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**KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

BIOLOGICAL ACTIVITY OF THE ROOT OF *Tribulus terrestris* L. GROWN IN NORTH OF IRAQ

SHNO ABDALQADIR SOFI

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ, TURKEY 2015

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DECLARATION

I hereby declare that all information in the thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all the material and results that are not original to this work.

Shno Abdalqadir Sofi

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IRAK’IN KUZEYİNDE YETİŞEN *Tribulus terrestris* L. KÖKLERİNİN BİYOLOJİK AKTİVİTESİ

(YÜKSEK LİSANS TEZİ)

SHNO ABDALQADIR SOFI

ÖZET

Bu çalışmanın amacı, *Tribulus terrestris* L. köklerinin verim yüzdesi, toplam kondanse tanen miktarı ile fenolik bileşiklerinin tanımlanması ve miktarlarının belirlenmesi olmuştur. Çalışmada ayrıca *Tribulus terrestris* L. kök ekstraktlarının antioksidant ve antimikrobiyal aktiviteleri de araştırılmıştır. Ekstraksiyonlar için çözücü olarak metanol, etanol ve su mikrodalga ekstraksiyonunda (ME), klasik ekstraksiyonda (CE) ve hızlandırılmış solvent ekstraksiyonunda (ASE) kullanılmıştır. Fenolik bileşiklerin tanımlanması ve miktar tayini LC-MS/MS ile gerçekleştirilmiştir. Kök ekstraktlarının antioksidant aktivitesi ve kondanse tanen miktarı UV-vis spektrofotometre ile ölçülmüştür. Kök ekstraktlarının antimikrobiyal aktivitesinin değerlendirilmesi disk difüzyon metodu kullanılarak yapılmıştır.

Toplam kondanse tanen miktarı 10.83 mg/L olarak bulunmuştur. En yüksek verim ASE yöntemiyle metanol ekstraktında elde edilmiştir (%12.06). En yüksek DPPH giderme etkisi ASE metodu ile tespit edilmiştir (%85.2). Etanol ekstraktlarında DPPH giderme gücü BHT’den daha yüksek bulunmuştur. Köklerin metanol ekstraktlarında fenolik bileşenlerden en fazla vanillin tespit edilmiştir (125.002 µg/g kuru ekstrakt). Ayrıca, *Micrococcus Luteus*’e karşı en yüksek inhibisyon bölgesi metanol ekstraktında mikrodalga ekstraksiyon (ME) yöntemiyle gözlenmiştir (19.33 mm).

Anahtar Kelimeler: Geleneksel tıp, *Tribulus terrestris* L., Antimikrobiyal aktivite, Antioksidant aktivite

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BIOLOGICAL ACTIVITY OF THE ROOT OF *Tribulus terrestris* L. GROWN IN NORTH OF IRAQ

(M.Sc. THESIS)

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ABSTRACT

The aims of present study were to determine the yield percentage, amount of total condensed tannin and identification and quantification of phenolic compounds in *Tribulus terrestris* L. root extracts. In addition, antioxidant and antimicrobial activities of the root extracts were also investigated. Methanol, ethanol and water were used as solvents in different extraction methods, such as microwave (ME), conventional (CE) and accelerated solvent extraction (ASE) techniques. Identification and quantification of the phenolic compounds were performed by LC-MS/MS. The antioxidant activity and amount of condensed tannin of the root extracts were measured by UV-vis spectrophotometer. The antimicrobial activity of root extracts were evaluated by disc diffusion method.

The amount of total condensed tannin was 10.83 mg/L. The highest yield was observed in methanol extract with ASE method (12.06%). The highest DPPH scavenging activity was detected with ASE method (85.2%). The DPPH scavenging power in ethanol extract was higher than BHT. Among the phenolic compounds, vanillin was higher in methanolic extract of roots (125.002 µg/g dry extract). Additionally, the highest inhibition zone was detected against *Micrococcus luteus* in methanol extract with microwave extraction (19.33 mm).

Keywords: Traditional medicine, *Tribulus terrestris* L., Antimicrobial activity, Antioxidant activity

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LIST OF ABBREVIATIONS

WHO:	World Health Organization
TT:	<i>Tribulus terrestris</i> L.
BHT:	Butylated hydroxytoluene
DPPH:	1,1-diphenyl-2 picrylhydrazyl
LAET:	Lyophilized aqueous extract of <i>Tribulus terrestris</i> L.dried fruits
HPLC:	High Performance Liquid Chromatography
LC:	Liquid Chromatography
MS:	Mass Spectrometry
MAE:	Microwave assisted extraction
ASE:	Accelerated solvent extraction
GSTT:	Gross saponins from <i>Tribulus terrestris</i>
NF- κ B:	Nuclear factor kappa-light-chain-enhancer of activated B cells
ATCC:	American Type Culture Collection
G-ve:	Gram-negative bacteria
G+ve:	Gram-positive bacteria
AAe:	Ascorbic acid equivalent
mg:	Milligram
mL:	Milliliter
μ g:	Microgram
μ l:	Microliter
mm:	Millimeter
ESBL:	Extended-spectrum beta-lactamases
FRAP:	Ferric reducing/antioxidant power
MHA:	Mueller-Hinton agar
SDA:	Sabouraud dextrose agar
AMU:	Atomic mass unit

1. INTRODUCTION

Traditional medicine plays a vital role for covering the basic health needs in developing countries. Moreover, using herbal remedies has been risen in developed countries in last decades. With this connection, plants continue to be a rich source of therapeutic agents. The remarkable contribution of plants to drug industry has been shown since large numbers of phytochemical and biological studies are present from all over the world. Herbal remedies used in folk medicine provide interesting and unexplored source for creation and development of new drugs for chemotherapy. This may help to overcome growing problems of drug resistance and the toxicity of the currently available commercial antibiotics (Kianbakht and Jahaniani, 2003).

Traditional medicinal plants have been known to possess diverse biological activities, such as antimicrobial, analgesics, anticancer, antipyrexial and antihypertensive along with important source of biological active compounds (Alma et al., 2003; Andrade et al., 2007; Webster et al., 2008). Traditional medicine has been used extensively in a large portion of the world for people's healthcare and remedy of diseases for many years. The data revealed that there is a high correlation between medicinal plant usage and laboratory analysis (Singh et al., 2008).

Phytotherapy is considered by using biological active compounds in plants (Garza et al., 2007). The most interesting area of application for medicinal plant extracts is either growth inhibition or reduction of the serious pathogens (Kuate et al., 2007; Kotzekidou et al., 2008). Several studies have been focused on plants as a significant source of new pharmaceuticals (Rabe and Staden 1997; Rates, 2001).

The plant kingdom is a good source of natural antioxidants. However, there is still not enough knowledge and data about the practical usefulness of them. Groups of secondary plant metabolites, such as phenolics and flavonoids are commonly found in various fruits, vegetables and herbs. They have been shown to provide a fruitful defense against oxidative stress from oxidizing agents and free radicals (Antolovich et al., 2000; Matkowski, 2006).

The oxidation process is an intrinsic factor in energy management of all living organisms and kept under strict control by several cellular mechanisms. However, the production of excessive free radicals and unbalanced antioxidant protection may result in

numerous diseases and accelerate ageing. The antioxidants are considered as protection agents by reducing oxidative damage in the body when internal enzymatic mechanisms fail or inadequately efficient (Halliwell, 1995).

Oxidation is also responsible for the rancidity of unpreserved food rich in unsaturated fatty acids. The natural antioxidants are suggested as a superior alternative for the synthetic ones such as BHA or BHT (Li et al., 2008). Therefore, there are growing interests in substances exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as specific preventative pharmaceuticals (Sarikurkcu et al., 2009).

Tribulus terrestris L. (Puncture Vine, Caltrop, Yellow Vine and Goathead) is a flowering plant of the Zygophyllaceae family native to warm temperature and tropical regions in Southern Europe, Southern Asia, Africa and Northern Australia. It can thrive even in desert climates and poor soil (Abeywickrama and Bean, 1991). The Arabic names of *Tribulus terrestris* L. “Al-Gutub” or “Qut’iba,” is a widely distributed in Iraq and the entire Mediterranean region. It is an herbal remedy used for various medicinal purposes including the treatment of kidney troubles, particularly stones (Al-Ali et al., 2003). In Iraq, it has been used in folk medicine as tonic, aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic, lithon-triptic and urinary anti-infective (Majeed and Mahmood, 1988).

Nowadays, due to increase of bacterial resistance to chemical drugs and their side effects tend to search for medicinal plants or herbs and identify their active ingredients. These active substances and their associated materials have a biological balance where they do not accumulate in the body, cause severe side effects and as a result have significant advantages over the chemical drugs (Walag and Stodula, 1996).

Although many potentially bioactive constituents have been isolated from *Tribulus terrestris* L., there has not been established a relationship between their structure and activity. A number of steroids (Wu et al., 1996; Yan et al., 1997), flavonoids (Bhutani et al., 1969; Saleh et al., 1982), lignanamides (Ren et al., 1994; Li et al., 1998; Wu et al., 1999) and alkaloids (Bourke et al., 1992) have been identified.

The foliage of *Tribulus terrestris* L. can be toxic to livestock especially sheep, when consumed in large quantities (more than 80 percent of the diet). Puncture vine has

also been used in folk medicine throughout the history far back to the Greeks, for headache, nervous disorders, constipation and sexual dysfunction (Sahelian, 2003). The fruits are the parts most often used in traditional medicine. They contain a number of different substances including saponins (protodioscin, furostanol), flavonoids, alkaloids, resins, tannins, sugars, sterols and essential oil (Sahelian, 2003).

A literature survey revealed that the plant and its constituent's are effective for hypotensive, cardiac depressant and contractile activities on smooth muscles (Mossa et al., 1983). It has been found to be effective in treating angina pectoris by dilating the coronary arteries and improving the cardiac circulation (Wag et al., 1990). In addition, it is effective for diuresis, colicky pains, hypertension and hypercholesterolemia reported by Arcasoy et al. (1998). It has been shown to increase free serum testosterone levels (Brown et al., 2001). It is effective for sexual and erectile dysfunctions (Adiomoelja, 2000), protective for genetic damage (Liu et al., 1995) and stimulates melanocyte proliferation in vitiligo treatment (Lin et al., 1999). Its antibacterial, cytotoxic and nematocidal activities were also evaluated by Ali et al. (2001) and (Nandal and Bhatti, 1983).

The aims of present study were to determine the yield percentage, amount of total condensed tannin and identification and quantification of phenolic compounds in *Tribulus terrestris* L. root extracts. In addition, antioxidant and antimicrobial activities of *Tribulus terrestris* L root extracts were also researched.

2. LITERATURE REVIEW

2.1. Traditional Medicine

Nature has been a source of medicinal agents for thousands of years. Plant derived substances have recently become of great interest owing to their versatile applications (Baris et al., 2006). Plants offer a considerably greater chemical diversity than microorganisms. With advancement in technology and analytical chemistry, more than one million compounds have been discovered from natural compounds. Among them 50-60% are produced by plants and just 5% is from microbial origin (Berdy, 2005).

There are more than 35,000 plant species used in various human cultures around the world for medicinal purpose. Biologically active compounds present in medicinal plants have always been of great interest to scientist working in this field (Raja and Venkataraman, 2011a).

Traditional medicines serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. More than 30% of the entire plant species has been used for medicinal purposes. Today, the herbal medicine trade is a booming business worldwide. It has been estimated that in developed countries, such as United States, plant drugs constitute as much as 25% of the modern medicine. While, in developing countries like China and India the contribution is as much as 70% (Choudhary, 2002).

Traditional medicine has been practiced for many centuries by a substantial proportion of the Iraqi population. The interest of Traditional medicine studies as a source of pharmacologically active compounds has increased worldwide. It is recognized that plants are the main medicinal sources for treating infectious diseases in developing countries. Plant extracts represent a continuous effort to find new compounds with a potential for acting against multi-resistant bacteria. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological test and a substantial number of new antibiotics introduced on the market are obtained from natural or semi synthetic resources (Mothana and Lindequist, 2005). One of the plant used by Arabs was *Tribulus terrestris* L. (Rowland and Tai, 2003). It has an important role in folk medicine of many countries (Xu et al., 2000).

2.2. Plants and Secondary Metabolites

Plants are rich in a variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides and volatile oils. These serve as natural plant defense mechanisms against invasion by microorganisms, insects and herbivores or for combating infectious or parasitic agent or generated in response to stress conditions. Plant secondary metabolites possess a wide range of applications, such as in food, flavors, poisons and perfumes, scented oils in aromatherapy, dyes, rubber and oil. It is estimated that 1/4th of prescription drugs contain at least one chemical originally identified and extracted from a plant. Herbal medicine has been used for centuries and their active compounds have been identified and purified with various analytical techniques (Cowan, 1999).

Mainstream medicine is increasingly receptive for using antimicrobial and other drugs derived from plants. However, traditional antibiotics become ineffective due to the rapid rate of plant species extinction. There is a feeling among natural products where chemists and microbiologists like them. The phytochemical structures, which could be synthesized chemically, are at risk of being lost irretrievably (Cowan, 1999).

2.2.1. Flavonoids

Flavonoids are ubiquitous in photosynthesizing cells and commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these compounds as active constituents have been used to treat human diseases (Tim Cushnie and Lamb, 2005).

The function of flavonoids in flowers is to provide colors attractive to plant pollinators in leaves. These compounds are increasingly believed to promote physiological survival of the plant protection from fungal pathogens (Harborne and Williams, 2000). Additionally, these compounds are also a component of disease treatment (phytotherapy) as they are present in seeds, stems, barks, roots and flowers of several medicinal plants (Nijveldt et al., 2001).

Flavonoids are water-soluble plant antioxidants and free radical scavengers, which prevent oxidative cell damage. Diets rich in fruits and vegetables have been reported to

associate with reducing the risk of tumors, especially epithelial cancers of the respiratory and gastrointestinal tract (Nanasombat and Teckchuen, 2009).

Pietta (2000) has investigated the wide range of therapeutic properties in clinical and preclinical studies including antioxidants. In addition, other therapeutic properties of flavonoids, such as anticancer, anti-inflammatory and anti-allergy activities were also studied by others (Chahar et al., 2011; Serafini et al., 2010; Kawai et al., 2007). Thus, authors reported that the flavonoids form complexes with extracellular soluble proteins and may disrupt microbial membranes. It has also been reported to possess antiulcer by Vidari et al. (2003), antibacterial by Xu and Lee (2001) and antiviral property by (Li et al., 2002).

Coneac et al. (2008) and Kasim et al. (2011) have reported the antimicrobial activity of flavonoids. The antioxidant capacity of the phenolic compounds, especially rutin and quercetin, was reported by Sun et al. (2007). Rutin, a natural flavone derivative quercetin-3- rhamnosylglucoside, is an important anti-lipoperoxidant agent (Negre-Salvayre et al., 1991). It has also been found to be a strong scavenger of hydroxyl and superoxide radicals (Metodiowa et al., 1997). It has been previously reported to possess anti-inflammatory (Kroes et al., 1992), antitumor (Miki et al., 2001), antimicrobial and antioxidant properties (Proestos et al., 2005).

2.2.2. Alkaloids

Alkaloids are natural products that contain heterocyclic nitrogen atoms. The name of alkaloid derives from the “alkaline” and used to describe any nitrogen-containing base. A large number of organisms including animals, plants, bacteria and fungi naturally synthesize them. Some of the natural products isolated from medicinal plants were alkaloids in 19th century. It was found that they were nitrogen-containing bases, which formed salts with acid. Alkaloids play a significant role in plant protection and survival. They ensure their survival against microorganisms (antibacterial and antifungal activity), insects and herbivores (feeding deterrents). They also contribute against other plants by means of all elopathically active chemicals (Saxena et al., 2013).

