ANTIBACTERIAL, ANTICANDIDAL, ANTIOXIDANT ACTIVITIES AND TOXICITY OF *Euphorbia hirta* L.

BASMA R. M. ABUARRA

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ANTIBACTERIAL, ANTICANDIDAL, ANTIOXIDANT ACTIVITIES AND TOXICITY OF *Euphorbia hirta* L.

By

BASMA R. M. ABUARRA

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
amu	Atomic mass units
ANOVA	One-Way Analysis of Variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CCD	Charge-coupled device
CE	Catechol equivalence
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EXD	Extreme or Extensive Drug-Resistance
FRPA	Ferric reducing power assay
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-mass spectrometry
GT	Germ tube
h	Hour(s)
HIV	Human immunodeficiency virus
HPTLC	High performance thin layer chromatography
IC_{50}	Inhibitory Concentration at 50%
IDSA	Infectious Disease Society of America
LC_{50}	Lethality Concentration at 50%
LD_{50}	Median lethal dose

MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MHA	Muller Hinton agar
MHB	Muller Hinton broth
MIC	Minimum inhibitory concentration
MDR	Multidrug resistance
MOPS	Morpholinepropanesulfonic acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NA	Nutrient Agar medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NIST	National Institute of Standard and Technology
OD	Optical density
OECD	Organization for Economic Cooperation and Development
PDR	Pan drug resistance
R_f	Retention factor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Round per minute
RPMI	Roswell Park Memorial Institute
t_R	Retention time
SDA	Sabouraud's dextrose agar
SEM	Scanning electron microscope
SPSB	Sterile phosphate saline buffer
SPSS	Statistical Package for the Social Sciences
TEM	Transmission electron microscope

TLC	Thin layer chromatography
TFC	Total flavonoid content
TNF−α	Tumor necrosis factor-alpha
TPC	Total phenolic content
UV	Ultraviolet
WHO	World health organization

ABSTRAK

Pelbagai usaha telah dilakukan untuk menunjukkan antimikrob dan konstituen fitokimia yang terdapat pada tumbuhan ubatan dan menggunakannya untuk rawatan pelbagai penyakit sebagai alternatif kepada drug kimia sintetik. Aktiviti antimikrob ekstrak metanol daun, bunga, batang dan akar *Euphorbia hirta* L. diuji dengan beberapa bakteria perubatan penting dan yis dengan menggunakan kaedah agar resapan cakera. Empat bakteria Gram positif (*Staphylococcus aureus*, *Micrococcus sp*., *Bacillus subtilis* dan *Bacillus thuringensis*), empat bakteria Gram negatif (*Escherichia coli*, *Klebsiella pneumonia, Salmonella typhi* dan *Proteous mirabilis*) dan satu yis (*Candida albicans*) disaring. Zon perencatan berkisar antara 9-29 mm. Ekstrak daun menghalang pertumbuhan semua mikroorganisma dengan zon perencatan yang luas, diikuti oleh bunga, yang menghalang semua bakteria kecuali *C. albicans.* Mikrob yang paling rentan terhadap semua ekstrak adalah *S. aureus* dan *Micrococcus sp*. Nilai MIC terendah diperolehi daripada *E. coli* dan *C. albicans* (3.12 mg/ml), diikuti oleh *S. aureus* (12.50 mg/ml) dan *P. mirabilis* (50.00 mg/ml). Semua bakteria lain mempunyai nilai MIC 100.00 mg/mL. Cerakin bunuh-masa terhadap *C. albicans* menunjukkan kesan fungisida pada 1 dan 2 kali ganda MIC. Kajian mikroskopik elektron pengimbasan dan transmisi (SEM dan TEM) ke atas *C. albicans* menunjukkan bahawa sel yang terdedah kepada ekstrak daun memaparkan perubahan drastik struktur dalaman dan luaran yang mana ianya semakin teruk dengan peningkatan masa rawatan. Analisis GC-MS menunjukkan kehadiran enam sebatian berbeza dalam ekstrak daun. Ekstrak *E. hirta* mempunyai aktiviti antioksidan yang signifikan apabila diukur menggunakan DPPH, FRAP, serta penentuar kandungan jumlah fenol dan flavonoid. Ekstrak daun *E. hirta* mempamerkan aktiviti pengautan

