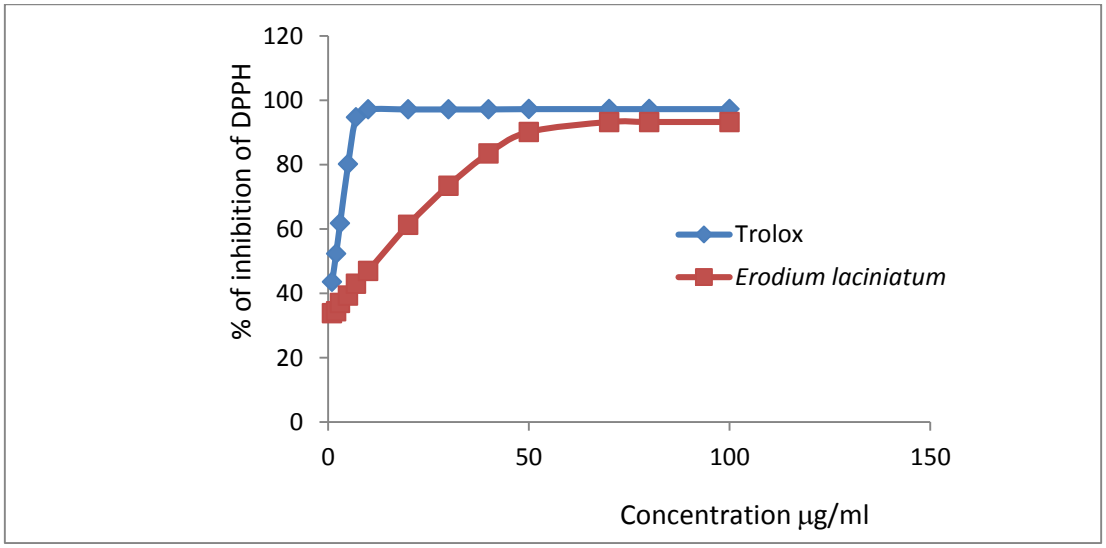

Figure 7: Antioxidant inhibition activity of Trolox standard and *E. laciniatum*

3.4. Anti bacterial activity

Antibacterial activities of plant extracts against reference strains, which were determined by agar diffusion disc-variant method, are presented in Table 10. There was no inhibition observed in most of plants extracts in this method except *E. laciniatum* organic extract, which had inhibition diameter about 10 mm against *Enterococcus faecium*. On the other hand, a lower level of inhibition (about 7mm diameter) of 100%DMSO solvent and the two organic plants extracts (dissolved in100%DMSO) was observed on *Escherichia coli* strain.

Table 10: Antibacterial activity of plants extracts against reference strains using agar diffusion disc-variant method:

Bacteria reference strain	Aqueous extracts		DMSO 100%	Organic extracts	
	L. orientalis	*E. laciniatum*		*L. orientalis*	*E. laciniatum*
Staphylococcus aureus (ATCC 25923)	NI	NI	NI	NI	NI
Escherichia coli (ATCC 25922)	NI	NI	7	7	7
Pseudomonas aeruginosa (ATCC 27853)	NI	NI	NI	NI	NI
Staphylococcus aureus (MRSA Positive)	NI	NI	NI	NI	NI
Enterococcus faecium (ATCC 700221)	NI	NI	NI	NI	10
Shigella sonnei (ATCC 25931)	NI	NI	NI	NI	NI

NI: no inhibition occurred

The results of antibacterial activities, which were determined by using agar diffusion well-variant method on reference strains, are presented in Table 11. Using this method, antibacterial activities of the two plant

extracts were detected against a bigger number of reference strains. For aqueous extracts the range of diameters of inhibition was 7-16mm while no inhibition was observed on *Enterococcus faecium* from both plants extracts and on *Pseudomonas aeruginosa* from *E. laciniatum* extract. The largest diameter of inhibition was 16 mm which was made by *L. orientalis* aqueous extract against *Staphylococcus aureus.* The inhibition diameters of the organic extracts were at the range of 9-15mm. The largest inhibition diameter was made by *E. laciniatum* organic extract against *Enterococcus faecium* and it was 15mm. Organic extracts exhibited low level of growth inhibition (diameter of inhibition= 9-10mm) of *Shigella sonnei* , *Staphylococcus aureus* and MRSA positive *Staphylococcus aureus*. The rest of inhibition cases were not confirmed to be as a result of plants extracts due to DMSO solvent inhibitions.

