T.R.
KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *Salix aegyptiaca* L. ROOTS

MAAROOF RASUL ABDALRAHMAN

MASTER THESIS
DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ, TURKEY 2015

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ANNOUNCEMENT

I hereby announce that this dissertation is my own work. It is being submitted in fulfillment for the degree of Master of biology at the University Kahramanmaraş Sütçü İmam University, Turkey. It has not been submitted before for any degree or examination at this or any other University except where due acknowledgment has been made in the text.

Maaroof Rasul Abdalrahman

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Salix aegyptiaca L. KÖKLERİNİN ANTİMİKROBİYEL VE ANTİOKSİDAN AKTİVİTELERİ

(YÜKSEK LİSANS TEZİ)

MAAROOF RASUL ABDALRAHMAN

ÖZET

Bu çalışmanın amacı, *Salix aegyptiaca* L. köklerinin ekstraksiyon verim yüzdesi, toplam kondanse tanen miktarı, fenolik bileşikleri ile antioksidan ve antimikrobiyal aktivitelerinin belirlenmesi olmuştur. Kökleri ekstrakte edebilmek için; su, etanol ve metanol gibi çözücüler hızlandırılmış solvent ekstraksiyonu (ASE), geleneksel ekstraksiyon (CE) ve mikrodalga ekstraksiyonu (ME) yöntemlerinde kullanılmıştır. Bileşen tanımlamaları LC-MS/MS metotlarıyla gerçekleştirilmiştir. Kök ekstraktlarının antioksidan aktiviteleri ve toplam kondanse tanen miktarları UV-vis spektrofotometre cihazında sırasıyla 517nm ve 580 nm olarak ölçülmüştür. Ekstraktların antimikrobiyal aktivitesi disk difüzyon testi kullanılarak değerlendirilmiştir.

En yüksek ekstraksiyon verimi metanol kullanılan ASE metodunda bulunmuştur (%17.1). Metanol kullanılarak yapılan kök ekstraktlarında toplam kondanse tanen ve kinik asit düzeyleri sırasıyla 35.14 mg/L ve 63894.761 µg/g olarak bulunmuştur. En yüksek DPPH giderme aktivitesi, metanol kullanılan ASE yönteminde belirlenmiştir (%98.8). En yüksek inhibisyon bölgesi *Candida albicans'* a karşı bulunmuştur (25.6 mm).

Anahtar Kelimeler: Tıbbi bitki, Sekonder metabolizma, *Salix aegyptiaca* L., Antioksidan aktivite, Antimikrobiyal aktivite.

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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *Salix aegyptiaca* L. ROOTS

(M.Sc. THESIS)

MAAROOF RASUL ABDALRAHMAN

ABSTRACT

The aim of current study was to determine antioxidant and antimicrobial activities of *Salix aegypyiaca* L. root extracts along with their yield percentages, total condensed tannin concentrations and identification of phenolic compounds. The root extractions were performed with water, ethanol and methanol. Used extraction methods were accelerated solvent extraction (ASE), conventional extraction (CE) and microwave extraction (ME). Identification of phenolic compounds in methanolic extract was done by LC-MS/MS. The antioxidant activities of root extracts and total condensed tannin concentrations were measured by UV-vis spectrophotometer at 517 nm and 580 nm, respectively. The antimicrobial activities of the extracts were evaluated based on disc diffusion assay.

The highest extraction yield was observed for methanol extracts obtained with ASE technique (17.1%). Furthermore, total condensed tannin and quinic acid concentrations in methanol root extracts were 35.14 mg/L and 63894.761 µg/g dry extract, respectively. Additionally, the highest DPPH scavenging activity was determined by methanol extract obtained with ASE method (98.8%). The highest inhibition zone was against *Candida albican* (25.6 mm).

Keywords: Medicinal plant, Secondary metabolism, *Salix aegyptiaca* L., Antioxidant activity, Antimicrobial activity

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LIST OF ABBREVIATIONS

WHO:	World Health Organization
Aspirin^{TM}:	Aspirin trademark
ASA:	Acetyl Salicylic acid
BC:	Before Christ
BHT:	Butylated hydroxytoluene
DPPH:	1, 1-diphenyl-2 picrylhydrazyl
HPLC:	High Performance Liquid Chromatography
EPM:	Elevated Plus Maze
LC:	Liquid Chromatography
MS:	Mass Spectrometry
MAE:	Microwave assisted extraction
ASE:	Accelerated solvent extraction
OH:	Hydroxyl
MHA:	Mueller-Hinton agar
SDA:	Sabouraud dextrose agar
ATCC:	American Type Culture Collection
MIC:	Minimum Inhibitory Concentration
L:	liter
ml:	Milliliter
µl:	Microliter
mg:	Milligram
µg:	Microgram
mm:	Milimeter
cm:	Centimeter
Kg:	Kilogram

1. INTRODUCTION

The vast majority of people on this planet still use traditional material in their everyday medical healthcare. It is also a fact that one quarter of the medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs. According to the WHO, 80% of the world's population primarily those in developing countries use plant derived medicines for their healthcare. People who use traditional remedies may not understand the scientific rationale behind their medicines. However, they know from personal experience that some plants can be effective if used at curative doses (Gurib-Fakim, 2006).

Medicinal plants are used as excellent antimicrobial agents. Recently, there are varieties of chemical constituents in nature. Much attention has been directed towards to those extracts and biologically active compounds isolated from plants (Prince and Prabakaran, 2011). Plants have ability to synthesize aromatic substances, such as phenolic (phenolic acids, flavonoids, quinones, and tannins) and nitrogenous (alkaloids, amines) compounds along with vitamins, terpenoids (i.e. carotenoids) and other endogenous metabolites. These substances serve as plant defense mechanisms against to predation of microorganisms, insects and herbivores (Bharathi et al., 2011).

Plant polyphenols (i.e. tannins) constitute a complex group of naturally occurring polymers. Chemical definition of these substances is usually difficult. Thus, tannins are considered polyphenolic metabolites of plants with a molecular weight larger than 500. They have ability to precipitate gelatin and other proteins in solutions (Mehansho et al., 1987). Plant polyphenols have an astringent taste. They are believed to be active agents in many traditional medicines due to their interactions with proteins. In such, herbal teas are in this group even though high consumption of these teas is known to lead medical problems (Whiting, 2001). Based on their structure, tannins can be divided into two groups, hydrolysable and condensed tannins.

Proanthocyanidins (PA) or condensed tannins are polymeric flavonoids (Wünsch et al., 1984). Catechins and some low-molecular weight PA have received considerable attention refer to their different biological activities, in particular their effects on arteriosclerosis (Masquellier, 1988) and oxygen free radical scavenger ability (Ricardo-da-Silva et al., 1991). Flavonoids are classified under phenolic groups in plants, which have

been known as antimicrobial activities (Cowan, 1999). The mechanism of flavonoids can be classified as the inhibition of nucleic acid synthesis, distraction of cytoplasm membrane function and disturbing energy metabolism (Cushnie and Lamb, 2005).

Willow bark is considered one of the first examples of modern drug improvement from an herbal medicine 160 years ago. The chemical oxidation of salicin is known to be a constituent of the bark of willow tree (Latin name: *Salix*). It turned to be a new substance called salicylic acid, and the acetylated derivative of this substance was turned to be the most successful drug in the history, Aspirin^{TM}. This development was pointed the anti-inflammatory, antipyretic and analgesic effects of willow bark in the classical antiquity (Aronson, 1994). Stone reported it in detail for the first time in 1763. Today, willow bark is included in the German pharmacopoeia and a monograph for the European pharmacopoeia is in preparation. The European Scientific Cooperative on Phytotherapy (ESCOP) recommends an equivalent of up to 240 mg salicin for a day (ESCOP, 1997).

The *Salix* family is famous due to its endogenous salicylate compounds e.g. salicylic acid and acetyl salicylic acid (ASA). This class of compounds is comprised as anti-inflammatory effect throughout the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) for leading the inhibition of prostaglandin synthesis (Yu et al., 2002; Mahdi et al., 2006). The species of genus *Salix* are deciduous trees and shrubs with simple, stipulate leaves alternately arranged on woody stems. Based on the several publications, 526 species are recognized for the genus worldwide (Fang-Zhen, 1987; Ohashi, 2000; Argus, 2007). In addition, *Salix* species probably originate from the Middle East, especially in Egypt and somewhere in Turkey, Iran, Iraq, Armenia, Turkmenistan and Afghanistan. However, it has spread as an ornamental plant in Europe, America and Australia (Rabbani et al., 2011).

Salix aegyptiaca L. commonly known as Musk Willow is a flowering plant and generally cultivated in the Middle East for hedge and ornamental purposes (Sonboli et al., 2010). It belongs to Salicaceae family in the order of Malpighiales that contains about 55 genera and more than 1000 species. Furthermore, *Salix aegyptiaca* L. is a deciduous plant growing up to 4-5 m. It is a strong, fast growing, and deciduous small tree with purplish-red, thick branches. Leaves are oblong, serrated, deep green above, underside hairy and up to 15 cm long. The inflorescence is catkin; catkins are fragrant and grey. Individual flowers are either male or female, but only one sex is found on one plant, consequently both male

and female plants must be grown if seed is required and are pollinated by bees. Male catkins are 4 cm long with yellow anthers and probably include one of the used parts of this plant; female catkins are 7.5 cm long. The plant is not self-fertile (Zargari, 1988).

The presence of high amounts of phenolics, such as gallic acid, caffeic acid, vanillin, p-coumaric acid, myricetin, catechin, epigallocatechin gallate and flavonoids, such as rutin, quercetin and salicin are indicated in the leaves of *Salix aegyptiaca* L. (Enayat and Banerjee, 2009). Plants play a vital role in maintaining human health and contribute towards to improvement of human life. Although hundreds of plant species were tested for their antimicrobial properties (Nair and Burke, 1990), they contribute important components of medicines, cosmetics, dyes, and beverages (Khare, 2004).

There are many cases of infection by drug resistant bacteria but few drugs are effective for curing such patients. Thus, it is necessary to discover or develop new drugs that are effective on those resistant bacteria. In addition, discovery of novel compounds (antimicrobials and inhibitors) is useful against multidrug-resistant bacteria (Horiuchi et al., 2007). Recently, a main concern among scientists and clinicians worldwide is to meet microbial drug resistance against available antimicrobial agents. In general, it is observed that pathogenic viruses, bacteria, fungi, and protozoa are more complex to treat with the existing drugs (Koomen et al., 2002).

Structural modification of antimicrobial drugs has been developed for extending the duration of antifungal and antibacterial agents, such as azoles (Jeu et al., 2003), lactams and quinolones (Poole, 2001). The screening of plant extracts for antimicrobial activity has been studied extensively with various plants containing active compounds. The presence of antibacterial, antifungal and other biological activities have been demonstrated in extracts of different plant species used in traditional medicine practices (Masoko et al., 2005).

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries. Therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially to microorganisms resistant to conventional antimicrobials (Silva and Junior, 2010). Many studies demonstrated concordance between selectivity of plants. Among those plants, many of them showed antibacterial and antifungal activities in the laboratory (McCutcheon et al., 1992; Webster et al., 2008).

The activity of plant extracts on bacteria and fungi has been studied by a very large number of researchers in different places of the world. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still include as part of the habitual treatment of a variety of maladies (Rios and Recio 2005). About 20% of the plants found in the world have been submitted to pharmaceutical or biological tests and a sustainable number of new antibiotics are obtained from natural or semi synthetic resources (Mothana and Lindequist, 2005). These are effective for infectious disease treatments. While, there may have many side effects that are often associated with synthetic antimicrobials (Harishchandra et al., 2012). Plants with possible antimicrobial activity should be tested against some microbes to confirm the activity (Duraipandiyan and Ignacimuthu 2011).

In last decade, the scientific interest has been focused on antimicrobial peptides or bacteriocins (Diez et al., 2012) particularly on polyphenols from natural sources towards to their possible antimicrobial activities (Garcia-Ruiz et al., 2008). However, the effect of polyphenols on bacterial growth is not yet well understood. The antimicrobial activity may differ according to microorganism, structure, and concentration of polyphenols. Large doses of phenolic compounds may be toxic for bacteria while low doses can be used as substrate (Campos et al., 2003). Some authors propose that these compounds can interact with the proteins of the bacterial cell membrane. These compounds may also cause damage to cell membranes and subsequent release of cytoplasm or interacting with cellular enzymes (Rozes and Perez, 1998; Garcia-Ruiz et al., 2009; Garcia- Ruiz et al., 2012).

Phenolic compounds are known to serve oxygen scavenging and redox potential reducer of wines (Vivas et al., 1997). Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species (ROS), which are continuously produced *in vivo*, result in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases including cancer, diabetes and cardiovascular diseases (Halliwell and Gutteridge, 1999).

Antioxidants are vital substances and capable of protecting body from free radical based oxidative stress (Ozsoy et al., 2008). There is an increasing interest in natural antioxidants as polyphenols present in medicinal and dietary plants, which might help to prevent oxidative damage (Silva et al., 2005). Polyphenols possess ideal structural

chemistry for free radical scavenging activity. They have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors. They stabilize or delocalize the unpaired electron (chain breaking function) and chelate the transition metal ions (Rice-Evans et al., 1997).

Human body has defense mechanisms. With enzymatic and non-enzymatic antioxidant systems, they can limit and protect the cellular molecules against reactive oxygen species (ROS) (Anderson, 1996). However, the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants for balancing the ROS in human body. Many synthetic antioxidants, such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxyltoluene (BHT) are very effective and used as antioxidants. The request for natural antioxidants has been increased due to side effects of synthetic antioxidants (Anagnostopoulou et al., 2006).

The aims of current study were to determine extract yield, amount of total condensed tannin and identification of phenolic compounds in *Salix aegyptiaca* L. roots. In addition, antioxidant and antimicrobial activities of root extracts were also performed.

2. LITERATURE REVIEW

2.1. Medicinal Plant

Since ancient times, plants have been continued to play a role for maintaining human health due to their medicinal compounds. According to World Health Organization, plant extracts or their active constituents are used in traditional therapies of 80% of the world's population as folk medicine. Over 50% of all modern clinical drugs are natural origin (Kirbag et al., 2009).

The specific purpose of many phytochemical is still unclear. However, many studies indicated that phytochemicals are involved in the interaction of (plants/epidemic/ diseases). Antimicrobial screening of plant extracts and phytochemicals represents a starting point for antimicrobial drug discovery. Phytochemical studies have been brought the attention of plant scientists due to the improvement of new and complicated techniques. These techniques played a significant role for additional resources of raw substances in pharmaceutical production (Shakeri et al., 2012).

Medicinal plants possess immune-modulator and antioxidant properties leading to antibacterial activity. They are known to have versatile immune-modulator activity by stimulating both non-specific and specific immunity (Pandey and Chowdhry, 2006). Using plant extracts and phytochemicals provide a great significance in therapeutic treatments. In last few years, numbers of studies have been conducted to verify such effectiveness. Many plants have been used for their antimicrobial characters due to the compounds synthesized in secondary metabolism of plants (Nascimento et al., 2000).

Using plants for medicinal purposes have been practiced for many centuries. As a source of pharmacologically active compounds, plants are continued to use for phytotherapeutic activities and other industrial applications. One of the important activities for some plants is to bring free radical scavenging power, also crucial for food industry (Thabti et al., 2014).

2.2. Secondary Metabolites

Plant cells synthesize a vast supply of natural compounds that are not strictly needed for their growth and reproduction. Those compounds are known secondary

metabolites and have a wide variety of chemical, physical and biological properties (Gottlieb, 1990).

The plant secondary metabolites grouped into three main groups; phenolic, nitrogen containing and terpenoid compounds. They are present in different parts of plants (roots, leaves, barks, seeds, fruit skins) and exhibit diverse functions, including plant chemical defense and attraction of beneficial animals for pollination and seed dispersal (Reichling, 1999).

The contents of secondary metabolites vary among plant species. They may contain as little as 1% or as much as one-third of their dry weight and vary in plant tissues and seasons. Plant metabolites have been used for thousands of years as dyes, flavors, fragrances and poisons medicines. However, using plant secondary metabolites in healthcare, personal care, and development of novel drugs led them to bring more interest for isolation and characterization. Special attention has been given to phenolic compounds due to their antioxidant properties and probable roles in prevention of various diseases (i.e. cancer, cardiovascular and neurodegenerative diseases) (Harborne and Williams, 2000; Kroon and Williamson, 2005).

2.2.1. Phenolic compounds

Phenolic compounds are the plant secondary metabolites originated from pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir et al., 2004).

These compounds play an important role in plant growth and reproduction, providing protection against ultraviolet radiation and pathogens. Structurally, phenolic compounds comprise of an aromatic ring, bearing one or more hydroxyl substituent and range from simple phenolic substances to highly polymerized compounds (Bravo, 1998).

They are usually found as conjugates with mono and polysaccharides, linked to one or more of the phenolic groups and may occur as functional derivatives, such as esters and methyl esters (Harborne et al., 1999).

2.2.1.1. Quinic acid

Quinic acid derivatives, which are polyphenol rich compounds, consist of a large family of esters formed between quinic acid and one or more of several phenylpropanoic

acids, such as caffeic, ferulic, coumaric, sinapic, and cinnamic acids (Lee et al., 2013). It has been exhibit various beneficial effects including antioxidant, anti-inflammatory, anti-hepatitis B virus, hypoglycaemic, the inhibition of mutagenesis and carcinogenesis (Farah and Donangelo, 2006; Gorzalczany et al., 2008).

Zhang et al. (2014) indicated that chromatograms for of the *Acanthopanax henryi* methanol extract in HPLC showed presence of 5-*O*-caffeoyl (27.54 mg/g dry leaves), 4-*O*-caffeoyl (5.91 mg/g dry leaves), 3,4-di-*O*-caffeoyl (0.66 mg/g dry leaves), 1,5-di-*O*-caffeoyl (0.853 mg/g dry leaves), 3,5-di-*O*-caffeoyl (5.81 mg/g dry leaves) and 4,5-di-*O*-caffeoyl quinic acids (3.81 mg/g dry leaves), respectively.

2.2.1.2. Tannins

D'Archivio, et al. (2007) and Quideau, et al. (2011) reported that polyphenols are present in plant tissues mainly as glycosides and associated with various organic acids or as complex polymerized molecules with high molecular weights. These are called tannins.