dengan DPPH maksima 72.96±0.78% pada 1.0 mg/ml diikuti oleh bunga, akar dan batang dengan aktiviti 52.45±0.66%, 48.59±0.97% dan 44.42±0.94% masing-masing. Piawai hidroksitoluena terbutil (BHT) didapati mempunyai 75.13±0.75% pada kepekatan yang sama. Kuasa penurunan ekstrak daun adalah setanding dengan asid askorbk dan didapati bergantung kepada dos. Kandungan fenol dilaporkan sebagai kesetaraan asid galik. Ekstrak daun mempunyai kandungan flavonoid tertinggi (206.17±1.95 mg GAE/g), diikuti oleh bunga, akar dan batang yang mempunyai 117.08±3.10 mg GAE/g, 83.15 ±1.19 mg GAE/g dan 65.70±1.72 mg GAE /g masing-masing. Daun juga mempunyai jumlah kandungan fenol tertinggi iaitu 37.97±0.003 mg CE/g, diikuti oleh ekstrak bunga, akar dan batang yang mempunyai 35.20±0.002 mg CE/g, 24.35±0.006 mg CE/g dan 24.12±0.004 mg CE/g masing-masing. Penyaringan fitokimia ekstrak metanol daun *E. hirta* menunjukkan kehadiran gula penurunan, terpenoid, alkaloid, steroid, tannin, flavonoid serta sebatian fenol. Kajian ketoksikan *in vitro* menunjukkan bahawa daun dan batang *E. hirta* tidak toksik terhadap anak udang air garam sedangkan bunga dan akar adalah beracun. Nilai LC_{50} 1.589, 1.420, 0.206 dan 0.0827 mg/ml masing-masing adalah diperolehi daripada batang, daun, bunga dan akar. Kajian *in vivo* ketoksikan akut oral pada tikus menunjukkan bahawa ekstrak daun mempunyani LD_{50} lebih daripada 5000 mg/kg. Tidak ada kesan toksik yang signifikan ataupun perubahan perilaku yang di dapati pada tikus yang dirawat dengan ekstrak daun berbanding dengan kumpulan kawalan. Tambahan lagi, analisis histopatologi makroskopik dan mikroskopik organ penting daripada tikus rawatan menunjukkan tiada keabnormalan signifikan berbanding kawalan. Oleh kerana itu daripada hasil kajian, tumbuhan *E. hirta* boleh digunakan untuk memperolehi bahan bioaktif semulajadi menarik yang boleh bertindak sebagai pemula dalam perkembangan farmaseutikal baru.

ABSTRACT

Much work has been done to reveal the antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of various ailments as alternatives to chemically synthetic drugs. The antimicrobial activities of the methanolic extracts of *Euphorbia hirta* L. leaves, flowers, stems and roots were evaluated against some medically important bacteria and yeast using the agar disc diffusion method. Four Gram positive bacteria (*Staphylococcus aureus, Micrococcus sp*., *Bacillus subtilis* and *Bacillus thuringensis*), four Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumonia, Salmonella typhi* and *Proteous mirabilis*) and one yeast (*Candida albicans*) were screened. Inhibition zones ranged between 16–29 mm. Leaves extract inhibited the growth of all tested microorganisms with large zones of inhibition, followed by flowers, which also inhibited all the bacteria except *C. albicans*. The most susceptible microbes to all extracts were *S. aureus* and *Micrococcus* sp. The lowest MIC values were obtained from *E. coli* and *C. albicans* (3.12 mg/ml), followed by *S. aureus* (12.50 mg/ml) and *P. mirabilis* (50.00 mg/ml). All the other bacteria had MIC values of 100.00 mg/ml. Time-kill assay of *C. albicans* indicated a fungicidal effect at 1 and 2 fold MIC. Scanning and transmission electron microscopic studies (SEM and TEM) of *C. albicans* revealed that cells exposed to the leaf extract displayed drastic external and internal structural changes which increased with increasing time of treatment. GC-MS analysis indicated the presence of six different compounds in the leaf extract. *E. hirta* extracts had significant antioxidant activity when measured using the DPPH, FRAP, and total phenolic and flavonoid contents. Leaf extract of *E. hirta* exhibited a maximum DPPH scavenging activity of 72.96 ± 0.78% at 1.0 mg/ml followed by the flowers, roots and stems whose scavenging activities were 52.45 ± 0.66%, 48.59 ± 0.97% and

44.42 ± 0.94% respectively. The standard butylated hydroxytoluene (BHT) had a scavenging activity of 75.13 ± 0.75% at the same concentration. The IC_{50} of the methanol extract of *E. hirta* leaves, flowers, roots, stems and BHT were found to be 0.803 mg/ml, 0.972 mg/ml, 0.989 mg/ml, 1.358 mg/ml and 0.794 mg/ml respectively. The reducing power of the leaf extract was comparable with that of ascorbic acid and found to be dose dependent. Leaf extract had the highest flavanoid content (206.17± 1.95 mg GAE/g), followed by flower, root and stem extracts which had 117.08 ± 3.10 mg GAE/g, 83.15 ± 1.19 mg GAE/g and 65.70 ± 1.72 mg GAE/g respectively. Leaves also had the highest total phenol content of 37.97 ± 0.003 mg CE/g, followed by flower, root and stem extracts which had 35.20 ± 0.002 mg CE/g, 24.35 ± 0.006 mg CE/g and 24.12 ± 0.004 mg CE/g respectively. Phytochemical screening of *E. hirta* leaf extract revealed the presence of reducing sugars, terpenoids, alkaloids, steroids, tannins, flavanoids and phenolic compounds. The *in vitro* toxicity study revealed that the leaves and stems of *E. hirta* were non toxic against the brine shrimp nauplii, whilst flowers and roots were toxic. LC_{50} values of 1.589, 1.420, 0.206 and 0.0827 mg/ml were obtained from stems, leaves, flowers and roots respectively. The *in vivo* acute oral toxicity study in mice showed that the leaf extract had LD_{50} of more than 5000 mg/kg. No significant toxic effects or behavioural changes appeared on the leaf extract treated mice as compared to the untreated groups. Furthermore, the macroscopic and microscopic histopathological analysis of the vital organs from the treated mice showed no significant abnormalities when compared with the control. Hence, *E. hirta* could be used to discover new bioactive natural products that may serve as leads in the development of new pharmaceuticals.

