ANTIBACTERIAL ACTIVITY OF EUPHORBIA HIRTA (L.): EUPHORBIACEAE AND ISOLATION OF ITS BIOACTIVE CONSTITUENTS

by

SHANMUGAPRIYA PERUMAL

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	Description	Symbol and Abbreviation
1	Absorbance	Abs
2	American type culture collection	ATCC
3	Arbitrary unit	AU
4	Colony forming units	CFU
5	Correlation coefficient	r^2
6	Degree Celcius	^oC
7	Fourier Transform Infra-Red	FTIR
8	Gram	g
9	High performance liquid chromatography	HPLC
10	Hour (s)	h
11	Litre	L
12	Mass spectrometry	MS
13	Microgram	µg
14	Microgram per millilitre	µg/mL
15	Microlitre	µl
16	Milligram	mg
17	Milligram per millilitre	mg/mL
18	Millilitre	ml
19	Millimolar	mM
20	Minimum inhibitory concentration	MIC

21	Minimum bactericidal concentration	MBC
22	Minute (s)	min
23	Nanometer	nm
24	Parts per million	ppm
25	Percentage	%
26	*p*-Iodonitrotetrazolium violet	INT
27	Potential hydrogen	pH
28	Retention factor	R_f
29	Revolution per minute	rpm
30	Standard deviation	S.D.
31	Scanning electron microscope	SEM
31	Ultra violet	UV
32	World Health Organization	WHO

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AKTIVITI ANTIBAKTERIA BAGI EUPHORBIA HIRTA (L.): EUPHORBIACEAE DAN PEMENCILAN JUZUK BIOAKTIFNYA

ABSTRAK

Produk semulajadi, terutamanya tumbuhan ubatan, kekal sebagai sumber berharga sebagai pendekatan revolusi hijau dalam penemuan sebatian antibakteria novel. Tumbuhan *Euphorbia hirta* (L.) digunakan secara tradisional di negara-negara tropika untuk rawatan gastrousus, bronkial dan penyakit pernafasan yang disebabkan oleh agen jangkitan nosokomial. Dalam kajian ini, pelbagai ekstrak pelarut (metanol, etanol, etil asetat, diklorometana dan heksana) daripada tumbuhan *E. hirta* telah dikaji dalam penyaringan fitokimia untuk menentukan jumlah kandungan fenolik dan flavonoid. Aktiviti antibakteria yang berpotensi daripada ekstrak mentah dinilai melalui ujian *in vitro* antibakteria dengan menggunakan kaedah mikropencairan medium brot terhadap panel patogen klinikal yang rintang dan strain jenis kultur koleksi Amerika (ATCC). Ekstrak yang paling bioaktif, ekstrak metanol, kemudiannya menjalani penulenan dengan kaedah fraksinasi dan pemencilan berdasarkan bioaktiviti menggunakan kromatografi turus, yang akhirnya menghasilkan tiga sebatian tulen antibakteria. Struktur kimia sebatian tulen telah dikenalpasti melalui kaedah analisis spektroskopi. Setiap sebatian antibakteria yang telah dipencilkan ini dikaji dalam mekanisme tindakan antibakterianya melalui beberapa manifestasi fisiologi bakteria yang melibatkan ketelapan membran luar, pengeluaran ion kalium intraselular, kebocoran nukleotida intraselular dan pengubahan morfologi sel bakteria. Kesan gabungan sebatian-sebatian antibakteria terpencil daripada *E. hirta* dengan ejen

antibakteria klinikal, cefepim, telah dinilai melalui kaedah "checkerboard" micropencairan medium brot. Ekstrak mentah metanol *E. hirta* memperolehi peratusan hasil tertinggi (12.5 %) bagi jumlah kandungan fenolik 213.4 ± 0.75 mg GAE/g ekstrak dan jumlah kandungan flavanoid 62.3 ± 1.01 mg CE/g ekstrak. Kajian ini memperlihatkan aktiviti antibakteria yang sangat ketara bagi ekstrak mentah metanol terhadap isolat klinikal *Pseudomonas aeruginosa* yang rintang, dengan nilai MIC dan MBC sebanyak 0.063 mg/mL and 0.125 mg/mL masing-masing. Fraksinasi dan pemencilan ekstrak mentah metanol selanjutnya telah mengeluarkan tiga sebatian antibakteria yang dikenalpasti sebagai asid kafeik (CA), epikatechin 3-gallat (ECG) dan quersitrin. ECG telah mempamerkan aktiviti anti-jangkitan yang paling menggalakkan terhadap *P. aeruginosa* dengan kesan potensi yang sama seperti kawalan positif, cefepim, pada nilai MIC dan MBC sebanyak 16 μg/mL dan 31 μg/ mL. CA kurang aktif daripada ECG dengan nilai MIC dan MBC yang rendah sebanyak dua kali ganda diikuti dengan quersitrin yang menunjukkan keberkesanan yang sama dengan ekstrak mentah metanol. ECG dipencilkan daripada *E. hirta* buat pertama kalinya dalam kajian ini manakala CA dan quersitrin dilaporkan buat kali pertama untuk aktiviti antibakteria terhadap *P. aeruginosa*. Kajian mekanisme tindakan menunjukkan kedua-dua ECG dan CA mempunyai kesan bakterisidal manakala quersitrin mempamer kesan bakteriostatik terhadap *P. aeruginosa*. ECG dan CA masing-masing berkemampuan untuk mengganggu membran luar dan membran sitoplasma sel *P. aeruginosa* melalui peningkatan ketelapan sel membran. Mikrograf pengimbas elektron menunjukkan ECG dan CA menyebabkan kerosakan ketara pada sel membran dan kebocoran kandungan sitoplasma yang membawa kepada kematian sel. Sebagai langkah untuk memaksimumkan lagi kesan antibakteria, suatu kombinasi

cefepim dengan ECG, CA dan quersitrin masing-masing telah diuji secara individu terhadap isolat *P. aeruginosa*. Cefepime digabungkan dengan ECG telah menghasilkan kesan sinergistik dengan purata indeks FIC sebanyak 0.24. Walau bagaimanapun, gabungan cefepim dengan CA menunjukkan kesan interaksi penambah dan gabungan cefepim dengan quersitrin pula memaparkan kesan berkecuali. Sebagai kesimpulan, juzuk antibakteria yang telah dipencilkan daripada tumbuhan ubatan *E. hirta* mempunyai potensi anti-jangkitan yang menonjol terhadap patogen resistan *P. aeruginosa*. Molekul bioaktif yang bersifat klinikal ini menanti pemajuan selanjutnya sebagai ejen kemoterapeutik yang baru.