2.2.3. Saponins

Saponins are widely distributed in plant species. They are naturally occurring in glycosides, which consist of non-sugar aglycone coupled with sugar chain units. These

sugars can be attached as one, two or three chains and the terms monodesmoside, bidesmoside and tridesmoside have been given to these saponins, respectively (Greek desmos=chain). According to nature of aglycone, they can be classified into steroidal or triterpene groups. Some authors also included the saponins within steroidal glycoalkaloids of solanidans and spirosolan classes. All classes of aglycones may have a number of functional groups (–OH, –COOH, –CH₃) causing a big natural diversity. Over 100 steroidal and probably even larger numbers of triterpene sapogenins have been identified (Oleszek, 2002).

Furthermore, saponins are toxic to a variety of organisms by possessing antifungal (Miyakoshi et al., 2000; Yang et al., 2006), antibacterial (Soetan et al., 2006), antioxidant (Haridas et al., 2001), antiviral (Mengoni et al., 2002), antiprotozoal (Wallace, 2004), chemo-preventive (Mujoo et al., 2001) and molluscicidal activities (Apers et al., 2001).

2.3. Taxonomic Classification

Scientific classification of this plant is presented in Table 2.1 (Gauthaman et al., 2003).

Table 2.1. Scientific classification of *Tribulus terrestris* L.

Kingdom	Planate
Division	Magnoliphyta
Class	Magnolipsida
Order	Zygophyllates
Family	Zygophyllaceae
Genus	<i>Tribulus</i>
Species	<i>terrestris</i> L.

2.4. Plant Used in This Study

2.4.1. *Tribulus terrestris* L.

Tribulus terrestris L. (Puncture Vine, Caltrop, Yellow Vine and Goathead) is a flowering plant of the Zygophyllaceae family, native to warm temperature and tropical regions of Southern Europe, Southern Asia, Africa and Northern Australia. It can thrive even in dessert climates and poor soil. In Iraq, it has been used in folk medicine as tonic,

aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic and urinary anti-infective (Al-Bayati and Al-Mola, 2008).

The Arabic names, of *Tribulus terrestris* L. “Al-Gutub” or “Qut‘iba,” is a widely distributed plant in Iraq and Mediterranean region. It is an herbal remedy used for various medicinal purposes including the treatment of kidney troubles, particularly stones (Al-Ali et al., 2003). Hakimi-Vala et al. (2014) suggested that TT is a strong diuretic and used to treat kidney and bladder stones, decrease blood pressure, prescribe as an anti-inflammatory, joint pain reliever and reduce fever (Hakimi-Vala et al., 2014).

2.4.2. Description

Tribulus terrestris L. is a small silky, hairy and prostrate herb with stems up to 2 m long (Figure 2.1). Leaves are pinnate, short (~1.25 cm length), opposite and consisting of 4-8 pairs of spear shaped leaflets. It is characterized by small (8-15 mm diameter) yellow petal flowers and thorny fruits. Fruits are woody burr about 1 cm diameter with sharp spines up to 6 mm long. Burr consists of 5 wedge shaped segments. Each segment has 2 unequal pairs of spines (Hashim et al., 2014).

Seeds are enclosed in a woody star-shaped structure (carpels) of around 5-7 mm length and 5-6 mm width. There are five seeds in each carpel and 1.5-3 mm long yellow color. Each plant can have up to 2000 seeds. They taste astringent and agreeable. The root is slender, fibrous, cylindrical and light brown color when it is fresh. The stomata of *Tribulus terrestris* L. is anomocytic in which the guard cells are not surrounded with any subsidiary cell (Hashim et al., 2014).



Figure 2.1. *Tribulus terrestris* L.

2.4.3. Distributions and habitat

Native *Tribulus terrestris* L. is grown in Mediterranean region but widely distributed in warm regions of Europe, Asia, America, Africa and Australia (Kostova et al., 2002). It is also in Morocco, Bulgaria, Romania and Slovakia (Salamon, et al., 2006). It is also found throughout Syria, Lebanon, Jordan, Yemen, Palestine and Egypt (Abdul-Wahed, 2002). *Tribulus terrestris* L. is common in the desert and forest zone of Iraq (Galala, Sulaimaniya, Mousil, Rutba and Habbania) (Al-Rubii, 2010).

2.4.4. Chemical constituents

The preliminary phytochemical study of *Tribulus terrestris* L. revealed the presence of saponins, flavonoids, glycosides, alkaloids and tannins (Usman et al., 2007)

Kostova and Dinchev (2005) studied the chemistry and bioactivity of saponins in *Tribulus terrestris* L. They reported that furostanol, spirostanol saponins, tigogenin, neotigogenin, gitogenin, neogitogenin, hecogenin, neohecogenin, diosgenin, chlorogenin, ruscogenin and sarsasapogenin are frequently found in this plant. In addition, four sulfated saponins of tigogenin and diosgenin were also isolated. Major ones are furostanol glycosides including protodioscin and protogracillin of which protodioscin is the most dominant saponin and spirostanol glycosides present in small quantities.

Bhutani et al. (1969) isolated kaempferol, kaempferol-3-glucoside, kaempferol-3-rutinoside and tribuloside [kaempferol-3- β -d-(6''-p-coumaroyl) glucoside] from leaves as well as fruits of TT.

Louveaux et al. (1998) detected 18 flavonoids in four *Tribulus* leaf extracts, such as caffeoyl derivatives, quercetin glycosides, rutin and kaempferol glycosides by high-performance liquid chromatography (HPLC).

Yekta et al. (2008) isolated and characterized three flavonoid glycosides based on quercetin and kaempferol. Flavonoid glycosides consisted of quercetin 3-O-glycoside, quercetin 3-O-rutinoside and kaempferol 3-O-glycoside.

Gas chromatography-mass spectrometry (GC-MS) analysis in methanol extract of whole plant *Tribulus terrestris* L. were revealed the presence of α -amyrin as the major constituent. In addition, seven minor constituents are 3,7,11,15-tetramethyl-2-hexadecen-1-

ol, n-hexadecadienoic acid, hexadecadienoic acid ethyl ester, phytol, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid and 1,2-benzenedicarboxylic acid dioctyl ester. Sterols, such as β -sitosterols and stigmasterols were also found to be present (Abirami and Rajendran, 2011).

Wu et al. (1999) identified three new compounds from dried TT fruits (terrestribisamide, 25R-spirost-4-en-3, 12-dione, and tribulusterine). In addition, 10 other known compounds (*N*-*p*-coumaroyltyramine, terrestriamide, hecogenin, aurantiamide acetate, xanthosine, fatty acid ester, ferulic acid, vanillin, *p*-hydroxybenzoic acid and β -sitosterol) were also detected.

Gomathi et al. (2012) showed that the aqueous and ethanolic extracts of *Tribulus terrestris* L. contain alkaloids, tannin, flavonoids, quinines and phenolic compounds.

Twenty five flavonoid glycosides were detected in *Tribulus pentandrus* and *Tribulus terrestris* L. The glycosides were belonging to common flavonols, kaempferol, quercetin and isorhamnetin with 3-gentiobiosides as the major glycosides. A trace of flavone (tricin) glycoside was also present in *T. Pentandrus* (Saleh et al., 1982).

2.4.5. Pharmacological activities of *Tribulus terrestris* L.

2.4.5.1. Diuretic activity

Al-Ali et al. (2003) tested the aqueous extract of *Tribulus terrestris* L. prepared from its fruit and leaves in rat diuretic model. The strips of Guinea pig ileum were used for contractility test. The oral dose of 5 g/kg aqueous *Tribulus terrestris* L. extract elicited a positive diuresis, which was more than furosemide. The increased smooth muscle tonicity produced by TT extract together with diuretic activity helped the propulsion of stones along the urinary tract.

2.4.5.2. Aphrodisiac activity

Singh et al. (2012) evaluated the dose administration of lyophilized *Tribulus terrestris* L. fruits (LAET) in aqueous extract at 50 and 100 mg/kg of body weight as a sexual enhancer in male rats. A dose dependent improvement in sexual behavior was observed with LAET treatment. A significant increase in serum testosterone level was also

observed. These findings confirm the traditional use of TT as a sexual enhancer for managing the sexual dysfunctions in males.

2.4.5.3. Activity in cardiac disorders

Zhang et al. (2010) evaluated the protective effect of tribulosin from *Tribulus terrestris* L. against cardiac ischemia/reperfusion injury in rats. Tribulosin protected myocardium against ischemia/reperfusion injury through protein kinase C epsilon activation.

2.4.5.4. Antiinflammatory activity

Baburao et al. (2009) observed a dose dependent inhibition of rat paw volume by methanol extract of *Tribulus terrestris* L. in carrageenan induced inflammation in rats.

2.4.5.5. Analgesic activity

Prusty et al. (2013) reported an analgesic activity caused by methanolic extract of *Tribulus terrestris* L. fruits.

2.4.5.6. Anticancer activity

Kim et al. (2011) showed that the *Tribulus terrestris* L. extract blocks proliferation and induces apoptosis in human liver cancer cells through the inhibition of NF- κ B signaling. Thus, TT has clinical therapeutic effects against liver cancer cells.

2.4.5.7. Immunomodulatory activity

Tilwari et al. (2011) evaluated that the alcoholic extract of whole plant *Tribulus terrestris* L. were exhibited a significant dose-dependent increase in humoral antibody titer and delayed hypersensitivity, which indicates increased specific immune response.

2.4.5.8. Antidiabetic activity

Li et al. (2002) evaluated that *Tribulus terrestris* L. was reduced serum glucose, triglyceride and cholesterol levels significantly. Serum superoxide dismutase activity was higher in alloxan-induced diabetic mice. In addition, the decoction of TT showed an inhibition for gluconeogenesis in mice.

2.5. Biological Activity

2.5.1. Antioxidant activity

Antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinon (TBHQ) and propyl gallate are widely used in many foods. These compounds have been added to oils and fats at low concentrations ranged from 50 to 200 ppm for preventing formation of peroxides during storage (Fki et al., 2005).

Eagappan et al. (2015) showed that the scavenging activity of *Tribulus terrestris* L. fruit extract (0.1-0.4 µ/mL) posses a significant free radical scavenging action against nitric oxide. The nitric oxide inhibition was 15-85% in cooked, 2-45% in aqueous, 22-68% in methanol and 3-35% in ethanol extracts compared to ascorbic acid as standard.

Zheleva-Dimitrova et al. (2012) demonstrated that *Tribulus terrestris* L. possess a significant antioxidant activity. The IC₅₀ for DPPH activity was varied from 2.84 to 4.56 mg/mL and from 0.28 to 0.31 mg/mL, respectively. All tested samples demonstrated strong FRAP activity compared to BHT (2.29 µg) and inhibit lipid peroxidation in linoleic acid system.

Lokhande et al. (2014) showed that the leaves of *Tribulus terrestris* L. were exhibited concentration dependent scavenging activity by inhibiting DPPH radicals. The IC₅₀ value in aqueous and alcoholic extract of *Tribulus terrestris* L. were 43.96 and 41.66 µg/ml, respectively.

Hemalatha and Hari (2013) found a high antioxidant activity of the saponin fraction of *Tribulus terrestris* L. (SFTT) extract at 0.90 abs when compared to crude extract of TT 0.75 abs with 100 µg/mL concentration. The antioxidant activity of the saponin fraction was similar to ascorbic acid. It was estimated that the SFTT contained 67 µg of ascorbic acid/mg of extract. Whereas, the crude extract of *Tribulus terrestris* L. contained 32.5 µg of ascorbic acid/mg of extract.

Hammoda et al. (2013) suggested the potential antioxidant activity of total ethanol extract. Different fractions of *Tribulus terrestris* L. were screened for their *in vitro* DPPH free radical scavenging activity. The results showed that 4 (4,5-di-p-transcoumaroylquinic acid) compound had the strongest DPPH free radical scavenging activity (661.98 mg AAe/g sample) followed by 3 (4,5-di-p-cis-coumaroylquinic acid) (589.916 mg AAe/g

sample). The remaining other compounds showed very weak DPPH free radical scavenging activities. It is worth to mention that compounds 3 and 4 showed stronger DPPH free radical scavenging activity than the total ethyl acetate fraction itself (33.928 mg AAe/g sample). Moreover, IC₅₀ of 3 (0.0216 mg) and 4 (0.0191 mg) compounds were very close to IC₅₀ of ascorbic acid (0.0125 mg) proving their potent antioxidant effect.

2.5.2. Antimicrobial activity

The total *Tribulus terrestris* L. fruit extracts were evaluated by Hakemi-vala et al. (2014). They showed a significant difference between the mean of inhibition zone by cup plate method with different concentrations against *E. coli*, *P. aeruginosa* and *B. subtilis*. The concentrations were 375 mg/mL (8.3-15.5 mm), 750 mg/mL (11.5-19.5 mm) and 1000 mg/mL (17.3-22 mm) of total extract in water solvent, respectively compared to penicillin (29.3-47 mm). In addition, there were significant differences between the mean of inhibition zones with different concentrations against similar bacteria. The concentrations were 375 mg/mL (10.8-15.3 mm), 750 mg/mL (13-19 mm) and 1000 mg/mL (14.7-20.7 mm) of total extract in 10% DMSO solvent, respectively compared to penicillin (29.3–47 mm).

Baburao et al. (2009) investigated the inhibition zones of methanol extract of *Tribulus terrestris* L. against gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) and gram-ve bacteria (*E. coli*, *Proteus vulgaris*) at 200 µg/mL (17, 18, 20 and 20 mm). While, the inhibition zones at 400 µg/mL were 19, 21, 23 and 22 mm against the same microorganisms mentioned above.

Deshwal (2012) evaluated that the ethanol extract of TT showed maximum inhibition zone (27.33 mm) as compared to water extract. Water extracts of TT showed inhibition zones from 13.66 to 25.66 mm but ethanol extract showed inhibition zones from 14.66 to 27.33 mm. It was shown that the TT has an effective antibacterial activity against *Pseudomonas aeruginosa*.

The organic and aqueous extracts from fruits, leaves and roots of TT were examined against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* by micro dilution method. All the extracts from different parts of plant showed

antimicrobial activity against most tested microorganisms. The most active extract against bacteria was ethanol extract for the fruits with a minimum inhibitory concentration (MIC) of 0.15 mg/mL against *B. subtilis*, *B. cereus*, *P. vulgaris* and *C. diphtheriae*. In addition, the same extract for the same plant part demonstrated the strongest antifungal activity (Al-Bayati and Al-Mola, 2008).

Sanjeeva et al. (2011) determined that the fruit extracts of *Tribulus terrestris* L. at 500 µg/mL concentration showed inhibition zones of 24.6, 20.4, 23.1, 22.1 and 14.6 mm against *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris*, respectively. The root extracts with the same concentration showed the inhibition zones of 11.5, 13.5, 16.3, 13.8 and 12.9 mm against the microorganisms mentioned above. Moreover, the inhibition zones of fruit extracts were 18.7, 13.4 and 10.6 mm against *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans*, respectively. The inhibition zones of root extracts against the same fungi at the same concentration were 13.36, 10.5 and 17.6 mm.

Mohammed (2008) evaluated that the saponin isolated from *Tribulus terrestris* L. fruit showed different inhibiting effect on all bacteria tested. The highest inhibition zones at 200 mg/mL against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis* and *Klebsiella pneumonia* were 26, 24, 23, 20 and 14 mm, respectively. While, the lowest inhibition zone against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* were 9, 10 and 12 mm, respectively. However, there was no effect against *Klebsiella pneumoniae* and *B. subtilis*.

Mohammed and Mohammed (2008) evaluated the alkaloids isolated from *Tribulus terrestris* L. fruits. The results showed different inhibiting effects at 200 mg/mL concentration against *Bacillus cereus* (22 mm), *Staphylococcus aureus* (20 mm), *Proteus vulgaris* (20 mm), *E. coli* (18 mm), *Pseudomonas aeruginosa* (18 mm) and *Serratia marcescens* (14 mm). With 100 mg/mL concentration, inhibition zones ranged from 10 to 16 mm. There was no antibacterial effect against the microorganisms tested at 12.5 mg/mL concentration.

Bedir et al. (2002) tested seven different saponins from *Tribulus terrestris* L. Only the spirostanol saponins showed antifungal activity against *C. albicans* and *Cryptococcus neoformans*. None of the furostanol derivatives exhibited inhibitory activity. Further, these

compounds were not effective against *S. aureus*, *Aspergillus fumigatus*, *P. aeruginosa* and *Mycobacterium intracellulare*.