Tannins are relatively high molecular weight compounds, which constitute an important group of phenolic structures and being synthesized in many plants. They can take place in wood, bark, leaves, fruits and galls. Tannins are subdivided into hydrolysable and condensed forms. The previous are gallic acid esters (gallo- and ellagi-tannins) while the latter are polymers of flavan-3-ol monomers also known as proanthocyanidins. The name proanthocyanidins came from the characteristic oxidative depolymerization effect in acidic medium, which yields colored anthocyanidins (Hümmer and Schreier, 2008).

There are varieties of different condensed tannins depending on the substitution pattern of the monomeric flavan-3-ol units. The condensed tannins, which exclusively consist of catechin (epi) units, are designated procyanidins, the most abundant condensed tannins in plants. The flavan-3-ol subunits may carry acyl or glycosyl substituent, the most common being gallic acid to form 3-*O*-gallates. Condensed tannins are quite sensitive to oxygen, light, acid and alkaline (Shi et al., 2005). For instance, monomers and dimmers are rapidly degraded at pH higher than 9.0 and formed brown colored degradation products (Zhu et al., 2002).

A large number of hydrolysable tannins exist in nature. The structural variations among these compounds are caused by oxidative coupling of adjoining gallic acid units or

by oxidation of aromatic rings. Gallo tannins consist of a central polyol, such as glucose, which is surrounded by several gallic acid units. Ellagitannins, which have a more complex structure, also derive biosynthetically from pentagalloylglucose by oxidative reactions between the gallic acid units (Mueller-Harvey, 2001).

2.2.1.3. Salicin

The active ingredient in willow bark is salicin, which is converted to salicylic acid in the body (Figure 2.1). Salicylic acid is also known aspirin. The presence of a phenol is recognized by the attachment of an OH group on the benzene ring. Substitutions on the phenolic group of various natural products include, but are not limited to methyl, acetyl, and ether linkages (Cooper and Nicola, 2014).

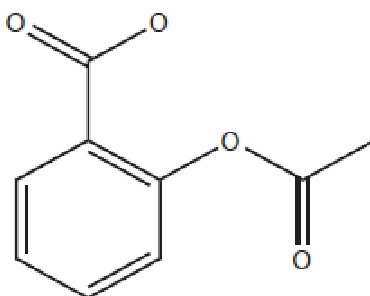

Figure 2.1. Salicylic acid

The Willow family consists of plants with notable amounts of endogenous salicylate compounds. Due to its active constituent salicin and its derivatives, willow bark (*Salix* species) has been famed and used throughout the world since ancient times. Specifically the discovery of aspirin has diverted important thought to this plant species (Barrett et al., 1999; Cragg and Newman, 2001).

Extracts from *Salix* species have been used for centuries as a medicinal plant in many parts of the world for various health disorders. Willow is mentioned as an herbal pain remedy in the *Ebers papyrus* of ancient Egypt. The bark from these plants is a rich source of salicin, the pro-drug form of acetyl salicylic acid (ASA; Aspirin). It has been associated with anti-nociceptive, anti-inflammatory properties and the induction of apoptosis in cancer cells (Chrubasik et al., 2001; Hostanska et al., 2007).

The *Salix* family is famous due to its endogenous salicylate compounds e.g. salicylic acid and ASA. This class of compounds exert anti-inflammatory effects

throughout the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin synthesis (Yu et al., 2002; Mahdi et al., 2006).

The anti-inflammatory and anti-nociceptive properties of the extracts of willow family may be related to its phytochemicals, such as salicin, myricetin, kaempferol, quercetin, rutin and luteolin. These compounds have immunomodulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors (Qin and Sun, 2005; Nahrstedt et al., 2007). Salicin, the major phenolic glycoside present in the bioactive extracts in *Salix* species, is considered the pharmacologically active principle due to its structure similarity to aspirin. In animal models, the extracts from *S. aegyptiaca* L. leaves and male flowers have shown anti-inflammatory effects in carrageenan-induced paw edema and hot plate tests (Karawya et al., 2010; Rabbani et al., 2010).

The aqueous extract of *Salix aegypyiaca* L. male flowers at 0.3, 0.6 and 1.2 mg/kg doses showed a significant analgesic effect compared to control group treated with ASA. In addition, it is demonstrated that the analgesic effect of 0.6 mg/kg of the extract was higher than 300 mg/kg of ASA (Karawya et al., 2010).

Chewing the willow bark from *Salix alba* reduced fever, inflammation, and was recognized by ancient Greek physician Hippocrates to possess health benefits. In the 1800s, pharmacists created salicylic acid in its acetylated form (ASA), more commonly known as aspirin. It was first isolated and synthesized by Felix Hoffmann, a chemist with the German company Bayer and marketed in 1897. Thus, the most widely used drug in the world continues to be aspirin (Cooper and Nicola, 2014).

Epidemiological studies have shown that patients under long-term aspirin therapy have lower propensity to develop colorectal cancer (Giardiello et al., 1998; Steinbach et al., 2000). However, recent studies have suggested that ASA may act through some COX-independent pathways; therefore, the mechanism of its anti-carcinogenic effect remains unclear (Law et al., 2000).

Since salicylic acid and other salicylates are naturally present in various fruits and vegetables, consuming these components in the daily diet might greatly reduce the risk of colorectal carcinoma. Studies have shown that the concentration of salicylic acid in the

serum of people consuming vegetables is greater than those who don‘t consume vegetables and those taking low dose aspirin (Paterson and Lawrence, 2001).

Salix bark samples were characterized by a diverse content of phenolic glycosides, expressed as salicin equivalent. The highest amount of salicin was found in the bark of *Salix myrsinifolia* 142.17 mg/g. In turn, a substantially lower content was noted for the bark of *Salix purpurea* 74.33 mg/g (Durak et al., 2014).

2.2.2. Biological effects of phenolic compounds

Zhang et al. (2013) have reported the antibacterial, antifungal and antioxidant activities of quinic acid. It was also reported that malic acid has high antimicrobial and antioxidant activities (Mokbel and Hashinaga, 2005). Rutin and caffeic acids have also antimicrobial activity (Coneac et al., 2008). The antioxidant capacity of the phenolic compounds, especially gallic acid, caffeic acid, rutin, quercetin, and p-coumaric acid in several models is well known (Sun et al., 2007). Rutin, a natural flavone derivative, is an important anti-lipoperoxidant agent (Negre-Salvayre et al., 1991). It was found to be a strong scavenger of hydroxyl and superoxide radicals (Metodiewa et al., 1997).

Gallic acid is a simple phenolic acid and possessing a single aromatic ring. Its derivatives are biologically active compounds, which are widely present in plants (Lee et al., 2000). It has been reported to possess an anti-inflammatory (Kroes et al., 1992) anti-tumor (Miki et al., 2001) antibacterial and antifungal activities (Akiyama et al., 2001; Panizzi et al., 2002). The phenolic acids, such as caffeic, salicylic, chlorogenic and 4-hydroxybenzoic acids are known to have antioxidant (Zheng and Wang, 2001) and antimicrobial properties (Proestos et al., 2005).

Kasim et al. (2011) reported that gallic, protocatechuic and chlorogenic acids along with flavonoids have antimicrobial activities. Furthermore, *p*-coumaric, protocatechuic, caffeic, salicylic, chlorogenic, gallic and 4-hydroxybenzoic acids have antioxidant and antimicrobial activities (Shabir et al., 2011). Moreover, hesperidin has antioxidant, anticancer and free radical scavenging properties reported by Miller and Rice (1997) and Lonchampt et al. (1989). As well as, naringenin has antioxidant effects that act as ROS scavengers (Agati et al., 2012; Ursini et al., 1994).

2.3. *Salix* species

Experience of both Assyrian (4000 BC) and Babylonian civilizations (605–562 BC) in Iraq has contributed to development of medicine (Barrett et al., 1999; Burns and Fulder, 2002). In ancient civilizations of that period, medicine was based on surgeons and physicians using herbal draughts to cure ailments; extracts of the willow tree used for curing pain and inflammation. Archaeologists have found clay tablets left by the Assyrians from the Sumerian period (2000-3500) describing the use of willow leaves for such conditions (Levesque and Lafont, 2000).

The Babylonians also used willow tree extracts to treat common fever, pain and inflammation. Sumerians were the first known civilization to register medical prescriptions for pain according to a clay tablet from 4000 years ago (Wells, 2003). In addition, the Code of Hammurabi (1750 BC) in Mesopotamia sheds light on a medical history that contributed to the long chain of pharmacological achievements prompted by man's attempt to survive disease. Such notions also prompted Egyptians (1300 BC) to use willow leaves to treat inflammatory conditions.

Furthermore, the Greeks also used willow as a form of medicine. The philosopher Hippocrates recommended chewing willow bark to patients suffering from high temperature and pain. Also, prescribed a brew of willow leaves to ease the aching pains of childbirth. Later, Greek physician Discords specified willow barks to reduce the symptoms of inflammation. Using willow bark has continued because of its analgesic and anti-inflammatory properties (Riddle, 1999).

2.4. *Salix aegyptiaca* L.

2.4.1. Definition

Salix aegyptiaca L., usually known as Musk Willow, is a flowering plant. It has an important area in drug development with some pharmacological activities. It has been used for a long time in traditional medicine for relief of anemia, vertigo, cardio tonic agent as well as fragrance additive and preparation of local candies. Recently it was shown to have antioxidant, anxiolytic and hypocholestrolemic effects. High amounts of phenols and flavonoids are reported from leaves of this plant (Asgarpanah, 2012).

2.4.2. Classification

It belongs to Salicaceae family in the order of Malpighiales that contains of 55 genera and more than 1000 species. The species of the genus *Salix* are deciduous plants and shrubs with simple, stipulate leaves alternately arranged on woody stems. Based on several publications (Fang-Zhen, 1987; Ohashi, 2000; Argus, 2007) 526 distinct species are recognized for the genus worldwide. Former Soviet Union includes 120 species, the New World 103, China 275, Europe 65, Pakistan 26, Iran 31 and 6 hybrids (Maassoumi, 2009).

2.4.3. Distribution

Salix species probably originates from the Middle East, especially in Egypt and somewhere in Turkey, Iran, Iraq, Armenia, Turkmenistan and Afghanistan. It has also spread as an ornamental plant up to Europe, America and Australia (Rabbani et al., 2011).

2.4.4. Morphology

S. aegyptiaca L. is a deciduous shrub growing up to 4-5 m. It is a vigorous, fast growing, bushy, deciduous small tree with purplish-red and thick branches (Figure 2.2). Leaves are oblong, serrated, deep green above, underside hairy and up to 15 cm long. The inflorescence is catkin; catkins are fragrant and grey. Individual flowers are either male or female, but only one sex is found on one plant. So that both male and female plants must be grown if seed is required and are pollinated by bees. Male catkins are 4 cm long with yellow anthers and considered as used part of this plant. Female catkins are 7.5 cm long. The plant is not self-fertile (Asgarpanah, 2012).

Figure 2.2. *Salix aegyptiaca* L.

2.4.5. Chemical compositions

Phytochemical compounds of *Salix aegyptiaca* L. are volatile substances, flavonoids and phenolics. Sonboli et al. (2010) showed that gallic acid concentration in butanol, methanol, water and chloroform fractions were 313.8, 129.6, 37.7 and 14.3 ppm, respectively.

Enayat and Banerjee (2009) reported that a number of chemical constituents such as flavonoids and volatile substances have been isolated from different parts of the plant. In addition, high amounts of phenolic compounds (gallic acid, caffeic acid, vanillin, p-coumaric acid, myricetin, catechin, and epigallocatechin gallate) and flavonoids (rutin, quercetin salicin) are found in leaves of *Salix aegyptiaca* L.

Salix aegyptiaca L. male catkins are rich in volatile components, such as p-methoxybenzene, eugenol, carvone, cedrene oxide, and geraniol. p-methoxybenzene (60%), eugenol (21%), decanol (4%), cedrene oxide (2.5%) and ocimene (2.3%) were identified as the major essential oils of *Salix aegyptiaca* L. male catkins collected from Urmia, North West of Iran (Salehi-Surmaghi, 2009). While, carvone (16%), cedrene oxide (16%), geraniol (10%), carvacrol (9%), citronellol (4%) and p-methoxybenzene (2.3%) were the major essential oils of *Salix aegyptiaca* L. male catkins collected from Shiraz, South of Iran (Salehi-Surmaghi, 2009).

Karimi et al. (2011) reported that essential oils of *Salix aegyptiaca* L. leaves contained 1,4-dimethoxybenzene (61.5%), methyleugenol (21%), phenylethyl alcohol (10.9%), citronellol (8%), carvone (6%), eugenol (6%) and 4-methoxyacetophenone (3%). 1,4-Dimethoxybenzene, also known as “hydroquinone dimethyl ether” is the para form of dimethoxybenzene, volatile aromatic ether with a sweet floral odor. It occurs naturally in *Salix* species (Dötterl et al., 2005).

Enayat et al. (2013) evaluated the bark of *Salix aegyptiaca* L. by ethanolic extraction. The extraction contained catechin 2311.55 ppm, salicin 944.7 ppm and catechol 502.57 ppm in 50 mg/ml of ethyl acetate fraction. In addition, gallic acid, epigalocatechin gallate (EGCG), quercetin, qoumaric acid, rutin, syringic acid and vanillin were also found in to smaller amounts.

Bravo (1998) reported that aqueous and ethanol extracts of willow leaves contain high portion of phenolic compounds, glycosides, tannins and salicin. Salicin is a salicylic acid related compound in willow leaves. Willow is famous as a source of salicylic acid, which is well known as systemic resistance inducer in many plants against several plant diseases (Claudia, 2003).

2.4.6. Medicinal usage

The male inflorescence of the plant has been used in folklore medicine as cardiotonic, anemia and vertigo treatments. The aqueous extracts and essential oils of these inflorescences are also used in confectionary, flavorful syrups and candies. Additionally, *Salix aegyptiaca* L. has been used as laxative, cardio protective, nervonic, sedative, hypnotic, somnolent, aphrodisiac and gastro protection. The decoction of *Salix aegyptiaca* L. leaves in honey is still used as a nervonic functional food. The decoction of leaves plus sugar has been used among Iranian and Turkish people for maladies like depression, neuropathic pain and rheumatoid arthritis (Karimi et al., 2011).

Based on current pharmaceutical studies and applications on *Salix aegyptiaca* L., authors revealed antioxidant, anti-inflammatory, analgesic, anxiolytic and anti-hyperchlosterolemic effects of this plant (Sonboli et al., 2010; Karawya et al., 2010; Rabbani et al., 2011; Karimi et al., 2011).

The flower extracts of *Salix aegyptiaca* L. on EPM model of anxiety in mice produced anxiolytic effects and reduced locomotors activities with 100 or 200 mg/kg doses. These doses did not affect ketamine induced-sleep intervals (Rabbani et al., 2010). On the other hand, the potent antioxidant activity of *Salix aegyptiaca* L. supports its possible use as a natural antioxidant in food industries and other pharmaceutical preparations (Sonboli et al., 2010).

2.5. Biological Effects

2.5.1. Antimicrobial activity

Current medicine has been used for many years successfully in treatment of infectious diseases. Improved sanitation, clean water, better living conditions and vaccines have been successful for controlling infectious diseases (Wilson, 1995). Many pathogens

are becoming resistant to antibiotic drugs in alarming situations where new drug improvements are needed (Dannaoui et al., 2001).

The screening of plant extracts for antimicrobial activity has shown that the great number of these plants contain active compounds. The presence of antibacterial, antifungal and other biological activities have been demonstrated in different plant extracts for traditional medicine practices (Masoko et al., 2005).

Previous papers by Dixon et al. (1998), Alcaraz et al. (2000) and Mirzoeva et al. (1997) indicated that the flavonoids have a strong antimicrobial activity.

Bonjar et al. (2004) reported that the methanol extract of 0.1 ml *Salix aegyptiaca* L. flower showed antibacterial activities against *Bordetella bronchiseptica* (PTCC1025), *Staphylococcus aureus* (PTCC1112) and *Bacillus cereus* (PTCC1115) with inhibition zones of 10, 9, 10 mm, respectively. However, there was no detected inhibition zone against *Escherichia coli* (PTCC1330), *Pseudomonas aeruginosa* (PTCC1074), *Pseudomonas fluorescen* (PTCC1181), *Klebsiella pneumoniae* (PTCC1053), *Serratia marcescens* (ATCC27117), *Staphylococcus epidermidis* (PTCC1114), *Micrococcus luteus* (PTCC1110) and *Bacillus pumilus* (PTCC1319). In addition, there was no inhibition zone against *Saccharomyces cerevisiae* (PTCC5052), *Candida albicans* (PTCC5027) and *Candida utilis* (PTCC5065) fungi.

Bonjar (2004a) evaluted the methanolic extract of *Salix aegyptiaca* L. flowers against *Bordetella bronchiseptica* PTCC1025. They found that the extract was effective at 20 and 15 mg/ml with 10 mm of an inhibition zone and minimal inhibitory concentration (MIC), respectively.

Bonjar (2004b) also evaluted the methanol extract of *Salix aegyptiaca* L. flowers (20 mg/ml) and showed an inhibition zone (9 mm) against *Staphylococcus aureus* (PTCC1337). However, there was no inhibiton zone against *Staphylococcus aureus* (PTCC 1112) and (PTCC 1113).

Varying concentrations (300, 400 and 500 mg/ml) and conditions (hot and cold) of methanol and aqueous extracts of *Salix acmophylla* showed antibacterial activities against *Shigella dysenteriae*, *Aeromona shydrophila*, *Escherichia coli*, *Klebsiella spp*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* with inhibition zones for methanol

(10-27 mm), hot aqueous (9-25 mm) and cold aqueous (12-30 mm) extracts. However, only *Enterobacter spp*. was resistant to all extracts (Ali and Aboud, 2010).

Likewise, Al-Kadum et al. (2008) studied the antimicrobial activity of *Salix acmophylla* leaves extract against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella spp*. and *Streptococcus pyogenes* in cold aqueous and ethanol at various concentrations. Both extracts showed the highest antimicrobial activity against *E. coli*, *S. aureus* and *St. pyogenes* (9, 12, and 14 mm inhibitory zone). There was no antimicrobial activity against *Klebsiella spp*. and *E. coli*. While, *S. aureus* and *St. pyogenes* were the most effective ones with aqueous and ethanolic extracts, respectively.