Table 11: Antibacterial activity of plants extracts against reference strains using agar diffusion well-variant method:

Bacteria reference strain	Aqueous extracts		DMSO 100%	Organic extracts	
	L. orientalis	*E. laciniatum*		*L. orientalis*	*E. laciniatum*
Staphylococcus aureus (ATCC 25923)	16	7	7	9	9.5
Escherichia coli (ATCC 25922)	7	7	12	12	12
Pseudomonas aeruginosa (ATCC 27853)	7	NI	12	12	12
Staphylococcus aureus (MRSA Positive)	7	7	8	9	9
Enterococcus faecium (ATCC 700221)	NI	NI	11	11	15
Shigella sonnei (ATCC 25931)	10	10	9	9	10

NI: no inhibition occurred

Table 12 shows the antibacterial activities of aqueous plants extracts against reference strains which were determined by broth micro-dilution method. The minimal inhibitory concentrations (**MIC**) on the bacterial strains were at the range of 6.25 – 25 mg/ml aqueous extract. Aqueous plant extracts of both *L. orientalis* and *E. laciniatum* exhibited strong activity (MIC=6.25 mg/ml) against *Staphylococcus aureus*.

Table 12: Minimal inhibitory concentrations for aqueous plants extracts in mg/ml

Reference strain	*L. orientalis* MIC (mg/ml)	*E. laciniatum* MIC (mg/ml)
Staphylococcus aureus (ATCC 25923)	6.25	6.25
Escherichia coli (ATCC 25922)	25	25
Pseudomonas aeruginosa (ATCC 27853)	25	25
Staphylococcus aureus (MRSA Positive)	12.5	12.5
Enterococcus faecium (ATCC 700221)	12.5	12.5
Shigella sonnei (ATCC 25931)	25	12.5

MIC values for organic plants extracts dissolved in 100% DMSO are presented in Table13. MIC for *E. laciniatum* against *Staphylococcus aureus* was 1.563 mg/ml. MIC values for the rest of organic plants extracts (range of 1.563-6.25 mg/ml) were not confirmed because of DMSO solvent inhibitory effect.

Table13: Minimal inhibitory concentrations for organic plants extracts in mg/ml

Reference strain	DMSO MIC (%)	*L. orientalis* MIC (mg/ml)	*E. laciniatum* MIC (mg/ml)
Staphylococcus aureus (ATCC 25923)	25%	6.25 (25%DMSO) *	1.563 (6.25%DMSO)
Escherichia coli (ATCC 25922)	12.5%	3.125 (12.5%DMSO)	3.125 (12.5%DMSO)
Pseudomonas aeruginosa (ATCC 27853)	6.25%	1.563 (6.25%DMSO)	1.563 (6.25%DMSO)
Staphylococcus aureus (MRSA Positive)	25%	3.125 (12.5%DMSO)	3.125 (12.5%DMSO)
Enterococcus faecium (ATCC 700221)	6.25%	1.563 (6.25%DMSO)	1.563 (6.25%DMSO)
Shigella sonnei (ATCC 25931)	12.5%	3.125 (12.5%DMSO)	3.125 (12.5%DMSO)

* 6.25 (25%DMSO): in the well, the concentration of plant extract was 6.25 mg/ml and the concentration of DMSO was 25%, which inhibits bacterial growth, so the antibacterial activity was not confirmed.

Table 14 shows comparison of antibiotics susceptibility and antibacterial activity of plant extracts against two multidrug resistant bacterial isolates using agar diffusion disc-variant method. For commercial antibiotics the diameters of inhibition were at the range 12-40 mm. The largest inhibition diameter against *Enterobacter cloacae* isolate was made by Ciprofloxacin commercial discs and it was 40mm(susceptible according to Clinical Laboratory Standard Institute [CLSI]). The largest inhibition diameter against *Klebsiella pneumonia* isolate was made by Imipenem

commercial discs and it was 32mm(susceptible according to CLSI). For plants aqueous and organic extracts no inhibition occurred except small inhibition by *L. orientalis* organic extract against *Klebsiella pneumonia* isolate and the diameter of inhibition was 8 mm.

Table 14: Plants extarcts' antibacterial activities against multidrug resistant isolates using agar diffusion disc-variant method in comparison with antibiotics.

Resistant isolates		*Enterobacter cloacae* (mm) CLSI Interp*.		*Klebsiella pneumonia* (mm) CLSI Interp.	
Tetracycline (TE)		22	S*	12	R*
Imipenem (IPM)		30	S	32	S
Levofloxacin (LEV)		38	S	12	R
Ciprofloxacin (Cip)		40	S	13	R
Nitrofurantoin(F)		18	S	15	I*
Cefotaxime (CTX)		33	S	14	R
Ceftazidime(CAZ)		28	S	14	R
Amikacin (AK)		22	S	16	I
Amoxicillin+ Clavulanic acid (AMC)		13	R	16	I
Azithromycin (AZM)		14	R	14	R
Aqueous Extracts	*L. orientalis*	NI*		NI	
	E. laciniatum	NI		NI	
DMSO		NI		NI	
ORGANIC EXTRACTS	*L. orientalis*	NI		8	
	E. laciniatum	NI		NI	

***NI: no inhibition occurred; CLSI interpretation; R, resistant; S, Susceptible; I, intermediate**

3.5. Anti fungal activity

3.5.1 . *Candida albicans* (ATCC 90028)

There was no observed inhibition of *L. orientalis* and *E. laciniatum* aqueous and organic extracts by using several methods: agar diffusion disc-variant method, agar diffusion well-variant method and broth micro-dilution method using RPMI-1640 broth. There was slight inhibition from DMSO only and MIC was 12.5% DMSO.