CHAPTER ONE

INTRODUCTION

1.1 General

The history of herbal medicines is as old as human civilization. Human has to rely on nature in order to survive and sustain. Nature provides a remedy for nearly every disease that may afflict a human being. During the ancient times, almost all medicines were from natural products. Since the beginning of humankind people have depended primarily on plants for nourishment and through trial and error they discovered that some plants are good for food, some are poisonous, and some produce physiological changes such as increased perspiration, bowel movement, fertility, urination, pain relief, hallucination, and healing. By time, these observations were passed orally from one generation to another, with each generation adding to and refining the body of knowledge (Chah *et al.,* 2006). However, nowadays, the use of medicinal plants is well known among the citizens of rural areas of many developing countries. While, in the developed countries, 25 percent of the medical drugs are derived from plants (Principe, 2005). The World Health Organization (WHO) estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Retnam and Martin, 2006).

In general, plants are found to be used as medicines or made into medicines in five traditional ways: by infusing the herb in water (as teas, infusions, decoctions, washes, beers, or steams), by infusing the herb in alcohol or an alcohol-and-water combination (tinctures, fluid extracts, and, when diluted, as washes or sprays), by transferring the power of the herb to an oil base, by using the plant itself (eaten whole, wound powders, in capsules, smoking, or smudging), or by distilling and

using the essential oil of the plant (Buhner, 1999). Majority of these extraction methods involve the isolation of the active constituents found in a particular medicinal plant and its subsequent modification.

Euphorbia hirta L. (Euphorbiaceae) is considered as one of the common medicinal plants in the tropics and subtropics. Recently, much research is focused on *Euphorbia* species which have been employed as a traditional cure for several indications such as gastrointestinal disorders (diarrhea, dysentery, intestinal parasitosis, etc), bronchial and respiratory diseases (asthma, cough, bronchitis, hay fever) (Bhaskara *et al.,* 2010), in addition to many other uses. Several pharmacological properties of *E. hirta* are reported in literature such as: sedative, anxiolytic, analgesic, antipyretic, antiinflammatory (Lanhers *et al*., 1991), antidiarrheic (Galvez *et al*., 1992), antibacterial (Abubakar, 2009; Doughari *et al*., 2008; Ogbulie *et al*., 2007) and diuretic (Johnson *et al*., 1999). This plant has also been shown to inhibit prostaglandin biosynthesis, platelet aggregation and carragenan-induced edema (Hiermann and Bucar, 1994).

The extraordinary values of *E. hirta* have attracted attention to investigate its natural properties. However, despite the various reports on the curative and preventive properties of *E. hirta*, this is the first time the plant is studied on its antimicrobial activities in such detailed manner and as separated parts (leaves, stems, flowers and roots).

1.2 Problem Statement

Despite the huge number of antimicrobial agents already exist for various purposes; the search for new drugs should be a continuous one since the target microorganisms often develop new genetic variants which subsequently become

resistant to available antimicrobial agents. Thus, the effective life span of any antibiotic is limited. The world's vision is now directing towards plant sources for developing antimicrobial drugs, since natural products are rich in secondary metabolites that may be used to develop new drugs with novel modes of action. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs (Nascimento *et al.,* 2000). Therefore, such plants should be investigated to better understand their properties, safety and efficacy. There are several reports published on antimicrobial activity of various crude plant extracts (Abubakar, 2009; Igoli *et al.,* 2005; Alzoreky and Nakahara, 2003). It is estimated that there are about 2.5 million species of higher plants and the majority of these have not been examined yet for their pharmacological activities (Ram *et al.,* 2003).

On the other hand, free radicals are usually generated *in vivo* from various biochemical reactions and also from the respiratory chain as a result of occasional leakage. However, excessive generation of these radicals that are not effectively scavenged by cellular antioxidant constituents may lead to a variety of disorders in biological systems including heart diseases, neurodegenerative diseases, cancer, diabetes, arthritis, inflammation, lung damage and the aging process itself (Ramamoorthy and Bono, 2007). Moreover, free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odours and flavours that decrease the food nutritional value (Walia *et al.*, 2010). There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources (Gulcin *et*

al., 2010; Esmaeili and Sonboli, 2010; Wang *et al.*, 2010). Concerns about the safety of the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have led to increase interest in natural antioxidants which occur in plants as secondary metabolites (Gulcin *et al.*, 2010). Many plants exhibit efficient antioxidant properties owing to their secondary metabolities, especially phenolic constituents (Lin *et al*., 2010).

