PHYTOCHEMICAL AND ANTIBACTERIAL STUDIES OF THE STEM BARK AND ROOT OF *ALCHORNEA CORDIFOLIA* (EUPHORBIACEAE)

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FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY, ZARIA.

DECEMBER 2012.

Declaration

I hereby declare that the work in this thesis entitled PHYTOCHEMICAL AND ANTIBACTERIAL STUDIES OF THE STEM BARK AND ROOT OF *ALCHORNEA CORDIFOLIA* (EUPHORBIACEAE) has been carried out by me in the Department of Pharmaceutics and Pharmaceutical Microbiology under the supervision of Dr. (Mrs) G. O. Adeshina and Prof. J. O. Ehinmidu.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work was previously presented for another degree or diploma in any institution.

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Name of Student	Signture	Date

Certification

This thesis entitled PHYTOCHEMICAL AND ANTIBACTERIAL STUDIES OF THE STEM BARK AND ROOT OF *ALCHORNEA CORDIFOLIA* (EUPHORBIACEAE) by Rabiah Orchewa Osumah meets the regulations governing the award of the degree of Master of Science (Pharmaceutical Microbiology) of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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Dedication

To Almighty Allah for giving me, wisdom, sound health, favour and life. To Him alone belong all the glory, adorations, worship and praises and my younger brother Mohammed who passed on December 5th, 2011. May almighty Allah have mercy on him, shower on him, his forgiveness, Love and admit him into Aljanah fidaous, Amin.

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Abstract

Stembark and root of *Alchornea cordifolia* were extracted with water and ethanol and the resulting extracts were further fractionated with n- butanol, hexane and ethylacetate. The extracts and fractions were screened for phytocompounds.

Antibacterial activities of the plant extracts and fractions against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi* were investigated using agar diffusion and broth dilution methods.

For comparism, the antibiotic susceptibility profiles of the bacterial isolates to standard antibiotics such as ciprofloxacin and gentamicin were determined using agar diffusion and broth dilution methods. The effect of temperature on the antibacterial activity of the plant extracts and rate of death/survival of the bacterial isolates on exposure to stembark extract and gentamicin were investigated

Phytochemical screening of *A. cordifolia* stembark and root extracts and fractions revealed the presence of sterols, flavonoids, tannins, saponin, glycosides, cardiac glycosides, anthroquinones, resins and carbohydrates.

The stem bark extracts and fractions of *A. cordifolia* demonstrated a high level of antibacterial activity on the various clinical test bacterial isolates than the root extracts.

The results of the zones of inhibition showed by the extracts indicated that there is no significant difference at $p > 0.05$ between the activities of the the root and stembark. The minimum inhibitory concentration values of the extracts and fractions against *S. aureus, S. typhi, Ps. aeruginosa* and *E. coli* ranged between 0.78 mg/ml – 25.0 mg/ml and 0.39 mg/ml – 1.53 mg/ml respectively. While the minimum bacericidal concentrations of the extracts and fractions were between 12.5 mg/ml – 100 mg/ml and 25.0 mg/ml – 100 mg/ml respectively. Antibacterial activity of the ethyl acetate fraction of the ethanol extract against test *Staphylococcus aureus* is comparable to that of ciprofloxacin. The result of the effect temperature on the antibacterial activity of the extracts revealed that the extracts maintained their activity at a lower temperature and the zones of inhibition decreased with increase in temperature.

The stembark extract showed a fast and consistent rate of kill against test *E. coli* similar to gentamicin biocidal activity.

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CHAPTER 1

INTRODUCTION

Higher plants, as sources of medicinal compounds continue to play a dominant role in maintenance of human health since antiquities. Over 50% of all modern clinical drugs are of natural product origin (Stuffness and Douros, 1982). Natural products from plants have been reported to play important role in drug development programs of the pharmaceutical industry, (Baker e*t al.*, 1995). Varieties of materials derived from plants have been used for the prevention and treatment of diseases, virtually in all cultures. Herbs have been reportedly used as sources of food and medicinal purposes for centuries and this knowledge have been passed from one generation to another (Adedapo *et al.,* 2002). Medicinal plants have been reported to also represent a rich source from which antimicrobial agents can be obtained (Kubmarawa *et al.,* 2007). Plants, which are observed to be efficacious and frequently used, may contain compounds that are potential drug candidates and could rightly be recomended for further examination. Scientific investigations of medicinal plants have been initiated in many countries because of their contributions to health care. The continual search for, and the interest in natural plant products, for use as medicines has acted as the catalyst for exploring methodologies involved in obtaining the required plant materials and thence probing their constituents.Many pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis and quinine (WHO, 2008). Pravin, (2006) reported that about 70% of the human population is dependent (wholly or partially) on plant-based medicines and the World Health Organization (2008) estimates that 80% of the world population use herbal medicine for some aspect of primary health care. In developing countries, especially in rural

areas, people usually turn to traditional healers when in diseased condition and plants of ethnobotanical origin are often presented for use.