3.5.2. *Epidermatophyton floccosum* (ATCC 52066)

The results of agar dilution method which was applied for plants extracts against *Epidermatophyton floccosum* are presented in Table 15. The minimal inhibitory concentrations of aqueous extracts were 25mg/ml for both plant extracts. For organic extracts minimal inhibitory concentration of *L. orientalis* was 0.781mg/ml and for *E. laciniatum* was 0.391mg/ml. The inhibition of aqueous extracts was low, while the best inhibition was found using *E. laciniatum* organic extract and the inhibition was until six folds. The method had been applied in duplicate.

Table 15: Plants extracts' antifungal activities against *Epidermatophyton floccosum* using agar dilution method

Plant extracts		**MIC (mg/ml)**
Aqueous extracts	*L. orientalis*	25
	E. laciniatum	25
Organic extracts (in 100% DMSO)*	*L. orientalis*	0.781(3.125% DMSO)
	E. laciniatum	0.391(1.563%DMSO)

***DMSO MIC(%) was 6.25%**

All antibacterial and antifungal tests showed in Figures 8-35.

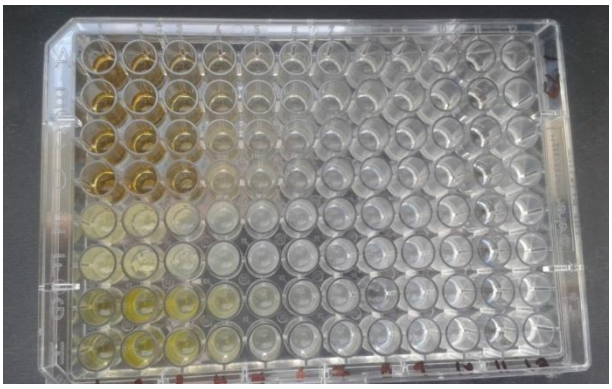

Figure 8: Antibacterial activity of aqueous and organic plants extracts against *Staphylococcus aureus* by minimum inhibitory concentration determination

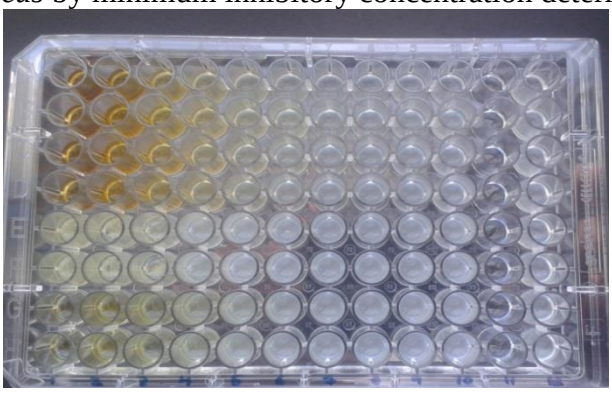

Figure 9: Antibacterial activity of aqueous and organic plants extracts against *Escherichia coli* by minimum inhibitory concentration determination

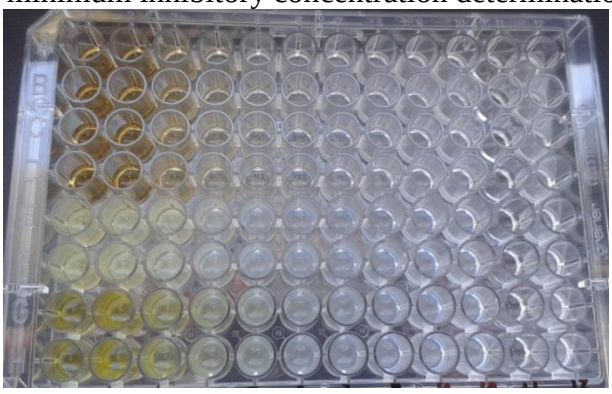

Figure 10: Antibacterial activity of aqueous and organic plants extracts against *Staphylococcus aureus* (MRSA Positive)by minimum inhibitory concentration determination

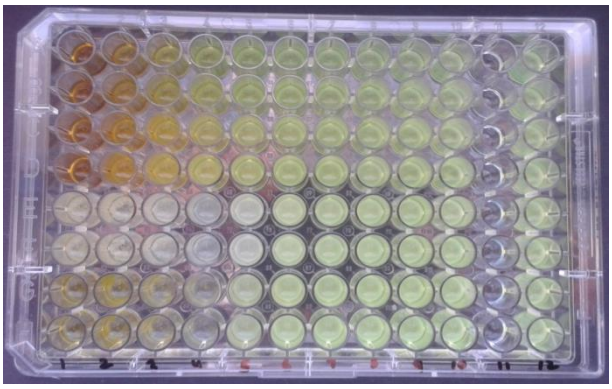

Figure 11: Antibacterial activity of aqueous and organic plants extracts against *Pseudomonas aeruginosa* by minimum inhibitory concentration determination