This study may contribute to finding new novel antimicrobial substances to control the pathogenic bacteria or *C. albicans*, especially those that are resistant to currently available antibiotics or even find new antioxidants against the destructive free radicals. It may also provide a clear image about the plant safety for consumption.

1.3 Objectives

This study aims to investigate the antibacterial, anticandidal activities of *E. hirta* against four Gram positive and four Gram negative bacteria in addition to *C. albicans*. The antioxidant potential of the plant parts is to be evaluated by DPPH radical scavenging, ferric reducing antioxidant power assay, total phenolic and flavonoid contents, whereas toxicological studies of the plant are conducted, both *in vitro* (*Artemia salina* napulii) and *in vivo* (mice acute oral toxicity). Finally, the chemical composition of the bioactive compounds in the most active part is determined using phytochemical screening, thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS).

1.4 Scope of Research

In this study, *E. hirta* separated parts (leaves, stems, flowers, and roots) will be screened for their *in vitro* antimicrobial, antioxidant activities and their toxicity will be evaluated using *in vitro* and *in vivo* methods. First, the plant composition will be studied using phytochemical screening to determine which secondary metabolites are present in the extract. Then, the antimicrobial activity of each part will be evaluated against a broad spectrum of bacteria and *C. albicans* using disc diffusion method, to know the most active extract. The lowest concentration of this part extract that can inhibit the microbial growth will be determined using the minimal inhibitory concentration broth dilution methods. Furthermore; the most susibtible microbe will be exposed to extensive studies such as the growth profile, electron microscopy observation and the active substances against this microbe will be identified using chromatography methods, such as TLC, agar overlay method and GC-MS analysis.

For antioxidant activity of *E. hirta*, four different methods will be used to study the antioxidant capacity of the plant parts such as DPPH scavenging, ferric reducing antioxidant power assay and the quantitative determination of the phenolic and flavonoid contents. This antioxidant activity will be confirmed using the high performance thin layer chromatography.

Finally, the plant toxicity will be evaluated using the *in vitro* brine shrimp lethality test and the *in vivo* Swiss albino mice acute oral toxicity test. Both tests will provide an idea about the toxicity levels of *E. hirta* extracts through determination of the median lethal concentration (LC_{50}) and median lethal dose (LD_{50}) values respectively.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antimicrobial Activity

2.1.1 Antimicrobial Agents

Antibiotic came from the ancient Greek word "antibios" where: *anti*= against, and *bios*= life. It is a natural or synthetic substance that stops microbes, both bacteria and fungi, by killing them or inhibiting their growth (Walsh, 2008). Antibiotics are one class of antimicrobials, a larger group which also includes anti-viral and anti-parasitic drugs. Antimicrobial agents could be biocidal or biostatic. The biocidal antimicrobials (bactericidal, fungicidal, virucidal) kill pathogenic microorganisms, while the biostatic ones (bacteriostatic, fungistatic) only slow down their growth and give the body the chance to use its immune system against the microorganisms (Ogundare and Onifade, 2009; Pankeyand Sabath, 2004). Antibiotics known as broad-spectrum antibiotics can be used to treat a wide range of infections caused by several types of bacteria and are usually used where the specific type of the microorganism is unknown. Antibiotics which are only active against particular types of Gram positive or Gram negative bacteria are called narrow-spectrum antibiotics. Antibiotics that target protein synthesis, such as the tetracyclines, aminoglycosides, and macrolides, are usually bacteriostatic in nature. Those that target the bacterial cell wall (penicillins, cephalosporins), or cell membrane (polymixins), or interfere with essential bacterial enzymes (sulphonamides, quinolones) are usually bactericidal (Finberg *et al*., 2004).

For a new antibiotic to be an effective chemotherapeutic agent, it should satisfy certain requirements, the most important is to be selective against different groups of microorganisms. It must have desirable antibacterial properties, that is, it must affect bacteria or other microorganisms that are not subject to the action of other antibiotics or synthetic chemical compounds, or it must be more potent or more effective than others in current use. The antibiotic must also be active in the presence of body fluids, not be destroyed by tissue enzymes, not too toxic to animals as a whole, or to tissues, or to individual cells. Preferably, the antibiotic should have desirable physical and chemical properties, such as solubility in water and a certain degree of stability. It has to be excreted readily, but not too fast, from the animal system and must not accumulate there and cause undesirable side effects (Todar, 2002).