Ranade et al. (2015) tested *Tribulus terrestris* L. fruits in aqueous and organic solvent extracts (ethanol, chloroform, isopropyl alcohol, acetone, ethyl acetate) against three multi-drug resistant uropathogenic *E. coli* (UPEC2, UPEC3 and UPEC4). The maximum activity was observed by ethanol extract for 3 *E. coli* cultures and the type strain (75%). The acetone extract showed 50% occurrence for inhibition zone. The acetone, ethyl acetate, isopropyl alcohol and petroleum ether extract showed 25% positive result. Whereas, the chloroform extract was unable to express any antimicrobial activity.

Usman et al. (2007) evaluated the activity of methanolic leaf extract of Nigerian *Tribulus terrestris* L. against *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans*. The extract exhibited considerable amount of inhibition against all test organisms. The highest and the lowest activities were against *Staphylococcus aureus* (18.5 ± 0.2 mm) and *E. coli* (10.5 ± 0.9 mm).

Jindal et al. (2012) suggested that flavonoid extracts in different parts of *Tribulus terrestris* L. had antibacterial activity. However, there was no activity against fungi. The most susceptible organism in the investigation was *E. coli*. Whereas, the most resistant bacteria was *P. mirabilis* against three extracts. The best activity against *E. coli*, *S. aureus* and *P. Mirabilis* was 12, 10.5 and 16.2 mm, respectively

Soleimanpour et al. (2015) determined *in vitro* antibacterial activity of *Tribulus terrestris* L. fruit extracts by using disk diffusion method. Results showed that the highest inhibition zone at 100 mg/mL concentration was 25.4 ± 1.3 mm against *Staphylococcus aureus* (ATCC 25923). The lowest inhibition the antibacterial activity zone was against *Streptococcus sanguis* PTCC1449 at 6.25 mg/mL concentration (6 mm). Moreover, no inhibition zone was observed against *Streptococcus sanguis* at 3.125 mg/mL concentration.

Raja and Venkataraman (2011a) showed that no inhibition zone was observed with water extract of *Tribulus terrestris* L. fruit. However, there were inhibition zones of 11, 15 and 18 mm in ethanol extract of fruits against *Bacillus cereus*, *Enterobacter spp.* and *Escherichia coli*, respectively. There was no effect against *Staphylococcus aureus*, *Aeromonas spp.*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Citrobactor spp.* However, chloroform extract had 14 mm inhibition zone against *Klebsiella pneumoniae*.

Kianbakht and Jahaniani (2003) evaluated the *in vitro* antibacterial activity of methanol extracts of different parts (fruits, stems plus leaves and roots) of *Tribulus terrestris* L. growing in Iran against four bacteria. The methanol extract of fruits showed inhibition zones of 27.8, 14.6, 25.7 and 26.2 mm; stems plus leaves 28.2, 15.7, 22.3 and 23.8 mm; and root 26.8, 14.3, 20.9 and 21.3 mm against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively.

Kiran et al. (2011) reported the antibacterial activity of aqueous (10-50%) and solvent (500, 1000, 1500 and 2000 ppm) extracts of *Tribulus terrestris* L. leaves against gram-positive (*Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*). Among the six pathogens tested, the maximum inhibition (31.0 mm) was observed for *B. Cereus* in aqueous extract. It was followed by *E. coli* (29.0 mm), *S. aureus* (27.0 mm), *P. aeruginosa* (23.0 mm), and *B. subtilis* (19.0 mm). The least inhibition was observed against *S. typhi* (18.0 mm). Among the four solvents tested, the methanol extract showed a maximum inhibition against *B. cereus* (33.0 mm), *E. coli* (31.0 mm) and *S. aureus* (29.0 mm) at 2000 ppm.

Dastagir et al. (2012) evaluated the antibacterial activity of methanol and n-hexane extracts of *Tribulus terrestris* L. whole parts. The inhibition zones against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* were 12, 17, 10, 19 and 19 mm, respectively. The inhibition zones for hexane extracts against same microorganisms tested were 25, 29, 30, 12 and 15 mm, respectively.

Sharma et al. (2013) tested the antibacterial activity of *Tribulus terrestris* L. leaf and fruit extracts and found inhibition zones ranging from 8 to 23 mm. The antibacterial activity on methanol extract of fruits was the highest against *P. vulgaris* (23 mm), *Staphylococcus aureus* (19 mm) and *E. faecalis* (16 mm). The least inhibition zone in aqueous extract of leaves was against *E. faecalis* (8 mm) and *E. cloacae* (9 mm).

Dıđrak et al. (2001) reported that the extracts of *Rhus coriaria* fruits were found to be effective against *Bacillus megaterium* DSM 32, *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, the *R. coriaria* did not have any effect against *E. coli* and *Candida albicans* CCM314.

2.5.3. The microorganisms tested in this study

2.5.3.1. *Klebsiella pneumoniae*

Klebsiella pneumoniae is gram-negative and a member of Enterobacteriaceae family. It is non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped and found in normal flora of mouth, skin, and intestines. It was recognized as a cause of community-acquired pneumonia. It is clinically the most important member and closely related to *Klebsiella oxytoca* distinguished by indole-negative and has ability to grow on both melezitose and 3-hydroxybutyrate (Njugu, 2011).

2.5.3.2. *Escherichia coli*

Escherichia coli is a gram-negative bacterium of Enterobacteriaceae family. It forms rod-shaped cells of 2.0-6.0 µm in length and 1.1-1.5 µm in width with rounded ends. These organisms are non-spore-forming and motile by peritrichous flagella and some are non-motile (Njugu, 2011). They are facultative anaerobes and produce gas from fermentable carbohydrates. They are capable of fermenting carbohydrates and producing both acid and gas (Abbott et al., 2003).

2.5.3.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is motile and rod-shaped. It is gram-negative and occurs as single bacteria, in pairs, and occasionally in short chains. They sometimes produce blue, yellow, or rust-colored pigments. The blue pigment, pyocyanin, is produced only by *P. aeruginosa*. Fluorescin, a yellow pigment is produced by *P. aeruginosa* and other less pathogenic *Pseudomonas* species (Jouda, 2013).

2.5.3.4. *Bacillus megaterium*

Bacillus megaterium is gram-positive and described by De Bary (1884). Due to its eponymous size (Greek: “big animal”) of 1.5 x 4 µm, this microorganism belongs to larger bacteria. Due to its dimensions in vegetative form and spores, it is suited for morphological studies, such as cell wall and cytoplasmic membrane biosynthesis, sporulation, spore structure and organization (Vary, 1992).

2.5.3.5. *Staphylococcus aureus*

Staphylococcus aureus is a spherical, gram-positive, non-motile, non-spore forming, occur singly or grouped in pairs, and grape-like clusters. Some *S. aureus* strains produce microcapsules. The colonies of most strains are pigmented, ranging from gray to deep golden yellow. Colonies of *S. aureus* on trypticase soy agar or blood agar are opaque and produce toxin that causes a wide zone of clear hemolysis on blood agar (Brooks et al., 2007).

2.5.3.6. *Bacillus subtilis*

Bacillus subtilis is a ubiquitous bacterium, rod-shaped, gram-positive, motile, and aerobic. Members of the genus are capable of producing endospores that are highly resistant to unfavorable environmental conditions. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. The organism is not biologically active under most conditions but exists in the spore form. It is not considered pathogenic or toxigenic to humans, animals and plants (EPA, 1997).

2.5.3.7. *Enterococcus faecium*

Enterococcus faecium is a gram-positive and spherical cell that can occur in pairs or chains. The cells are non-motile, facultative anaerobic and alpha-hemolytic (Kang and Lee, 2005). The cells have a thick peptidoglycan layer along with teichoic and lipoteichoic acids. It has circular DNA as well as several plasmids (Van Wamel et al., 2007; Gülhan et al., 2007).

2.5.3.8. *Micrococcus luteus*

Micrococcus luteus is obligate aerobic, gram-positive cocci with ability of surviving in diverse and stress conditions. Alexander Fleming first isolated it in 1929 as *Micrococcus lysodeikticus* (Umadevi and Krishnaveni, 2013). *M. luteus* oxidizes carbohydrates to CO₂ and water. It does not produce acid from glucose as well not make arginine dihydrolase or b-galactosidase. *M. luteus* produces yellow colonies (Smith et al. 1999).

2.5.3.9. *Candida albicans*

Candida albicans is thought to be the major fungal pathogen of humans. Severe *Candida* infections cause weakness in immune defense mechanism of individuals. *C. albicans* can colonize on skin and mucosal surfaces of healthy people. Therefore it occurs commensally in gastrointestinal tract, oral cavity and vagina infections. Moreover, it can enter the bloodstream by direct penetration from the epithelium after tissue damage, or by dissemination from biofilms formed on medical devices (catheters, dental implants, endoprostheses, artificial joints or central nervous system shunts) (Karkowska-Kuleta et al., 2009).

2.5.3.10. *Candida utilis*

Candida utilis is a yeast strain that can be utilized as a food substance. Other names of this strain are *Torula yeast*, *Torula utilis*, and *Torulopsis utilis*. *Candida utilis* grows rapidly and uses almost all of the carbohydrates. It has high level of protein than other food yeast (Lukic-Grlic et al., 2011). As a pathogen, *C. utilis* has been reported as a rare agent of fungemia (Alsina et al., 1988).

2.5.3.11. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular microorganism, ellipsoid shape and multi-polar budding. Yeasts have been known for many years and used in fermentation processes. Recently, the scientific research and technology allow the isolation, construction and industrial production of yeast strains for baking and fermentation industry (beer, wine, cider and distillates) (Bekatorou et al., 2006).

2.5.3.12. *Yarrowia lipolytica*

Yarrowia lipolytica is one of the most extensively studied yeasts, being a strictly aerobic microorganism. It is capable of producing important metabolites and having a secretory activity. This yeast became a reference in research dealing with non-polar substrate metabolism in last years. Many industrial applications of *Y. lipolytica* have been proposed. One of the most important products secreted by this microorganism is lipase, which can be induced by many substrates, including olive mill wastewaters (Gonçalves et al., 2009).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Raw material

The roots of *Tribulus terrestris* L. were collected in July 2014 from Shewashok locality of Koysinjak-Erbil city in Northern Iraq. The plant was identified by Assist. Prof. Dr. Abdulghani Omer Sarmamy from Department of Biology, Faculty of Science, Salaheldin University in Erbil, Iraq.

3.1.2. The chemical substances and materials

The purpose of chemical substances and materials used in the study was presented in Table 3.1.

Table 3.1. The chemical substances and materials used

Substances	Purpose
N-butanol	Determination of condensed tannin
Fe ₂ SO ₄	Determination of condensed tannin
Mimossa	Used for standard reference
Methanol (M)	Used for sample extraction
Ethanol (E)	Used for sample extraction
Distilled water (W)	Used for sample extraction
SDA	Used for fungi cultivation
DPPH	Estimating antioxidant activity
BHT	Determination of antioxidant
MHA	Used in antimicrobial susceptibility testing
Petri dish	Used for culturing bacteria and fungi
Micro pipette	Transporting smaller volumes of extracts
Test tube	Hold and mix small quantities of extracts
Nutrient Broth	Used for microbial cultivation
Whatman No.1	Used for sample filtration

3.1.3. The microorganisms tested in this study

All bacteria and fungi used in the study were obtained from microbiology laboratory of Biology Department in KSU Kahramanmaraş, Turkey. The names and the types of tested microorganisms were presented in Table 3.2.

Table 3.2. The microorganisms used in this study

Microorganisms	Type
<i>Klebsiella pneumoniae</i> (Kp) FMC5	Gram-negative bacteria
<i>Escherichia coli</i> (Ec) DM	Gram-negative bacteria
<i>Pseudomonas aeruginosa</i> (Pa) DSM50071	Gram-negative bacteria
<i>Bacillus megaterium</i> (Bm) DSM32	Gram-positive bacteria
<i>Staphylococcus aureus</i> (Sa) Cowan1	Gram-positive bacteria
<i>Bacillus subtilis</i> (Bs) IMG22	Gram-positive bacteria
<i>Enterococcus faecium</i> (Ef) Clinical isolate	Gram-positive bacteria
<i>Micrococcus luteus</i> (Ml) LA2971	Gram-positive bacteria
<i>Candida albicans</i> (Ca) ATCC1023	Fungi
<i>Candida utilis</i> (Cu) NRRL-Y-900	Fungi
<i>Saccharomyces cerevisiae</i> (Sc) WET136	Fungi
<i>Yarrowia lipolytica</i> (Yl) NCIM3589	Fungi

3.1.4. The instruments used in this study

The instrument names and their usage were listed in Table 3.3.

3.2. Methods

3.2.1. Preparation of sample

Tribulus terrestris L. roots were dried at room temperature. The roots were crushed by blender to form powder, then sieved to different mesh size and stored in a box until analysis.

Table 3.3. List of instruments used here and their usage

Name of instrument	Purpose
Microwave NEOS system	Used for extraction
Dionex ASE 350	Used for extraction
Shimadzu UVvis 1240 spectrophotometer	Determination of condensed tannin Determination of antioxidant activity
Vortex mixer	Used for mixing reagents and samples
Oven	Drying
Incubator	For growing microorganism
Electric blender	Used for sample crushing
Gravimeter	Used for weighing sample
Nexera model Shimadzu UHPLC	Used for identification of phenolic compounds
Hiedolph evaporator	Solvent removal by evaporation
Autoclave OT O12	Pressurized steam for destroying microorganisms

3.2.2. Extraction

3.2.2.1. Microwave extraction method

The extraction of *Tribulus terrestris* L. was carried out according to Laghari et al. (2011) using Microwave milestone NEOS system with slight modification. Ten gram of sample was mixed with 100 mL of methanol (95%), ethanol (95%) and water separately in beakers for stirring. The extraction was performed for 30 min at different temperatures and powers for solvents. At the end of extraction, the liquid was separated from the solid residue by filtration using Whatman No.1 filter paper. The liquid extract was collected and then evaporated by using rotary vacuum evaporator.

3.2.2.2. Conventional extraction method

The extraction of *Tribulus terrestris* L. roots was carried out according to the method of Crozier et al. (1997) with some modifications. Briefly, powder sample of the

roots (10 g) was mixed with 100 mL of methanol in a 250 ml conical flask. The extraction was performed at 40 °C for 2 h. At the end of extraction, the liquid extract was separated from the solid residue by filtration using Whatman No.1 filter paper. Finally, the liquid was collected and then evaporated by using a vacuum rotary evaporator for getting purified extract. The ethanol and water extracts were also performed with same preparation, respectively.

3.2.2.3. Accelerated solvent extraction (ASE) method

The extraction of *Tribulus terrestris* L. roots was carried out according to method of Comlekcioglu et al. (2013) using Dionex ASE 350. Dried powder roots (10 g) were weighed and put in a specific flask with a glass fiber filter (10 mm). Extraction was carried out automatically with ethanol and methanol separately at 60 °C for 40 min pressure. At the end of extraction, the liquids were collected and evaporated by using rotary vacuum evaporator for getting purified extract.

3.2.3. Determination of yield extract

The percentage yield extract of *Tribulus terrestris* L roots was obtained by the following formula for each one of the extraction techniques mentioned in section 3.2.2 (Anokwuru et al., 2011):

$$\text{Percentage yield} = \left(\frac{W_2 - W_1}{W_0} \right) \times 100 \quad (3.1)$$

where,

W₂ is the extract dry weight with container (g), W₁ is the weight of container alone (g) and W₀ is the oven dry weight of sample (g).

3.2.4. Determination of total condensed tannin in *Tribulus terrestris* L. roots

Total tannin determination of *Tribulus terrestris* L. roots was carried out according to the method of Makkar et al. (1995) using spectrophotometer (Shimadzu UV-vis 1240). Extraction solution was prepared by mixing 0.05 g of Fe₂SO₄, 95 mL N-butanol and 5 mL HCl (35%). For evaluating the condensed tannin, TT roots and mimosa tannin were weighed (0.01 g) separately and put in a test tube. Then, 10 mL of extraction solution was added to each tube and placed in a water bath for 1 h. Finally, 1 mL of extraction solution

was taken and measured at 580 nm wavelength of absorbance. The mean values of condensed tannin were calculated by three replications of the concentration.