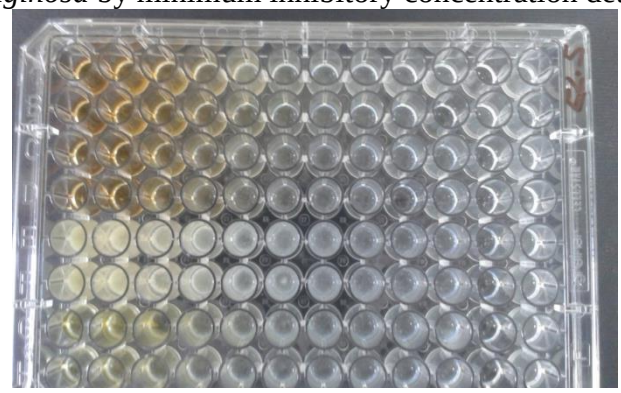

Figure 12: Antibacterial activity of aqueous and organic plants extracts against *Shigella sonnei* by minimum inhibitory concentration determination

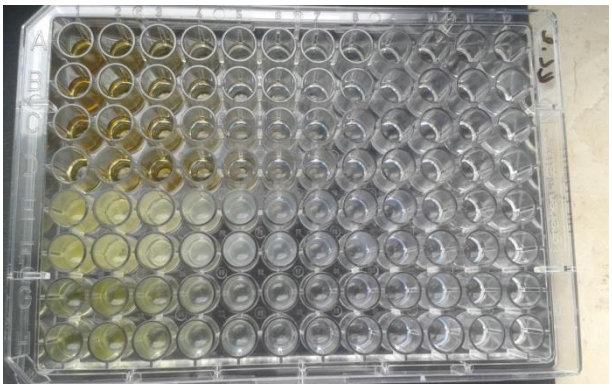

Figure 13: Antibacterial activity of aqueous and organic plants extracts against *Enterococcus faecium* by minimum inhibitory concentration determination

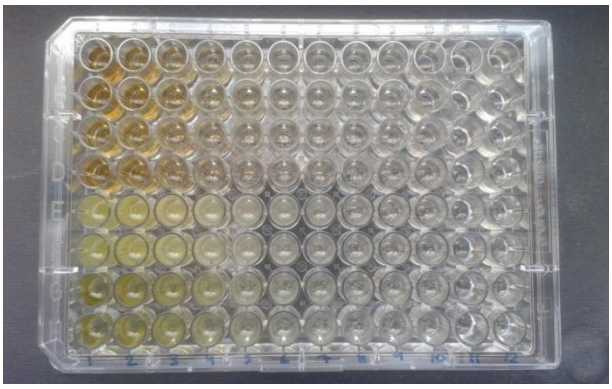

Figure 14: Antifungal activity of aqueous and organic plants extracts against *Candida albicans* by minimum inhibitory concentration determination using RPMI-1640 media

Figure 15: Antibacterial activity of organic plants extracts and DMSO against *Enterococcus faecium* using agar diffusion disc-variant method and agar diffusion well-variant method

Figure 16: Antibacterial activity of organic plants extracts and DMSO against *Staphylococcus aureus* using agar diffusion disc-variant method and agar diffusion well-variant method

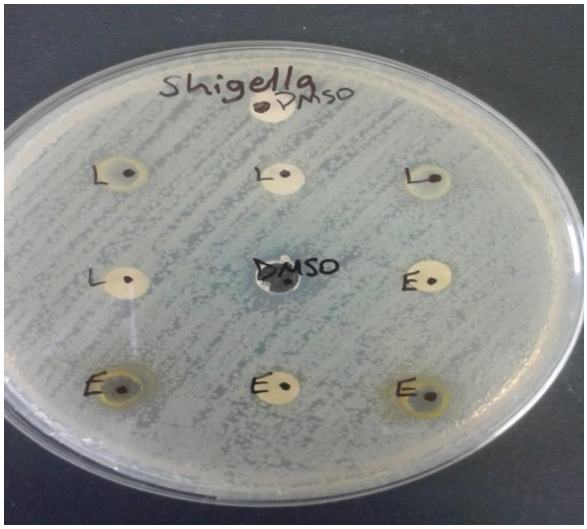

Figure 17: Antibacterial activity of organic plants extracts and DMSO against *Shigella sonnei* using agar diffusion disc-variant method and agar diffusion well-variant method

Figure 18: Antibacterial activity of organic plants extracts and DMSO against *Pseudomonas aeruginosa* using agar diffusion disc-variant method and agar diffusion well-variant method