2.1.2 History of Antibiotics

The first antibiotic was discovered by a British microbiologist, Alexander Fleming, in 1928 from a mold called *Penicillium notatum*. Fleming observed that a plate culture of the bacterium *Staphylococcus aureus* had been contaminated by the blue-green mold *Penicillium notatum* and that colonies of bacteria adjacent to the mold were inhibited. Therefore, Fleming grew the mold in a pure culture and found that it produced a substance that killed a number of disease-causing bacteria. However, the value of Alexander Fleming's discovery was not recognized and the use of penicillin did not start until the 1940s when Howard Florey and Ernst Chain isolated the active ingredient and prepared a powder form of the antibiotic. Since then, antibiotics have been recognized as important chemotherapeutic agents and their role in the treatment of numerous diseases became permanently established.

More efforts for the production of antibiotics from new sources resulted in the isolation of actinomycin from the soil bacteria *Actinomycetes* in 1940 (Waksman, 1949). Nowadays, most of the currently available classes of antimicrobial drugs are of natural or semisynthetic origins, and only three synthetic classes including the sulfa drugs, quinolones and oxazolidinones are in practical use (Walsh, 2003).

The almost exponential increasing of the total number of discovered bioactive microbial compounds in the last decades amazingly became constant. In 1940 only 10-20, in 1950 around 300-400, in 1960 approximately 800-1000 and in 1970 already 2500 antibiotics were known. From that time the total number of known bioactive microbial metabolites has doubled in each ten years. In 1980 about 5000, in 1990 around 10000 and in 2000 already almost 20000 antibiotic compounds were discovered. By the end of 2002, more than 22000 bioactive secondary metabolites, including antibiotics, were published in the literature (Berdy, 2005). Despite the huge number of natural antimicrobial products, most of them are already useless because they are not specific for bacteria, toxic, too weak, or lack the desired pharmacokinetic properties (Pelaez, 2006). From the 22500 known antibiotics and similar bioactive microbial compounds, less than one percent which accounts for only about 150 compounds, is in direct use in human and veterinary medicine, and agriculture. In the human therapy about one hundred compounds, most of them derived from Actinomycetales, are in direct practical use (Berdy, 2005). Since 1960s, only two new classes of antibiotics have been introduced for use, linezolid in 2000 and daptomycin in 2003 (Bubnoff, 2006).

2.1.3 Action Mechanisms of Antibiotics

Most antimicrobial agents used for the treatment of bacterial infections may be classified according to their principal mechanism of action. The five major modes of action are: (1) inhibition of cell wall synthesis, (2) alteration in cell membrane permeability, (3) inhibition of ribosomal protein synthesis, (4) suppression of nucleic acid (DNA) synthesis, and (5) inhibition of folic acid synthesis. Beta-lactam (penicillins, cephalosporins, carbapenems, and monobactams) and glycopeptide (vancomycin, teicoplanin) cell wall inhibitors prevent the formation of cross-linking steps required for stable cell wall synthesis to prevent micro-organisms from maintaining their internal osmotic pressure and consequently causing their lysis. Polymyxin B and our natural cationic peptides destruct cell membrane integrity with the cationic peptides literally putting pores in the membrane. Ribosomal proteins synthesis inhibitors (macrolides, clindamycin, tetracyclines, streptogramins and oxazolidinediones) inhibit protein synthesis at various ribosomal receptor sites. Since bacterial ribosomes structure differs from those in eukaryotic cells, antibacterial agents employ these differences to selectively inhibit bacterial growth. Metronidazole and fluorquinolones exert their antibacterial activities by disrupting DNA synthesis and producing fatal double-strand DNA breaks during DNA replication, while sulfonamides and trimethoprim block successive stages in the synthesis of folic acid necessary for the survival of certain bacteria. Disruption of bacterial membrane structure may be a fifth mode of action. It is suggested that polymyxins exhibit their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents (Tenover, 2006; Pallasch, 2003).

2.1.4 Antibiotic Resistance

Antibiotic resistance is a natural biological phenomenon where a microorganism is able to tolerate exposure to an antibiotic. The use of an antimicrobial for any infection, in any dose and for any time period, obligates microbes to either die or adapt in a condition known as selective pressure. Antimicrobial use kills the susceptible microorganisms but permits the newly resistant ones to survive and grow. Microbes which adapt and survive carry genes for resistance (WHO, 2010). Once obtained, resistance genes are not easily lost. Instead, they become a relatively stable part of the organism's genetic material and can be passed on. With time, additional resistance determinants join those currently available, thus widening the multidrug resistance (MDR) phenotype and further limiting treatment options (Levy, 2005). MDR means resistance to at least three of the five main groups of agents currently used for treatment, which include the broad-spectrum penicillins, broad spectrum cephalosporins, carbapenems, aminoglycosides and 4-fluoroquinolones. The terms pan drug resistance (PDR) and extreme or extensive drug-resistance (XDR) has also been used where PDR implies resistance to all licensed antimicrobial agents, while XDR implies something between MDR and PDR (Gould, 2008).