3.2.5. Identification and quantification of phenolic compounds in *Tribulus terrestris* L. roots by LC-MS/MS

LC-MS/MS analysis of the phenolic compounds was performed using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument as shown in Figure 3.1. 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 40 °C. 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) (Ertas et al., 2015).



Figure 3.1. Nexera model Shimadzu UHPLC

The MS instrumentation: MS detection was performed using Shimadzu LC-MS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC-MS/MS data was collected and processed by Lab-Solutions software (Shimadzu, Kyoto, Japan).

After different combinations of trials, gradient elution was achieved using two solvents as water (5 mM ammonium formate and 0.1% formic acid) and methanol (5 mM ammonium formate and 0.1% formic acid). The most commonly used atmospheric pressure ionization sources are electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization. Electrospray ionization source was chosen since the phenolic compounds are small and relatively polar molecules. Furthermore, liquid chromatography tandem mass spectrometry was decided to used in current study due to its ion fragmentation stability (Ertas et al., 2014a and 2014b).

In this study, 24 phenolic compounds (flavonoids, phenolic acids, phenolic) and 3 non-phenolic organic acids were quantified in *Tribulus terrestris* L. Rectilinear regression equations and the linearity ranges of the studied standard compounds were given in appendix Table A1 (Ertas et al., 2014c). The limit of detection (LOD) and limit of quantitation (LOQ) for reported analytical method was presented in appendix (Table A1). The results were calculated by the equation below:

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

where,

R is the LC-MS/MS results ($\mu\text{g/g}$),

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

3.2.6. Antimicrobial activity of *Tribulus terrestris* L. root extracts

3.2.6.1. Culture of test microorganisms

For cultivation of the microorganisms, Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) were used for bacteria and fungi, respectively. Then, petri plates were inoculated with 10 μl of bacteria and 20 μl of fungi, respectively.

3.2.6.2. Disc diffusion agar

The antimicrobial activities of the root extracts were tested against 8 bacteria and 4 fungi mentioned in Table 3.2. One hundred fifty μl of each *Tribulus terrestris* L. root extract (20 mg/mL) was absorbed to sterile disc with 10 mm in diameter. Subsequently, discs prepared from plant extracts were placed on these inoculated media and kept at room

temperature for diffusion. The methanol, ethanol and water were used as negative controls. Two standard synthetic antibiotic, Ampicillin (AM-10 µg/mL) and Gentamicin (GN-10 µg/mL) were used as positive controls against bacteria. In addition, discs impregnated with 150 µl of Flukozanol and Itraknazol were prepared as antifungal antibiotics. All petri dishes were incubated at 37 °C for 18-24 h, except for fungi which were incubated at 27 °C for 24-48 h. The inhibition zones were measured by a ruler and recorded as the mean diameter of three replications (Shankar et al., 2010).

3.2.7. Antioxidant activity of *Tribulus terrestris* L. root extracts

The antioxidant activity of *Tribulus terrestris* L. roots in ethanol and methanol extracts was assessed based on the radical scavenging effect of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical power described by Blois (1958). Briefly, 0.1 mM of DPPH for E and M were prepared. Then 0.1, 0.2 and 0.3 mL of sample solution was mixed with methanol and ethanol in a test tube separately. Then 1 mL of DPPH was added. The mixture was shaken vigorously and allowed to stand in dark at room temperature for 30 min. Later, changes in absorbance were measured at 517 nm wavelength by Shimadzu UV-vis spectrophotometer. The BHT was used as a reference. The optical density was recorded and the DPPH radical scavenging power percentage was calculated using the following formula (Bors et al., 1992):

$$\text{DPPH radical scavenging power (\%)} = \frac{X-Y}{X} \times 100 \quad (3.3)$$

where,

X is the optical density of DPPH,

Y is the optical density of sample and BHT.

3.3. Statistical Analyses

The antimicrobial activity data was analyzed with ANOVA test using SPSS statistical program. The differences were classified as significant at $P < 0.05$ level.

4. RESULTS AND DISSCUSION

4.1. Yield Percentage of *Tribulus terrestris* L. Root Extracts

Biologically active compounds in plants usually occur in low concentrations. The yield determinations of *Tribulus terrestris* L. root extracts in methanol, ethanol and water were presented in Table 4.1.

Table 4.1. The percentage yield of *Tribulus terrestris* L. root extracts

Methods	Solvents	Yield* (%)
Microwave (ME)	M	9.23 ± .15
	E	8.3 ± 0.1
	W	7.3 ± 0.15
Conventional extraction (CE)	M	6.65 ± 0.06
	E	6.7 ± 0.06
	W	6.6 ± 0.1
Accelerated solvent extraction (ASE)	M	12.06 ± 0.1
	E	10.03 ± 0.1
	W	NT

Note: W: water; E: ethanol; M: methanol; NT: not tested

*The values presented as mean ± SD of three measurements

A wide range of yields among extracts was observed depending on the extraction methods and solvents. The lowest extraction yield of *Tribulus terrestris* L. roots was 6.6% in water by using conventional extraction, whereas the highest extraction yield was observed in methanol with ASE (12.06%) as shown in Table 4.1. The variations in extract yields with different solvents might be explained by the polarity of extracted components and solvents applied (Hsu et al., 2006; Shabir et al., 2011). The results showed that among the extraction methods, the highest yield was obtained in methanol with ASE method. This finding was agreed with previous study (Chatha et al., 2006) where the maximum extract yield from rice bran was obtained by methanol.

An extraction technique should provide extracts with high yield and minimal change for functional properties of extract required (Quispe Candori et al., 2008).

4.2. Determination of Total Condensed Tannin in *Tribulus terrestris* L. Roots

Table 4.2 illustrates the total condensed tannin concentration of *Tribulus terrestris* L. roots analyzed with standard mimosa calibration. The most common standard used in this assay is mimosa tannin calculated by the regression equation ($y = 66.35x + 0.411$, $R^2 = 0.999$) obtained from linear calibration curve (Figure 4.1).

Table 4.2. Total condensed tannin concentration of *Tribulus terrestris* L. roots

Experiment	Condensed Tannin Concentration (mg/L)	Average mg/L	Standard Deviation	Variation
1	9.90	10.83	±1.39	12.79
2	10.17			
3	12.42			

Condensed tannins are polymeric flavonoid molecules that are found in higher plant species. These secondary compounds have particular value in forage crops. Their presence in herbage legumes up to 3-4% dry weight is regarded as a beneficial agronomic trait (Robbins et al., 1998).

The average total condensed tannin percentage of *Tribulus terrestris* L. roots was 10.83 mg/L. This result was agreed with the previous literature by Sebata et al. (2005) who evaluated the condensed tannin as 0.039 mg/g in dry matter of TT. In addition, Mohammed et al. (2013) reported low concentration of tannin in ethanol extract of *Tribulus terrestris* L. fruits. On the other hand, many authors supported the presence of tannin in different section of *Tribulus terrestris* L. (Usman et al., 2007; Sanjeeva et al., 2011; Gomathi et al., 2012; Sharma et al., 2013).

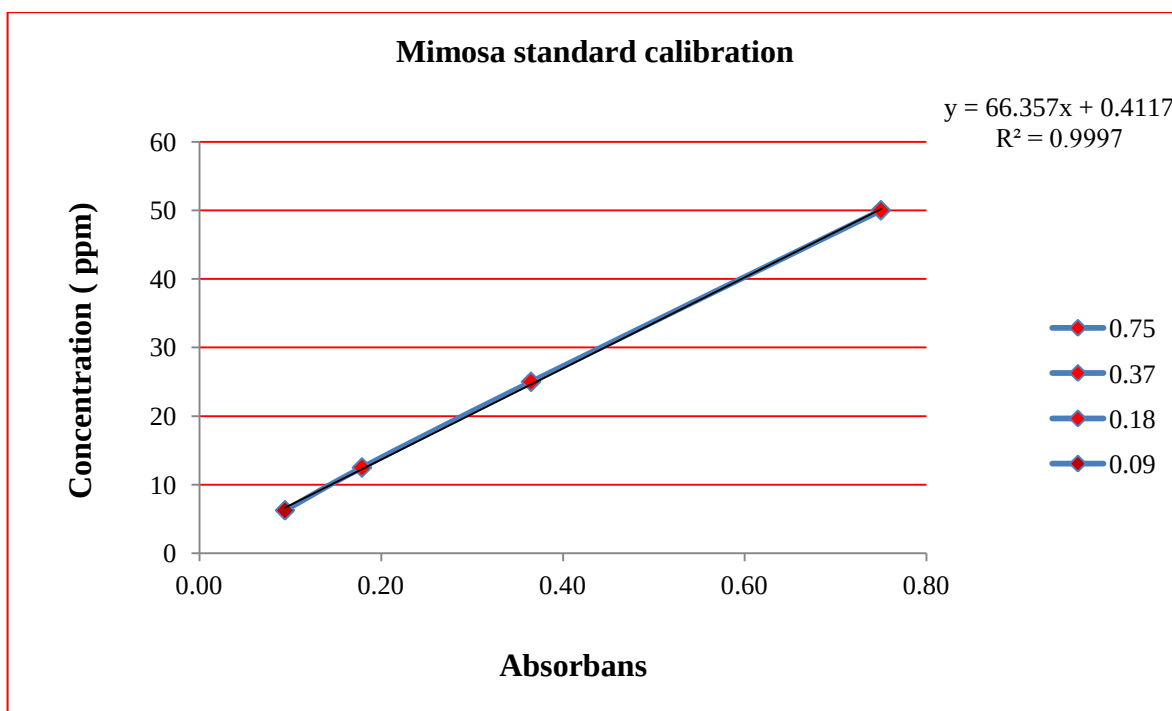


Figure 4.1. Mimosa tannin standard calibration curve

4.3. Identification and Quantification of Phenolic Compounds by LC–MS/MS

Quantitative analysis of phenolic and flavonoid compounds was performed by LC-MS/MS. These compounds have the same general structure with an aromatic hydroxyl nucleus. There are almost 8000 phenolic compounds exist in nature. Phenolic compounds that exist in plants constitute one of the most important groups acting as free radical terminators and primary antioxidants. Plant polyphenols are multifunctional properties and they act as reducing agents, hydrogen atom donors and singlet oxygen scavengers. Besides, being the most diverse and prevalent natural compounds, flavonoids are the most important phenolic structures. Flavonoids have more than 4000 types that exist in roots, flowers, and leaves of plants (Gulcin et al., 2010).

There are many analytical studies for various part of *Tribulus terrestris* L. In the present study, Twenty-seven non-phenolic, phenolic and flavonoid compounds of *Tribulus terrestris* L. in methanol extract were analyzed by a mass spectrometer equipped with triple-quadrupole analyzer. Due to its sensitivity and selectivity for phenolic and flavonoids, negative ionization mode was preferred in the current study. The quantified results in methanol extracts of *Tribulus terrestris* L. root were summarized in Table 4.3.

Table 4.3. LC–MS/MS identification of phenolic compounds in *Tribulus terrestris* L. roots

Compounds		RT ^a	Parent ion(m/z) ^b	LOD/LOQ (µg/L) ^c	U ^f	Quantification (µg /g) ^g
Phenolic acids	Vanillin	8.77	151.05	10.1 / 33.7	4.9	125.002
	Gallic acid	4.29	169.05	4.8 / 15.9	5.1	10.105
	Chlorogenic acid	5.43	353	7.3 / 24.3	4.9	1.462
	tr- caffeic acid	7.37	178.95	4.4 / 14.7	5.2	2.084
	Tannic acid	6.46	182.95	10.2 / 34.2	5.1	98.027
	Protocatechuic acid	5.63	152.95	25.8 / 85.9	5.1	4.048
	p-Coumaric acid	9.53	162.95	15.2 / 50.8	5.1	2.914
	Rosmarinic acid	9.57	358.9	10.4 / 34.8	4.9	ND
	4-OH Benzoic acid	11.72	136.95	3.0 / 10.0	5.2	34.527
	Salicylic acid	11.72	136.95	4 / 13.3	5.0	37.437
Non-Phenolic	Quinic acid	3.32	190.95	22.3 / 74.5	4.8	12.043
	Malic acid	3.54	133.05	19.2 / 64.1	5.3	78.159
	tr-Aconitic acid	4.13	172.85	15.6 / 51.9	4.9	6.628
Flavonoids	Myricetin	11.94	317	9.9 / 32.9	5.9	0.317
	Fisetin	12.61	284.95	10.7 / 35.6	5.5	0.724
	Coumarin	12.52	146.95	9.1 / 30.4	4.9	1.077
	Quercetin	14.48	300.9	2.0 / 6.8	7.1	0.164
	Naringenin	14.66	270.95	2.6 / 8.8	5.5	2.229
	Rutin	10.18	609.1	17.0 / 56.6	5.0	1.528
	Hesperidin	9.69	611.1	21.6 / 71.9	4.9	10.798
	Hyperoside	10.43	463.1	12.4 / 41.4	4.9	7.473
	Kaempferol	15.43	284.95	2.0 / 6.6	5.2	0.467
	Apigenin	17.31	268.95	0.1 / 0.3	5.3	0.477
	Rhamnetin	18.94	314.95	0.2 / 0.7	6.1	0.524
	Chrysin	21.18	253	0.05 / 0.17	5.3	ND
	Hesperetin	15.29	300.95	3.3/ 11.0	5.3	0.227
	Luteolin	15.43	284.95	5.8 / 19.4	6.9	ND

Note: RT^a: Retention time; Parent ion (m/z)^b: Molecular ions of the standard compounds (mass to charge ratio); LOD/LOQ^c (µg/L): Limit of detection/Limit of quantification; U^f (%): Percent relative uncertainty at 95% confidence level (k=2). ; N.D: not detected.

Among 27 compounds, there were 24 non-phenolic, phenolic and flavanoid compounds detected in methanol extracts of *Tribulus terrestris* L. roots. The vanillin was found to be the most abundant phenolic compound (Table 4.3). Many studies suggested that the main components of this plant were saponin, α -Amyrin, alkaloid and flavanoid as described by Wu et al. (1999), Usman et al. (2007), Abirami and Rajendran (2011) and Gomathi et al. (2012), respectively. The differences among results relating to main components of *Tribulus terrestris* L. might due to different geographic sources of plant origin, method techniques and types of solvent used.

The LC–MS/MS analysis of *Tribulus terrestris* L. roots in methanol extract was rich in terms of phenolic acid compounds. It contained high amount of vanillin (125.002 µg/g). While, the low amount of chlorogenic acid was detected and quantified within ten phenolic acids (1.462 µg/g). Rosmarinic acid was not detected and refers to low amount than LOD/LOQ as shown in Table 4.3 and Figure 4.2.