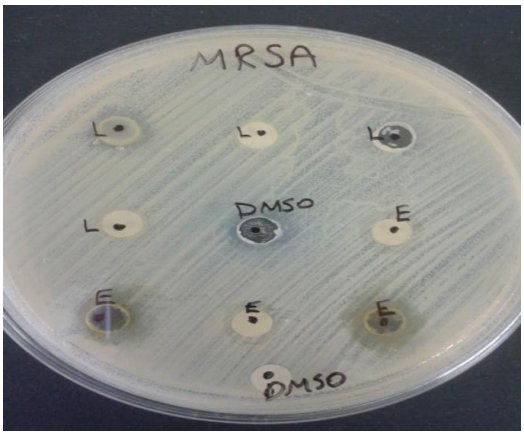

Figure 19: Antibacterial activity of organic plants extracts and DMSO against *Staphylococcus aureus* (MRSA positive)using agar diffusion disc-variant method and agar diffusion well-variant method

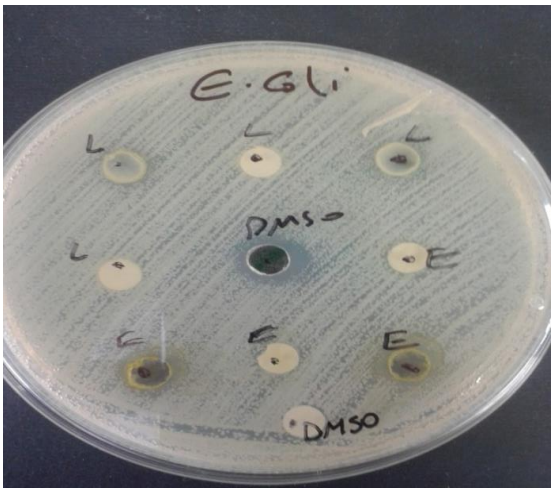

Figure 20: Antibacterial activity of organic plant extracts and DMSO against *Escherichia coli* using agar diffusion disc-variant method and agar diffusion well-variant method

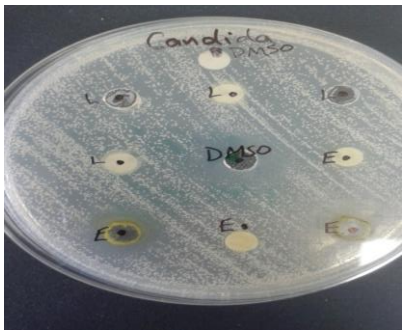

Figure 21: Antifungal activity of organic plants extracts and DMSO against *Candida albicans* using agar diffusion disc-variant method and agar diffusion well-variant method

Figure 22: Antibacterial activity of aqueous plants extracts against *Escherichia coli* using agar diffusion disc-variant method and agar diffusion well-variant method

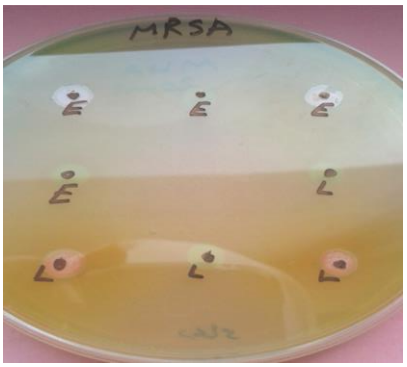

Figure 23: Antibacterial activity of aqueous plants extracts against *Staphylococcus aureus* (MRSA positive)using agar diffusion disc-variant method and agar diffusion well-variant method

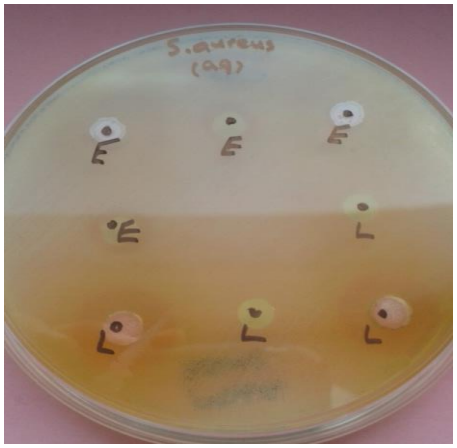

Figure 24: Antibacterial activity of aqueous plants extracts against *Staphylococcus aureus* using agar diffusion disc-variant method and agar diffusion well-variant method

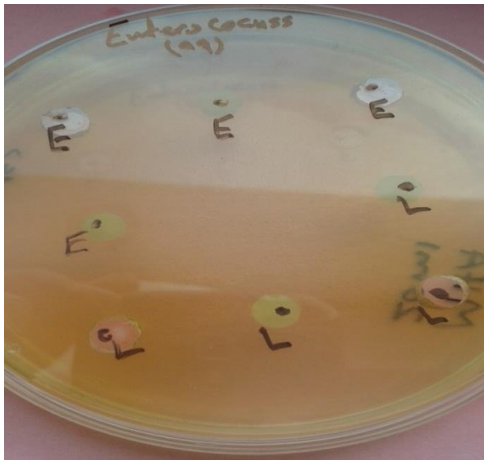

Figure 25: Antibacterial activity of aqueous plants extracts against *Enterococcus faecium* using agar diffusion disc-variant method and agar diffusion well-variant method

Figure 26: Antibacterial activity of aqueous plants extracts against *Shigella sonnei* using agar diffusion disc-variant method and agar diffusion well-variant method