Hussain et.al. (2011) suggested the antimicrobial activity of the crude extracts (CH_2Cl_2 and EtOAc) for leaves and bark of *Salix subserrata* against *Escherichia coli*, *Bacillus megaterium* and *Microbotryum violaceum*. All the extracts showed promising antibacterial activity against *Bacillus megaterium* (7-10 mm) inhibition zone and *Escherichia coli* (7-8 mm) inhibition zone except for CH_2Cl_2 exract of bark. Both EtOAC extracts of leaf and bark were inactive against *Microbotryum violaceum*.

Moreover, Sulaiman et al. (2013) evaluated the hot ethanol extract of *Salix alba* bark and showed antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The highest effect was on *C. albicans* with 23.66±0.33 mm inhibition zone at 80 µg/ml, followed by *S. aureus* (22.33 mm) and *P. aeruginosa* (17 mm). While, there was no inhibition zone observed against *Escherichia coli* and *Klebsiella pneumonia*.

Hussien (2011) evaluated the effect of ethanol and nanoparticales extracts of *Salix alba* on *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth. The highest effect of ethanolic extract was observed on *Staphylococcus aureus* (16 mm inhibition zone) and *Pseudomonas aeruginosa* (10 mm inhibition zone). However, the inhibition zones were increased with nanoparticales extract on both *Pseudomonas aeruginosa* (27 mm) and *Staphylococcus aureus* (22 mm).

Dığrak et al. (2001) reported that the *Rhus coriaria* fruit extracts were effective against *B. megaterium* DSM32, *M. luteus* LA2971, *B. cereus* FMC19, *E. aerogenes* CCM2531, *P. aeruginosa* DSM50071, *L. monocytogenes* ScootA, and *S. aureus* Cowan1

with inhibition zones between 36 and 51 mm. However, *R. coriaria* fruit extracts had no effect against *E. coli* and *Candida albicans* CCM314.

Furthermore, the *Laurus nobilis* extracts had an antimicrobial activity against *S. aureus* Cowan1, *P. aeruginosa* DSM50071, *B. brevis* FMC *B. megaterium* DSM32, *E. aerogenes* CCM2531 *M.* luteus LA2971 and *B. Cereus* FMC19 (6–13 mm). These extracts were also effective against *C. tropicalis* with a sufficient inhibition zone (32 mm). However, *Laurus nobilis* was not effective against *B. subtilis* IMG22, *E. coli* DM, *L. monocytogenes* Scott A and *C. albicans* CCM (Dığrak et al., 2001).

Ateş and Erdoğrul (2003) determined the antimicrobial activity of alcohol extract of *Pimpinella anisum* seeds. There was an antibacterial activity against *Micrococcus luteus* LA2971 (8 mm inhibition zone). Alcohol, ethyl acetate, acetone and chloroform extracts of *Glycyrrhiza glabra* roots also showed antibacterial activities against *Micrococcus luteus* LA2971 (8, 10, 7 and 10 mm inhibition zones, respectively). On the other hand, the *Cinnamomum cassia* bark extracts showed an antibacterial activity against *M. luteus* (9-15 mm). Whereas, the alcohol extract did not inhibit *M. luteus*. Likewise, the *Coriandrum sativum* seeds extract showed no inhibition zone against *M. luteus*.

Aneja and Joshi (2009) observed that the acetone, ethanol and methanol extracts of *A. subulatum* showed an antimicrobial activity against tested all microorganisms except *L. acidophilus* and *S. aureus*. The extracts of *E. cardamomum* showed high inhibitory effect against *S. aureus*, *C. albicans, S. cerevisiae* and *S. mutans*. The highest inhibition zone was observed with ethanolic extract of *A. subulatum* (16.32 mm) and acetonic extract of *E. cardamomum* (20.96 mm) against *S. aureus*. Minimum inhibitory concentration (MIC) of these extracts against the selected microorganisms was ≥10 mm.

Saravanan et al. (2010) showed that the aqueous garlic extract inhibited the growth of both Gram positive and negative bacterial test cultures. The maximum antibacterial activity was noted against *Klebsiella pneumoniae* (8 mm), *Bacillus cereus* (7 mm), *Escherichia coli* (6 mm) and *Streptococcus mutans* (6 mm). The minimum antibacterial activity was against *Salmonella typhi* (4 mm) inhibition zone.

Eloff (1999) investigated the antibacterial activity of 27 southern African members of Combretaceae and found that selected plant extracts inhibited the growth of *Escherichia*

coli, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* with MIC values between 0.10 and 6.0 mg/ml.

Katerere et al. (2003) reported that pentacyclic triterpenes isolated from members of African Combretaceae were active against *S. aureus*, *Candida albicans*, *Mycobacterium fortuitum* and *E. coli*.

Ayepola and Adeniyi (2008) investigated that methanolic extracts of the *Eucalyptus camaldulensis* leaf showed greater antibacterial activity against *Salmonella typhi, Staphylococcus aureus* and *Bacillus subtilis* (15-16 mm) compared to *Klebsiella spp, Yersinia enterocolitica* and *Pseudomonas aeruginosa* (14 mm). In addition, dichloromethane fraction exhibited higher activity against *Klebsiella spp, Salmonella typhi, Yersinia enterocolitica* and *Bacillus subtilis* (15–16 mm) than *Staphylococcus aureus* and *Pseudomonas aeruginosa* (13-14 mm). However, the methanol residue had a lower activity against all the test organisms except *Klebsiella spp* and *Salmonella typhi*.

Additionally, Abubakar (2010) evaluated the antibacterial potential of crude leaf extracts of *Eucalyptus camaldulensis* against some pathogenic bacteria. The least activity in terms of growth inhibition zone was in aqueous extract against *E. coli* (7 mm), *K. pneumoniae* (9 mm), *P. mirabilis* (13 mm), *S. typhi* (12 mm) and *S. aureus* (12 mm). While, the highest activity was in acetone extract for *E. coli (*12 mm), *K. pneumoniae* (13 mm), *S. typhyi* (14 mm), *P. mirabilis* (15 mm) and *S. aureus* (14 mm).

Moreover, Arora et al. (2004) evaluated the *in vitro* antibacterial activities of *Withania somnifera* extracts. The results showed that methanol extract of leaves had a higher activity against *S. typhimurtum* than *E. coli*. While, in roots the activity was more for *E. coli* than *S. typhimurtum*. However, hexane extracts of both leaves and roots had a low activity against *S. typhimurtum* than *E. coli*.

Hussain and Gorsi (2004) evaluated that the ethanolic extracts of *Nerium oleander* roots exhibited moderate activity against *Bacillus pumillus* and *S. aureus*. The antimicrobial activity was high against *E. coli* but low against *B. subtilis*. Moreover, methanol extracts of *Nerium oleander* roots revealed marked activity against all tested bacteria. None of the crude extracts showed activity against *A. niger*. The chloroformic leaf and root extracts of *Nerium oleander* did not show any appreciable activity against any of the microbes tested.

Ahmed et al. (2013) investigated the antimicrobial activity of *Nerium oleander* root and leaf extract against *Staphylococcus epidermis, Escherichia coli*, *Protus mirabilis* and *Staphylococcus aureus.* The ethanol and methanol extracts of *Nerium oleander* showed high activity against the bacteria with different inhibition zones. The ethanol extract of *Nerium oleander* (2.5, 5, 10, and 20 mg/ml) showed a higher antibacterial activity against all tested microorganisms (12-25 mm inhibition zone). While, methanol leaf extracts with same concentrations showed inhibition zones between 10 and 22 mm.

Derwich et al. (2010) evaluated the antibacterial activity and chemical composition of essential oil of *Nerium oleander* flowers. The results indicated that *E. coli* was the most sensitive tested strain to *Nerium oleander* oil (28.89 mm inhibition zone). *P. aeruginosa* was also sensitive (18.22 mm). The modest activity was observed against *S. aureus* (6.32 mm). The component of this oil (1,8-cineole) has been shown to exhibit antimicrobial activity against *E. coli*, *P. aeruginosa*, *S. typhi*, *S. aureus*, *S. intermedius*, and *B. subtilis*.

Shobana et al. (2009) evaluated the antibacterial activities of two Garlic varieties (*Ophioscordon* and *Sativum*) on enteric pathogens, such as *Escherichia coli*, *Proteus mirabilis, Salmonella typhi, Shigella flexineri and Enterobacter aerogenes*. The results showed that aqueous extract of both garlic varieties inhibited the growth of enteric pathogens at 200, 300, 400 and 500 mg concentrations. However, *Enterobacter aerogenes* was not susceptible to aqueous extract of both garlic varieties. Ethanol extract of *Sativum* was highly effective against all tested bacteria.

Elbashiti et al. (2011) evaluated the crude extracts of five plants obtained with different extraction methods against *E. coil* and S. *aureus*. There was no antibacterial activity in any plant extracts against *E. coil* except *Cakile maritima* seeds extracted with ethanol (13 mm). However, *Withania sonmifera* leaf extracts had an antibacterial potential against *S. aureus* (25 mm). In addition, *Marrubium vulgare* stems and leaves had an antibacterial potential against *S. aureus* with inhibition zones of 15 and 13 mm, respectively.

2.5.2. Antioxidant activity

An antioxidant is defined as any substance that is present at low concentrations and delays or prevents oxidation of the substrate (Rhee et al., 2009). Antioxidants are of interest to biologists and clinicians because they help to protect the human body against

damages induced by reactive free radicals generated in atherosclerosis, ischemic heart disease, cancer, and aging process (Aruoma, 2003).

Enayat and Banerjee (2009) suggested that the different extracts of *Salix aegyptiaca* L. catkins, leaves and bark with high antioxidant activity was capable of quenching the DPPH free radicals at lower concentrations. The results showed that the highest antioxidant activity was observed in ethanol extract of bark with an IC_{50} value of 19 g/ml. The lowest activity was observed in cyclohexane extract of bark with an IC_{50} value of 319 g/ml. The IC_{50} standards were 3.1 and 26l g/ml for quercetin and BHT, respectively.

Sonboli et al. (2010) reported the most antioxidant activity for different fractions of *Salix* extract in methanol with an IC_{50} value of 27.7 µg/ml which is comparable to synthetic antioxidant BHT (IC_{50} = 26.5 µg/ml). The antioxidant activities of other fractions decreased in the order of ME > WF > CF. The potent antioxidant activity of *Salix aegyptiaca* L. supported its possible use as a natural antioxidant in food industries and other pharmaceutical preparations.

The molecular mechanism of radical scavenging activity of *Salix aegyptiaca* L. could be attributed to presence of polyphenolic compounds. It has been exhibited that polyphenolic compounds are responsible for radical scavenging activity, due to the ease of their hydrogen atom donation to active free radicals (Ho et al., 1994).

The free radical scavenging activities (FRSA) of *Salix alba* bark extract at different concentrations was studied by Sulaiman et al. (2013). The DPPH radical inhibition pattern showed a concentration dependent manner for *S. alba* extract. The results showed that the FRSA was increased with increasing the concentration (12.5% at 10 µg/ml, 37.5% at 50 µg/ml and 80% at 100 µg/ml).

Soumia et al. (2014) indicated that IC_{50} values for DPPH method ranged from 4.3-48.6 µg/ml. The total antioxidant activity by β-carotene/linoleic acid bleaching assay ranged from 17.03 to 86.13%. *Pistacia lentiscus* showed the highest antioxidant activity by DPPH assay (IC_{50} 4.30 µg/mL). *Populus trimula*, *Origanum glandulosum*, *Centaurea calcitrapa*, *Sysimbrium officinalis* and *Rhamnus alaternus* also showed a higher total antioxidant activity by β- carotene/linoleic acid bleaching assay.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material: The roots of *Salix aegyptiaca* L. were collected in Feb. 2014 from Sarsang localy- Dohuk city in the North of Iraq. Further, taxonomic identification was done by Dr. Zeravan Abdulkaliq Sadeeq, Faculty of Agriculture-University of Dohuk.

3.1.2. Substances

Table 3.1. List of substances and their using purpose in the study

Substance	Usage purpose
Methanol	Used for extraction
Ethanol	Used for extraction
Distilled water	Used for extraction
MHA	Used in antimicrobial assay
SDA	Used in antimicrobial assay
Nutrient Broth	Used for the cultivation of a wide variety of microorganisms
DPPH	Antioxidant evaluation
BHT	Used as a reference in antioxidant assay
HCl	Condensed tannin determination
N-butanol	Condensed tannin determination
Fe_2SO_4	Condensed tannin determination
Mimosa	Standard Reference
Standard Ampicillin 10 µg/ml	Used in antibacterial test
Standard Gentamicin 10 µg/ml	Used in antibacterial test
Nystatin syrup	Used in antifungal test
Petri dish	Used to culture bacteria and fungi
Test tube	Hold the extracts
Conical flask	Heating and boiling of liquid Distillation Contain chemical reactions

3.1.3. Instruments

Table 3.2. The instruments and their usage in this study

Instruments	Usage purpose
Electric blender	For sample crushing
Gravimeter	For sample weighting
Microwave NEOS system	For sample extraction
Dionex ASE 350	For sample extraction
SHIMADZU UVvis 1240 spectrophotometer	For condensed tannin measurement For antioxidant measurement
Incubator	For growing microorganisms
Oven	For drying
Autoclave OT O12	For sterilization
Hiedolph evaporator	For evaporation
Nexera model Shimadzu UHPLC	Identification of phenolic compound

3.2. Methods

3.2.1. Sample preparation

The *Salix aegyptiaca* L. roots were dried at room temperature. The samples were crushed to form powder using steel blender. The powder sample was sieved to different mesh, and stored in a refrigerator until analysis.

3.2.2. Extraction methods

3.2.2.1. Conventional extraction

For conventional extraction, 8 g of powder sample and 80 ml of water, ethanol (95%) and methanol (95%) were mixed in a round bottom flask separately. The ratio of plant material mass (g) to solvent volume (ml) was 1:10. Additional, stirring was applied. The extraction was performed at 40 °C for 3 h. At the end of the extraction cycle, the liquid extract was separated from the solid residue by filtration. Finally, filtrates were collected and evaporated in a rotary vacum evaporator (Dhanani et al., 2013).

3.2.2.2. Microwave extraction

This assay was performed by Microwave milestone NEOS system. Eight gram of sample was mixed with 80 ml of water, ethanol (95%) and methanol (95%) separately in beakers. Additionally, magnetic stirrer bar was applied. Extraction was carried out for 25 min and temperatures were standard for solvents and different powers were used. Finally, the liquid was separated from the solid residue by filtration. Then the liquid extracts were evaporated by using rotary vacum evaporator (Laghari et al., 2011).

3.2.2.3. Accelerated solvent extraction (ASE)

Dried root samples (8 g) were put in a specific flask. The extraction was performed by Dionex ASE 350 under the following conditions: 60 ℃ for 40 min and pressure. At the end of extraction, the liquid was evaporated by using rotary vacum evaporator (Comlekcioglu et al., 2013).

3.2.3. Yield determination

The extraction yield is a measure of solvent efficiency to extract specific components from the original material and it was defined as the amount of extract recovered in mass compared with the initial amount of dry sample (Zhang et al., 2007).

Powder roots (0.5 g) were extracted with 50 ml water, ethanol and methanol separately by using varies extraction methods described in 3.2.2. The yield percentage of the extract was determined by using the following formula:

$$\text{Yield percentage } (\%) = \frac{X}{Y} \times 100 \quad (3.1)$$

where,

X is the oven dry weight of extract (g),

Y is the oven dry weight of the sample (g).

3.2.4. Determination of total condensed tannin

This assay was carried out by Shimadzu UV-vis spectrophotometer as shown in Figure 3.1. Extraction solution was prepared by mixing 0.05 g of Fe_2SO_4, 95 ml *N*-butanol

and 5 ml HCl (35%). For determining the condensed tannin, 0.01 g of both *Salix aegyptiaca* L. roots and mimosa tannin put separately in a test tube and 10 ml of extraction solution was added and placed in water bath for heating 1 h. The absorbance was measured at 580 nm wavelength (Makkar et al., 1995).

Figure 3.1. Shimadzu UV-vis spectrophotometer

3.2.5. Identification and quantification of the compounds in methanol extract of *Salix aegyptiaca* L. roots by LC–MS/MS

3.2.5.1. Instruments and chromatographic conditions for LC–MS/MS

LC–MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled with a tandem MS instrument. The liquid chromatography was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO- 10Asvp column oven and SIL-30AC auto sampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3 µm) analytical column. The column temperature was fixed at 40ºC. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and Mobile Phase B (methanol, 5 mM ammonium formate and 0.1% formic acid). The solvent

flow rate was maintained at 0.5 ml/min and injection volume was settled as 4 µl (Ertas et al., 2015).

3.2.5.2. MS instrumentation

MS detection was carried out using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in negative ionization modes. LC–MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) modes were used to quantify the analyses.

3.2.5.3. Method validation parameters for LC–MS/MS

In this study, 27 compounds were quantified in *Salix aegyptiaca* L. Rectilinear regression equations and the linearity ranges of the studied standard compounds were given in Table 3.3 (Ertas et al., 2014a). Correlation coefficients were found to be higher than 0.99. The limits of detection (LOD) and quantitation (LOQ) of reported analytical method were presented in Table 3.3. For the studied compounds, LOD ranged from 0.05 to 25.8 µg/L and LOQ ranged from 0.17 to 85.9 µg/L. Moreover, the recoveries of the phenolic compounds ranged from 96.9% to 106.2%. The results were calculated by the equation below:

$$\text{Quantification of compound } (\mu g/g) = \frac{R x U^f}{100} \quad (3.2)$$

where,

R is the result from LC–MS/MS (µg),

U^f is the percent relative uncertainty at 95% confidence level (%).