Investigation into the chemical and biological activities of plants during the past two centuries have yielded compounds like Artemisinin and quinine, which are used for the development of major novel and more effective therapeutic agents (Roja and Rao, 2000). Presently, many scientist and organizations are in search of traditional remedies as alternative medicine (Cortella and Pochettino, 1999). Thus, many plants that are used in traditional practices are sold in urban settlement to meet the need of public desire.

The African continent is one of the continents endowed with the richest biodiversity in the world, with an avalanche of many food plants used as herbs and for therapeutic purposes. This is largely due to the geographical spread spanning a land mass of approximately 216,634,000 hectares of closed forest areas, over 5,000 different species of plant substances have been recognized to occur in these areas and many of them have been found to be useful in traditional medicine for prophylaxis and cure of diseases (Iwu, 1993). This great biodiversity, therefore, offers economic promise and in rapid growing Pharmaceutical industry. The potential of herbal medicines and medicinal plant research results in health care is no longer in doubt, having gained recognition in several nations of the world and the World Health Organisation (WHO). Infections due to pathogenic bacteria and fungi have been reported to represent a critical problem to human health (Eswarappa, 2009). It has been reported that despite the extensive use of antibiotics and vaccines, infectious diseases continue to be a leading cause of morbidity and mortality worldwide (Bloom, 2000). Widespread antibiotic resistance, the emergence of new pathogens in addition to the resurgence of old ones and the lack of effective new therapeutics exacerbate the

problems (Bloom, 2000). Microbial infections are repeatedly reported to be the major cause of inflammation (Du-Shieng *et al.,* 2005).

In-spite of the heterogeneous nature of the continent and delays of information on the compositions and biological activity of many plant substances, there has been little effort devoted to the development of chemotherapeutic and prophylactic agents from these plants. In view of the emerging competitive world, therefore, the evolution of the constituents, pharmacological properties, detailed screening of bioactive substances for chemotherapeutic purposes are warranted urgently.

1.1 STATEMENT OF PROBLEM

The resistance of disease causing organism against antibacterial agents has been repeatedly reported to be the major cause of death worldwide and in tropical countries, it accounts for approximately 50% of death.Since the era of World War II, these drugs were widely used to save countless lives and reduced serious complications of many feared diseases and infections. However, after a long period of usage, organisms begin to develop resistance to these antimicrobial agents and equally developed way to circumvert the effect of these drugs, which may probably be due to;

- Indiscrimate use of antibiotic that was thought to have spurred these powerful adaptations that enabled microorganism to survive those drugs.

Infectious disease is the number one cause of death worldwide, microbial resistance to antibacterial agent has proved difficult in the treatment of infectious diseases. It increases the risk of nosocomial infections (NIAID, 2004).

Antibacterial resistance has been reported to a difficult problem for hospital immunocompromised patients. The use of heavy antibacterial agents on patients has resulted in the adaptation of mutants' organisms, which can be very resistance to the most potent drug, thus scaling up health care cost and for this reason, the masses can not afford it, the result is increase in disease severity and death from certain infections (NIAID, 2004).

- Microorganisms are singled–celled, when compared to higher life forms, having small number of gene. A single gene mutant can affect their ability to cause disease. Mutation help microorganism to survive the effect of antibacterial agents. Bacteria are known to acquire resistance gene from other resistant bacterial strains. Hospitals provide a fertile ground for transmission and selections of antibacterial drug resistance and the addition of antibacterial agents to agricultural food have been reported to promote microorganism resistances (NIAID, 2004). Antibacterial resistance has been a problem since the time immemorial. For example, *S. aureus* has been reported to develop resistance to penicillin, not long after its introduction.

Medicinal plant have continued to play dominant role since time immorial for the protection, prevention and treatment of diseases. Inamol, (2004) reported that approximately, one hundred and seventy herbal drugs were officially recognized in the United State Pharmacopia. It has equally been reported that 80% of the world population rely on herbal medicine for primary health care need (Akerele, 1993). Traditional herbal therapy has no doubt the potential of contributing to a better health care system (WHO, 1998), therefore *Alchornea cordifolia* with its proven ethnotraditional uses will be an added tool for therapeutic use.