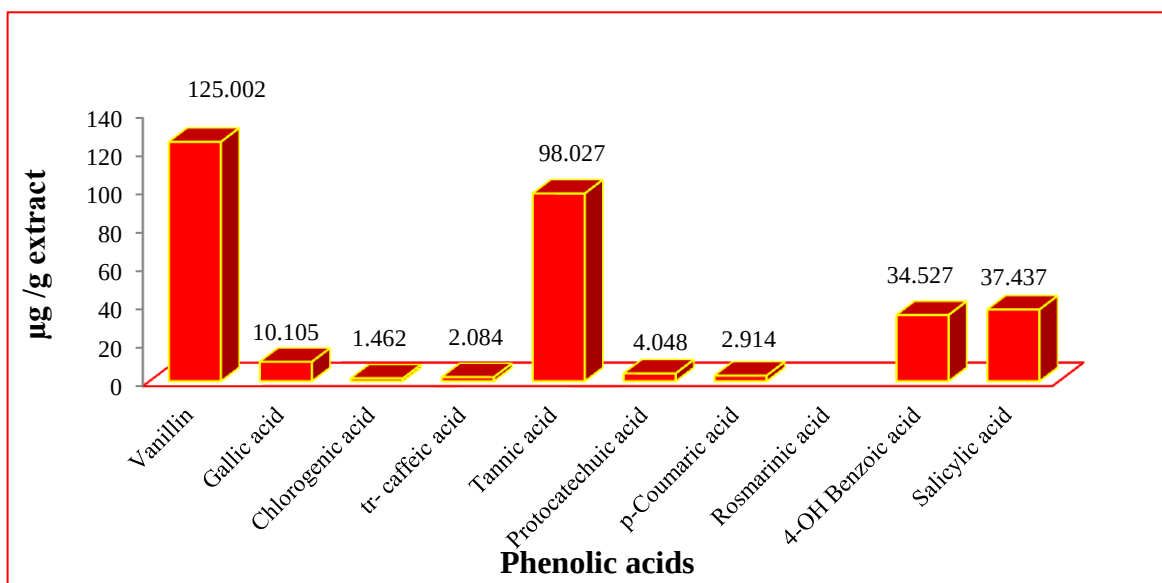


Figure 4.2. LC–MS/MS identification of phenolic acids of *Tribulus terrestris* L. roots

Caffeic, chlorogenic and 4- hydroxybenzoic acids were different than the another research by Reshma et al. (2015). They determined that the HPLC analysis revealed the presence of phenolic compounds like caffeic, chlorogenic and 4-hydroxybenzoic acids. Furthermore, the amount of gallic acid in our study was lower than the study by Mishra and Bisht (2012). The dissimilarity between the results might be due to differences in origin, assay method and plant parts used. Hammada et al. (2013) reported that 4,5-di-p-cis-coumaroylquinic and 4,5-di-p-trans-coumaroylquinic acids isolated in ethanol extract of *Tribulus terrestris* L. had the strongest DPPH free radical scavenging activity.

Abirami and Rajendran (2011) showed that methanol extract of whole plant *Tribulus terrestris* L. revealed the presence of α -Amyrin, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, n-hexadecadienoic acid, hexadecadienoic acid ethyl ester, phytol, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid and 1,2-benzenedicarboxylic acid diisooctyl ester by GC-MS analysis. In addition, there were β -sitosterol and stigma sterol.

Kostova and Dinchev (2005) reported that furostanol and spirostanol saponins of tigogenin, neotigogenin, gitogenin, neogitogenin, hecogenin, neohecogenin, diosgenin,

chlorogenin, ruscogenin and sarsasapogenin were frequently found in this plant. In addition, four sulfated saponins of tigogenin and diosgenin were also isolated. In the present study, furostanol glycosides including protodioscin and protogracillin were present in small quantities. The presence of vanillin in our study was agreed with the study of Wu et al. (1999) who isolated three new compounds. These were terrestribisamide, 25R-spirost-4-en-3,12-dione, and tribulusterine. In addition, 10 other compounds of dried *Tribulus terrestris* L. fruits (*N-p*-coumaroyltyramine, terrestriamide, hecogenin, aurantiamide acetate, xanthosine, fatty acid ester, ferulic acid, vanillin, *p*-hydroxybenzoic acid and β -sitosterol) were also detected.

The LC–MS/MS analyses of non-phenolic acid concentrations ranged from 78.159 to 6.628 $\mu\text{g/g}$. The highest and lowest concentrations were determined for malic and tr-Aconitic acids as shown in Table 4.3 and Figure 4.3, respectively. Hammada et al. (2013) also supported the presence of quinic acid. They reported that 4,5-di-*p*-cis-coumaroylquinic and 4,5-di-*p*-trans-coumaroylquinic acids were isolated from ethanol extract of *Tribulus terrestris* L. In addition, Gopalakrishnan and Vadivel (2011) reported that quinic acid was the major chemical constituent (32.87%) in ethanol extract of *Mussaenda frondosa* by GC-MS analysis. Wu and Zhao (2013) evaluated that amount of malic acid in *Broussonetia papyrifera* root extract was greater than that in *Morus alba* (86% vs. 60%). Malic acid in *Orychophragmus violaceus* was higher than that of *Brassica napus* (81% and 79%).

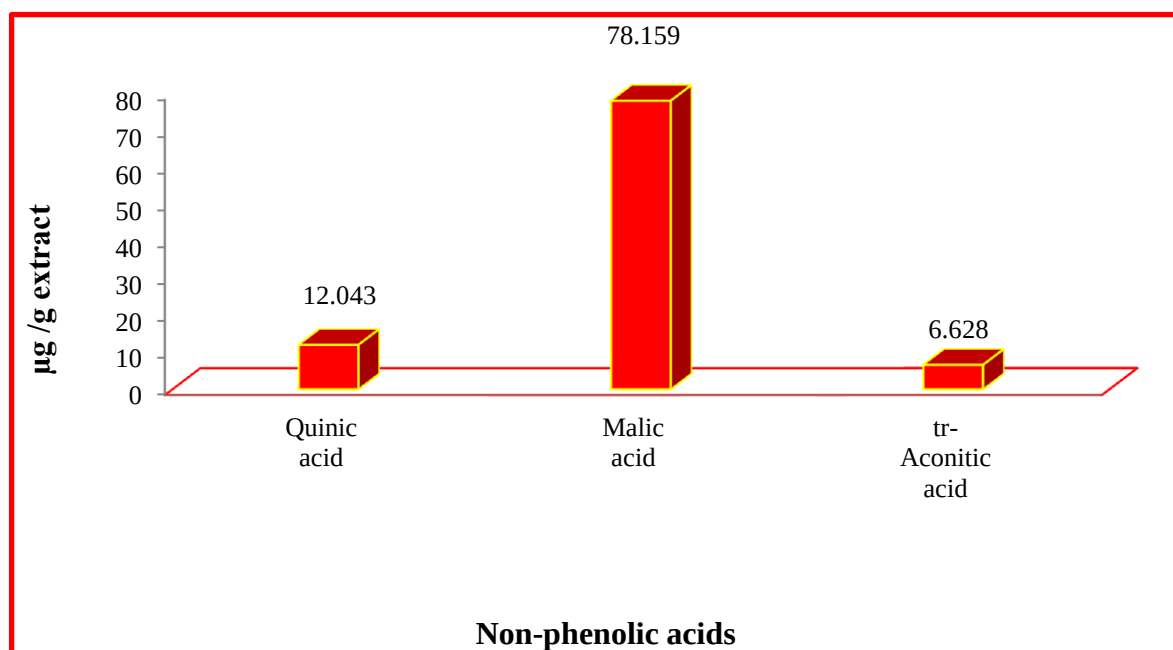


Figure 4.3. LC–MS/MS identification of non-phenolic acids of *Tribulus terrestris* L. roots

The flavonoids are valuable secondary metabolites and effectively used in chemosystematics (Raja and Venkatarman, 2011b). They occur widely in plants as biologically and chemically diverse groups of secondary metabolites for chemotaxonomic surveys of plant genera and families (Mitra, 2012). In the present study, 12 flavanoids were detected and quantified. However, chrysin and luteolin were not detected as shown in Table 4.3 and Figure 4.4. The results showed high and low concentrations of hesperidin (10.798 $\mu\text{g/g}$) and quercetin (0.164 $\mu\text{g/g}$), respectively.

Detection of hesperidin as a main flavanoid in this work was differed from previous literature studied by Mitra (2012) who reported that the concentration of chrysin was the highest in leaf of TT. The absence of chrysin and luteolin in our study was agreed with the study done by Mitra (2012). The quercetin and kaempferol were low in concentration in the current study. However, Qin et al. (1999) found that the quercetin, kaempferol, isorhamnetin, and quercetin had the highest flavonoids in TT. Louveaux et al. (1998) also showed that the *Tribulus terrestris* L. leaf extracts were rich in quercetin glycoside. Table 4.3 illustrates concentrations of apigenin, fisetin, hesperetin and naringenin. This was disagreed with the study of Mitra (2012) who reported the absence of flavanoids in roots, fruits and leaves of TT.

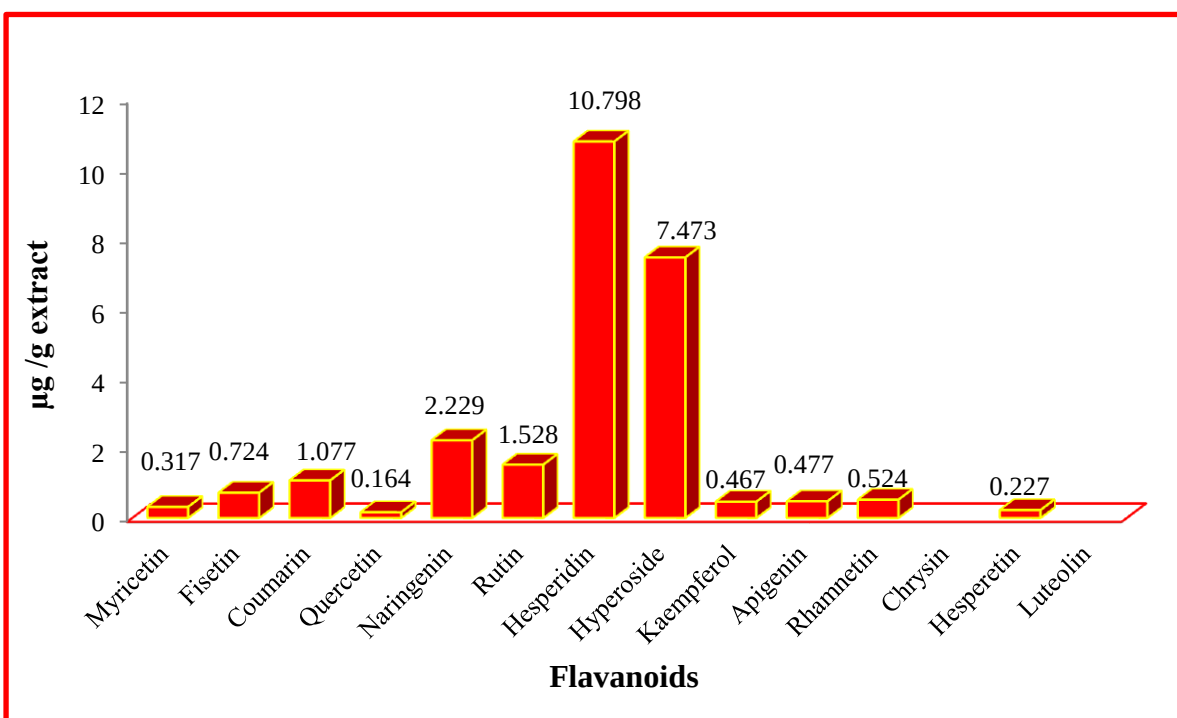


Figure 4.4. LC–MS/MS identification of flavanoids in *Tribulus terrestris* L. roots

The rutin in this study had a lower concentration than that of Ivanova et al. (2014). The rutin concentrations in their study were 1.02, 1.51, 0.34, 0.11 and 0.11 mg/g for Hungary cultivated, Turkey cultivated, Vedral wild growing, Vedral in culture and Plovdiv wild growing, respectively. Furthermore, a high concentration of coumarin in fruit of TT in another study (Mohammed et al., 2013) was disagreed with our result for roots of TT. The variances between current and other studies for flavanoid concentrations might be due to different plant parts, solvents, extraction methods and weight of samples used. Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenols. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties (Patil et al. 2012).

4.4. Antimicrobial Activity of *Tribulus terrestris* L. Root Extracts

The activity of plant extracts on bacteria and fungi has been studied by a large number of researchers in different place of the world. The specific plants used and the methods of application for particular ailments were passed through oral tradition. Plants with possible antimicrobial activity should be tested against some microbes to confirm the activity (Duraipandian and Ignacimuthu, 2011). Thus, the antimicrobial activity at 20 mg/mL of solvent extract of *Tribulus terrestris* L. roots were determined against eight bacteria and four fungi by using disc diffusion method. The different inhibition zones are presented in Table 4.4. Furthermore, the activity of synthetic drug against bacteria and fungi are also presented in Table 4.5. Likewise, the activity of solvents control against bacteria and fungi are summarized in Table 4.6.

The various extracts of *Tribulus terrestris* L. were significant against all the microorganisms tested as presented in Table 4.4 ($P < 0.05$). The *in vitro* antimicrobial screening of the root extracts exhibited high inhibitions against *Micrococcus luteus* LA2971, *Candida albicans* ATCC1023 and *Yarrowia lipolytica* NCIM35. The moderate inhibition zones were found against *Bacillus megaterium* DSM32, *Staphylococcus aureus* Cowan1 *Pseudomonas aeruginosa* DSM50071 and *Bacillus subtilis* IMG22. The low inhibition zones were observed against *Klebsiella pneumoniae* FMC5, *Escherichia coli* DM, *Enterococcus faecium* and *Saccharomyces cerevisiae* WET136. On the other hand, no inhibition was seen against *Candida utilis* NRRL-Y-900 (Table 4.4).

Table 4.4. Antimicrobial activity of *Tribulus terrestris* L. root extracts

Microorganisms		Inhibition zones (mm)*							
		ME			CE			ASE	
		M	E	W	M	E	W	M	E
<i>Kp</i>	Mean	11	11	-	12.67	—	—	11.67	11
	SD	±0.0	±0.0	-	±0.57	—	—	±0.57	±0.0
<i>Ec</i>	Mean	11.67	12	11	11.67	11	—	12.67	11.67
	SD	±0.57	±0.0	±0.0	±0.57	±0.0	—	±0.57	±0.57
<i>Pa</i>	Mean	14.67	11.67	11.0	12.67	12.67	—	14.67	12.67
	SD	±0.57	±0.57	±0.0	±0.57	±0.57	—	±0.57	±0.57
<i>Bm</i>	Mean	12.67	11	12.67	13	12.67	—	13.67	12.67
	SD	±0.57	±0.0	±0.57	±0.0	±0.57	—	±0.57	±0.57
<i>Sa</i>	Mean	11	14.67	11.67	11	13.67	—	11.67	11
	SD	±0.0	±0.57	±0.57	±0.0	±0.57	—	±0.57	±0.0
<i>Bs</i>	mean	11	11	11	12.67	11	—	13.33	13
	SD	±0.0	±0.0	±0.0	±0.57	±0.0	—	±0.57	±0.0
<i>Ef</i>	Mean	—	—	—	11.67	11	11	12.67	12
	SD	—	—	—	±0.57	±0.0	±0.0	±0.57	±0.0
<i>Ml</i>	Mean	19.33	15.67	14.67	15	16	13.67	14.67	14.67
	SD	±0.57	±0.57	±0.57	±1.0	±1.0	0.57	±0.57	±0.57
<i>Ca</i>	Mean	17.67	15.67	11.67	15.67	14.67	11	16.33	15.67
	SD	±0.57	±0.57	±0.57	±0.57	±0.57	±0.0	±0.57	±0.57
<i>Cu</i>	Mean	—	—	—	—	—	—	—	—
	SD	—	—	—	—	—	—	—	—
<i>Sc</i>	Mean	—	11.67	—	—	12	11	—	12
	SD	—	±0.57	—	—	±0.0	±0.0	—	±0.0
<i>Yl</i>	Mean	15.67	16.67	13.67	11	11.67	15.67	11.67	11.67
	SD	±0.57	±0.57	±0.57	±0.0	±0.57	±0.57	±0.57	±0.57

*The values presented as mean ± SD of three replication Mean differences were statistically significant ($P < 0.05$). p-value (0.000) and the value of f-test (5.184);.