Table 3.3. Analytical parameters of LC–MS/MS method

No	Analyses	RT[a]	Parent ion (m/z)[b]	Ionization Mode	R^{2c}	RSD %[d]	Linearity Range (mg/L)	LOD/LOQ (µg/L)[e]	Recovery (%)	U[f]
1	Quinic acid	3.32	190.95	Neg	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8
2	Malic acid	3.54	133.05	Neg	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3
3	tr-Aconitic acid	4.13	172.85	Neg	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9
4	Gallic acid	4.29	169.05	Neg	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1
5	Chlorogenic acid	5.43	353	Neg	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9
6	Protocatechuic acid	5.63	152.95	Neg	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1
7	Tannic acid	6.46	182.95	Neg	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1
8	tr- caffeic acid	7.37	178.95	Neg	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2
9	Vanillin	8.77	151.05	Neg	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9
10	p-Coumaric acid	9.53	162.95	Neg	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1
11	Rosmarinic acid	9.57	358.9	Neg	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9
12	Rutin	10.18	609.1	Neg	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0
13	Hesperidin	9.69	611.1	Poz	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9
14	Hyperoside	10.43	463.1	Neg	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9
15	4-OH Benzoic acid	11.72	136.95	Neg	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2
16	Salicylic acid	11.72	136.95	Neg	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0
17	Myricetin	11.94	317	Neg	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9
18	Fisetin	12.61	284.95	Neg	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5
19	Coumarin	12.52	146.95	Poz	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9
20	Quercetin	14.48	300.9	Neg	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1
21	Naringenin	14.66	270.95	Neg	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5
22	Hesperetin	15.29	300.95	Neg	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3
23	Luteolin	15.43	284.95	Neg	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9
24	Kaempferol	15.43	284.95	Neg	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2
25	Apigenin	17.31	268.95	Neg	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3
26	Rhamnetin	18.94	314.95	Neg	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1
27	Chrysin	21.18	253	Neg	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3

Note: RT[a]: Retention time, parent ion (*m/z*) [b]: Molecular ions of the standard compounds (mass to charge ratio), R^{2c}: coefficient of determination, RSD[d]: relative standard deviation, LOD/LOQ (µg/L) [e]: Limit of detection/Limit of quantification, U[f] (%): Percent relative uncertainty at 95% confidence level.

3.2.6. DPPH radical scavenging activity

The free radical scavenging activity of ethanol and methanol extracts of the *Salix aegyptiaca* L.roots was determined by the DPPH assay described by Blois (1958) with slight modification. In its radical form, 1,1-diphenyl-2-picrylhydrazyl (DPPH) absorption level decreases at 517 nm with reduction of an antioxidant or a radical specie. Briefly, 0.1 mM DPPH was prepared for ethanol and methanol extractions. Then 0.1, 0.2 and 0.3 ml of sample solutions mixed with methanol and ethanol up to 3 ml in a test tube, separately. Then, 1 ml of DPPH was added. The mixture was then shaken vigorously and placed in a dark room temperature for 30 min. Later, the absorbance was measured by Shimadzu UV-vis 1240 spectrophotometer at 517 nm. Butylated hydroxyltoluene (BHT) was used as a reference. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and calculated with the following equation (Gülçın et al., 2003):

$$\text{Inhibition of DPPH radical scavenging activity (\%)} = \frac{A\text{-}B}{A} \times 100 \qquad (3.3)$$

where,

A is the absorbance of DPPH,

B is the absorbance in the presence of sample and BHT.

3.2.7. Antimicrobial activity

3.2.7.1. Microorganisms

Seven bacteria and four fungi were used in the study. All the microorganisms were obtained from microbiology laboratory at Biology Department of Kahramanmaraş Sütçü İmam University, Turkey. The names, types and source of tested organisms are listed in Table 3.4.

3.2.7.2. Disc diffusion assay

Antimicrobial activity was carried out using 7 bacteria and 4 fungi (Table 3.4). Ethanol, methanol and water activities of *Salix aegyptiaca* L. root extracts were tested separately against all microorganisms using disc diffusion method (CLSI, 2012). The Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) were sterilized by autoclaving at 121 ºC for 15 min. The medium was transferred aseptically into each

sterilized petri plate. Discs (10 mm in diameter) were impregnated with 100 µl of plant extracts and allowed to dry. Discs were then placed on MHA medium, which is inoculated with 10 µl of bacteria. The SDA medium was inoculated with 20 µl of fungi suspension. Methanol, ethanol and water were used as negative controls. Ampicillin and gentamicin (10 µg/ml) were used as positive controls for antibacterial activity (6 mm in diameter). In addition, 100 µl of Nystatin unit/disc (10 mm in diameter) was used to test antifungal activity. Plates were incubated at 37 ºC for 24 h and 25-27 ºC for 48 h for each test, respectively. Antimicrobial activities were evaluated by measuring the inhibition zones against all tested bacteria and fungi. All tests were performed in triplicate

Table 3.4. List of microorganisms tested in this study

Organism code	**Organisms**	**Type**	**Source**
B 2	*Bacillus megaterium*	G+ bacteria	DSM32
B 11	*Klebsiella pneumoniae*	G- bacteria	FMC5
B 13	*Escherichia coli*	G- bacteria	DM
B 19	*Pseudomonas aeruginosa*	G- bacteria	DSM50071
B 20	*Staphylococcus aureus*	G+ bacteria	Cowan1
B 25	*Micrococcus luteus*	G+ bacteria	LA2971
B 29	*Bacillus subtilis*	G+ bacteria	IMG22
M 1	*Candida albicans*	Fungi	ATCC1023
M 2	*Candida utilis*	Fungi	NRRL-Y-900
M 3	*Saccharomyces cerevisiae*	Fungi	WET136
M 4	*Yarrowia lipolytica*	Fungi	NCIM3589

3.3. Statistical Analyses

The data for antimicrobial activity were analysed with ANOVA using SPSS (version 18) statistical program. The mean differences were compared as significant ($P < 0.05$) or non-significant ($P > 0.05$).

4. RESULTS AND DISCUSSION

4.1. Yield Determination

The yield percentage of the *Salix aegyptiaca* L. root extracts prepared by conventional, microwave and accelerated solvent extraction (ASE) methods by using water, ethanol and methanol is summarized in Table 4.1.

Table 4.1. Yield percentage in the root extracts of *Salix aegyptiaca* L.

Method	Solvent	Yield (%)	
		Mean[1]	SD[2]
Conventional extraction	W	9.10	±0.1
	E	10.56	±0.15
	M	11.66	±0.57
Microwave extraction	W	10.40	±0.1
	E	12.03	±0.57
	M	12.56	±0.15
Accelerated solvent extraction	E	15.0	±0.1
	M	17.16	±0.15

Note: W: water, E: ethanol, M: methanol
[1]Values presented as mean ± SD of three measurements
[2]SD: Standard deviation

The highest extraction yield was found in M with ASE technique (17.3%). While, the lowest extraction yield (9.2%) was observed in W with conventional extraction method (Table 4.1). The results were agreed with the previous study reported by Anokwuru et al. (2011) who found the highest yield with methanolic extract (17.23%). Among the extraction methods, the extracts obtained by ASE method from *S. aegyptiaca* L. roots contained the highest extracts compared to other extraction methods given in Figure 4.1. The yield with preserved extracts is an indication of the fact that this method can be adapted as a standard method for extract preparation.

The differences in extract yields from the plant material in the analysis might be due to availability of extractable components, resulting from the varied chemical compositions and polarity of the solvents (Sultana et al., 2009)

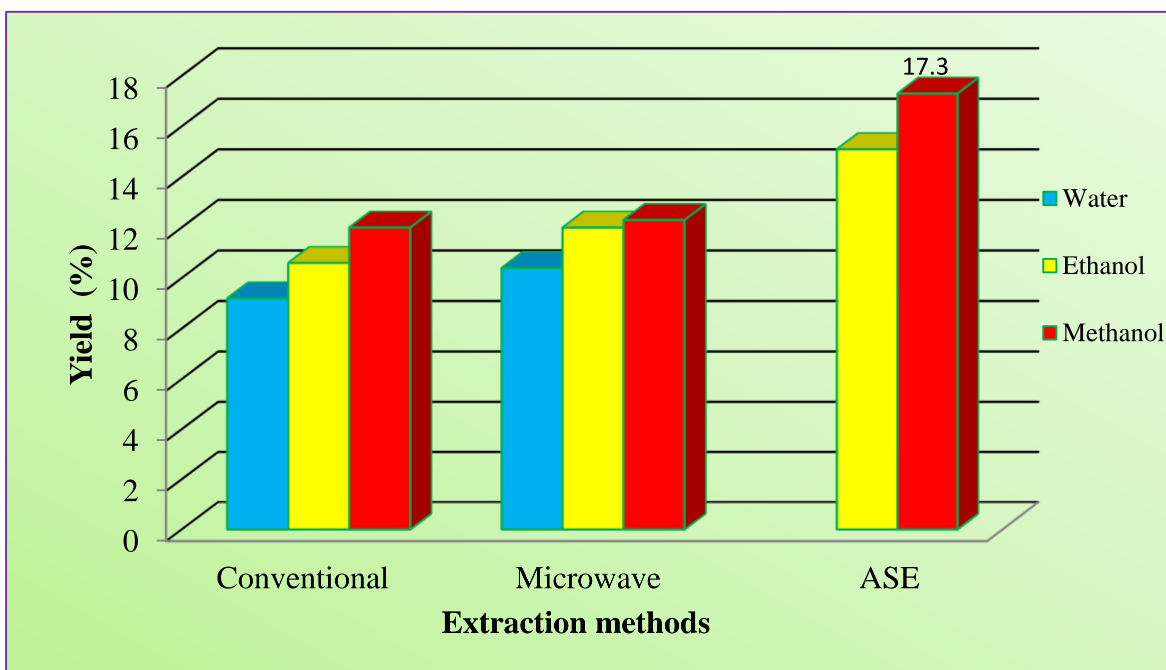

Figure 4.1. The highest extraction yield (%)

Note: ASE: Accelerated solvent extraction

4.2. Total Condensed Tannin

Total condensed tannin concentration of the *Salix aegyptiaca* L roots is presented in Table 4.2.

The tannin concentration was calculated by using standard calibration curve (R^2= 0.999) with concentration ranged from 6.25 mg/L to 50 mg/L detailed in Table 4.3 and Figure 4.2.

Table 4.2. Total condensed tannin concentration in roots of *Salix aegyptiaca* L. roots

Salix aegyptiaca L.	Condensed Tannin (mg/L)			Average	Standard deviation	Variation (V)
	35.32	34.85	35.25	35.14	±0.25	0.71

The average total condensed tannin concentration from triplicate measurement was 35.14 mg/L. Tannins are water-soluble antioxidant with molecular weight of 500-3000 g/mol. Tannins are natural polyphenols ubiquitously distributed in plants, such as vegetables, fruits and seeds. Tannins are widely used in wine industry for color stabilizer; balancing the complexity in wines, inhibit certain enzymes in infected fruits and act as wine fining agents (Sanz et al., 2008). The presence of tannin in the current study was

proven by Bravo (1998) previously. The value in this study was lower than the study of Hayashia et al. (2005). They reported that the leaves of *Salix sachalinensis* contained high concentration of condensed tannin 8.2 ± 0.79 mg/g.

Table 4.3. Calibration standard of mimosa tannin

Mimosa tannin calibration	
Concentration (mg/L)	**Absorbance**
50	0.75
25	0.365
12.5	0.179
6.75	0.094

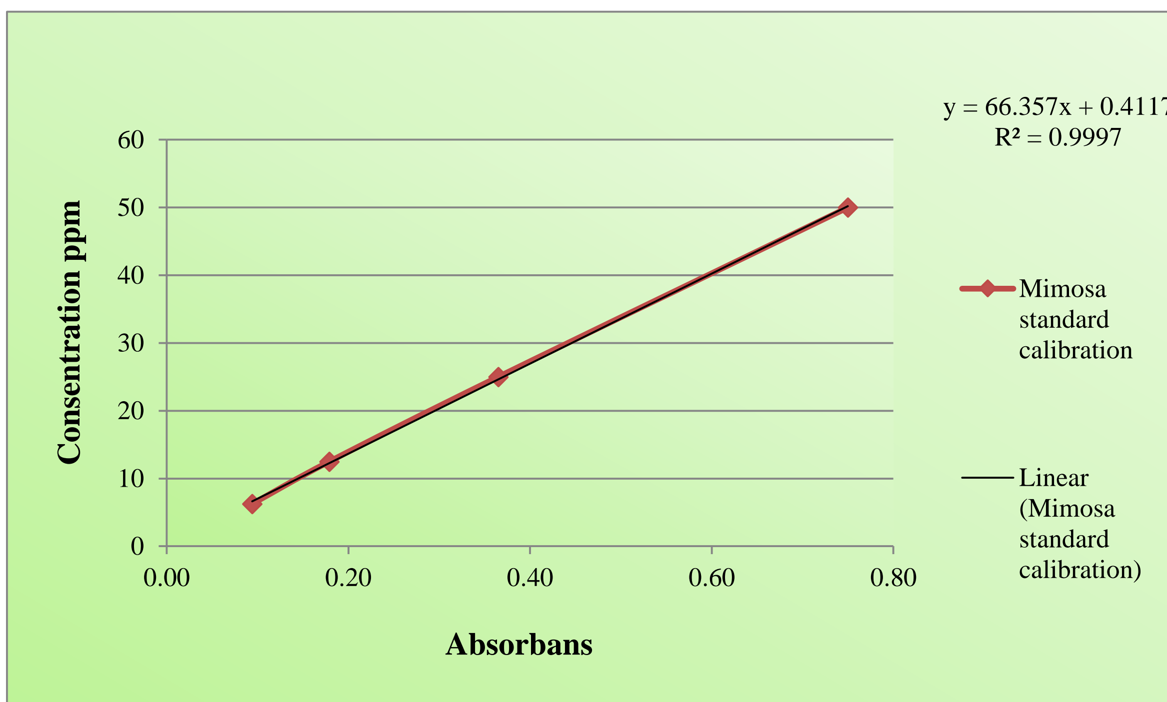

Figure 4.2. Mimosa tannin calibration curve

Tannins have antifungal activity (Otshudi et al., 2005). Their activity is possibly due to their capacity to join with extracellular and soluble proteins or combine with cell wall of fungi. The character of these compounds may also interrupt fungal membranes (Tsuchiya et al., 1996).

4.3. Identification of the Compounds in Methanol Extract of *Salix aegyptiaca* L. Roots by LC–MS/MS

In recent literature, there are several studies about LC–MS/MS based quantitative analysis (Cavaliere et al. 2005; Ertas et al. 2014b). Further, numerous studies have provided evidence for fundamental role of phenolic glycosides with the interactions of plant species of the Salicaceae family against their natural herbivore enemies. Hence, an accurate quantitative LC-MS/MS method was prepared for analysis of 27 compounds (3 non phenolic acids, 10 phenolic acids and 14 flavanoids) in the root methanol extracts. In the present study, negative ionization modes were used for analyzing the compounds. The LOD/LOQ of the phenolic compounds and the quantified results for methanol extracts of the *Salix aegyptiaca* L. root are presented in Table 4.4.

Overall, the results are indicated that non-phenolic compounds are rich in methanol extract of *Salix aegyptiaca* L. roots by LC–MS/MS analysis. It contained high amount of quinic acid (63894.76 µg/g) as presented in Table 4.4 and Figure 4.3. This was somewhat disagree with previous studies on *Salix spp*. Many authors reported that the main component of *Salix aegyptiaca* L. and other *Salix* species was salicylic acid (Paterson and Lawrence, 2001; Yu et al., 2002; Mahdi et al., 2006; Karawya et al., 2010; Rabbani et al., 2010 Cooper and Nicola, 2014) and 1,4- Dimethoxybenzene (Karimi et al., 2011). On the other hand, the results were in agreement with the study of Zhang et al. (2014). In addition, the results showed higher amounts of malic and tr-aconitic acids (Table 4.4 and Figure 4.3). The disparity in the results might be due to the time of collection, part of plant sections, origin, extraction methods and solvents used in the study. Huang et al. (2005) who reported that the presence of phenolics and flavonoids is affected by the type of plant parts, maturity at harvest, growing conditions and soil conditions.

In the terms of phenolic acids, a high amount of tannic acid was found in the roots of plant 554.682 µg/g. While, a low amount of tr-caffeic acid was detected 8.695 µg/g. On the other hand, there was no rosmarinic acid in the methanol extract of *Salix aegyptiaca* L roots (Table 4.4 and Figure 4.4). These results are supported by the studies of Bravo (1998), Enayat and Banerjee (2009), Sonboli et al. (2010) and Enayat et al. (2013). Differences in compound analysis between this study and previous studies might be due to the various analytical methods and solvents used in present work. Moreover, a moderate amount of salicylic acid 204.889 µg/g was detected in the methanol extract. The result was

similar to that for ethanol extract catkin 0.2 ± 0.002 mg/g (Enayat and Banerjee, 2009; Karawya et al., 2010; Enayat et al., 2013). While, Enayat and Banerjee (2009) and Durak et al. (2014) studied butanol and water extract of catkin. The dissimilarities between the results might be referring to the solvents used in previous studies.

Table 4.4. Quantification of methanol extracted compounds of *Salix aegyptiaca* L. roots by LC–MS/MS

NO	Compounds	RT[a]	Parent ion(m/z)[b]	LOD/LOQ (µg/L)[e]	U[f]	Quantification µg/g extract
1	Quinic acid	3.32	190.95	22.3 / 74.5	4.8	63894.761
2	Malic acid	3.54	133.05	19.2 / 64.1	5.3	38019.793
3	tr-Aconitic acid	4.13	172.85	15.6 / 51.9	4.9	815.661
4	Gallic acid	4.29	169.05	4.8 / 15.9	5.1	183.914
5	Chlorogenic acid	5.43	353	7.3 / 24.3	4.9	29.938
6	Protocatechuic acid	5.63	152.95	25.8 / 85.9	5.1	286.963
7	Tannic acid	6.46	182.95	10.2 / 34.2	5.1	554.682
8	tr- caffeic acid	7.37	178.95	4.4 / 14.7	5.2	8.695
9	Vanillin	8.77	151.05	10.1 / 33.7	4.9	440.992
10	p-Coumaric acid	9.53	162.95	15.2 / 50.8	5.1	220.868
11	Rosmarinic acid	9.57	358.9	10.4 / 34.8	4.9	N.D
12	Rutin	10.18	609.1	17.0 / 56.6	5.0	52.374
13	Hesperidin	9.69	611.1	21.6 / 71.9	4.9	67.949
14	Hyperoside	10.43	463.1	12.4 / 41.4	4.9	275.275
15	4-OH Benzoic acid	11.72	136.95	3.0 / 10.0	5.2	201.260
16	Salicylic acid	11.72	136.95	4 / 13.3	5.0	204.889
17	Myricetin	11.94	317	9.9 / 32.9	5.9	28.393
18	Fisetin	12.61	284.95	10.7 / 35.6	5.5	4.830
19	Coumarin	12.52	146.95	9.1 / 30.4	4.9	0.626
20	Quercetin	14.48	300.9	2.0 / 6.8	7.1	18.964
21	Naringenin	14.66	270.95	2.6 / 8.8	5.5	105.198
22	Hesperetin	15.29	300.95	3.3/ 11.0	5.3	0.832
23	Luteolin	15.43	284.95	5.8 / 19.4	6.9	4.385
24	Kaempferol	15.43	284.95	2.0 / 6.6	5.2	8.588
25	Apigenin	17.31	268.95	0.1 / 0.3	5.3	1.120
26	Rhamnetin	18.94	314.95	0.2 / 0.7	6.1	0.681
27	Chrysin	21.18	253	0.05 / 0.17	5.3	N.D

Note: RT[a]: Retention time, parent ion *(m/z)* [b]: Molecular ions of the standard compounds (mass to charge ratio), R^{2c}: coefficient of determination, RSD[d]: relative standard deviation, LOD/LOQ (µg/L) [e]: Limit of detection/Limit of quantification, U [f] (%): Percent relative uncertainty at 95% confidence level, Values in µg/g (w/w) [g] of plant methanol extract, N.D: not detected.