ANTIBACTERIAL ACTIVITY OF EUPHORBIA HIRTA (L.): EUPHORBIACEAE AND ISOLATION OF ITS BIOACTIVE CONSTITUENTS

ABSTRACT

Natural products, especially medicinal plants, remain as a valuable source offering a revolutionary green approach to the discovery of novel antibacterial compounds. *Euphorbia hirta* (L.) plant is traditionally used in many tropical countries for the treatment of gastrointestinal, bronchial and respiratory ailments caused by nosocomial infectious agents. In this study, various solvent extracts (methanol, ethanol, ethyl acetate, dichloromethane and hexane) of *E. hirta* were subjected to phytochemical screening to determine total phenolic and flavonoid contents. The potential antibacterial activities of the crude extracts were assessed via *in vitro* antibacterial test using broth microdilution method against a panel of clinically resistant pathogens and American Type Culture Collection (ATCC) strains. The most bioactive extract, the methanol extract was further purified in a bioactivity-guided fractionation and isolation method using column chromatography, which finally yielded three pure antibacterial compounds. The chemical structures of these purified compounds were characterized and identified through analyses of spectroscopic methods. The isolated antibacterial constituents were investigated for the antibacterial mechanisms by several bacterial physiological manifestations involving outer membrane permeabilization, intracellular potassium ion efflux, intracellular nucleotide leakage and alteration of bacterial cell morphology. The combination effects of isolated antibacterial compounds from *E. hirta* with clinically established antibacterial

agent, cefepime were evaluated via checkerboard broth microdilution method. The crude methanol extract of *E. hirta* obtained the highest percentage yield (12.5 %) with total phenolic content of 213.4 ± 0.75 mg GAE/g extract and total flavonoid contents of 62.3 ± 1.01 mg CE/g extract. This study observed remarkable antibacterial activity of the crude methanol extract against the resistant clinical isolate of *Pseudomonas aeruginosa* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.063 mg/mL and 0.125 mg/mL. Further fractionation and isolation of the crude methanol extract produced three antibacterial compounds which were identified as caffeic acid (CA), epicatechin 3-gallate (ECG) and quercitrin. ECG had displayed the most promising anti-infective activity against *P. aeruginosa* with similar potency as positive control, cefepime with MIC and MBC values of 16 µg/mL and 31 µg/mL respectively. CA was less active than ECG, at two fold lower MIC and MBC values followed by quercitrin which showed the same efficacy as the crude methanol extract. ECG is being isolated from *E. hirta* for the first time in this study while CA and quercitrin are reported for the first time as having antibacterial activity against *P. aeruginosa*. Study on the mechanism of action showed both ECG and CA possessed bactericidal effect whereas quercitrin exerted bacteriostatic effect against *P. aeruginosa*. ECG and CA were capable in disrupting the outer membrane and cytoplasmic membrane of *P. aeruginosa* cell by enhancing the cell membrane permeability. Scanning electron micrographs showed ECG and CA each caused apparent cell membrane damages and leakages of cytoplasmic contents had eventually led to cell death. In an attempt to further maximize antibacterial potency, combinations of cefepime with ECG, CA and quercitrin respectively were tested individually against *P. aeruginosa* isolate. Cefepime combined with ECG had produced synergistic effect

with average FIC index of 0.24. However, combination of cefepime and CA showed additive interaction while combination of cefepime and quercitrin displayed indifference effect. To conclude, antibacterial constituents isolated from the ethnomedicinal plant *E. hirta*, possessed remarkable anti-infective effect against resistant *P. aeruginosa* pathogen. These bioactive molecules of clinical value await further development as a new chemotherapeutic agent.

CHAPTER ONE
LITERATURE REVIEW

1.1 Medicinal plant

Ever since the advent of humanity on earth, plants have served as unlimited source of phytotherapeuticals for various diseases (Clardy and Walsh, 2004). Medicinal plant is any plant used to prevent, ease or cure a disease or any plant utilized as a source of drugs or their precursors (Kotze and Eloff, 2002). Plant derived medicines have been of great influence to human health and well-being. According to the World Health Organization, 80 % of the world population depends primarily on traditional medicines and therapy which involves the consumption of plant extracts or their active constituents (WHO, 1993).

Since 1978, The World Health Organization (WHO) and dozens of collaborating institution worldwide have sought to assess the value and extent of the use of plants in health care systems. Observing traditional, historic, folkloric, or ethnobotanical uses of plants are regarded as a useful approach for targeting research leads in the development of new drugs from plants. A recent survey of medicinal plants used in therapy worldwide found that 119 distinct chemical substances derived from 91 species are used as drugs in one or more countries (Mahomoodally, 2013). Of these plant-derived substances, 74 % were discovered following chemical studies to determine the active compounds responsible for the use of the plant in traditional medicine. While many traditional uses may not be validated as safe or efficacious by

current scientific methodology, they can provide valuable leads for new drug compounds or expanded utilization in the future.