1.2 PROSPECTS (JUSTIFICATION)

Alchornea cordifolia is an important medicinal plant in traditional medicine. Since many plants show antibacterial properties, synthetic antibacterial agent should not be encouraged due to its

side effects and resistance problem. However, the link between activity and particular compounds is often not clear. More research work is needed to elucidate these relations. It is probable that *Alchornea cordifolia* will remain a major medicinal plant.

1.3 AIM

This study was undertaken evaluate the antibacterial activity and report scientific evidences to justify the ethnomedicinal use of *Alchornea cordifolia.*

1.4 SPECIFIC OBJECTIVES

- To collect the stem bark and root of *Alchornea cordifolia*.
- To carry out solvent extraction, preliminary fractionation and phytochemical analysis of the stem bark and root of *Alchornea cordifolia* using water and ethanol as solvent system.
- To determine the antibacterial activities of the *Alchornea cordifolia* stembark, root extracts and fractions against the clinical isolates of *S. aureus, Ps. aeruginosa, S. typhi, Escherichia coli* using the agar diffusion and agar dilution techniques.
- To determine the Death/Survival rate of some resistant isolates to different concentrations of the most active extract.
- To determine the effect of temperature on the antibacterial activities of plant extracts.

1.5 LIMITATION

The problem encountered in these work was getting the location of this plant, which was observed to be unavailable in the Northern part of Nigeria. From the geographical distribution, *Alchornea cordifolia* grow best in the swamping area, which happened to be in the Middle belt and Southern part of Nigeria. In addition, the problem of *Alchornea cordifolia* plant collection and transportation was difficult.

CHAPTER 2

LITERATURE REVIEW

2.1 DESCRIPTION OF PLANT

Alchornea cordifolia (Schum. and Thonn. Mull. Arg.) is of the Family – Euphorbiaceae. The common names are Christmas bush and dovewood. In Nigeria, it is called: 'Unwanwe' in Edo; 'Ububo' in Igbo; 'Osokpo' in Urhobo; 'Ipa, esinyin' in Yoruba, 'Bambami' in Hausa, and'mbom' in Efik.

Alchornea cordifolia plant is an evergreen dioeciously shrub measuring up to 8 m tall with young erect shoots later becoming horizontal, hollow, glabrous. The leaves are alternate, simple, stipules and triangular, the is base cordate, with basal lobes slightly auriculate and overlapping, apex acute to acuminate, margins toothed, shortly hairy when young.

The male inflorescence has an axillary panicle, sparingly hairy; bracts minute.While the female inflorescence has an auxiliary spike, bracts broadly triangular-ovate. Flowers unisexual, sessile; male flowers with sepals, petals absent, stamens, the united filaments forming a basal plate; female flowers with calyx, lobes obtuse, hairy, petals absent, ovary superior, conical, styles. Fruit is some what compressed, smooth, shortly hairy, green to red, 2-seeded.



PlateI *Alchornea cordifolia* plant

2.2 ORIGIN AND GEOGRAPHYIC LOCATIONS.

Alchornea cordifolia occurs widely in Africa from Senegal to Kenya, Tanzania, and throughout Central Africa to Angola. It is cultivated in Democratic Republic of Congo for its medicinal use (Latham, 2004).

2.3 PHYTOCHEMICAL PROPERTIES OF *A. CORDIFOLIA*

The leaves, roots and stem bark of *Achornea cordifolia* have been reported by previous worker to contain terpenoids, steroid glycosides, and flavonoids (2–3%), tannins (about 10%), saponins, carbohydrates and the imidazopyrimidine alkaloids alchorneine, alchornidine and several guanidine alkaloids (Macfoy and Sama, 1990). The leaves have also been reported to contain a range of hydroxybenzoic acids, gallic acid and its ethyl ester, gentisic acid, anthranilic acid

(vitamin) and protocatechuic acid, and ellagic acid (alizarine yellow). A C_{20} homologue of vernolic acid, named alchornoic acid was found in the seed oil (Osadebe and Okoye, 2003; Manga, *et al.,* 2004; Mavar – Manga *et al.,* 2008).

2.4 MEDICINAL PROPERTIES

Alchornea cordifolia has been reported to be commonly used as a medicinal plant throughout its area of distribution. The leaves, stem bark, stem pith, leafy stems, root bark, roots and fruits have been reported to be commonly used in local medicine (Neuwinger, 2000).