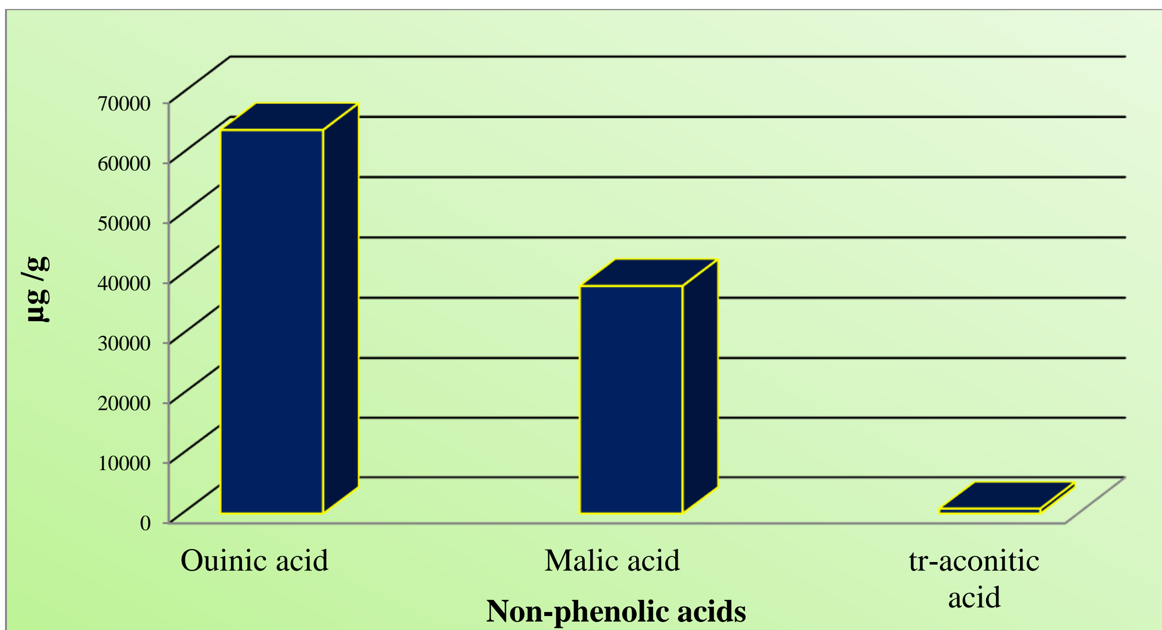

Figure 4.3. Non-phenolic acids in roots of *Salix aegyptiaca* L.

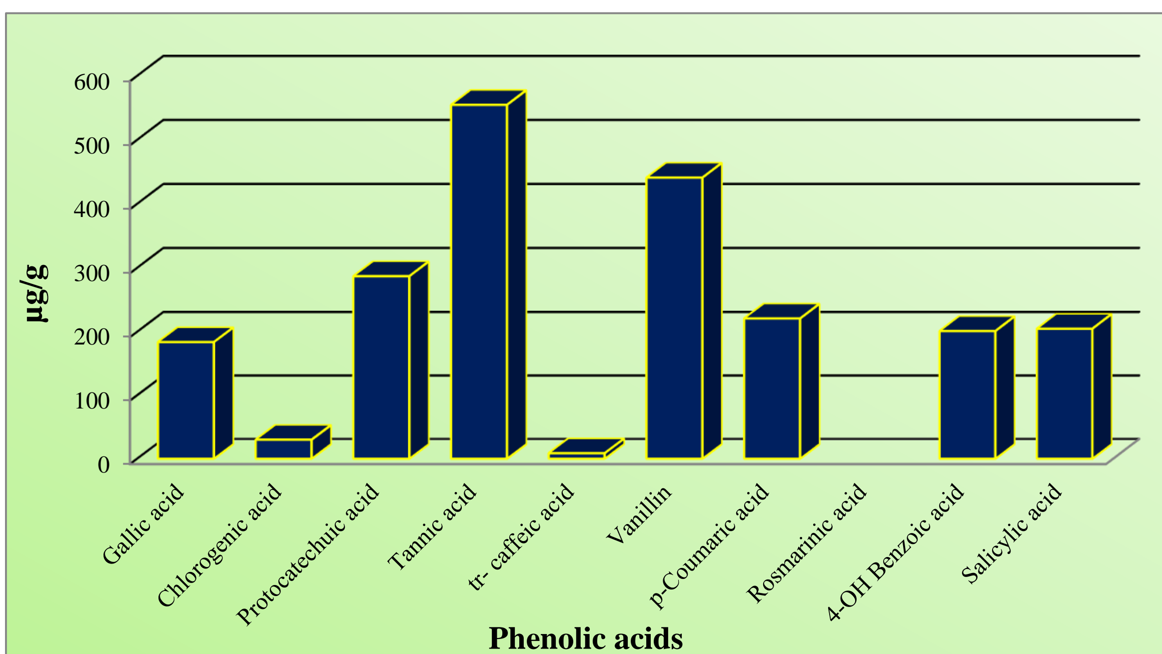

Figure 4.4. Phenolic acids in roots of *Salix aegyptiaca* L.

The flavanoids were examined by LC–MS/MS. The highest and the lowest compounds were hyperoside 275.27 µg/g and coumarin 0.626 µg/g. Whereas, chrysin was no found in the *Salix aegypyiaca* L. roots shown in Table 4.3 and Figure 4.6. The results and presences of these compounds are in agreement with other studies (Qin and Sun, 2005; Nahrstedt et al., 2007; Enayat and Banerjee, 2009; Enayat et al., 2013).

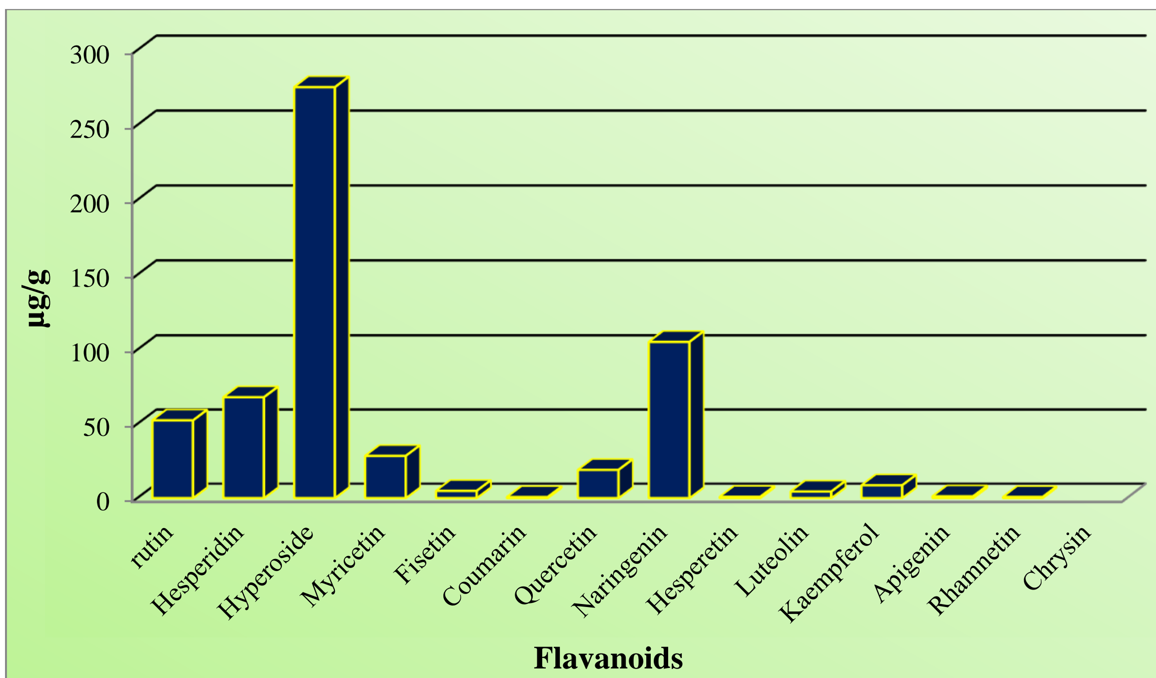

Figure 4.5. Flavanoids in roots of *Salix aegyptiaca* L.

4.4. DPPH Radical Scavenging Activity

The free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) has a characteristic absorption at 517 nm (purple in color) and decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron donation. A lower absorbance at 517 nm indicates a higher radical scavenging activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical scavenging ability of specific compounds or extracts (Amarowicz et al., 2004). The radical scavenging activity of the *Salix aegyptiaca* L. root extracts with various extraction methods and BHT results are presented in Table 4.5.

The highest DPPH scavenging activity (98.8%) was determined in M extract with accelerated solvent extraction (ASE) method at 0.1 µl concentration (0.0264 mg/ml). The lowest DPPH scavenging activity (97.52%) was observed in E extract with microwave extraction method at 0.3 µl concentration 0.0162 mg/ml. As shown in Table 4.5, the DPPH radical scavenging activity of whole extracts were higher than that were obtained by butylated hydroxyltoluene (BHT) at 53 mg/L concentration.

The mechanism of radical scavenging activity of *Salix aegyptiaca* L. could be attributed to the presence of polyphenolic compounds. It has already been exhibited that

the polyphenolic compounds are responsible for radical scavenging activity due to their hydrogen atom donation to active free radicals (Ho et al., 1994). Phenols are very important plant constituents because of their scavenging (Hatano et al., 1989) or antioxidative abilities (Duh et al., 1999).

Table 4.5. DPPH scavenging activities in root extracts of *Salix aegyptiaca* L.

Methods	**Solvents**[1]	**DPPH Radical Scavenging activity (%)**					
		Extract volume (ml)			**BHT volume (ml)**[2]		
		0.1 ml	0.2 ml	0.3 ml	0.1 ml	0.2 ml	0.3 ml
Conventional[3]	M	98.49	97.93	97.53	90.4	91.9	90.0
Microwave[4]	M	98.73	98.33	98.49	90.4	91.9	90.0
ASE[5]	M	98.80	98.33	97.61	90.4	91.9	90.0
Conventional[6]	E	98.51	98.23	97.53	67.4	65.6	78.3
Microwave[7]	E	98.41	97.86	97.52	67.4	65.6	78.3
ASE[8]	E	98.51	98.51	98.04	67.4	65.6	78.3

[1]M: Methanol; E: ethanol; ASE: Accelerated solvent extraction; [2]53 mg/L; [3]0.0183 mg/ml; [4]0.0188 mg/ml; [5]0.0264 mg/ml; [6]0.0162 mg/ml; [7]0.0183 mg/ml; [8]0.0231 mg/ml

The scavenging effects of *Salix aegyptiaca* L. roots in E and M extracts with DPPH radicals increased with increasing the concentrations separately. This was also observed by Sulaiman et al. (2013). The results indicated that DPPH radical scavenging at 0.2 µl was more than 0.3 µl but less than 0.1 µl in M extract with microwave extraction. This was agreed with previous studies done by Enayat and Banerjee (2009) and Sonboli et al. (2010). With this result, the root extracts exhibited antioxidant potential as supported by (Sonboli et al., 2010).

The results suggested that high levels of antioxidant activity may be due to the presence of phenolic components. The significant antioxidant activity of methanol and ethanol extracts of the *Salix aegyptiaca* L. root might be due to presence of high amounts of various phenolic compounds. These can be listed quinic acid (Farah and Donangelo, 2006; Gorzalczany et al., 2008; Zhang et al., 2013), gallic acid (Li et al., 2000; Shabir et al., 2011), malic acid (Mokbel and Hashinaga, 2005), chlorogenic acid (Zheng and Wang, 2001; Shabir et al., 2011), caffeic and salicylic acids (Proestos et al., 2005).

4.5. Antimicrobial Activity of the *Salix aegyptiaca* L. Root Extracts

Many plants contain microbial inhibitors (Voravuthinkuchai, 2006). Antimicrobial properties are useful tools for controlling microorganisms in infection treatments and food spoilage (Abaoba et al, 2004). There have been many studies on the antimicrobial activities of plant extracts and their biological properties. In our experiment, disc diffusion method was used to assess the antimicrobial effects of ethanol, methanol and water extracts of *Salix aegyptiaca* L. roots. Disc concentration of 25 mg/disc was tested against 11 microorganisms including 3 Gram negative, 4 Gram positive and 4 fungi (Table 4.6). In addition, the effects of synthetic antibiotic activities and solvent activities against the microorganisms are summarized in Table 4.7 and 4.8, respectively. This study is the first report for antimicrobial activity of the root extracts.

The various extract of the of *Salix aegyptiaca* L. they were statically significant by the differnet diameters of inhibition zones ($P < 0.05$) of all the activities against the organisms tested were given in Table 4.6. The mean inhibition zones of *Salix aegyptiaca* L. against all test bacteria ranged from 11-24 mm and for fungi 11.6-25.6 mm. The highest inhibitory zone 25.6 mm was against *Candida albicans* ATCC1023 obtained in methanol extract with ASE method at 25 mg/disc. While, the lowest inhibitory zone 11 mm was against *Staphylococcus aureus* Cowan1 in water extract with conventional extraction method at the same concentration. Furthermore, there was no inhibitory zone against *Candida utilis* NRRL-Y-900 and *Saccharomyces cerevisiae* WET136 (Table 4.6).

Table 4.6. Inhibition zones (mm) of the root extracts against the microorganisms

Organisms		Inhibition zone (mm)[1]							
		Conventional extraction			Microwave extraction			ASE	
		Water	Ethanol	Methanol	Water	Ethanol	Methanol	Ethanol	Methanol
B2	Mean	11.6	17.6	18.6	–	16.3	17.0	18.6	24.0
	SD	±0.57	±0.57	±0.57	–	±0.57	±1.0	±0.57	±1.0
B11	Mean	–	15.0	14.6	11.3	15.3	15.3	16.3	17.3
	SD	–	±1.0	±0.57	±0.57	±0.57	±0.57	±1.15	±1.15
B13	Mean	–	14.3	17.0	–	13.6	13.0	16.0	18.6
	SD	–	±0.57	±1.0	–	±1.52	±1.0	±1.0	±0.57
B19	Mean	–	17.0	16.0	–	13.6	15.3	18.0	17.3
	SD	–	±1.0	±1.0	–	±0.57	±1.15	±1.0	±0.57
B20	Mean	11	15.6	14.6	–	14.0	17.3	14.6	19.3
	SD	±0.0	±0.57	±0.57	–	±1.0	±0.57	±0.57	±1.52
B25	Mean	–	18.3	16.0	12.0	14.3	11.6	19.3	21.0
	SD	–	±0.57	±1.0	±1.0	±0.57	±0.57	±0.57	±1.73
B29	Mean	13.0	16.3	19.3	–	14.6	15.0	19.0	22.3
	SD	±0.0	±0.57	±0.57	–	±0.57	±1.0	±1.73	±1.52
M1	Mean	15.3	23.3	23.3	17.6	20.3	23.3	24.3	25.6
	SD	±0.57	±1.52	±0.57	±0.57	±0.57	±1.52	±1.52	±4.04
M2	Mean	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–
M3	Mean	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–
M4	Mean	–	13.6	11.6	12.6	11.6	14.3	16.6	19.6
	SD	–	±0.57	±0.57	±0.57	±0.57	±0.57	±0.57	±0.57

Note:*B2: Bacillus megaterium; B11: Klebsiella pneumoniae; B13: Escherichia coli; B19: Pseudomonas aeruginosa; B20: Staphylococcus aureus; B25: Micrococcus luteus; B29: Bacillus subtilis; M1: Candida albicans; M2: Candida utilis; M3: Saccharomyces cerevisiae; M4: Yarrowia lipolytica*; ASE: Accelerated solvent extraction**;** (–): no inhibition zone

[1]The values presented as mean ± SD of three replication; Mean differences were statistically classified as significant (P< 0.05). P- value (0.00)

Table 4.7. Synthetic antibiotic activities against the microorganisms

	Inhibition zone (mm)[1]		
Organism	**Ampicillin 10µg/ml**	**Gentamicin 10µg/ml**	**Nystatin /units**
B2	6.6±0.57	34.6±0.57	
B11	–	44.0±2.0	
B13	–	34.3±1.52	
B19	6.0±0.0	34.3±1.15	
B20	6.3±0.57	36.3±1.52	
B25	9.3±0.57	34.6±1.15	
B29	6.3±0.57	38.6±1.15	
M1			14.6±0.57
M2			17.6±0.57
M3			13.6±0.57
M4			13.0±0.0

Note: B2: *Bacillus megaterium*; *B11*: *Klebsiella pneumoniae*; *B13*: *Escherichia coli*; *B19*: *Pseudomonas aeruginosa*; *B20*: *Staphylococcus aureus*; *B25*: *Micrococcusluteus*; *B29*: *Bacillus subtilis*; *M1*: *Candida albicans*; *M2*: *Candida utilis*; *M3*: *Saccharomyces cerevisiae*; *M4*: *Yarrowia lipolytica*; (–): no inhibition zone.
[1]The values presented as mean ± SD of three replication

Table 4.8. Solvent activities on the microorganisms

	Inhibition zone (mm)		
Organism	**Water**	**Ethanol**	**Methanol**
B2	–	–	–
B11	–	–	–
B13	–	–	–
B19	–	–	–
B20	–	–	–
B25	–	–	–
B29	–	–	–
M1	–	–	–
M2	–	–	–
M3	–	–	–
M4	–	–	–

Note: *B2*: *Bacillus megaterium*; *B11*: *Klebsiella pneumoniae*; *B13*: *Escherichia coli*; *B19*: *Pseudomonas aeruginosa*; *B20*: *Staphylococcus aureus*; *B25*: *Micrococcus luteus*; *B29*: *Bacillus subtilis*; *M1*: *Candida albicans*; *M2*: *Candida utilis*; *M3*: *Saccharomyces cerevisiae*; *M4*: *Yarrowia lipolytica*; (–): no inhibition zone

The results showed that the methanol extract of the roots by ASE method had a strong antimicrobial activity against *Bacillus megaterium* DSM32 (24 mm) zone of inhibition (Figure 4.6). These result is lower than that of Dığrak et al. (2001) but higher than that of Hussain et al. (2011). This dissimilarity might be due to the plant species, origin and plant parts which were used in this study. The inhibitory zones were obtained by conventional and microwave water extracts against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Micrococcus luteus* were also supported by previous studies (Bonjar, 2004a; Bonjar et al., 2004).