1.2 Phytochemicals

Phytochemicals are known as plant secondary metabolites which evolve naturally in plants. Secondary metabolites are organic compounds that are not found in all species of plant cells. Unlike primary metabolites, they are not essential to sustain growth, development, reproduction or survival of an organism. According to Edeoga et al. (2005), secondary metabolites are synthesized to enhance the strength of the plant and play an important role in deterrence against predators and pathogens. Secondary metabolites often play an important role in plant defence against herbivorous and other interspecies defences (Samuni-Blank *et al.,* 2012). Humans use secondary metabolites as medicines, flavorings, and recreational drugs. Plants phytochemicals have been classified in several categories including alkaloids, terpenoids, polyphenols, flavonoids, tannins and glycosides (Kennedy and Wightman 2005). Majority of secondary metabolites such as terpenoids, phenolics, alkaloids and tannins are of potential medicinal interest in drug discovery (Cowan, 1999). For example, aspirin was produced synthetically inspired from anti-pyretic effect of salicin found in the bark of the white willow tree.

1.3 Medicinal plants and antimicrobial properties

Plants products have been used since ancient time for medicinal reasons to cure and prevent diseases. Traditional herbal practitioners have extensively used plants to prevent or treat infectious conditions. However, since the launch of antibiotics in the

1950s from microorganism source, the use of plants as antimicrobial has been diminished. Annually, two or three antibiotics on average derived from microorganisms are being launched (Nikaido, 2009). In recent decades, the effective life span of any antibiotic has become limited due to misuse and abuse of antibiotics. Increased resistance of many microorganisms toward established drugs has made investigation of chemical compounds within traditional plants a necessity. Discovering new anti-infective agents particularly from plant sources has gained recognition again in the 1980s (David and Gorgon, 2012).

According to Borris (1996), there are an estimated of 250,000 to 500,000 plant species on earth and only one-tenth of these plants had been explored. Infectious diseases caused by pathogen are a problem yet to be controlled adequately, which necessitates the exploration of alternative sources of safe, effective and acceptable natural antibacterial. Plants enclose immeasurable phytoconstituents and are precious sources of novel compounds possessing antibacterial properties. For example, cranberry juice (*Vaccinium macrocarpon*), garlic (*Allium sativum*), tee tree (*Melaleuca alternifolia*) and lemon balm (*Melissa officinalis*) are depicted as broad-spectrum antimicrobial agents (Heinrich *et al.,* 2004). Plant extracts as source of either standardized extracts or pure compounds provides unlimited possibilities to inhibit and eradicate bacterial growth owing to their diverse phytochemicals. Medicinal use of the various plant extracts vary significantly depending on the potency and quantity of bioactive constituents comprised in it. Medicinal plants possess both potential antimicrobial crude drugs as well as a source for natural compounds that act as new anti-infective agents in the future.

1.4 Major classes of antibacterial phytocompounds

Various types of medicinal plants possessing significant antibacterial properties with correlative phytoconstituents are as shown in Table 1.1. Structures of common antibacterial plant phytochemicals are as depicted in Figure 1.1.

1.4.1 Phenolics and polyphenols

Simple phenols and phenolic acids - Cinnamic and caffeic acids are simple phenols consist of a single substituted phenolic ring. Both compounds were reported effective against bacteria (Brantner *et al.,* 1996). Catechol and pyrogallol are hydroxylated phenols with two and three −OH groups each, respectively. Some authors have found that more number of hydroxyl groups on the phenol ring can contribute higher inhibitory effect to the bacteria (Scalbert, 1991; Filomena *et al.,* 2013). Eugenol belongs to essential oils group possessing a C_3 side chain containing no oxygen as in penicillin and therefore contemplated having bacteriostatic action against bacteria (Rastogi *et al.,* 2008).

Flavones, flavonoids, and flavonols - Flavones originated from phenolic structures with one carbonyl group. The inclusion of a 3-hydroxyl group produces a flavonol. Flavonoids on the other hand, contain hydroxylated phenolic substances at C_6-C_3 unit linked to an aromatic ring. They are known to be effective as antimicrobial substances against a wide array of microorganisms. Catechin belongs to the group of flavan-3-ols. Catechins extracted from teas were reported to have antibacterial activities (Friedman *et al.,* 2006). Phloretin, isoflavone and galangin were also

observed for antibacterial activities against a wide range of Gram-positive and Gram-negative bacteria (Manner *et al.*, 2013).

Quinones - Quinones derived from aromatic compounds with two ketone substitutions. Quinones react with microbial cell surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes. It also causes bacteria inaccessible to the substrate (Alam, 2009).

Tannins - Tannin is a group of polymeric phenolic substances. Tannin is separated into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are multiple esters with d-glucose such as gallic acid, while condensed tannins are derived from flavonoid monomers. Tannins are known to inhibit bacteria by deactivating the microbial enzymes and proteins and form composite with polysaccharide (Frutos *et al.*, 2004).

Coumarins - Coumarins belong to benzopyrone chemical class. Rehman et al. (2005) found in vitro antibacterial activity from group of coumarins. Hydroxycinnamic acid, associated to coumarins was observed to inhibit the growth of Gram-positive bacteria (Cheung *et al.*, 2014).

1.4.2 Terpenoids and essential oils

These compounds are based on an isoprene structure. The general molecular formula of a terpene is $C_{10}H_{16.}$ Terpenes exist as diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), hemiterpenes (C_5) and sesquiterpenes (C_{15}). Terpenoid

compounds comprise oxygen as additional element and share their derivation with fatty acids. Many authors have reported the antibacterial activities of terpenenes and terpenoids (Verica *et al.,* 2014; Barre *et al.,* 1997; Scortichini *et al.,* 1991).

1.4.3 Alkaloids

Alkaloids are heterocyclic nitrogen-containing compounds. Omulokoli et al. (1997) had observed that diterpenoid alkaloids from the plants of Ranunculaceae family possessed effective antimicrobial properties. Glycoalkaloids also have been witnessed to have microbiocidal effects against intestinal infections (McDevitt *et al.,* 1996; Ghoshal *et al.,* 1996). Berberine had been found as effective antimicrobial agent with its mode of action attributed to the ability to intercalate with microbial DNA (Phillipson and O'Neill, 1987).