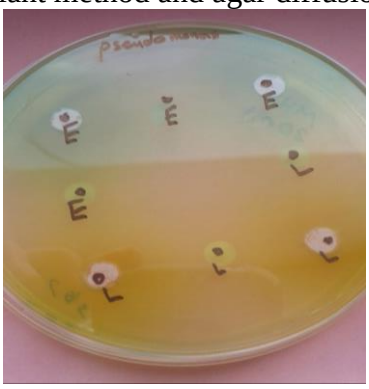

Figure 27: Antibacterial activity of aqueous plants extracts against *Pseudomonas aeruginosa* using agar diffusion disc-variant method and agar diffusion well-variant method

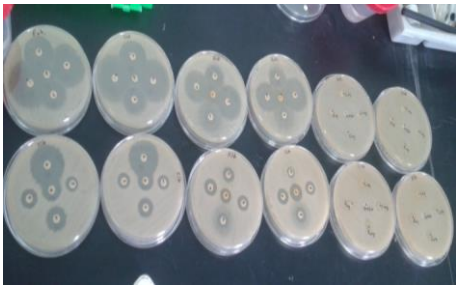

Figure 28: Antibacterial activity of aqueous and organic plants extracts , DMSO and commercial discs of antibiotics against bacterial isolates *Enterobacter cloacae* and *Klebsiella pneumonia.*

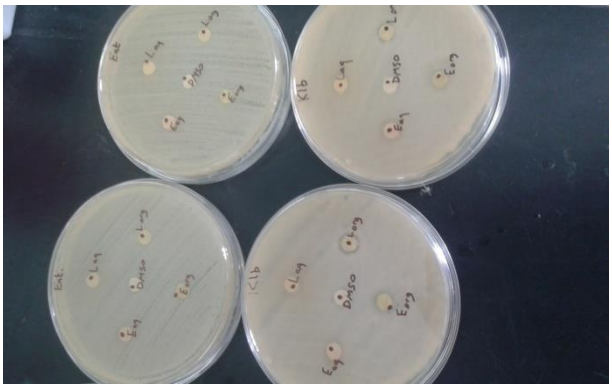

Figure 29: Antibacterial activity of aqueous and organic plants extracts and DMSO against bacterial isolates *Enterobacter cloacae* and *Klebsiella pneumonia.*

Figure 30: Antifungal activity of *L. orientalis* aqueous extract against *Epidermatophyton floccosum* using agar dilution method

Figure 31: Antifungal activity of *E. laciniatum* aqueous extract against *Epidermatophyton floccosum* using agar dilution method

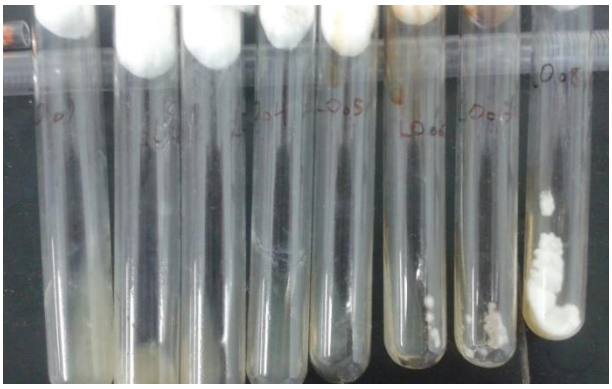

Figure 32: Antifungal activity of *L. orientalis* organic extract against *Epidermatophyton floccosum* using agar dilution method

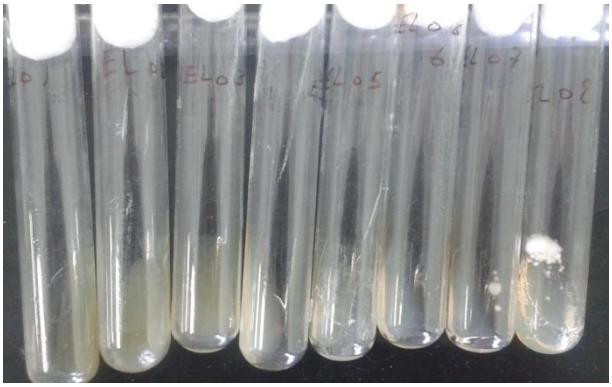

Figure 33: Antifungal activity of *E. laciniatum* organic extract against *Epidermatophyton floccosum* using agar dilution method

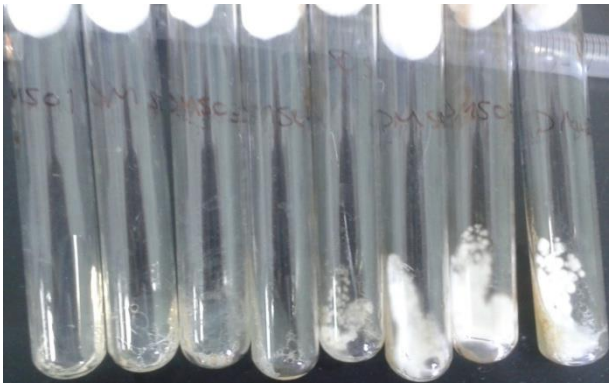

Figure 34: Antifungal activity of DMSO against *Epidermatophyton floccosum* using agar dilution method