Bacterial infections which cause most human diseases are also those in which emerging and microbial resistance is most evident: diarrhoeal diseases, meningitis, respiratory tract infections, sexually transmitted infections, and hospital-acquired infections. Some important examples of antibiotic resistant bacteria include penicillin-resistant *Streptococcus pneumoniae,* vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus,* multi-resistant salmonella, multi-

resistant *Mycobacterium tuberculosis* (WHO, 2010), broad spectrum ß-lactama resistant *K. pneumoniae* or *E. coli* and some cephalosporin resistant species of Enterobacteriaceae (Jones and Pfaller, 1998). Additionally, antimicrobial resistance is not restricted to only bacteria, resistance development among some fungi, particularly those fungi that cause infections in transplant patients with weakened immune systems, resistance emergence to common anti-malaria and anti-HIV drugs are also of great concerns (WHO, 2010).

Resistance of microbial pathogens to an increasing number of antibiotics is a serious problem (Bubnoff, 2006). It is considered one of the most serious issues in hospital infections, where small but increasing numbers of isolates, mainly Gram-negative nonfermenters of the genera *Acinetobacter* and *Pseudomonas*, are resistant to all good antibiotics and where growing numbers of Enterobacteriaceae are resistant to all except carbapenems. However, despite there is a lesser decrease of agents active against staphylococci, the prevalence of infections with methicillin-resistant *S. aureus* (MRSA) remains extensively high in many countries (Livermore, 2004). For instance, in July 2004, the Infectious Disease Society of America (IDSA) reported that 90,000 people of the two million patients, who are infected with bacteria in US hospitals annually, have died. Seventy percent of the deaths acquired strains that are resistant to at least one of the commonly used antibiotics, especially, methicillin-resistant *Staphylococcus aureus* (Cushnie and Lamb, 2005). At present, the antibiotic resistance problem has grown to include all of the major bacterial pathogens and all classes of antibiotic compounds worldwide (Barrett, 2005). This may mark the end of the antibiotic era; therefore, it is our responsibility as researchers to prevent such a trend by searching new sources of antimicrobial agents with different mechanisms of action from the already available drugs.

2.1.5 Mechanisms of Resistance to Antibacterial Agents

The normally susceptible bacteria may acquire resistance to antimicrobial agents by mutation and selection, or through acquiring the genetic material that encodes resistance from other bacteria. Genetic exchange may occur through one of various genetic mechanisms including transformation, conjugation or transduction. Through genetic exchange mechanisms, many bacteria have become resistant to several classes of antibacterial agents. These multidrug resistant bacteria have become a cause for serious concern, especially in hospitals and other healthcare centres where they tend to occur most commonly (Tenover, 2006).

Sensitive bacteria may turn resistant to an antimicrobial agent via new mutations. Such spontaneous mutations may cause resistance by (1) modification of the target protein to which the antibacterial agent binds by altering or eliminating the binding site, (2) upregulating the production of enzymes that inactivate the antimicrobial agent before it reaches its target site, (3) downregulating or altering an outer membrane protein channel that the drug involves to enter the cell, (4) upregulating efflux pumps that expel the drug from the cell or (5) development of alternate growth requirements (Tenover, 2006; Pallasch, 2003). In all of these cases, strains of bacteria carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is called *vertical evolution* (Tenover, 2006).

Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is called *horizontal evolution*, and can occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and

transformation. During conjugation, a gram-negative bacterium transfers plasmid-containing resistance genes to a nearby bacterium, usually through an elongated proteinaceous structure called a *pilus*, which connects the two organisms. During transduction, resistance genes are moved from one bacterial cell to another by bacterial viruses or bacteriophages. Conjugation among gram-positive bacteria is usually initiated by production of sex pheromones by the mating pair, which ease the clumping of donor and recipient organisms, permitting DNA exchange. Lastly, transformation can transfer resistance genes into previously susceptible strains when bacteria acquire and incorporate DNA segments from other bacteria that have lysed and released their DNA complement into the environment. Therefore, mutation and selection, together with the mechanisms of genetic material exchange, allow many bacterial species to tolerate the introduction of antibacterial agents into the environment (Tenover, 2006).

2.1.6 Plants as a Potential Source of New Antibiotics

Despite previous successes of antibiotic drug discoveries, infectious diseases still become the second-leading cause of mortality worldwide. Bacterial infections cause 17 million deaths globally, especially in children and elderly people (Butler and Buss, 2006). On the other hand, the rapid onset of resistance to most antibacterial drugs shortens their effectiveness and necessitates a constant supply of new antibiotics with new modes of action for efficient treatment of infections (Butler and Buss, 2006; Tenover, 2006).

Natural products have played a central role in antibiotic drug discovery with most antibacterial drugs being derived from a natural product or natural product lead (Nascimento *et al.*, 2000). In the last few decades, several reports have been

published about the antimicrobial properties of different compounds from herbs and spices, fruits and vegetables, leaves and bark, animal tissues and microorganisms. Many herbs and spices have been reported to possess significant antimicrobial properties, which are due to compounds synthesized in the secondary metabolism of the plant (Nascimento *et al*., 2000). The effective components of plant antimicrobial phytochemicals include alkaloids, flavones (flavonoids, flavonols, and quinones), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Samy and Gopalakrishnakone, 2008).