1.4.4 Lectins and polypeptides

Peptides are positively charged compounds and contain disulfide bonds. Thionins are peptides found in barley and wheat which have been observed to be toxic to Gram-negative and Gram-positive bacteria (Pelegrini and Franco, 2005). Fabatin, from fava beans appeared to inhibit *E. coli* and *P. aeruginosa* (Zhang and Lewis, 1997).

Table 1.1 Plants containing antibacterial activity and its corresponding phytocompounds.

Plant	Compound	Class
Pimenta dioica	Eugenol	Terpenoid
Aloe vera	Latex	Complex mixture
Malus sylvestris	Phloretin	Flavonoid derivative
Withania somniferum	Withafarin A	Lactone
Berberis vulgaris	Berberine	Alkaloid
Piper nigrum	Piperine	Alkaloid
Anacardium pulsatilla	Salicylic acids	Polyphenols
Matricaria chamomilla	Anthemic acid	Phenolic acid
Capsicum annuum	Capsaicin	Terpenoid
Syzygium aromaticum	Eugenol	Terpenoid
Erythroxylum coca	Cocaine	Alkaloid
Eucalyptus globulus	Tannin	Polyphenol
Allium sativum	Allicin, ajoene	Sulfoxide
Camellia sinensis	Catechin	Flavonoid
Lawsonia inermis	Gallic acid	Phenolic
Allium cepa	Allicin	Sulfoxide
Satureja montana	Carvacrol	Terpenoid
Thymus vulgaris	Caffeic acid	Phenolic acid
Curcuma longa	Curcumin	Terpenoids
Gaultheria procumbens	Tannins	Polyphenols

(Adapted and modified from Duke, 1985)

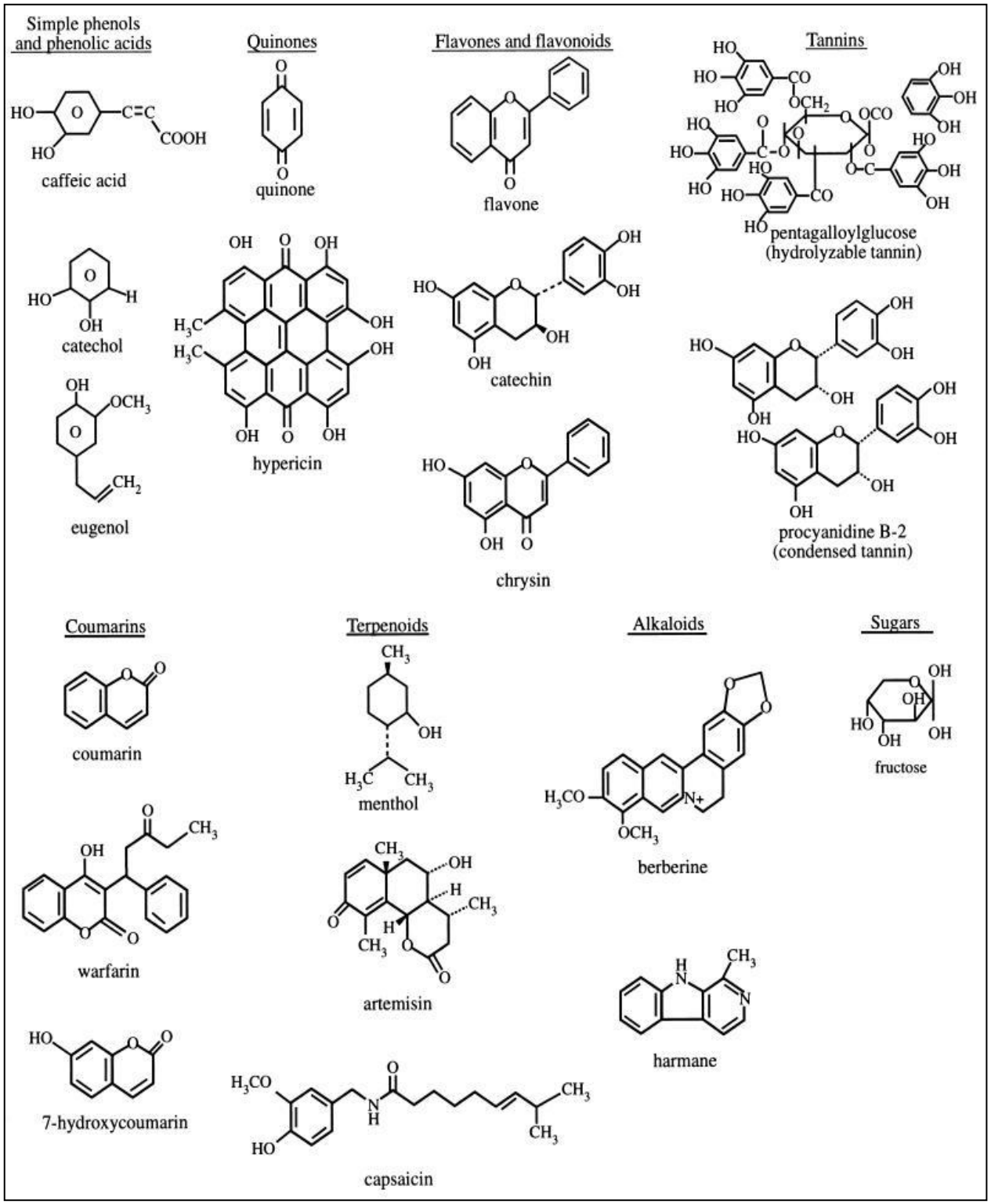

Figure 1.1 Structures of common antibacterial plant phytochemicals. Adapted from Cowan (1999).