Note: M: Methanol; E: Ethanol; W: Water; ME: Microwave extraction; CE: conventional extraction; ASE: Accelerated solvent extraction *Kp*: *Klebsiella pneumoniae* FMC5; *Ec*: *Escherichia coli* DM; *Pa*: *Pseudomonas aeruginosa* DSM50071; *Bm*: *Bacillus megaterium* DSM32; *Sa*: *Staphylococcus aureus* Cowan1; *Bs*: *Bacillus subtilis* IMG22; *Ef*: *Enterococcus faecium* Clinical isolate; *Ml*: *Micrococcus luteus* LA2971; *Ca*: *Candida albicans* ATCC1023; *Cu*: *Candida utilis* NRRL-Y-900; *Sc*: *Saccharomyces cerevisiae* WET136; *Yl*: *Yarrowia lipolytica* NCIM3589; (—) means no inhibition zone; 150 µL/disc (20 mg/mL) extracts were used

Table 4.5. Antibiotic activity against bacteria and fungi

Inhibition zones (mm)*				
Microorganism	Gentamicin	Ampicillin	Flukozanol	Itraknazol
<i>Kp</i>	43.33±1.15	—		
<i>Ec</i>	35.67±1.15	—		
<i>Pa</i>	33.67±0.57	7.0±0.0		
<i>Bm</i>	33.33±0.57	6.33±0.57		
<i>Sa</i>	35.67±0.57	6.0±0.0		
<i>Bs</i>	40.00±1.0	6.67±0.57		
<i>Ef</i>	31.00±1.0	—		
<i>Ml</i>	34.33±0.57	8.66±0.57		
<i>Ca</i>			12.67±0.57	13.00±1.0
<i>Cu</i>			16.33±0.57	18.33±0.57
<i>Sc</i>			13.67±0.57	13.67±0.57
<i>Yl</i>			12.67±0.57	12±0.0

*The values presented as mean ± SD of three replication

Note: (—) means no inhibition zone

Table 4.6. The solvents control activity against bacteria and fungi

Inhibition zones (mm)*			
Microorganism	Methanol	Ethanol	Water
<i>Kp</i>	—	—	—
<i>Ec</i>	—	—	—
<i>Pa</i>	—	—	—
<i>Bm</i>	—	—	—
<i>Sa</i>	—	—	—
<i>Bs</i>	—	—	—
<i>Ef</i>	—	—	—
<i>Ml</i>	—	—	—
<i>Ca</i>	—	—	—
<i>Cu</i>	—	—	—
<i>Sc</i>	—	—	—
<i>Yl</i>	—	—	—

*The values presented as mean ± SD of three replication

Note: (—) means no inhibition zone

The inhibition zones against gram-positive bacteria (*Bacillus megaterium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecium* and *Micrococcus luteus*) ranged from 11 to 19.33 mm (Figure 4.5). The highest inhibition zone was against *Micrococcus luteus* in methanol extract with microwave extraction. The effect of *Tribulus terrestris* L. extracts against *Micrococcus luteus* was also supported by Ali et al. (2015). They reported that *Micrococcus luteus* was inhibited by one of the non-polar fraction. Our inhibition zone result was lower than the result of Upadhyay et al. (2010) where the essential oil from olive inhibited the growth of *Micrococcus luteus* (27.1 mm). The inhibition zone against *M. luteus* was higher than the reference antibiotic ampicillin and lower than gentamicin.

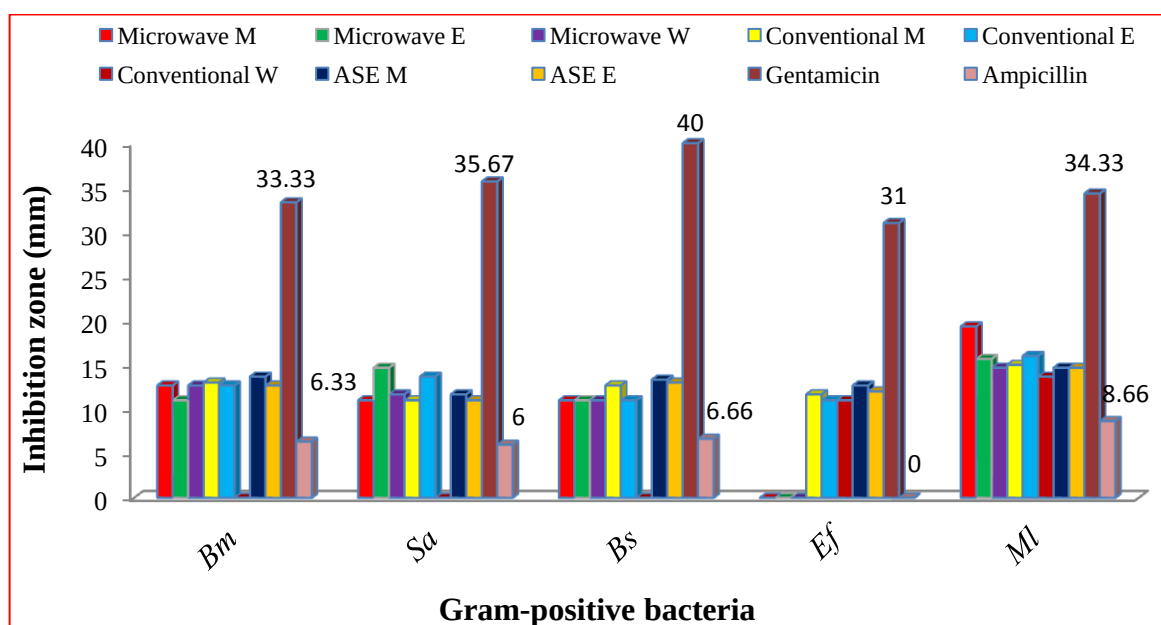


Figure 4.5. Inhibition zones against gram-positive bacteria compared with antibiotic
Note: M: Methanol; E: Ethanol; W: Water; ASE: Accelerated solvent extraction; Bm: *Bacillus megaterium* DSM32; Sa: *Staphylococcus aureus* Cowan1; Bs: *Bacillus subtilis* IMG22; Ef: *Enterococcus faecium* Clinical isolate; Ml: *Micrococcus luteus* LA2971

The highest inhibition zone was against *Micrococcus luteus*. This might be due to the presence of phenolic and flavonoids in the methanol extract. Many authors reported that the phenolic compounds had a good antibacterial activity, such as vanillin (Vaghasiya et al., 2004), quinic acid (Zhang et al., 2013) and flavonoids (Kasim et al., 2011). Antibacterial activity results against gram-positive strains showed that the lowest inhibition zone (11 mm) was obtained by ethanol ME, methanol CE, ethanol ASE, methanol ME, ethanol ME, water ME, ethanol CE and water CE (Table 4.4 and Figure 4.5). The inhibition zones of *Bacillus subtilis* affected by all the extracts were ranged from 11 to

13.33 mm except for water CE having no inhibition zone. This result was lower than that of Mohammed (2008), Mohammed and Mohammed (2008) and Baburao et al. (2009) but similar to the study of Sanjeeva et al. (2011). These variations might be due to different plant parts or methods used.

Staphylococcus aureus was affected by all the extracts at 20 mg/disc with inhibition zones ranged from 11 to 14.67 mm except for water CE with no inhibition. This result was disagreed with other studies by Kianbakht and Jahaniani (2003), Kiran et al. (2011) and Soleimanpour et al. (2015). Zou et al. (2014) concluded that the antimicrobial activity varies from one plant part to another. However, Sanjeeva et al. (2011) and Dastagir et al. (2012) observed a similar growth inhibition pattern. The antibacterial activity results of all the root extracts of TT against *Bacillus megaterium* were lower than that were determined by (Dıđrak et al., 2001).

All *Tribulus terrestris* L. root extracts at 20 mg/disc inhibited the growth of gram-negative bacteria *Klebsiella pneumoniae* (11-12.67 mm), *Escherichia coli* (11-12.67 mm) and *Pseudomonas aeruginosa* (11-14.67 mm). The water ME did not show an inhibition against *E. coli*. In addition, water conventional, ethanol and water microwave extracts did not have any effect against G-ve bacteria were (Table 4.4). The antibacterial activity of methanol extract with conventional extraction showed 12.67 mm inhibitory zone against *Klebsiella pneumoniae*. This was consistent with the studies of Mohammed (2008), Raja and Venkataraman (2011a) and Dastagir et al. (2012). The methanol extract with ASE extraction inhibited the growth of *E. coli* (12.67 mm) as agreed with Usman et al. (2007) and Jindal et al. (2012). Raja and Venkataraman (2011a) indicated a lower antibacterial activity in ethanol extract of TT fruit. Kiran et al. (2011) reported a maximum inhibition against *E. coli* in aqueous (29.0 mm) and methanol extracts (31.0 mm). Dıđrak et al. (2001) reported that the variations between antimicrobial activities might depend on plant species and concentrations.

The antibacterial activity of TT roots against *Pseudomonas aeruginosa* was 14.67 mm of inhibition (Table 4.4). This result was compatible with the results of Mohammed and Mohammed (2008) and Deshwal (2012). On the other hand, it was lower than that of Kianbakht and Jahaniani (2003). These variations might be due to different locations or plants used (Angioni et al., 2006). The water extract of conventional extraction did not have antimicrobial activity against *Pseudomonas aeruginosa* as agreed with Raja and

Venkataraman (2011a). The standard drug gentamicin showed a greater antibacterial activity against gram-negative bacteria than plant extracts. However, the results of plant extracts were higher than the standard drug ampicillin (Figure 4.6).

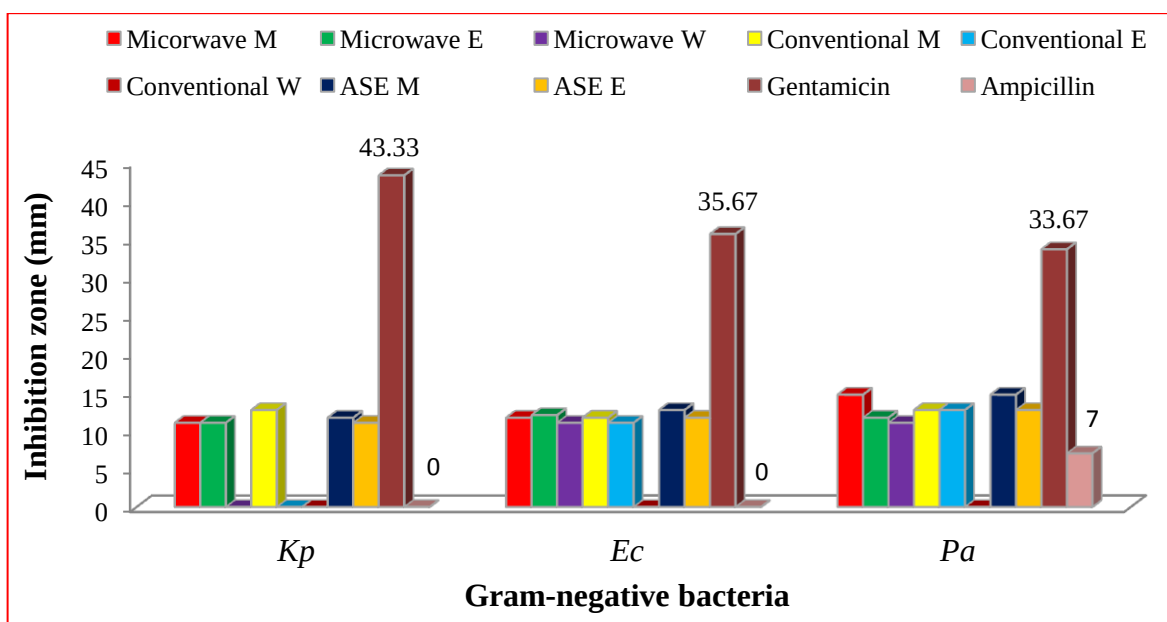


Figure 4.6. Inhibition zones against gram-negative bacteria compared with antibiotic
Note M: Methanol; E: Ethanol; W: Water; ASE: Accelerated solvent extraction; Kp: *Klebsiella pneumoniae* FMC5; Ec: *Escherichia coli* DM; Pa: *Pseudomonas aeruginosa* DSM50071

The plant antimicrobial activity against gram-positive and negative bacteria might be indicative to presence of broad-spectrum antibiotic compounds or general metabolic toxins in the plant. In addition, plant pharmacological metabolites like furostanol and spirostanol saponins (Kostova and Dinchev, 2005), flavonoid glycosides (Saleh et al., 1982), phytosterols and amides (Xu et al., 2000) may be considered for antimicrobial activity. The plants also contain a mixture of b-carboline, harmine, norharmine, tetrahydroharmine, harmine, harmaline, harmol, harmalol, ruin and dihydroruin (Bourke et al., 1990).

All the parts of Turkish and Iranian *T. terrestris* L. (fruits, stems, leaves and roots) showed antibacterial activity against *Enterococcus faecalis*, *S. aureus*, *E. coli* and *P. aeruginosa* (Abbasoğlu and Tosun, 1994; Kianbakht and Jahaniani, 2003) in contrast to aerial parts of Yemeni *T. terrestris* which had no antibacterial activity against these bacteria (Awadh- Ali et al., 2001). It can be argued that antibacterial activity of Northern Iraqi *Tribulus terrestris* L. can be similar to Turkish and Iranian *Tribulus terrestris* L. Different results concerning the antibacterial activity of TT might be due to different

geographic sources of plant, different types of strains and different assay methods used (Alo et al., 2012; Okwu et al., 2014).

Regarding antifungal activity, all the extracts from *T. terrestris* L. roots were resisted by *Candida utilis*. The strongest antifungal activity of *T. terrestris* L. roots was observed for methanol extract using microwave extraction with an inhibition zone of 17.67 mm (Table 4.4). It was followed by ethanol extract with microwave extraction of *Yarrowia lipolytica* (16.67 mm). The weakest inhibition zone was against *Saccharomyces cerevisiae*. The results showed that methanol and ethanol extracts of *T. terrestris* L. roots had potent antifungal activity as supported by (Al-Bayati and AL-Mola, 2008).

The antifungal activity of *T. terrestris* L. may be attributed to various chemicals detectable in its extracts, such as saponins (Zhang et al., 2006) or vanillin (Fitzgerald et al., 2005). The action mechanisms of saponins may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death (Mshvildadze et al., 2000). Also the antifungal activity of gallic acid has been reported by Akiyama et al. (2001) and Panizzi et al. (2002). All the results are presented in Table 4.4. It was observed that the inhibition zone against *C. albicans* was higher than both Flukozanol and Itraknazol. Additionally, the antimicrobial activity of TT root extracts against *Saccharomyces cerevisiae* and *Yarrowia lipolytica* had relative value with both standard drugs (Figure 4.7).

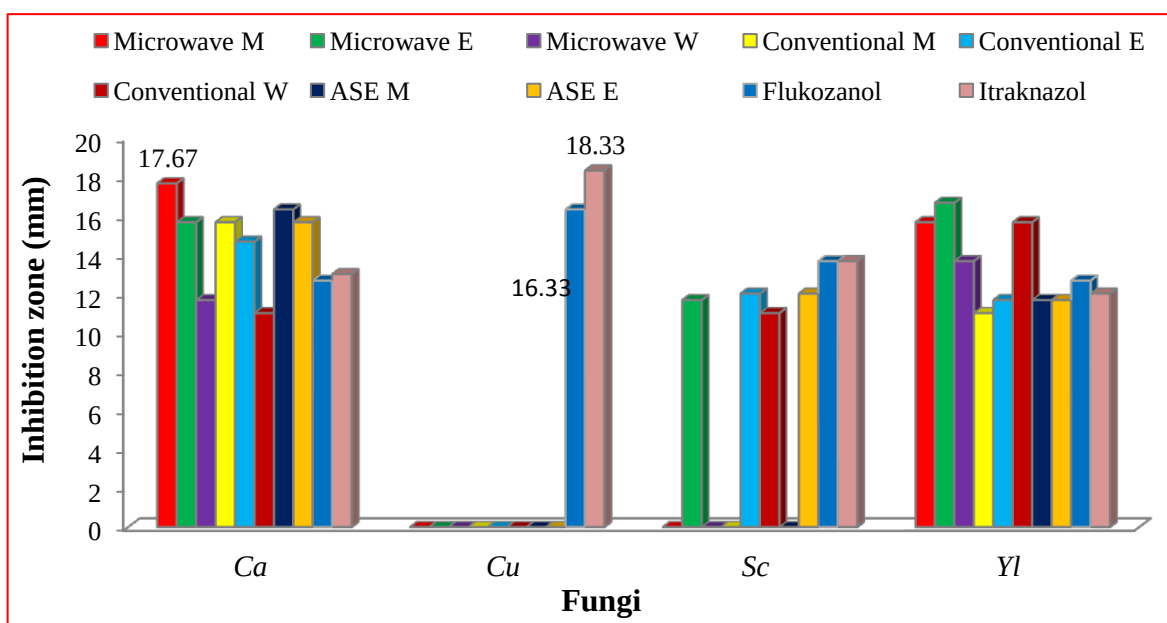


Figure 4.7. Antifungal activity of root extracts compared to antibiotic

Note: Ca: *Candida albicans* ATCC1023; Cu: *Candida utilis* NRRL-Y-900; Sc: *Saccharomyces cerevisiae* WET136; Yl: *Yarrowia lipolytica* NCIM3589; M: Methanol; E: Ethanol; W: Water; ASE: Accelerated solvent extraction

It was observed that different extracts of TT roots exhibited high, moderate and weak antimicrobial activities against tested bacteria and fungi. The methanol, ethanol and water extracts showed high, moderate and weak antimicrobial activities against most of the tested organisms. It was observed that plant extractions with organic solvents provided higher antimicrobial activity than those extracted with water. This could be explained by different active compounds been extracted with any solvents. The results supported the idea that water is not apposite for extraction of antimicrobials from plants compared to other solvents, such as methanol or ethanol (Karaman et al., 2003; Moniharapon and Hashinaga, 2004; Parekh et al., 2005).