Flavonoids have been reported to possess numerous therapeutic properties, including antimicrobial, antioxidant, anti-inflammatory, enzyme inhibition, oestrogenic, antiallergic, vascular and cytotoxic antitumour activities (Cushnie and Lamb, 2005). Interesting effects of oleuropein, the main glycoside present in olives, has been shown against both Gram negative and Gram-positive bacteria as well as mycoplasma (Omar, 2010). Oleuropein and hydrolysis products are able to inhibit the development and production of enterotoxin B by *Staphylococcus aureus*, the development of *Salmonella enteritidis* and the germination and consequent development of *Bacillus cereus* spores. Oleuropein and other phenolic compounds (*p*-hydroxybenzoic, vanillic and *p*-coumaric acids) also completely inhibit the development of *K. pneumoniae, E. coli* and *B. cereus*. Recent studies showed the antimicrobial activity of commercial *Olea europaea* (olive) leaf extracts (mainly oleuropein) against *Campylobacter jejuni, Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Omar, 2010). Despite the wide antimicrobial activity, the exact mechanism of action of oleuropein is still not completely established. However, some authors have suggested that it may be due to the presence of the *ortho*-diphenolic system (catechol), the ability of the glycoside

group of oleuropein to modify and penetrate the cell membrane and reaches the target site, the effective interference with the production procedures of particular amino acids necessary for the growth of certain microorganisms or the direct stimulation of phagocytosis as a response of the immune system to microbes of all types (Omar, 2010).

This and other studies suggest screening of plant extracts and/or their phytochemicals for antimicrobial properties as a great source for developing new promising antimicrobial agents. More efforts in this field may lead to significant discovery of novel antibiotics to replace the existing ones to whom most bacteria became resistant.

2.1.7 Evaluation of Antimicrobial Susceptibility

There are various techniques that are widely used in screening plants for antimicrobial activity. Although a variety of methods exist, the goal of all the *in vitro* antimicrobial susceptibility testing methods is to measure the inhibitory effect of the antimicrobial agent and to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy *in vivo*. The common antimicrobial test methods are agar diffusion, agar dilution and broth dilution. In order to be effective, an *in vitro* susceptibility testing technique has to be reproducible, accurate and reliable, but not time-consuming, expensive or tedious.

2.1.7.1 Agar Diffusion Method

This is a qualitative method that is divided into disc diffusion and well diffusion methods which can be performed according to the properties of the substances to be tested. The disc diffusion method also known as the Kirby-Bauer

disc-diffusion, or the zone of inhibition method, is probably the most widely used method for testing antibacterial activity, because it uses only small amounts of the test substance (10–30 μL). It can be performed with minimal training. Briefly, the method involves the preparation of a Petri dish containing 15–25 ml agar; and microorganism at a known concentration is then spread across the agar surface and allowed to establish for 10-30 min. A filter paper disc of 6 - 8 mm diameter, containing a known volume of the test substance is then applied on the agar surface and the dish incubated for 24 h or more. Discs should be evenly distributed so that zones of inhibition do not overlap to such a degree that the zone of inhibition diameter cannot be determined. This can be obtained if the discs are not less than 24 mm far from centre to centre. On the other hand, when the extract is viscous or semi-solid such as honey, a well can be made in the agar and the substance allowed to diffuse out of the well instead of away from a disc. Generally, the compound diffuses into the agar. The diffusion of the antimicrobial agent into the seeded agar media results in a gradient of the antimicrobial agent. The concentration of the compound will be highest next to the disc/well, and will decrease as distance from the disc/well increases. If the compound is effective against bacteria at a certain concentration, a clear area of no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area, the more effective the compound. Data from these assays are typically presented as a mean size of zone of inhibition (mm), a ranking system of +, ++, and +++ or slight/ moderate/ strong, and sensitive/ intermediate/ resistant, to indicate levels of activity. Agar diffusion test ought to be standardized since the zone size is affected by many factors such as inoculum size, medium composition, temperature of incubation, excess moisture and

thickness of the agar. If these conditions are controlled, reproducible tests can be achieved and zone diameter is only a function of the test organism susceptibility (OIE, 2008).

2.1.7.2 Agar Dilution Method

Agar dilution method is a quantitative method, being used to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being tested. MIC values are used to determine susceptibilities of bacteria to drugs and also to evaluate the activity of new antimicrobial agents. In this method the antimicrobial agent of known concentrations is added into the agar at two fold serial dilution and, once set, bacteria are applied to its surface. Replicate dishes can be set up with a range of concentrations of the test substance. In this way, a large number of bacteria may be screened within a single assay run. The dishes are incubated for 24 hrs or more and the growth of the bacteria on the extract/agar mix is scored either as present, absent, or a percentage of inhibition as a proportion of the control (e.g. 0, 25%, 50%, 75%, 100%). No growth of the test organism indicates that it is susceptible at the antimicrobial concentration incorporated into the medium (OIE, 2008; Wiegand et al., 2008). Agar dilution is often recommended by the Clinical and Laboratory Standards Institute (CLSI) as a standardised antimicrobial susceptibility method for fastidious organisms, such as anaerobes, *Campylobacter* and *Helicobacter* species (OIE, 2008).