Figure 35: Blank tubes for *Epidermatophyton floccosum* on SDA media

In this study, phytoconstituents screening revealed the presence of pharmacological active compounds in both of the *L. orientalis* and *E. laciniatum* such as phenols, tannins, flavonoids, saponins, monosaccharides, reducing sugars, carbohydrates, cardiac glycosides and steroids, while alkaloids were not detected in both of studied species. Proteins, starch and terpenoids were detected in *E. laciniatum* but not detected in *L. orientalis* which means that *E. laciniatum* had more phyto-constituents than *L. orientalis*.

Our studies showed that the total flavonoid content in *E. laciniatum* extract was 455.7±35.8mg/g equivalent of quercetin whereas the total flavonoid content in *L. orientalis* extract was 418.6±28.1mg/g equivalent of quercetin. These results of total flavonoids content showed that *E. laciniatum* extract had higher content of flavonoids than *L. orientalis* extract.

On the other hand, the total phenols content was higher in *E. laciniatum* extract than *L. orientalis* which were 10.3 mg/g equivalent of gallic acid and 5.2 mg/g equivalent gallic acid respectively. In fact, the total tannin content was also much higher in *E. laciniatum* than *L. orientalis* extracts

which were 123.33±11.5 mg/g equivalent of catechin and 62.33±5.68 mg/g equivalent of catechin respectively.

The IC_{50} values of the antioxidant activity were almost the same values in *L. orientalis* and *E. laciniatum* extracts which were 28.94 µg/ml and 30.49 µg/ml respectively.

In a study which was conducted by Bouaziz et al. in Tunisia, 2009 on another species of Erodium plant which called *Erodium glaucophyllum*, the total phenols content in the methanol extract was 2225±267 mgPyE/100g of the methanolic extract which equal to 22.25 mg/g equivalent of catechin, and the total flavonoid content in this species was 203mg of RuE/100g methanolic extract which equal to 20.3mg/g equivalent of quercetin. Our studies on *Erodium* species have revealed that the total flavonoid and total phenol content were 455.7±35.8mg/g equivalent of quercetin and 10.3mg/g equivalent of gallic acid respectively. The same study on the *Erodium glaucophyllum* methanolic extract showed a weak antioxidant activity in comparison with Trolox standard compound [84].

Accordingly, the final results of antibacterial activity of the MIC test of organic and aqueous extracts of *L. orientalis* and *E. laciniatum* showed that the lowest value of MIC was 1.563 mg/ml and it was for organic extract of *E. laciniatum* against *Staphylococcus aureus*. The organic extract of *E. laciniatum* also showed the largest diameter of inhibition against *Enterococcus faecium* using agar diffusion well-variant method and agar diffusion disc-variant method .

However, *L. orientalis* aqueous extract showed the largest diameter of inhibition against *Staphylococcus aureus* using agar diffusion well-variant method.

The organic extract of *L. orientalis* was found to have a small diameter of inhibition against *Klebsiella pneumoniae* isolate (8mm) and this inhibition was the only inhibition that was detected by testing plants extracts against multidrug resistant bacterial isolates *Enterobacter cloacae* and *Klebsiella pneumoniae* using agar diffusion disc-variant method.

The antifungal activity which was tested by using agar dilution method against *Epidermatophyton floccosum* showed that organic extract of *E. laciniatum* had the lowest MIC which was 0.391mg/ml. In fact, the organic extract of *E. laciniatum* was the most effective extract against *Epidermatophyton floccosum* compared with other plants extracts that were tested in this study.

Chapter Four

4. Conclusion and Future work

The results of these evaluations and screenings confirm the great potential of *E. laciniatum* and *L. orientalis* plants as a source for the production of bio-active compounds, natural food's preservatives and the rationalization of medicinal plants usage in primary health care systems. The identification and characterization of the active phytochemical compounds of the studied plants extracts and qualitative standardizations are necessary as well as safety issues and toxicity studies.

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جامعة النجاح الوطنية
كلية الدراسات العليا

الفحص الحيوي و الكيميائي لنوعين من الأعشاب *Erodium laciniatum* و *Lactuca orientalis*

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إشراف
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قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين .
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الملخص

منذ العصور الأولى للحياة على الأرض ,كانت مختلف الأدوية العشبية والنباتات الطبية المحتوية على الفلافانويدات والتنينات والفينولات تستخدم للوقاية والعلاج من مختلف الأمراض والعلل. علاوة على ذلك، فهذه المجموعات من المركبات الفعالة حيوياً تتصدر حالياً مواضيع أبحاث المضادات الميكروبية لامتلاكها فعاليات مضادة للفيروسات والفطريات والبكتيريا. بالإضافة إلى أن الكثير من الدراسات عالية الجودة عملت على تقييم العلاقة بين التركيبة الكيميائية لكل من الفلافانويدات والتنينات والفينولات و فعاليتها المضادة للبكتيريا ودرست أيضاً آليات عملها على نمو الميكروبات. هذه الأبحاث أكدت أن تلك الأنواع من المركبات الطبيعية يمكن أن تمتلك الكثير من المواصفات العلاجية التي تشمل الفعاليات المشابهة للإستروجينات والمضادة للالتهابات والمثبطة للأنزيمات والمضادة للميكروبات.