4.5. Antioxidant Activity of *Tribulus terrestris* L. Root Extracts

The DPPH is a stable free radical at ambient temperature and often used to evaluate the antioxidant activity of several natural compounds. It accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Reduction capability of DPPH radicals is determined by the decrease in its absorbance at 517 nm. An antioxidant interacting with DPPH, either transfers electron or hydrogen to DPPH or neutralizes its free radical character. This electron becomes paired in the presence of a free radical scavenger, absorption vanishes and resulting decolourisation with respect to number of electrons taken up (Banerjee and Bonde, 2011). The scavenging power of methanol and ethanol extracts of TT root examined and compared against BHT is presented in Table 4.7.

Table 4.7. DPPH scavenging power of *Tribulus terrestris* L. root extracts

Methods	Solvents	DPPH Radical Scavenging Power (%)					
		Extract volume (ml)			BHT volume (mL) ¹		
		0.1 mL	0.2 mL	0.3 mL	0.1 mL	0.2 mL	0.3 mL
Microwave ²	M	79.68	80.47	83.01	91.0	92.0	90.5
Conventional ³	M	79.52	82.93	84.52	91.0	92.0	90.5
ASE ⁴	M	83.73	82.38	85.23	91.0	92.0	90.5
Microwave ⁵	E	77.11	79.44	80.18	68	66.4	78.0
Conventional ⁶	E	76.0	76.18	76.93	68	66.4	78.0
ASE ⁷	E	78.51	78.32	79.81	68	66.4	78.0

Note: ASE; Accelerated solvent extraction method; M: methanol; E: ethanol; BHT: Butylated hydroxytoluene; ¹:53 mg/L; ²:0.0073 mg/mL; ³: 0.0101 mg/mL; ⁴: 0.0131 mg/mL; ⁵:0.0073 mg/mL; ⁶:0.0091 mg/mL; ⁷:0.0109 mg/mL;

The data showed the highest DPPH scavenging power of *Tribulus terrestris* L. (85.2%) determined with methanol extract of ASE method. Whereas, the lowest DPPH scavenging power (76.0%) was observed for ethanol extract with conventional extraction method. Pietrzak et al. (2014) investigated that ASE had more effective scavenging capability than other extraction methods. The DPPH scavenging power of ethanol fraction in the present study was higher than the BHT used as positive control. However, the DPPH scavenging power of methanol extract was lower than BHT (Table 4.7). The results of current study based on different solvents used were disagreed with the study of Eagappan et al. (2015).

On the other hand, when the results compared to standard reference BHT they were agreed with other researchers Zheleva-Dimitrova et al. (2012) and Hemalatha and Hari (2013). The ethanol extracts of TT roots had a potent antioxidant activity with different extraction methods. This might be due to presence of polyphenolic compounds in *Tribulus terrestris* L roots. High phenolic compounds in TT extract could be taken into account for strong free radical scavenging activity. This may refer to presence of vanillin in methanolic extract of the roots. The TT root extract has a good antioxidant activity and reported by Tai et al. (2011). Manach et al. (2004) and Giovanelli and Buratti (2009) have reported the bioavailability of poly-phenols. It has been known that total poly-phenols (including phenolic acids, flavonols and anthocyanins) are highly correlated with antioxidant activity. This antioxidant activity might be due to presence of either gallic (Li et al., 2000) or malic acids (Mokbel and Hashinaga, 2005). High phenolic compounds in TT root extracts may suggest that further research should be performed for isolation and identification of the individual antioxidant components in *Tribulus terrestris* L. (Lokhande et al., 2014).

5. CONCLUSIONS

This study was involved to determine the yield percentage and total condensed tannin concentrations of TT extracts. The identification of phenolic compounds and biological activity of *Tribulus terrestris* L. roots, such as antioxidant and antimicrobial activities were also studied.

The methanol extract of accelerated solvent extraction technique has better yields than that were obtained by other extracts. Moreover, there was total condensed tannin present in TT root extracts. There were twenty-seven compounds identified in methanol extract of *Tribulus terrestris* L. roots by LC-MS/MS analysis. The most abundant phenolic was vanillin.

The highest DPPH scavenging power was found in methanol extract obtained by ASE method. Whereas, the lowest DPPH scavenging power was observed in ethanol extract obtained by conventional extraction method. The DPPH scavenging power of all ethanol extract fractions was higher than that of BHT reference. This plant (TT) is a possible natural antioxidant source and might be useful in therapy of free radical pathology. Therefore, this research is useful for clarifying the pharmacological activity of *Tribulus terrestris* L. as a biological therapy.

The antimicrobial activity of the extracts was evaluated based on disc diffusion method. The results indicated that the different extracts of *Tribulus terrestris* L. showed good antibacterial and antifungal activities with stated methods. Among the extracts, the methanol extract with microwave extraction exerted a good antibacterial inhibition zone against *Micrococcus luteus* and antifungal activity against *Candida albicans*.

The present study tends to confirm the use of selected plant's roots in folk medicine as being safe and useful for treating several diseases caused by microorganisms. The results indicate that the microwave assisted extraction method can be a viable alternative for traditional extraction methods. The presence of phenolic compounds and tannins in roots may be responsible for these activities. Hence, further study is required to isolate and characterize new phyto-constituents responsible for these activities.

The Northern Iraq originated *Tribulus terrestris* L. demonstrated an antimicrobial activity against the most prevalent gram-negative bacteria. Furthermore, it may help to

discover new chemical classes of antibiotics that could serve as selective agents for maintenance of animal and human health.

Consequently, consumption of *Tribulus terrestris* L. protects people against oxidative stress and pathogenic microorganisms without any side effect. However, further *in vivo* studies are needed to understand the possible activities of this plant in biological systems. The presence of various compounds in TT roots might be justified by traditional practitioners for potential source of natural bioactive chemicals.

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APPENDIX

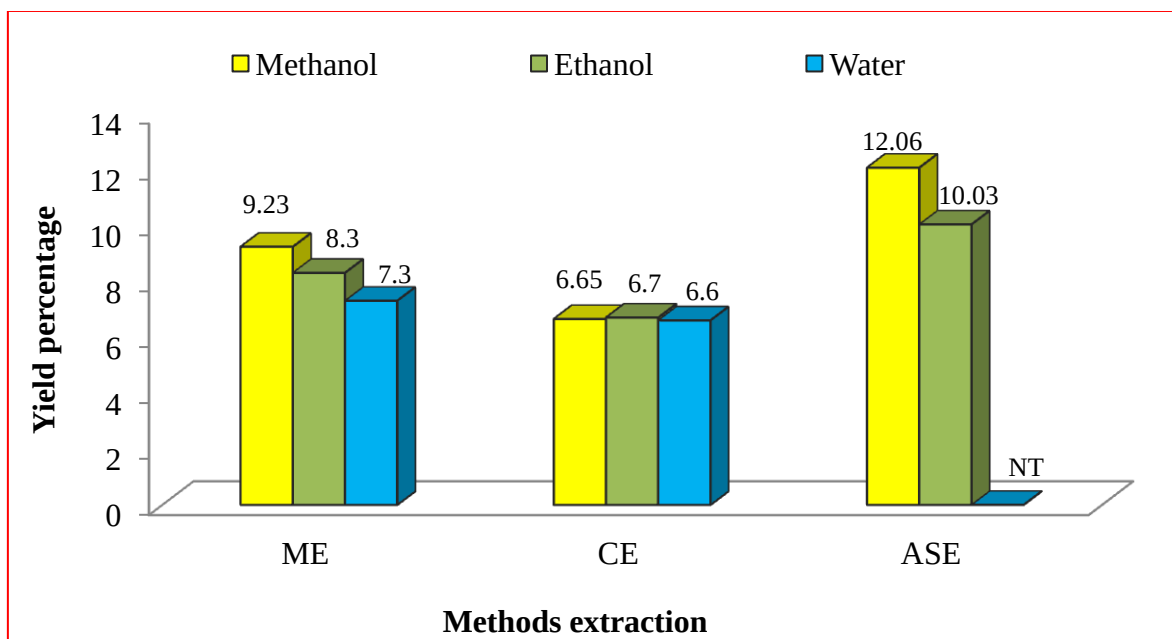


Figure A1. The yield percentage of *Tribulus terrestris* L. roots

Note: CE: conventional extraction; ME: Microwave extraction; ASE: Accelerated solvent extraction



Figure A2 . The yeild extract of *Tribulus terrestris* L.

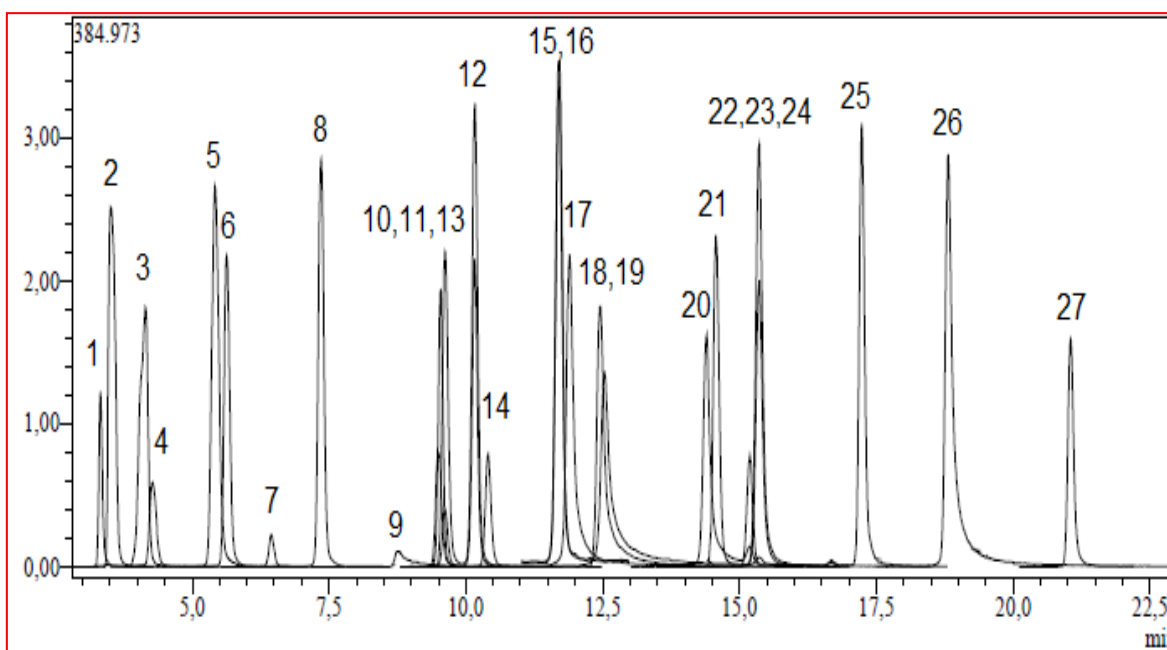


Figure A3. Illustration of LC-MS/MS chromatogram reference calibration

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.