The results indicated that the *Micrococcus luteus* LA2971 was affected by all the extracts with zone inhibition ranging between 11.6 and 21 mm except for water extract with conventional extraction (Figure 4.6). This result was higher than that of Ateş and Erdoğrul (2003). In addition, the inhibition zones were higher in methanol and ethanol extracts with ASE and conventional extractions against *Bacillus subtilis* 16.0-22.3 mm. This result was higher than that of Ayepola and Adeniyi (2008). The difference in antibacterial activity of a plant extract might be attributable to age of plant, freshness of plant material, physical factors (temperature, water) and time of plant collection and extraction process. Angioni et al. (2006) who reported that the antimicrobial effect of plant extract varies from different researches carried out in varies regions. This may be due to many factors such as: the effect of climate, quality, quantity and composition of extracted product, different source of bacterial strains However, the inhibition zone results 13-15 mm in methanol and ethanol extracts with microwave extraction as well as in water extract with conventional extraction against *Bacillus subtilis* were similar to the previous study (Ayepola and Adeniyi, 2008).

The antibacterial activities of *Salix aegyptiaca* L. roots in methanol extracts with all extraction methods against *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* as shown in Figure 4.6, were similar to that of observed by Ali and Aboud (2010). Similarly, the results obtained in ethanol extracts with all extraction methods against *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus* were similar to the results of Hussein (2011) and Sulaiman et al. (2013). However, the results were higher than that of Al-Kadum et al. (2008). The variances between results might owe to solvents, plant species and plant sections used in this study.

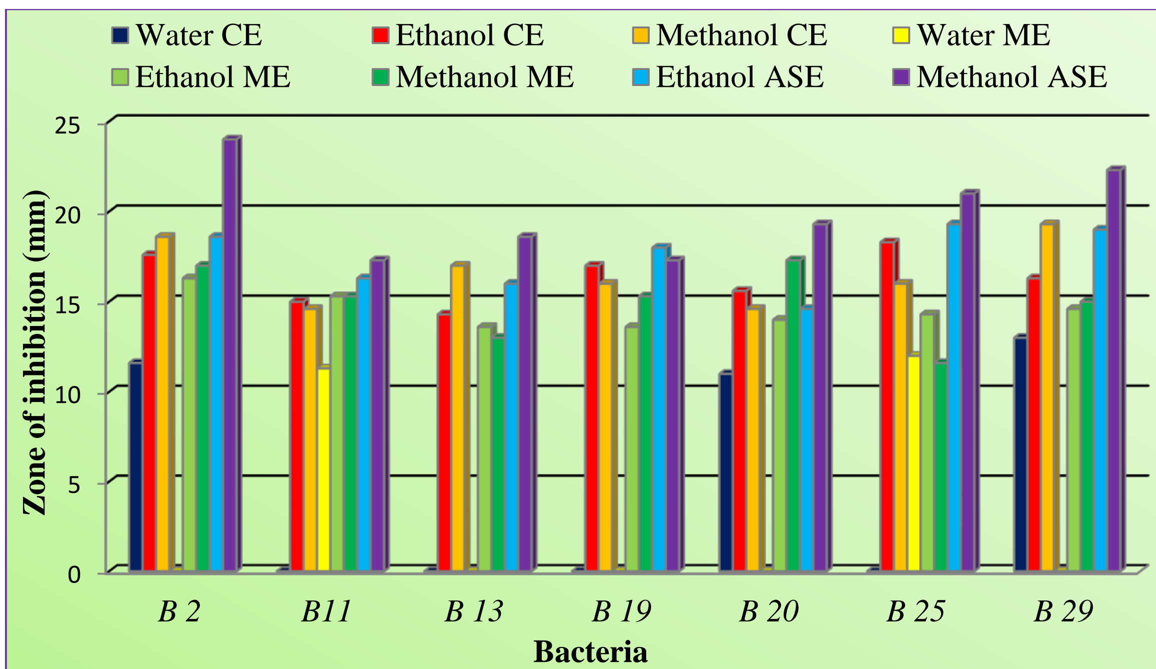

Figure 4.6. Antibacterial activity of *salix aegyptiaca* L. root extracts
Note: *B2*: *Bacillus megaterium* DSM32; *B11*: *Klebsiella pneumoniae* FMC5; *B13*: *Escherichia coli* DM; *B19*: *Pseudomonas aeruginosa* DSM50071; *B20*: *Staphylococcus aureus* Cowan1; *B25*: *Micrococcus luteus* LA2971; *B29*: *Bacillus subtilis* IMG22; CE: conventional extraction; ME: microwave extraction; ASE: Accelerated solvent extraction

The plant extractions with organic solvents provided strong antibacterial activity than water. As it can be seen in Table 4.6 and Figure 4.6, the methanol and ethanol extracts of the roots showed vigorous antibacterial activity against all bacterial strains. The different active compounds extracted with various solvents could explain these observations. Many studies confirmed that water is not apposite for extraction of antibacterials from medical plants compared to other solvents, such as methanol or ethanol (Karaman et al., 2003; Moniharapon and Hashinaga, 2004; Parekh et al., 2005). Among all results, it was observed that the G- bacteria were more resistant than G+ bacteria when exposed to plant extracts (Lin et al., 1999; Palombo and Semple, 2011). This may be attributed to the differences in cell wall structures of bacteria. G-ve bacteria are considered to be more resistant due to their outer membrane (Kaye et al., 2004).

The *Salix aegyptiaca* L. had a potent antibacterial activity against most of the bacteria when compared with Ampicillin 10 µg/ml (refer to Table 4.6.). While, there was a weak antibacterial activity compared to Gentamicin 10 µg/ml. The presence of antibacterial activity of this plant could be explained by the presence of higher amount of phenolic compounds in the roots. These compounds are quinic acid (Farah and Donangelo, 2006; Gorzalczany et al., 2008; Zhang et al., 2013), malic acid (Mokbel and Hashinaga,

2005), rutin and caffeic (Coneac et al., 2008), gallic acid (Akiyama et al., 2001; Panizzi et al., 2002), salicylic acid, chlorogenic acid, 4-hydroxybenzoic acid (Proestos et al., 2005; Shabir et al., 2011), protocatechuic acid and flavonoids (Kasim et al., 2011) and *p*-coumaric acids (Shabir et al., 2011).

The highest inhibition zone 25.6 mm was observed against *Candida albicans* determined by methanol extracts with ASE method (Figure 4.7). On the other hand, the lowest inhibition zone was recorded against *Yarrowia lipolytica* 11.6 mm which obtained by methanol and ethanol extracts for conventional and microwave extraction technique, respectively. Further, *Candida utilis* and *Saccharomyces cerevisiae* were found to be more resistant to the plant extracts. The antifungal activity of root extracts is illustrated in Figure 4.8. The activity of tannins is possibly due to their capacity of joining extracellular and soluble proteins or combining to cell wall of fungi. These compounds may also interrupt fungal membranes (Tsuchiya et al., 1996).

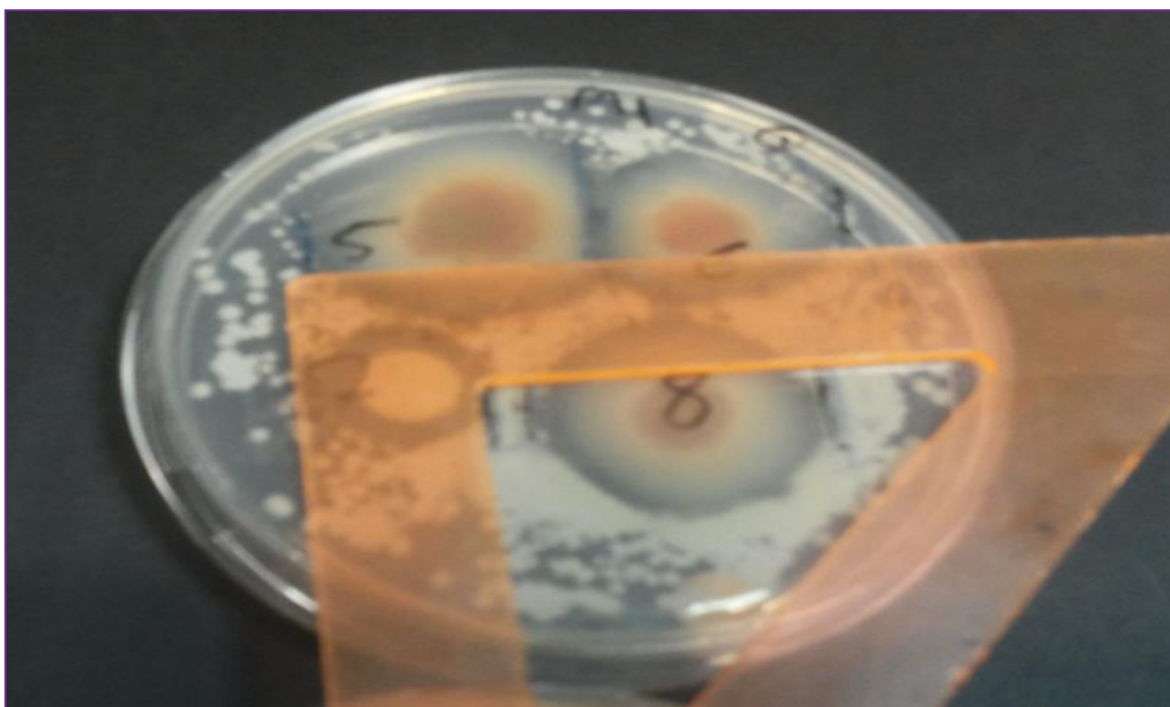

Figure 4.7. Inhibition zone (mm) against *Candida albicans* ATCC1023
Note: M1: *Candida albicans*; 5: Methanol extract for accelerated solvent extraction of *salix aegyptiaca* L. root

Antifungal activity results are summarized in Table 4.6. The *Salix aegyptiaca* L. roots showed a varying degree of antifungal activity. It was found that methanol extracts with ASE method obtained the highest inhibition zone against *C. albicans* (25.6 mm). This was incompatible with the study of Bonjar et al. (2004) where they reported no inhibition

against *C. albicans*. Dığrak et al. (2001) reported that *Laurus nobilis* extract was not effective against *C. albicans*. However, the antifungal activity of the extracts depends on plant species, type of extracts and concentrations.

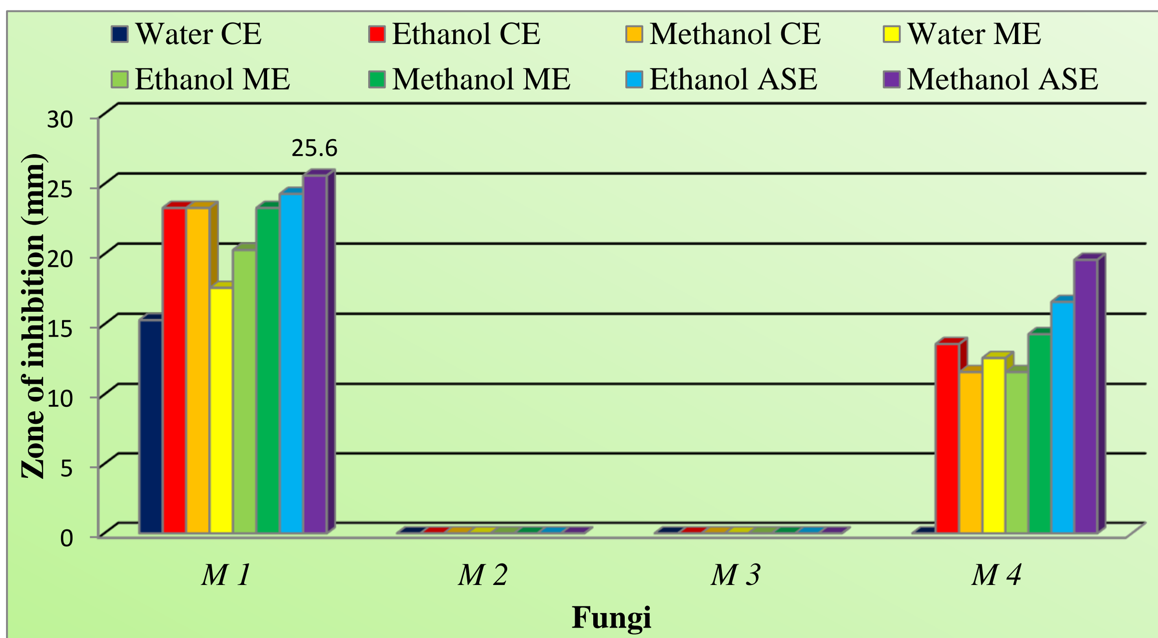

Figure 4.8. Antifungal activity of *Salix aegyptiaca* L. root extracts
Note: *M1*: *Candida albicans* ATCC1023; *M2*: *Candida utilis* NRRL-Y-900; *M3*: *Saccharomyces cerevisiae* WET136; *M4*: *Yarrowia lipolytica* NCIM3589; CE: conventional extraction; ME: microwave extraction; ASE: Accelerated solvent extraction

The methanol and ethanol extracts with all tested methods against *Candida albicans* showed inhibition zones ranging from 20.3 to 25.6 mm. This was in agreement with the studies of Aneja and Joshi (2009) and Sulaiman et al. (2013). They reported that the fruit extracts of *Amomum subulatum* showed an inhibition against *C. albicans* (29.3 mm). Similarly, Ertürk (2006) reported that *M. piperita* and *E. arborea* extracts showed an antifungal activity against *A. niger* and *C. albicans* 18-23 mm. The water extracts with conventional and microwave extractions showed a moderate antifungal activity against *C. albicans* and *Y. lipolytica* 12.6-17.6 mm. This result was somewhat similar to the results of Ertürk (2006) who reported that the extracts of *M. officinalis*, *P. nigrum*, C. *annum*, and *C. cyminum* showed antifungal activity against *C. albicans* with inhibition zones ranged from 10 to 16 mm. Our results also indicated that *Candida utilis* and *Saccharomyces cerevisiae* were more resistant to all extracts performed with various methods. Similarly, this result was also confirmed by Bonjar et al. (2004).

The antifungal activities of tested methanol and ethanol extracts against *C. albicans* and *Yarrowia lipolytica* were more potent than the standard antifungal nystatin. Whereas,

the results obtained by water extract were similar to synthetic drug nystatin (Figure 4.9). This may be attributed to the presence of flavonoids and phenolic compounds (Zidon et al., 2005) or may be due to the presence of quinic acid (Zhang et al., 2013) and gallic acids (Akiyama et al., 2001; Panizzi et al., 2002). Likewise, it may be due to the presence of caffeic, salicylic, chlorogenic and 4-hydroxybenzoic acids. All of the detected phenolic compounds are known to have antimicrobial properties (Proestos et al., 2005).

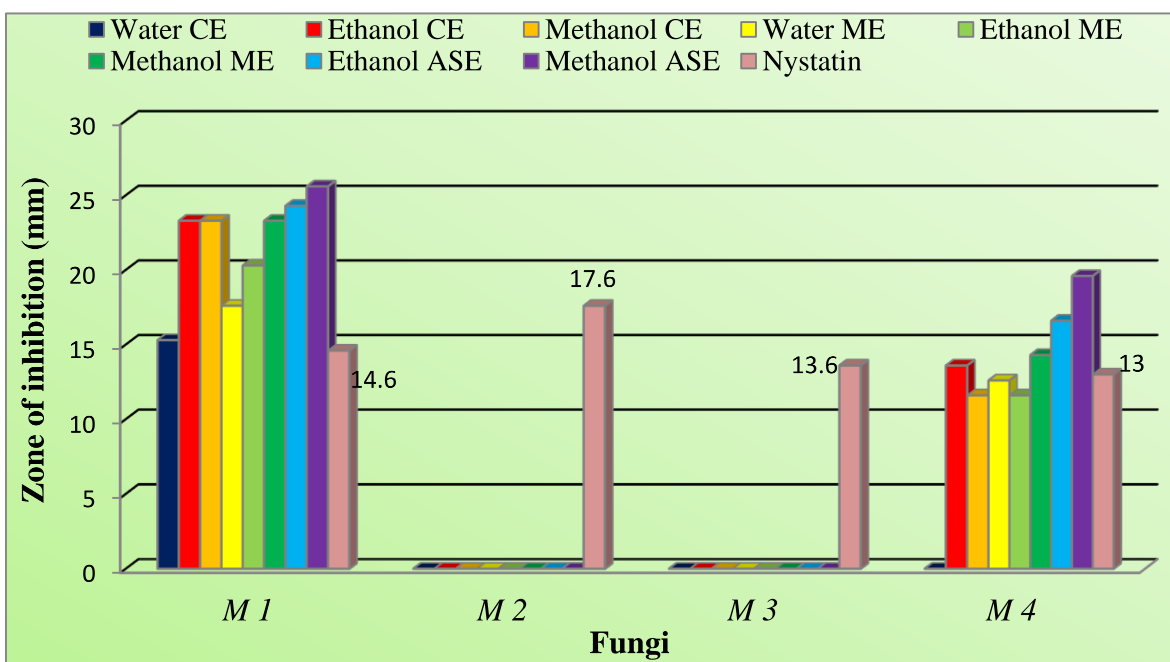

Figure 4.9. Antifungal activity of root extracts compared with nystatin
Note: *M1*: *Candida albicans* ATCC1023; *M2*: *Candida utilis* NRRL-Y-900; *M3*: *Saccharomyces cerevisiae* WET136; *M4*: *Yarrowia lipolytica* NCIM3589; CE: conventional extraction; ME: microwave extraction; ASE: Accelerated solvent extraction

5. CONCLUSIONS

The highest extraction yield was found in methanol extract with ASE technique (17.1%). Furthermore, total condensed tannin concentration was 35.14 mg/L.