1.5 Medicinal plant selection for drug discovery

The main objective of using ethnomedicinal plants as sources of therapeutic agent are to isolate biologically active compounds for direct use as drugs, for example morphine, taxol, vincristine and vinblastine. The medicinal plants are also exploited to create novel lead bioactive compounds of known molecular structures for further modification in semisynthesis. These new modified molecular structures harvest patentable constituents with higher desirable biological activities and lower or nil toxicological activity, for instance metformin, verapamil, nabilone and oxycodon. These ethnomedicinal plant extracts are also consumed for herbal remedy, as example cranberry, feverfew and ginkgo biloba (Farnsworth *et al.,* 1985).

According to Verpoorte (2000), there are a few approaches available for selecting plants as lead drugs. Firstly, the plants can be selected randomly followed by chemical screening. This approach is also known as phytochemical screening and has been exercised in the past and at present practised mainly in developing countries. The tests are carried out to detect the presence of important phytocompounds like alkaloids, flavonoids, iridoids and cardenolides. Although the tests are simple to perform, more often false-positive and false-negative tests provide results that are difficult to assess. It can also pose complexity to establish relationship between specific class of phytochemical to particular biological target (Fabricant and Farnsworth, 2001).

The second approach involves random selection of plants followed by biological assay. Plant extracts are evaluated for several biological activities, including

antibacterial, antifungal, antidiabetic, antitumor, antifertility, anti-inflammatory, antihypercholesteremic, cytotocixity and others. However, no medically useful active compounds for human use have been developed from this approach even though substantial number of known and novel bioactive compounds were isolated from the active plants (Wall and Wani, 1996).

Follow-up of biological activity report is another consideration opted for plant selection to facilitate drug discovery. The reports that showed plant extracts with remarkable pharmacological activities will be sensible to investigate and study for their responsible active principles. A further approach is the follow-up of ethnomedical uses of plants. There are a number of ethnomedicinal information available in this method and among them is plants used in traditional medical system such as Ayurveda, Unani, Kampo and traditional Chinese medicine. These traditional medical systems have existed and are in use for thousands of years. They are frequently seen as a revised body of written knowledge and theory. These medical systems are being practiced till today because of their organizational strengths which concentrate mainly on multi-component mixtures of the plant extracts (Kinghorn, 1994). However, these system lacks credibility owing to poor documentation of adverse effects of those widely used plants. In addition, the efficacy of the plant mixtures often poses difficulty to be assessed by Western scientific methods.

Folklore, herbalism and shamanism are novice medical system where information is passed to the next generation through a herbalist or shaman. In this system, trivial information about the plant is recorded as they are often kept secret by the healers.

The herbalist or shaman playing the roles of pharmacist and medical doctor combines the spiritual, religious and cultural beliefs to treat patients, where treatments are often regarded as mysticism or magic. Such medical system is well known in Africa and South America (Bonet *et al.*, 1999). In this type of screening method based on biological activity report, plant selection for potential drug discovery can also be accomplished through books on medical botany and herbals, review articles, field work reports and computer databases search (Siddique *et al.*, 2000).

1.6 Natural product isolation

Natural product research with regards to the isolation of novel biactive constituents has captured massive interest of many reserachers since exceptional developments in areas of chemical separation and purification methods, spectroscopic techniques and sensitive bioassays (Sticher, 2008). The conventional methods of isolation of natural products such as medicinal plants usually begins with identification, collection and preparation of the biological material by drying. Next, extraction can be proceeded with various extraction solvent systems of low to high polarity. The solvent extraction methods include maceration, soxhlet extraction, percolation, ultrasound-assisted and turbo-assisted extraction. Maceration can be done at room temperature by immersing the plant material with the extracting solvent. Even though this method undergo long extraction times and high solvent consumption, it has the advantage of mild extraction conditions with high extraction yields (Seidel, 2012).

Extraction methods are applied as pre-purification step to selectively remove interfering components in isolation process. Pre-purification procedures can involve filtration, precipitation, removal selectively of chlorophyll, tannins or waxes from plant extacts by the use of solid-phase extraction (SPE) cartridges. Pre-packed SPE cartridges function by the principle of a liquid-solid extraction where the interfering matrix substances of a sample are retained on the solid sorbent of the cartridge while the components of interest are eluted as eluent or the components of interest are retained on the solid sorbent while the interfering matrix substances are eluted out (Sarker *et al.,* 2006).

Liquid-solid separation techniques are the most widely performed separation in liquid chromatography. This technique materializes in the forms of planar chromatography and column chromatography. Planar chromatography techniques include thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC) where mobile phase is eluted through a stationary phase by capillary forces. The common adsorbent of TLC and PTLC is silica gel and has been utilized for the separation of both lipophilic and hydrophilic substance mixtures. As for the final purification step, gel filtration on Sephadex LH-20 is highly advised to remove binders or fluorescent indicators which may be extracted together with the scraped off compounds (Peng *et al.,* 2006).

Column chromatography is usually used for preparative separations of extract. In this method, a glass column is occupied and packed with an adsorbent material of defined particle size. The principle of the column chromatography is that when a sample

mixture to be separated is introduced together with the eluted mobile phase from the top of the column, the individual components of the mixture is partitioned between the solid sorbent phase and the solvent phase. Thus based on the respective components polarity, the individual components migrate at respective different and specific rates. Those substances with lower affinity and little adsorption to stationary phase migrate faster and eluted out first while those with strong adsorption affinity travel slower and eluted out last. Column chromatography can offer a rapid preliminary fractionation of complex mixtures (Fulzele and Satdive, 2005).

Bioactivity-guided isolation technique is frequently employed to significantly reduce the time for hit discovery. This approach typically links information on the chemical profiles of extracts and fractions with their respective activity data of *in vitro* bioassays. Completing isolation of a pure substance, chemical structure elucidation needs to be addressed using chemical identification and characterization techniques such as ultra-violet and visible (UV-Vis) spectroscopy, Fourier transform infra-red (FTIR) spectroscopy and nuclear magnetic resonance (NMR).