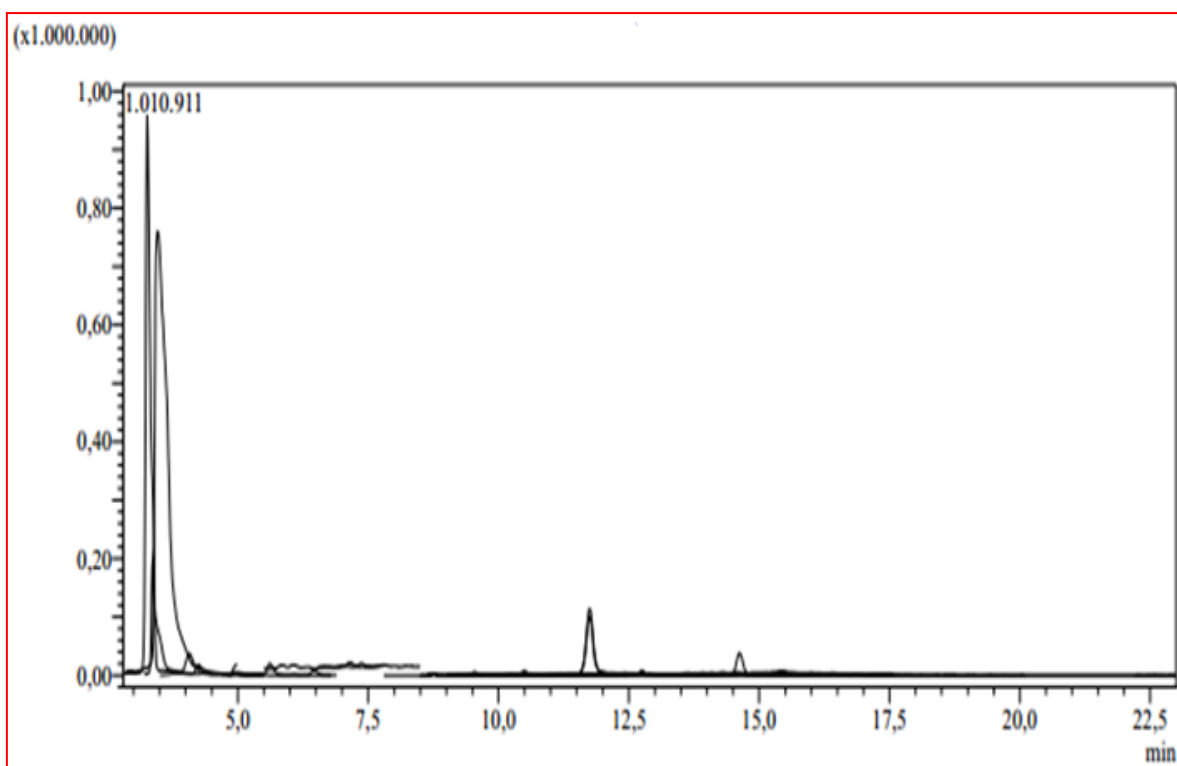


Figure A4. LC-MS/MS chromatogram of *Tribulus terrestris* L. roots

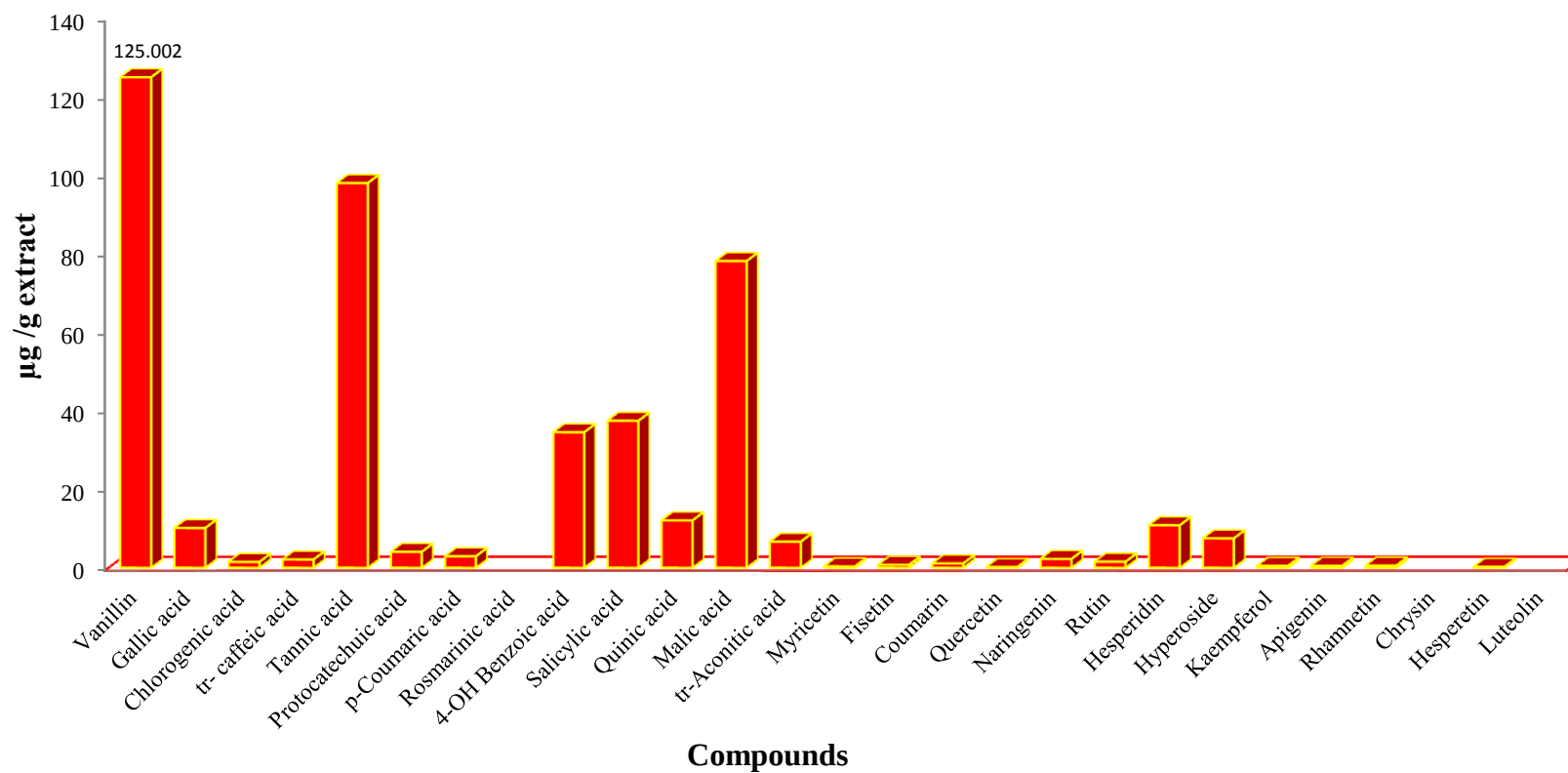


Figure A5. LC–MS/MS identification of phenolic compounds in *Tribulus terrestris* L. roots

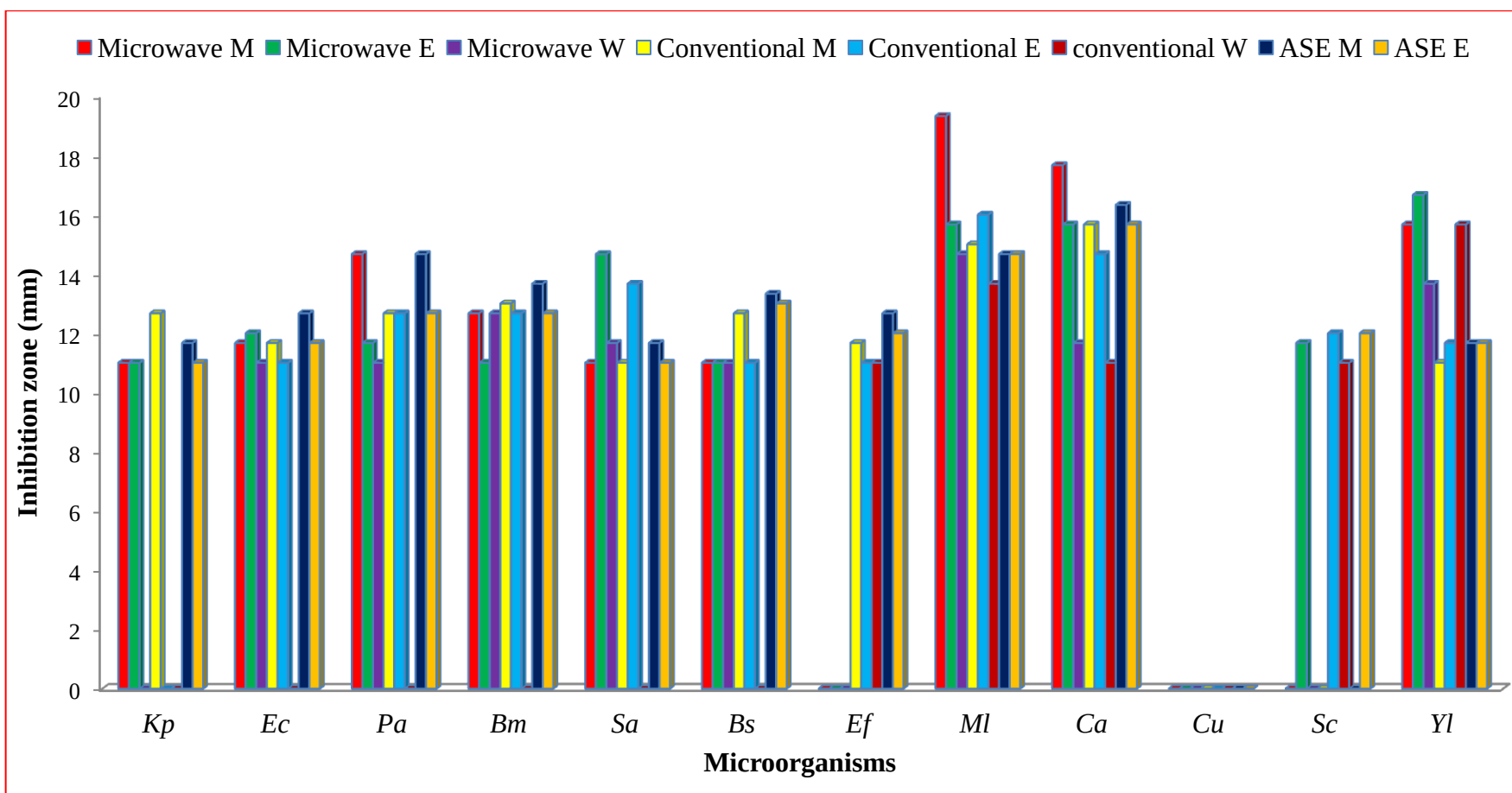


Figure A6. The inhibition zones of *Tribulus terrestris* L. extracts against microorganisms

Note M: Methanol; E: Ethanol; W: Water; ASE: Accelerated solvent extraction; Kp: *Klebsiella pneumoniae* FMC5; Ec: *Escherichia coli* DM; Pa: *Pseudomonas aeruginosa* DSM50071; Bm: *Bacillus megaterium* DSM32; Sa: *Staphylococcus aureus* Cowan1; Bs: *Bacillus subtilis* IMG22; Ef: *Enterococcus faecium* Clinical isolate; Ml: *Micrococcus luteus* LA2971; Ca: *Candida albicans* ATCC1023; Cu: *Candida utilis* NRRL-Y-900; Sc: *Saccharomyces cerevisiae* WET136; Yl: *Yarrowia lipolytica* NCIM3589



Figure A7. Inhibition zone of *Candida albicans* ATCC1023

Note: 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 A8. Inhibition zone of *Yarrowia lipolytica*

Note: 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. Control solvents against *Enterococcus faecium*

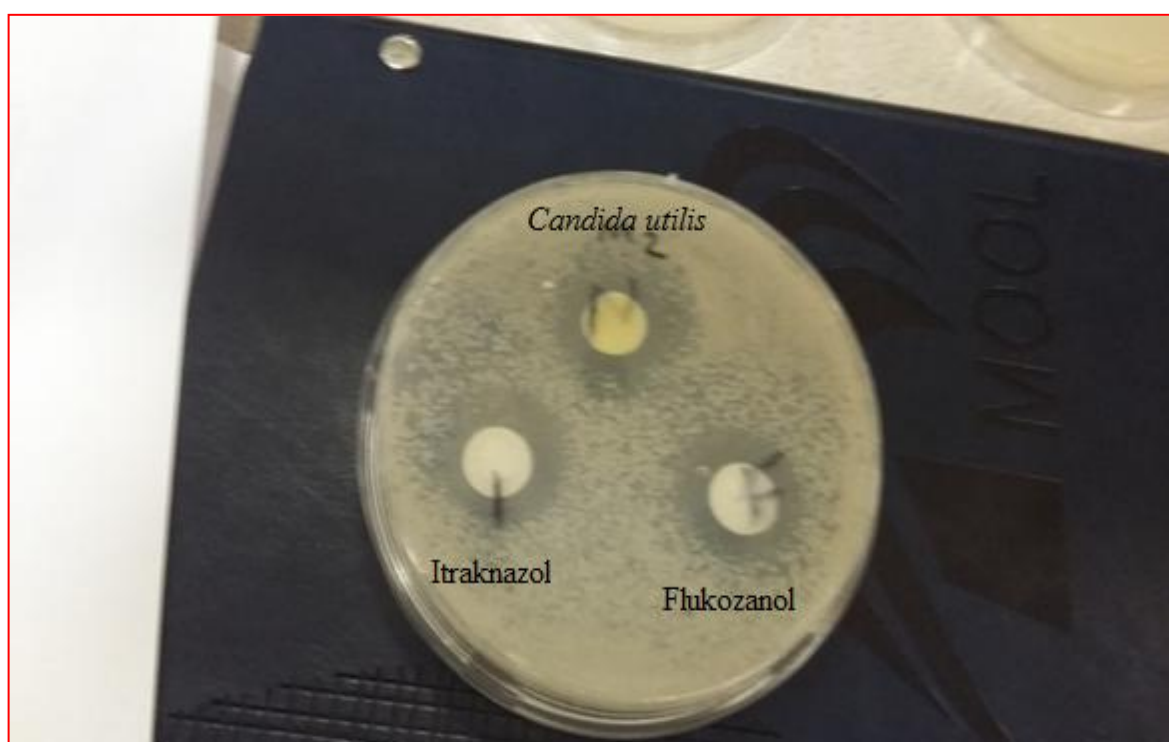


Figure A10. Flukozanol and Itraknazol activity against *Candida utilis*

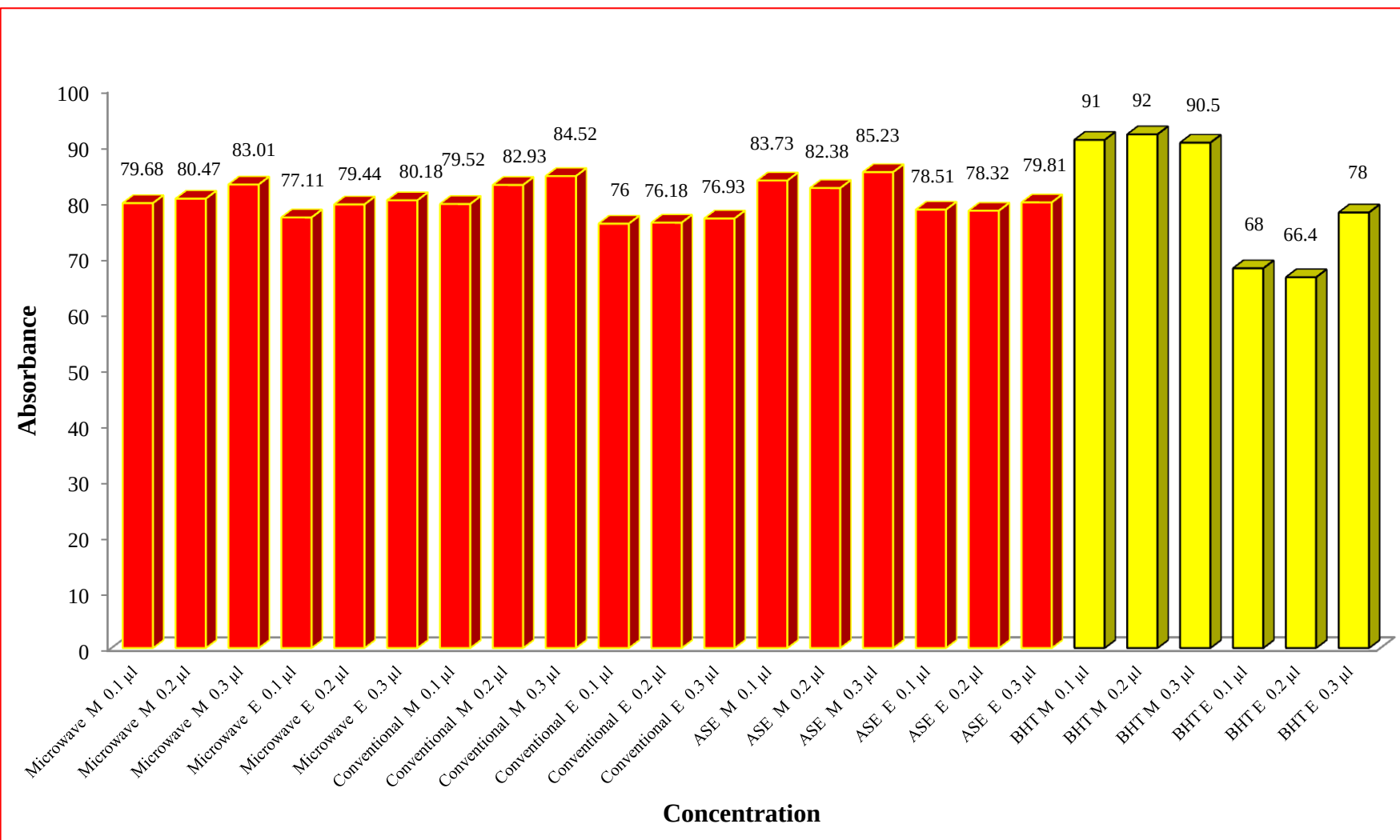


Figure A11. The DPPH radical scavenging power with BHT reference

Note: M: Methanol; E: Ethanol; ASE: Accelerated solvent extraction; BHT: Butylated hydroxytoluene

Table A1. 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.

Table A2. ANOVA table for antimicrobial activity

Methods	Solvents			Sum of Squares	Df	Mean Square	F	Sig
Microwave extraction	Methanol	Between groups	Combined	1532.17	11	139.28	695.48	0.00
		Within groups		4.8	24	0.2		
	Ethanol	Between groups	Combined	998.75	11	90.79	544.7	0.00
		Within groups		4.0	24	0.16		
	Water	Between groups	Combined	1224.2	11	111.29	801.3	0.00
		Within groups		3.33	24	0.139		
Conventional extraction	Methanol	Between groups	Combined	872.75	11	79.34	317.36	0.00
		Within groups		6.0	24	0.25		
	Ethanol	Between groups	Combined	875.63	11	79.6	358.21	0.00
		Within groups		5.3	24	0.22		
	Water	Between groups	Combined	1414.3	11	128.57	2314.3	0.00
		Within groups		1.33	24	0.05		
ASE	Ethanol	Between groups	Combined	950.08	11	86.37	310.93	0.00
		Within groups		6.6	24	0.27		
	Methanol	Between groups	Combined	467.0	11	45.18	271.09	0.00
		Within groups		4.0	24	0.16		

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

Table A3. Standard mimosa tannin with concentration

Concentration (mg/L)	Absorbance
50	0.75
25	0.365
12.5	0.179
6.75	0.094

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