This plant has a great medicinal value having versatile phytochemical constituents, such as non-phenolic, phenolic acid and flavanoids. Quinic acid was the most abundant polyphenol present in the methanol root extract 63894.761 µg/g. It is certain that the highest amount of quinic acid is reported for the first time. It can be concluded that the *Salix aegyptiaca* L. is a new source for quinic acid.

The antioxidant activity of *Salix aegyptiaca* L. root extracts has been detected by the means of free radical scavenging assays. The plants containing high phenols and flavonoids are an indication of antioxidant activity in their roots. Summing up the results, the highest DPPH scavenging activity was found in methanol extract obtained with ASE method (98.8%).

The antimicrobial activity of the extracts was evaluated based on the inhibition zones of disc diffusion assay. Among all the extracts, the methanolic fraction had a better antibacterial and antifungal activity against microorganism. The highest inhibition zone was against *Candida albicans* 25.6 mm. In addition, the highest antibacterial inhibition zone was against *Bacillus megaterium* 24.0 mm.

The *Salix aegyptiaca* L. root extracts obtained by the ASE method contained large amount of bioactive compounds (total phenols and flavonoids). They also showed strong antioxidant and antimicrobial activities. Thus, methanolic extracts of *Salix aegyptiaca* L. roots can be used as an accessible source of natural antioxidants and antimicrobials.

The isolated compounds found in this study could be useful for the development of new antimicrobial drugs. Therefore, the *Salix aegyptiaca* L. root is a promising plant material and could be researched further for its potential antioxidative and antimicrobial activities.

We can propose that additional cultivations of this plant species should take place. Other biological effects of this plant may be studied for preventing disease agents.

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APPENDIX

Figure A1. The extract yield of *Salix aegyptiaca* L. roots

Figure A2. Comparing the roots with standard Mimoza tannin

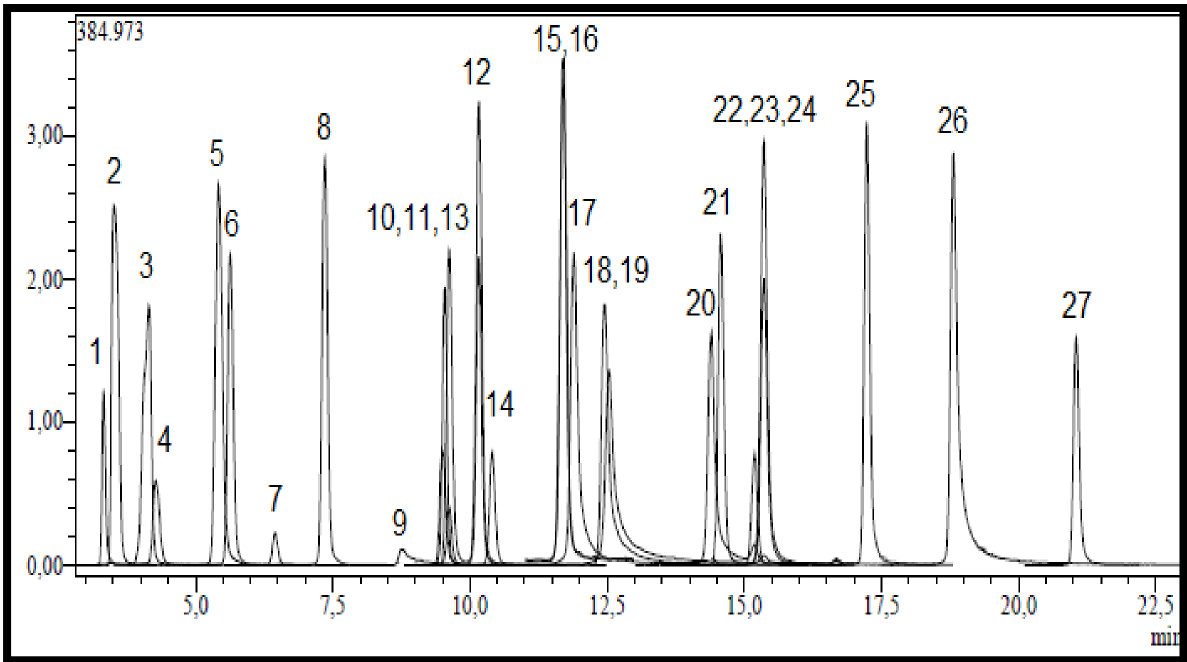

Figure A3. LC-MS/MS chromatogram of the standard calibration

Note: (1): Quinic acid; (2): Malic acid; (3): tr-Aconitic acid;(4): Gallic acid; (5): Chlorogenic acid; (6): Protocatechuic acid; (7): Tannic acid; (8): tr-caffeic acid; (9): Vanillin; (10): *p*-Coumaric acid; (11): Rosmarinic acid; (12): Rutin; (13): Hesperidin; (14): Hyperoside; (15): 4-OH Benzoic acid; (16): Salicylic acid; (17): Myricetin; (18): Fisetin; (19): Coumarin; (20): Quercetin; (21): Naringenin; (22): Hesperetin; (23): Luteolin; (24): Kaempferol; (25): Apigenin; (26): Rhamnetin; (27): Chrysin

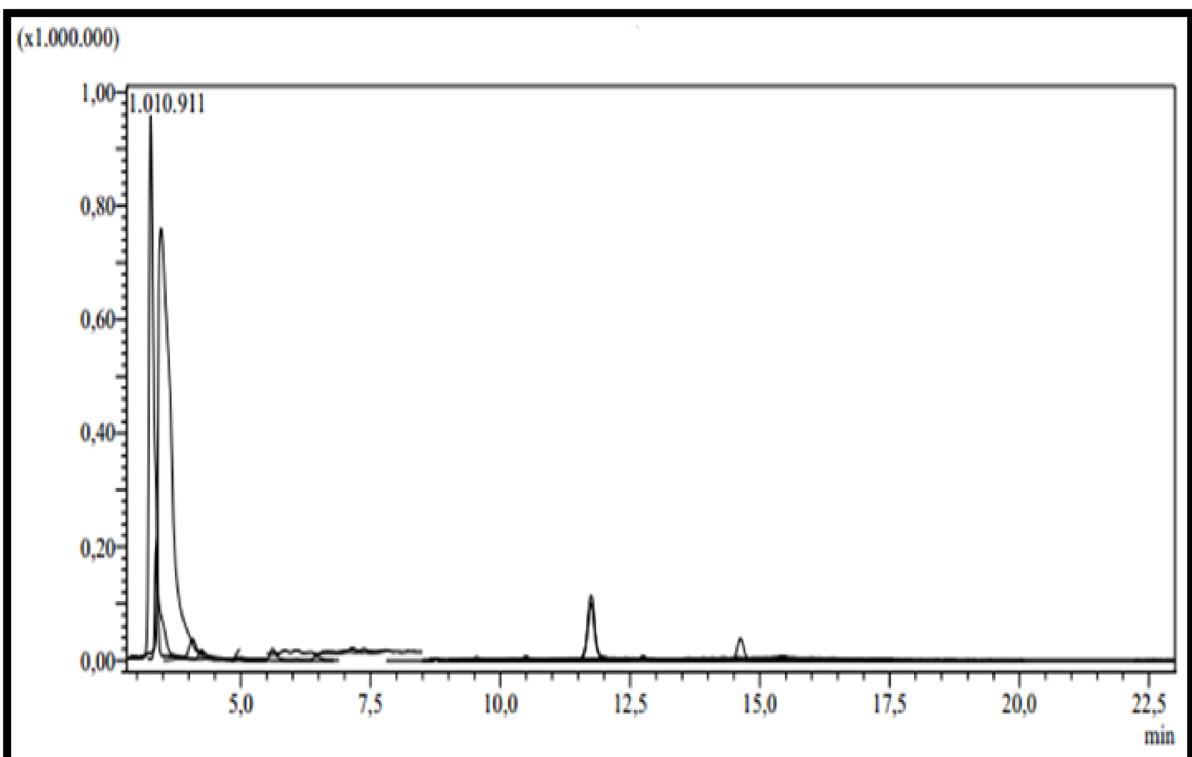

Figure A4. LC-MS/MS chromatogram of the *Salix aegyptiaca* L. roots

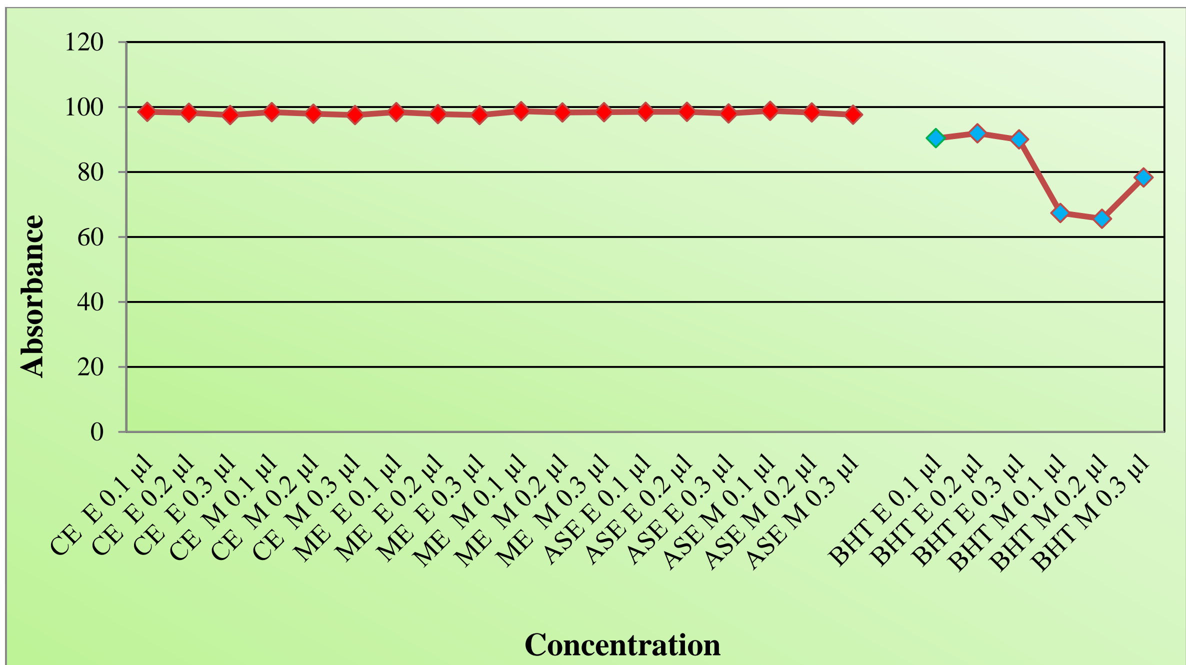

Figure A5. DPPH scavenging activities of root extracts of *Salix aegyptiaca* L.
Note: M: Methanol; E: ethanol; CE: Conventional extraction; ME: Microwave extraction; ASE: Accelerated solvent extraction; BHT: Butylated hydroxytoluene

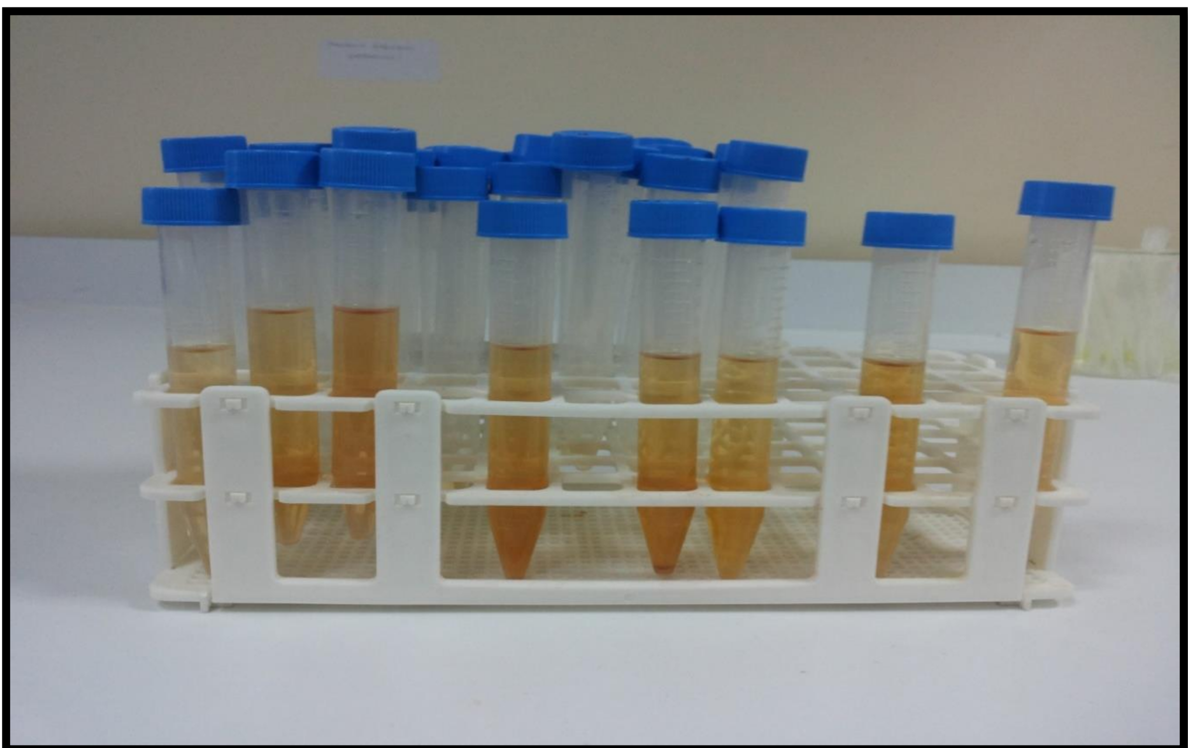

Figure A6. Methanol and ethanol extracts of the roots without DPPH solution

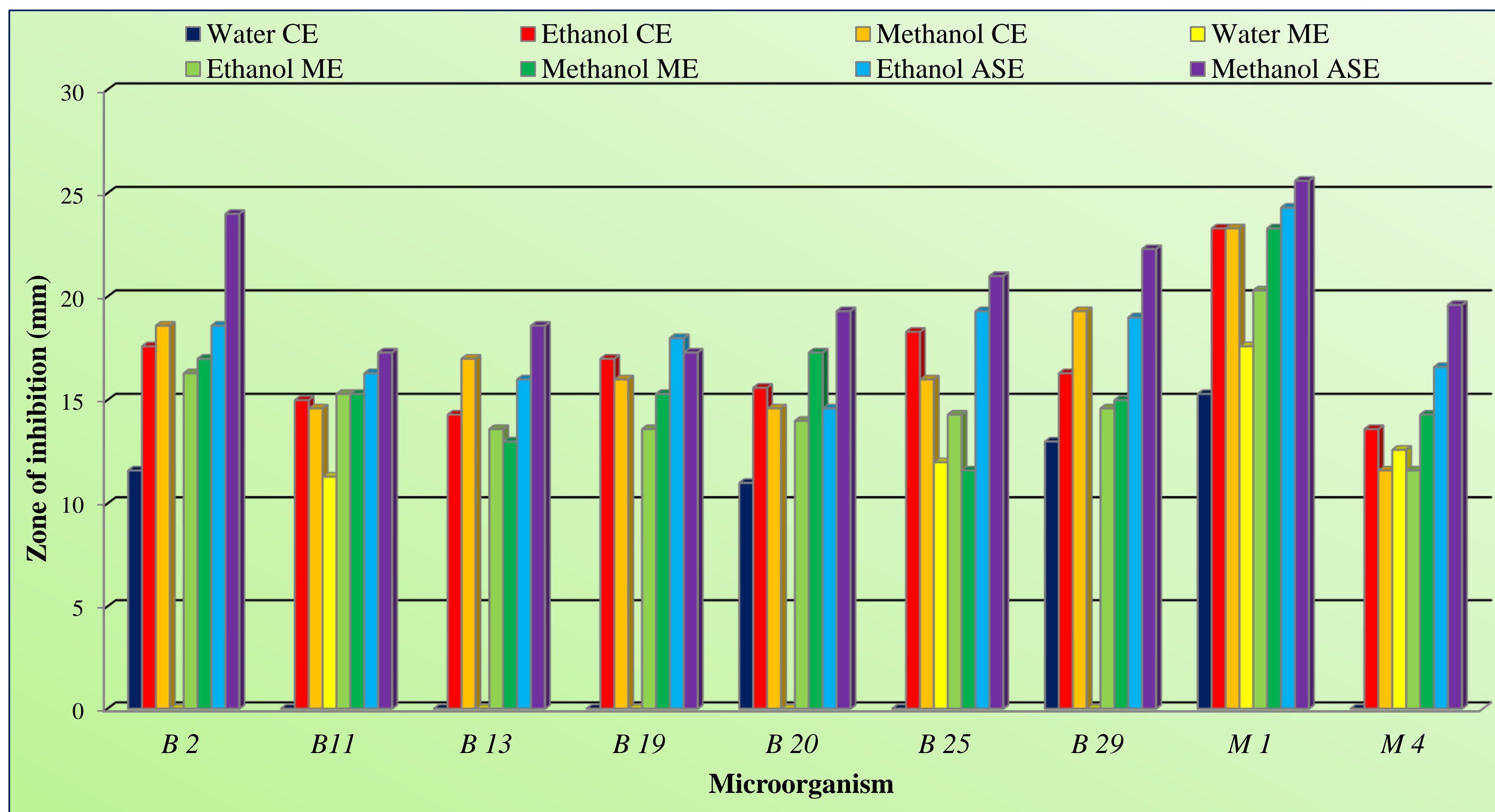

Figure A7. Antimicrobial activity of *Salix aegyptiaca* L. root extracts

Note: *B2*: *Bacillus megaterium* DSM32; *B11*: *Klebsiella pneumoniae* FMC5; *B13*: *Escherichia coli* DM; *B19*: *Pseudomonas aeruginosa* DSM50071; *B20*: *Staphylococcus aureus* Cowan1; *B25*: *Micrococcus luteus* LA2971; *B29*: *Bacillus subtilis* IMG22; *M1*: *Candida albicans* ATCC1023; *M2*: *Candida utilis* NRRL-Y-900; *M3*: *Saccharomyces cerevisiae* WET136; *M4*: *Yarrowia lipolytica* NCIM3589; CE: conventional extraction; ME: microwave extraction; ASE: Accelerated solvent extraction

Table A1. ANOVA table

Methods	Solvents		Sum of Squares	Df	Mean Square	F	Sig
Conventional extraction	Water	Between Groups	1261.879	10	126.18	1388.06	0.00
		Within Groups	2000	22	0.091		
	Ethanol	Between Groups	1587.394	10	158.73	275.7	0.00
		Within Groups	12.667	22	0.576		
	Methanol	Between Groups	1656.061	10	165.6	797.23	0.00
		Within Groups	10.000	22	0.455		
Microwave extraction	Water	Between Groups	1449.515	10	144.95	797.23	0.00
		Within Groups	4.000	22			
	Ethanol	Between Groups	1227.576	10	122.75	238.29	0.00
		Within Groups	11.333	22	0.515		
	Methanol	Between Groups	1493.879	10	149.38	205.4	0.00
		Within Groups	16.000	22	0.727		
ASE	Ethanol	Between Groups	1798.909	10	179.89	1797.88	0.00
		Within Groups	20.00	22	0.909		
	Methanol	Between Groups	2285.576	10	228.55	91.98	0.00
		Within Groups	54.667	22	2.485		

Note: Df: Degree of freedom; F: F-value; Sig: significant.