1.7 Bacteria

Bacteria are single celled living things. They live in every climate and location on earth. These unicellular microorganisms are known as prokaryotes that without a nucleus or membrane bound organelles. The genetic material in a prokaryote is contained within a DNA-protein complex called nucleoid. In addition, prokaryotes also have circular DNA structures termed as plasmids which often contains beneficial genes. Prokaryote cells exist in five basic shapes; spherical (cocci), rod

(bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). Bacteria reproduce through asexual reproduction usually by binary fission where a single cell, divides into two identical daughter cells.

Bacteria are distinguished into two classes, Gram-positives and Gram-negatives which derived from the reaction of bacterial cells to the Gram stain. Gram staining is a bacteriological laboratory technique used to differentiate bacteria by the physical properties of their cell walls (Ryan and Ray, 2004). Gram-positive and Gram-negative bacteria have very distinct external structures. Gram-positive bacteria possess multilayered cell wall consisting of peptidoglycan whereas Gram-negative bacteria have only few layers of peptidoglycan. However, Gram-negative bacteria have an outer membrane external to the peptidoglycan which is mainly containing lipopolysaccharides and lipoproteins. The presence of outer membrane helps the Gram-negative bacteria to maintain the cell structure and act as a permeability barrier to large molecules and hydrophobic molecules. The cell wall structures of Gram-positive and Gram-negative bacteria are shown in the Figure 1.2.

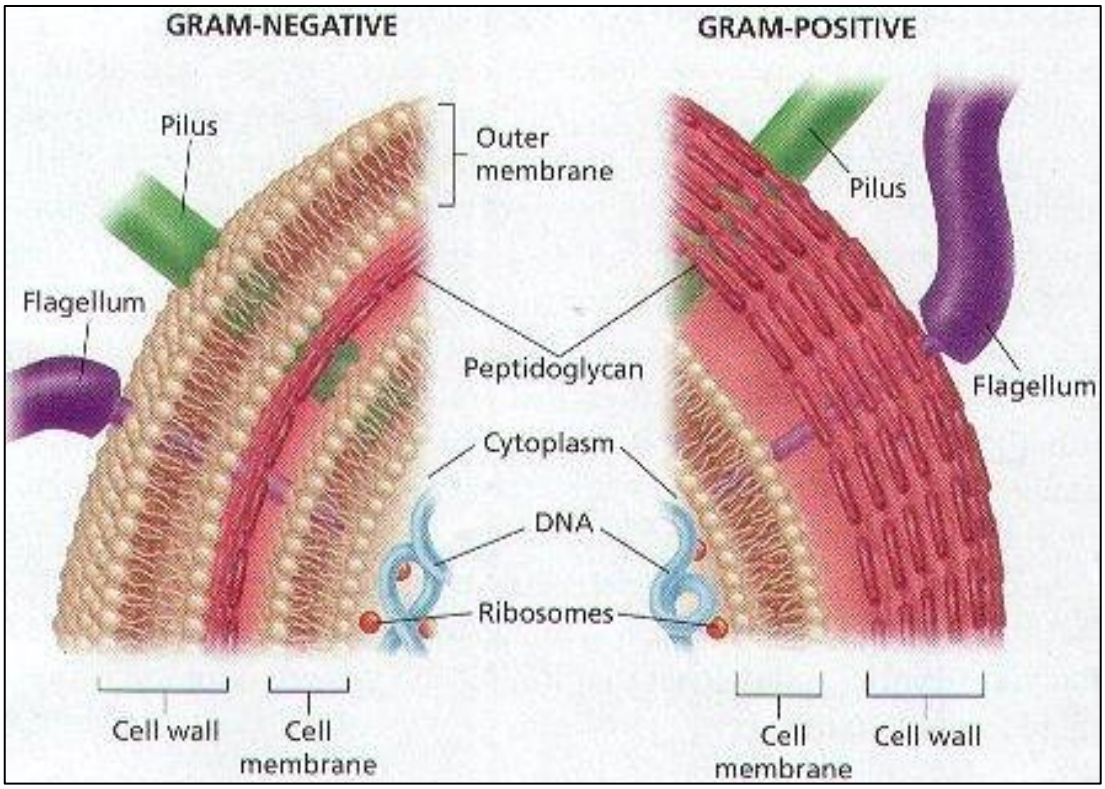

Figure 1.2 Cell wall structures of Gram-negative and Gram-positive bacteria.

(Source:http://healyourselfathome.com/SUPPORTING_INFORMATION/MICROBES/BACTERIA/ABOUT/BACTERIA_characteristics_gram_pos_or_gram_neg.asp)

1.7.1 Bacterial infection

Most bacteria are free-living species that are harmless and highly beneficial. Comparatively there a few species of bacteria that is pathogenic. Pathogenic bacteria often form a parasitic coalition with other organisms and obtain most of its requirements from those organisms. This parasitic action causes infections and diseases to the host. Pathogenic bacteria are a major cause of infectious diseases and human death. World Health Organization (WHO) recently estimates that there are more infections than people because many people acquire more than one infection which are are commonly instigated by streptococci, pneumococci, staphylococci, klebsiella and pseudomonas strains (Braine, 2011).

1.8 Infectious disease

Infectious diseases caused by bacteria present a massive threat to human life worldwide. Infectious diseases have become the second major cause of death worldwide and bacteria are the most frequent source of infection-related death. Based on worldwide death toll, annually about 12 million people are deceased from infections (WHO, 2013). An infection is the invasion and replication of infectious bacteria in the host body and the subsequent host tissues response to the presence of the pathogen or the toxins they produced in the host. Infectious disease, by contrast, a process that destructs a person's entire health. However, a person whom is infected with pathogens does not necessarily have infectious disease. The process when the health is not impaired after an infection is called as subclinical infection. According to Burt (2004), infectious disease arises when a person's body lacks natural immunity towards the bacterial infection or when the amount of pathogens invading

the body exceeds the capability of the host immune system to handle. Pathogenic bacteria causes various infectious diseases globally such as tuberculosis, pneumonia, tetanus, typhoid fever, meningitis, diphtheria, syphilis, cholera, food borne illness and leprosy. Table 1.2 illustrated the list of infectious diseases associated with infectious bacteria.