2.1.7.3 Broth Dilution Method

Broth dilution is a technique in which a suspension of microorganism of an appropriate concentration is tested against varying concentrations of an antimicrobial

agent in a liquid medium. The broth dilution method usually uses serial twofold dilutions and can be performed either in tubes containing a minimum volume of 2.0 ml (macrodilution) or in smaller volumes using microtitration plates (microdilution) (OIE, 2008). Serial dilutions of the antimicrobial agent are made in a liquid medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. The lowest concentration of antibiotic preventing appearance of turbidity is considered to be the minimal inhibitory concentration (MIC). At this dilution the antimicrobial agent is microstatic. Also, the minimal bactericidal/fungicidal concentration (MBC/MFC) can be determined by subculturing some of the tube contents onto antibiotic-free agar medium and examining for microbial growth. Despite, the tube dilution test is fairly precise, the test is laborious because serial dilutions of the antimicrobial agent must be prepared and only one isolate can be tested in each series of dilutions (Ncube et al., 2008). In contrast, the microdilution method appears to be more applicable since it gives reproducible results, requires only small volumes of extract to determine minimal inhibitory concentrations and works well with fungi. This method is useful in screening plants for antimicrobial activity and for the bioassay-guided isolation of antimicrobial compounds from plants (Ahmad *et al.,* 2006).

2.2 *Candida*

Candida is thin-walled, small yeast of 4 to 6 microns that reproduces by budding (Spencer and Spencer, 1997). It is an asexual dimorphic organism and can exist in two shapes and forms simultaneously. One form is a yeast-like state that is a non-invasive and sugar fermenting organism. The other is the invasive fungal form that produces very long root-like structures, called rhizoids which can penetrate the mucosa (McCullough *et al.,* 1996).

The genus *Candida* includes between 150 and 200 species. Amongst these, seven are most commonly isolated in human infections. They are divided into two broad categories; albicans and non-albicans species. *C. albicans* is the most abundant and significant species, due to its ability to adhere to host tissues, to produce aspartyl proteases and phospholipase enzymes, and to transformate from yeast to hyphal phase, the major determinants of its pathogenicity. *Candida glabrata, Candida tropicalis, Candida stellatoidea, Candida parapsilosis, Candida krusei,* and *Candida kyfer* are also isolated as causative agents of *Candida* infections (Kikani *et al.,* 2008; McCullough *et al.,* 1996). Due to the relatively high DNA homology between *C. albicans* and *C. stellatoidea,* the second has been reclassified as a sucrose negative variant of *C. albicans* (McCullough *et al.,* 1996).

Candida can be found on or in the human body with the gastrointestinal tract, the vagina, and skin being the common sites, and *C. albicans* being the most common species isolated from these locations (Kikani *et al*., 2008). *Candida* is also counted as the most common cause of opportunistic mycoses worldwide. The infections caused by all species of *Candida* are called candidiasis, and are the fourth most causable of nosocomial blood stream infections associated with a significant mortality (Pfaller and Diekema, 2007). One of the main reasons for the increase in

Candida infections is the development of its resistant strains to azole drugs, such as fluconazole used in the prophylaxis and treatment of candidiasis (Bhavan *et al*., 2010). Therefore considerable interest in the screening for new anticandidal agents would be useful in the diagnosis and treatment of *Candida* infections.

On the other hand, the formation of *Candida* biofilms carries significant clinical importance because of their increased resistance to antifungal therapy and the ability of cells within biofilms to tolerate host immune defenses. *C. albicans* in biofilms on polyvinyl chloride disks has been reported to be 30 to 2,000 times more resistant to antifungal agents than free-floating cells (Bachmann *et al*., 2002).

C. albicans biofilm formation usually involves the presence of implantable devices and has three developmental phases; early phase that requires the adherence of yeast cells to the host cells or device surface, intermediate phase with the formation of a matrix with dimorphic switching from yeast to hyphal forms, and maturation phase where increase in the matrix material taking on a three-dimensional architecture takes place. Fully mature *Candida* biofilms contain a mixture of morphological forms and composed of a dense network of yeasts, hyphae, and pseudohyphae in a matrix of polysaccharides, carbohydrate, protein and unknown constituents (Ramage *et al*., 2005, 2001; Kojic and Darouiche, 2004).

2.2.1 *Candida albicans*

C. albicans is a dimorphic organism that commonly inhabits in gastrointestinal tract of human beings, oral and vaginal mucosa as one of the commensal organisms. It causes opportunistic infections in immunocompromised patients, and produces allergic reactions (Naglik *et al*., 2003). It also causes a variety of infections ranging from non-life threatening mucosal candidiasis like vaginal yeast