في هذه الدراسة كان هدفنا فحص المكونات الطبيعية لكل من *E. laciniatum* و *L. orientalis* وتقييم كمية محتواها من الفلافانويدات والتنينات والفينولات. بالإضافة إلى ذلك هدفت هذه الدراسة إلى تقييم فعالياتها المضادة للأكسدة والمضادة للميكروبات. المكونات الطبيعية ومجموع المحتوى من الفلافانويدات والتنينات والفينولات جرى فحصها وتقييمها باستخدام طرق تحليلية وكيميائية نموذجية، بحيث تم تقييم الفعالية المضادة للأكسدة باستخدام طريقة فحص تثبيط DPPH، بينما تم فحص الفعاليات المضادة للبكتيريا باستخدام عدد من السلالات البكتيرية المرجعية التي تم الحصول عليها من مجموعة الأنواع المستنبتة الأمريكية ATCC وعزلات بكتيرية سريرية مقاومة متعددة للأدوية. السلالات التي تم فحصها شملت

Escherichia coli (ATCC 25922) و*Staphylococcus aureus* (ATCC 25923) و*Pseudomonas aeruginosa* (ATCC 27853) (سلالة *Staphylococcus aureus* مقاومة للمثيسيلين) و*Enterococcus faecium* (ATCC 700221) و *Shigella sonnei* (ATCC 25931) و*Enterobacter cloacae* (عزلة سريرية) و *Klebsiella pneumoniae* (عزلة سريرية).

بالإضافة إلى ذلك، فإن الفعالية المضادة للفطريات لتلك المستخلصات العشبية تم فحصها ضد نوعين من السلالات الفطرية المرجعية *Candida albicans* (ATCC 90028) و*Epidermatophyton floccosum* (ATCC 52066). الفعاليات المضادة للبكتيريا والفطريات لكل المستخلصات المائية والعضوية للنبتتين *Erodium laciniatum* و *Lactuca orientalis* جرى تقييمها باستخدام طرق فحص مختلفة شملت طريقة فحص فروقات الانتشار في الآجر عن طريق الفجوات (agar diffusion well-variant method) وطريقة فحص فروقات الانتشار في الآجر عن طريق الأقراص(agar diffusion disc-variant method) وطريقة التخفيف (broth micro-dilution method). نتائج فحص المواد الكيميائية النباتية أظهرت أن النبتتين *E. laciniatum* و*L. orientalis* فيهما فينولات وتانينات وفلافونويدات وصابونينات وسكريات أحادية وسكريات مختزلة وكربوهيدرات وجلايكوسيدات وستيرويدات فيما لم يتم إيجاد مركبات الألكالويد في أي من النبتتين, بالإضافة إلى أنه تم إيجاد بروتينات ونشا وتيربينويدات في *E. laciniatum* ولم توجد في *L. orientalis* وهذا يعني أن *E. laciniatum* فيها مكونات كيميائية نباتية أكثر من *L. orientalis*. في نفس الوقت كان مجموع المحتوى لكل من الفلافونويدات و التنينات و الفينولات في مستخلص *E. laciniatum* أكثر منه في مستخلص *L. orientalis*، بينما كانت قيمة IC_{50} (تثبيط نصف التركيز) لمضاد الأكسدة شبيهة تقريباً لكلتا النبتتين. بالإضافة إلى ذلك فإن أقل قيمة لأدنى تركيز مثبط (MIC) كانت للمستخلص العضوي لنبتة *E. laciniatum* ضد سلالة *Staphylococcus aureus*.

المستخلص العضوي نفسه أظهر أن لديه أكبر مساحة من التثبيط ضد *Enterococcus faecium*، بينما تبين أن المستخلص المائي للنبتة *L. orientalis* أظهر أن له أكبر مساحة من

التثبيط ضد *Staphylococcus aureus*. في ذات السياق، الفعالية المضادة للفطريات التي تم فحصها باستخدام طريقة التخفيف بالآجر agar dilution method ضد *Epidermatophyton floccosum* أظهرت أن المستخلص العضوي للنبتة *E. laciniatum* له أقل قيمة لأدنى تركيز مثبط والتي كانت 0.391 مغم/مل. في المحصلة فإن كلتا النبتتين موضع الدراسة احتوتا على خليط من المواد الكيميائية النباتية وكانتا غنيتين بالفلافونويدات والفينولات والتنينات ولديهما فعاليات محتملة مضادة للأكسدة والميكروبات والتي من الممكن استخدامها كمواد علاجية أو يمكن أن تستخدم في التصنيع التجميلي أو الصيدلاني.