1.9 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped Gram-negative bacterium and one of the leading pathogen causing nosocomial infections worldwide (Tanya and Daniel, 2009). The high resistance rates of *P. aeruginosa* are owing to the frequent implications in severe infections. *P. aeruginosa* is an opportunistic pathogen since it only infects sick or immunocompromised persons. It rarely causes disease in healthy persons and does not ordinarily initiate disease in immunocompromised persons as it only multiply freely in them.

Hospitalized patients are highly risky to *P. aeruginosa* infections as it is often found in hospitals. Infections that have been acquired in the hospital are called nosocomial diseases. Nosocomial infection is related to any disease contracted by a patient while under hospitalization. Nosocomial infections are hospital-acquired infections where bacteria resistant to multiple antibiotics exist vastly. Typically, a nosocomial infection occurs within 48 hours after admittance to the hospital or within 30 days after discharge. People with cystic fibrosis and AIDS frequently die from infections produced by *P. aeruginosa*. Those who have undergone chemotherapy, have had

transplants or have any immunosuppressed conditions are far more at risk for developing *P. aeruginosa* infections (Rafla and Tredget, 2011).

The infections triggered by these bacteria are often tough to treat because of the intrinsic resistance of the pseudomonal species to many drug classes and its capability to acquire further resistance towards β-lactams, aminoglycosides and fluoroquinolones via mutation (McGowan, 2006). Multidrug resistance (MDR) is now very common and ever-increasing among *P. aeruginosa*. Numeral strains have now been isolated that display resistance to basically all consistent antipseudomonal antibiotics. *P. aeruginosa* is intrinsically resistant to multiple antibacterial agents due to the low outer membrane permeability (Mesaros *et al.*, 2007).

P. aeruginosa has several advantages that makes it a strong opponent. The ability of this bacterium to adhere to the host cells, minimal food requirement, multiple antibiotic resistancy, rigid cell wall along with outer membrane and capability to generate tissue harming proteins empower this bacterium to infect various parts of the human body. Infections of *P. aeruginosa* in specific body sites include heart and blood, bones and joints, central nervous system, eye and ear, urinary tract, skin and soft tissue and lung.

Table 1.2 List of infectious diseases and associated bacterial pathogens.

Bacteria	**Diseases**
Staphylococcus aureus	Skin and wound infection, abscess, toxic shock syndrome
Streptococcus pneumoniae	Pneumonia, otitis, sinusitis
Escherichia coli	Bacteremia, gastrointestinal infection
Bacillus anthracis	Pulmonary anthrax
Campylobacter jejuni	Acute enteritis
Corynebacterium diphtheriae	Diphtheria
Enterococcus faecalis	Nosocomial infections, urinary tract infection, peritonitis
Helicobacter pylori	Peptic ulcer
Leptospira interrogans	Leptospirosis
Mycobacterium leprae	Leprosy
Mycobacterium tuberculosis	Tuberculosis
Mycoplasma pneumoniae	Mycoplasma pneumonia
Neisseria meningitidis	Meningitis
Pseudomonas aeruginosa	Pseudomonas infection, bacteremia
Salmonella typhi	Typhoid fever
Treponema pallidum	Syphilis
Vibrio cholerae	Cholera

(Adapted and modified from O'Connor *et al.*, 2006)

1.10 Antibacterial agent

An antibacterial agent is also termed as an antibiotic. Antibacterial agents are widely used either to kill or inhibit the growth of bacteria. These agents are relatively very small molecules. Antibiotics are generally categorized based on their chemical structure, mechanism of action and spectrum of activity. These chemical agents aimed for destructing bacterial growth processes or bacterial functions (Calderon and Sabundayo, 2007).

Antibiotics are segregated into two clusters that are bactericidal agent and bacteriostatic agent based on their biological activity on bacteria. Bactericidal antibiotics kill bacteria meanwhile bacteriostatic antibiotics inhibit bacterial growth. Antibiotics with bactericidal effect would target the bacterial cell membrane (polymyxins), cell wall (penicillins and cephalosporins) or intervene with bacterial enzymes (rifamycins and sulphonamides). Those that obstruct with bacterial protein synthesis (tetracyclines and macrolides) are typically bacteriostatic (Sharma *et al.*, 2002).

Certain antibiotics which act only on specific types of bacteria such as Gram-negative or Gram-positive bacteria are known to have narrow-spectrum, whereas broad spectrum antibiotics target a wide array of bacteria. An ideal antibacterial should have the following requirements which includes; selective target, bactericidal, narrow spectrum, high therapeutic index, few adverse reactions like toxicity and allergy, various routes of administration, good absorption, and good distribution to the site of infection and finally emergence of resistance is slow.

Many modern antibiotics are derived from the semisynthetic alteration of various antibacterial compounds from natural products such as the cephalosporins, carbapenems, ketolides and glycylcyclines (Von Nussbaum *et al.,* 2006). It is reported that on average, 2 or 3 new antibiotics are being launched every year, but the effective life span of any antibiotic is limited to 1 to 2 years due to the emergence of antibiotic resistance.

1.11 Cefepime

Cefepime is a newer cephalosporin (aminothiazolylacetamido) antibiotic with broaden spectrum and greater potency than many other cephalosporins (Bonapace, 1999). This fourth-generation cephalosporin was developed in 1994 and is very effective against multiple drug resistant pathogens such as *P. aeruginosa* and *S. aureus* than third generation agents. Cephalosporins are class of β-lactam antibiotics originally derived from fungus *Acremonium*. Cephalosporins are bactericidal agents and possess the same mode of action as other beta-lactam antibiotics such as penicillin. This agent disrupts the synthesis of the peptidoglycan layer of bacterial cell wall.