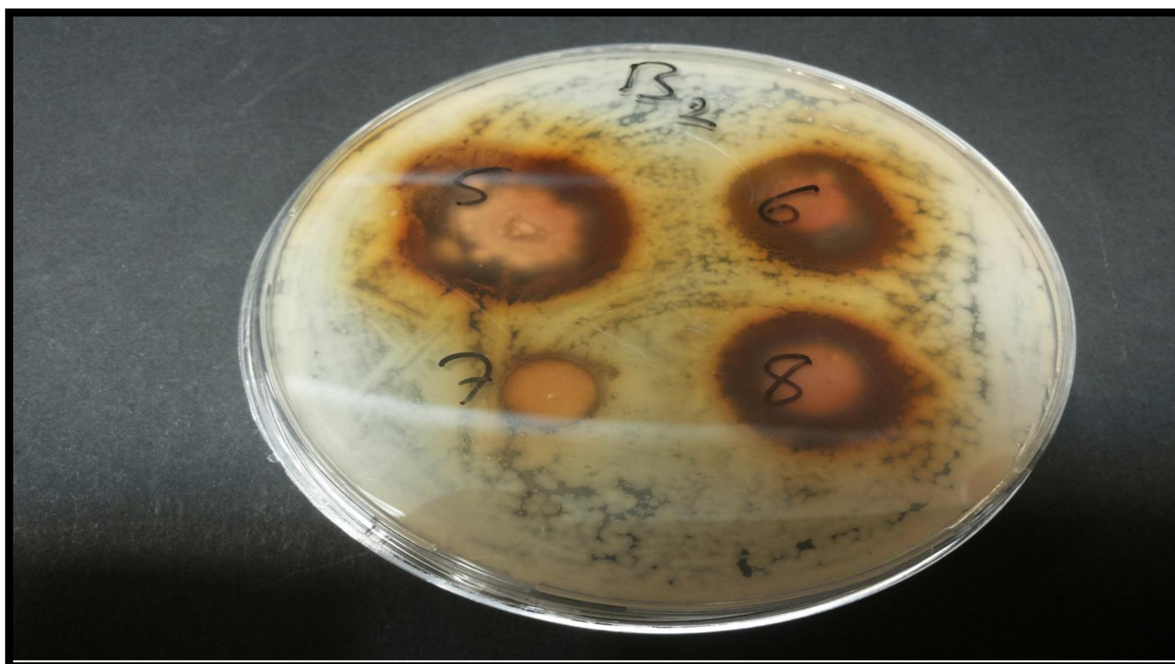

Figure A8. *Bacillus megaterium* DSM32 affected by the root extracts.
Note: B2: *Bacillus megaterium*; 5: Methanol extract for accelerated solvent extraction; 6: Methanol extract for conventional extraction; 7: Water extract for conventional extraction; 8: Ethanol extract for accelerated solvent extraction

Figure A9. *Klebsiella pneumoniae* FMC5 affected by the root extracts
Note: *B11*: *Klebsiella pneumoniae*; 1: Ethanol extract for microwave extraction; 2: Methanol extract for microwave extraction; 3: Water extract for microwave extraction; 4: Ethanol extract for conventional extraction

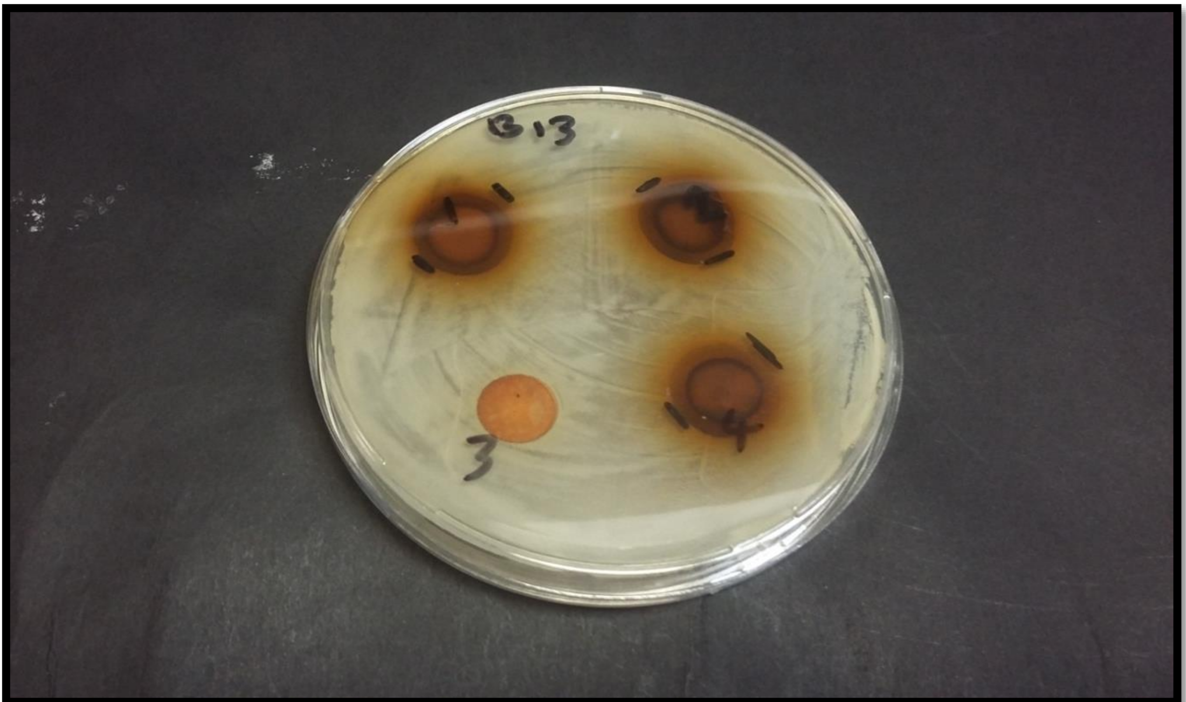

Figure A10. *Escherichia coli* DM affected by the root extracts.
Note: *B13*: *Escherichia coli*; 1: Ethanol extract for microwave extraction; 2: Methanol extract for microwave extraction; 3: Water extract for microwave extraction; 4: Ethanol extract for conventional extraction

Figure A11. Negative control against *Bacillus megaterium* DSM32
Note: *B2*: *Bacillus megaterium*; Et: Ethanol; W: Water; Met: Methanol

Figure A12. Negative control against *Bacillus subtilis* IMG22
Note: *B29*: *Bacillus subtilis*; Et: Ethanol; W: Water; Met: Methanol

Figure A13. *Yarrowia lipolytica* NCIM3589 affected by the root extracts.
Note: M4: *Yarrowia lipolytica*; 5: Methanol extract for accelerated solvent extraction; 6: Methanol extract for conventional extraction; 7: Water extract for conventional extraction; 8: Ethanol extract for accelerated solvent extraction

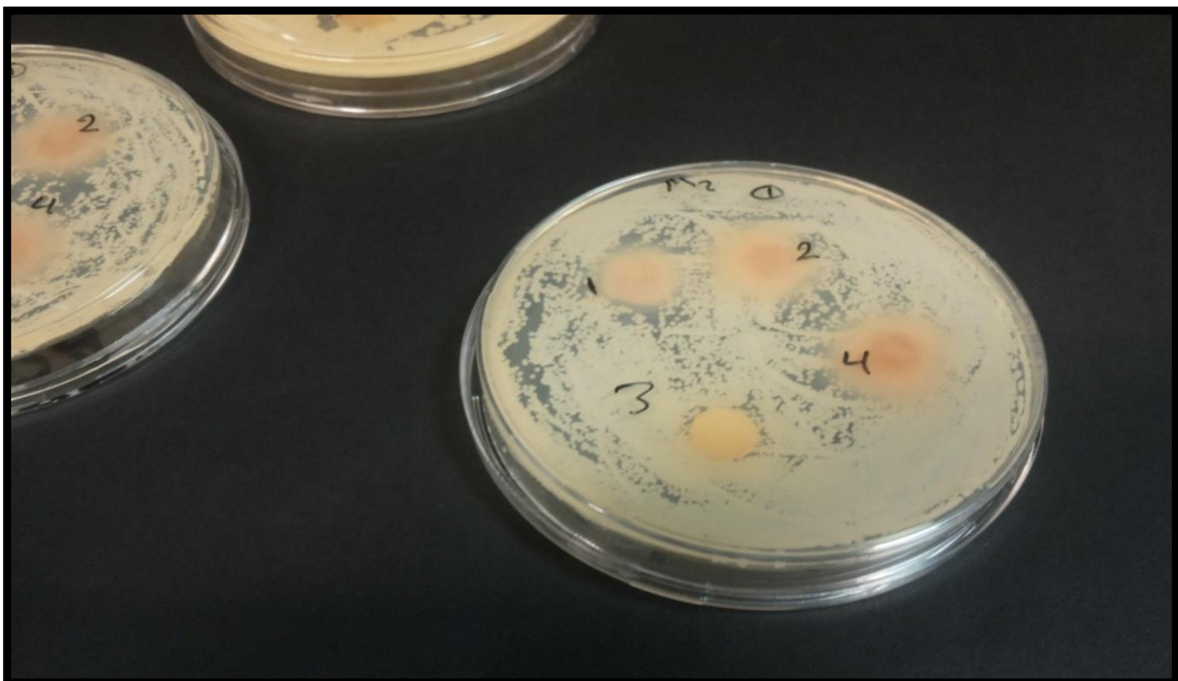

Figure A14. *Candida utilis* NRRL-Y-900 non-affected by the root extracts.
Note: *M2*: *Candida utilis*; 1: Ethanol extract for microwave extraction; 2: Methanol extract for microwave extraction; 3: Water extract for microwave extraction; 4: Ethanol extract for conventional extraction

Figure A15. *Candida utilis* NRRL-Y-900 affected by Nystatin
Note: *M2: Candida utilis*; N: Nystatin

Figure A16. *Candida albicans* ATCC1023 affected by Nystatin
Note: *M1: Candida albicans*; N: Nystatin

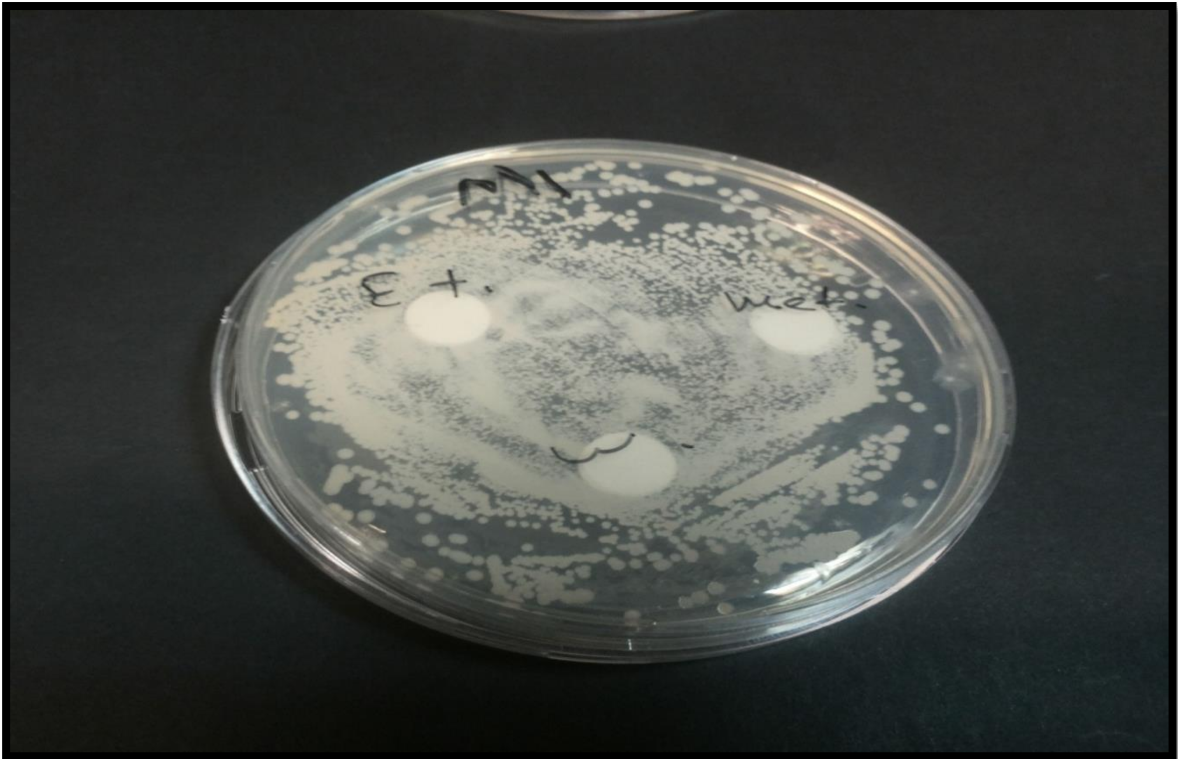

Figure A17. Negative control against *Candida albicans* ATCC1023
Note: *M1: Candida albicans*; Et: Ethanol; W: Water; Met: Methanol

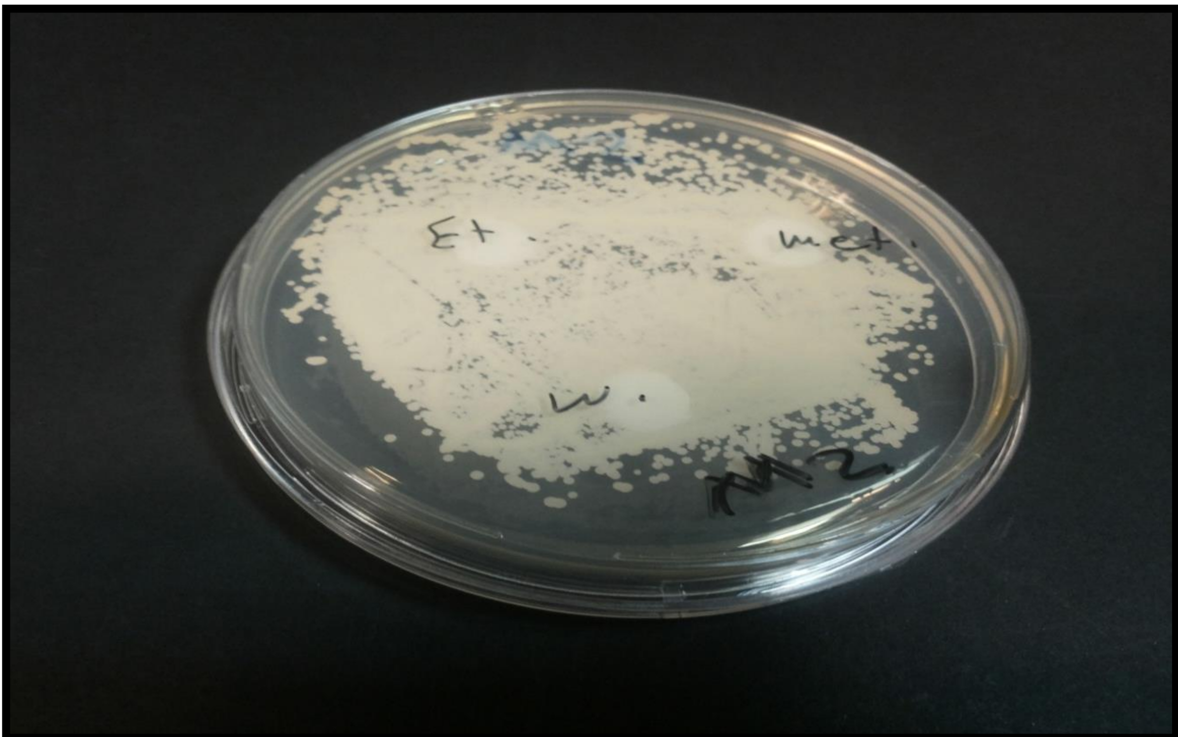

Figure A18. Negative control against *Candida utilis* NRRL-Y-900
Note: *M2: Candida utilis*; Et: Ethanol; W: Water; Met: Methanol

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