Cefepime has extended activity against *P. aeruginosa* comparable to that of ceftazidime. Those strains of *P. aeruginosa* that have shown resistance to cefotaxime and/or ceftazidime are therefore susceptible to cefepime (Fung-Tomc *et al.,* 1991). This agent is in a zwitterionic form that can penetrate the outer membrane of Gram-negative bacteria. In contrast to other cephalosporins, cefepime more promptly enters the Gram-negative bacterial cell hence targets multiple essential penicillin-binding

proteins. It exerts the antibacterial activity by appending to specific penicillin-binding proteins and therefore disrupts the cell wall synthesis. This antibacterial agent also capable to escape the effects of many beta-lactamases because of the drug's low affinity for the enzymes. The structure of cefepime is shown in Figure 1.3.

Cefepime, has a positively charged quaternary nitrogen at the 3-position of the cefepime molecule which contribute to the zwitterion characteristics of the molecule. In the side chain at the 7-position of the cefepime molecule, a 2-aminothiazolyl-acetamido group with an alpha-oxyimino substitution augment the stability against beta-lactamases by hindering the enzymes' approach to the main nucleus (Yahav *et al.,* 2007). Cefepime is not active *in vitro* against *Enterococcus faecalis*, *Clostridium difficile*, and methicillin-resistant and cefazolin-resistant *S. aureus*. The most common mechanism of resistance to cefepime is the excess production of beta-lactamases by pathogens.

Cefepime is generally reserved for treating severe nosocomial pneumonia, as well as empirical treatment of febrile neutropenia. It is also often administered for complicated urinary tract infections, skin structure infections and intra-abdominal infections. In neutropenic patients, cefepime alone reduces fever as effectively as combination of antibacterial agents such as ceftazidime or piperacillin plus gentamicin (Chapman and Perry, 2003). For life-threatening infections, the dosage of cefepime proposed is 1000-2000 mg i.v. every 8-24 hours and for severe infections, the dosage of cefepime is 500-2000 mg i.v. every 12-24 hours.

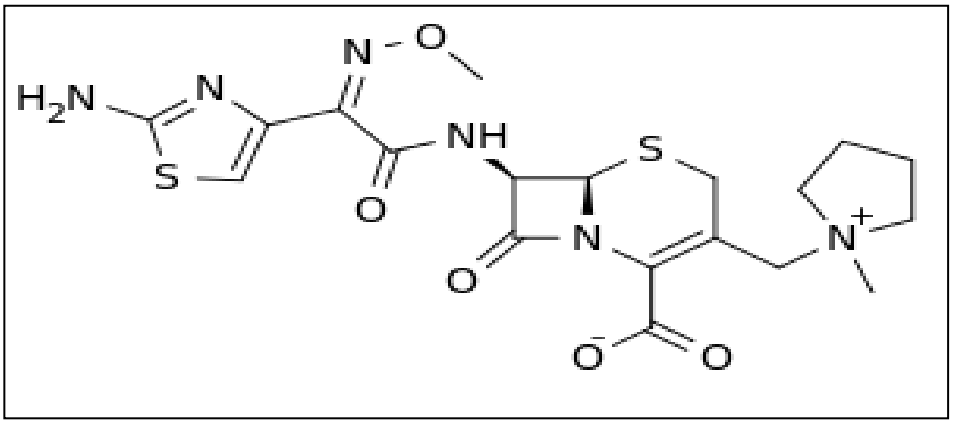

Figure 1.3 Chemical structure of cefepime.

(Source:http://pubchem.ncbi.nlm.nih.gov/compound/cefepime#section= Related-Compounds-with-Annotation)

1.12 Mechanism of action of antibacterial agents

Antibacterial agents operate by precluding crucial life sustaining processes in the bacteria such as the synthesis of cell wall material, the synthesis of DNA, RNA, ribosomes and proteins. The mode of action of antibiotics depends on the nature of their structure and degree of affinity towards target sites in bacterial cells. Inhibition of bacterial cell wall synthesis by antibacterial agent is very selective because cells of humans and animals do not have cell wall. The cell wall structure is vital for the life and survival of bacterial species. Antibiotics that aimed at cell walls can therefore selectively kill or inhibit bacteria. Examples of cell wall inhibitors are cephalosporins, penicillins, vancomycin and bacitracin.

Inhibition of cellular membrane function by antibiotics is frequently poorly selective and can be toxic for systemic use in the mammalian host. This is because cell membranes are found in both eukaryotic and prokaryotic cells. Membrane structure

is an essential partition that segregates and regulates the intra and extracellular flow of substances. A break or impairment to this structure could give rise to leakage of important solutes crucial for the cell survival. Therefore, clinical usage of this class of antibiotic is limited to topical applications. Examples of cellular membrane inhibitors are polymixin B and colistin.

Protein synthesis is a vital process compulsory for the multiplication and survival of all bacterial cells. All the cellular structures and enzymes are fundamentally made of proteins. Inhibition of protein synthesis by antibacterial agents targets the binding sites of either the 30S or 50S subunits of the intracellular ribosome. This action interrupts the normal cellular metabolism of the bacteria and consequently leads to the death of the organism or the inhibition of its growth and multiplication. Examples of protein synthesis inhibitors are chloramphenicol, tetracyclines, aminoglycosides, macrolides, lincosamides and streptogramins.

All living forms including bacteria require DNA and RNA for the replication processes. Inhibition of nucleic acid synthesis involves the binding process of antibacterial agent to the mechanism components involved in the process of DNA or RNA synthesis. This exploitation result in intrusion of the normal cellular processes which will influence bacterial multiplication and survival. Examples of nucleic acid synthesis inhibitors are rifampin, metronidazole and quinolones.