The leaves or leafy stems has been observed for their sedative and antispasmodic activities to treat a variety of respiratory problems including sore throat, cough and bronchitis, genital / urinary problems including venereal diseases and female sterility (Tona *et al*., 2000; Agbor *et al.,* 2004). Decoction of bruised fruit has be reportedly used to prevent miscarriage. The sap of the fruit was observed to cure eye problems and skin diseases.

The leaf or root infusion has been reportedly given to livestock to treat trypanosomiasis in veterinary medicine (Adewunmi, 2001). Leaves and root bark are externally applied to treat leprosy and as an antidote to snake venom (Burkill, 1994).

The leaves are eaten as an emmenagogue and to facilitate delivery and as an abortifacient (Burkil, 1994). Leaf and root decoctions have been reported widely used as mouthwash to treat ulcers of the mouth, toothache and caries, and twigs are chewed for the same purposes (Obadoni and Ochuko, 2002). Crushed fresh leaves or powdered dry leaves have been reported to be applied externally as a cicatrisant to wounds, to relieve pain, e.g. backache and headache, to fractures to improve healing.

A decoction or pastes of leafy twigs have been reportedly applied as a wash to treat fever, malaria, rheumatic pains, and enlarged spleen and as a lotion or poultice to sore feet. A decoction of roots or leaves is applied in the vagina to stop post-partum hemorrhage and to treat vaginitis (Kona *et al*., 2005; Ebi, 2000). Bruised leaves are applied as an enema to treat impotency (Latham, 2004). A root decoction or maceration is taken to treat amoebic dysentery and diarrhea and used as eye drops to cure conjunctivitis (Agbor *et al*., 2004).

2.5 OTHER USES

In West and East Africa *Alchornea cordifolia* has been reported to be used for alley cropping for *in-situ* mulch production in banana or maize plantations and leaves are used as forage for small ruminants and poultry. Burkill, (1994) and Neuwinger, (2000) reported that chicken produce egg yolks with a deeper yellow color when fed regularly with the leaves. It has been reported that fishermen use leaves and fruits for dyeing and preserving fishing nets (Adewunmi *et al.,* 2001). The Iwo people of Nigeria chew the leaves as an appetizer. Dried leaves of *A. cordifolia* have been reported used as tea substitute. Mats and cloth are cooked with the fruits and natron to color them black. The acidulous fruits are considered edible and used as bait to trap birds (Kanmegne *et al*., 1999). The wood ash serves as a mordant. The wood is light, soft and perishable and is used for house construction, stakes and kitchen utensils, and benches when large stems are available. The wood has been reportedly also used as fuel; the plant is used as a windbreak around crops (Ruhigwa *et al.,* 1994).



Plate II *A. cordifolia* stem showing the seeds arrangement.

2.6 SECONDARY METABOLITES

Secondary metabolites are molecules that are not necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency, or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat (Adewunmi *et al.*, 2001). Secondary compounds are often involved in key interactions between plants and their abiotic and biotic environments (Edward *et al.,* 1999). Relationship between intermediate metabolism and secondary compounds in plants throughout history has been utilized by humanity. There are approximately four major classes of secondary compounds that are significant to humans. These classes are the flavonoids, alkaloids, phenylpropanoids and terpenoids (Edwards *et al.,* 1999).

2.6.1 Flavonoids

The flavonoids are a large group of natural products widespread in higher plants, and are found in some lower plants including algae. The flavonoids are phenolic compounds possessing 15 carbon atoms and comprise two benzene rings joined by a linear three-carbon chain (Osadebe and Okoye, 2003).

Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most angiosperm families (flowering plants).

However, their occurrence is not restricted to flowers but includes all parts of the plant. They are low molecular weight substances found in all vascular plants. In the broad sense, they are virtually universal plant pigments. The anthocyanidins are responsible for flower colour in the majority of angiosperms, but colourless flavonoids are also wide spread and abundant, they are phenyl benzopyrones with an assortment of basic structures usually found conjugated to sugars. Flavonoids have been reported to occur in several structurally and biosynthetically related classes and are important constituents of the human diet, being derived largely from fruits, vegetables, nuts, seeds, stems and flowers (Harborne, 1977).

Several members of the flavonoids family have been reported to possess antiviral and anti-inflammatory properties, vasculo-protector and anti-thrombotic action, spasmolytic activity, estrogenic actions, antioxidant and liver protecting effects (Middleton and Kandaswami, 1994).

Very little was known before 1989 on the effects of this class of compounds on the central nervous system (CNS). Some flavonoids, like quercetin and gossypin, have recently been shown to possess sedative and analgesic effects (Picq *et al*., 1991). Another flavonoid, and biflavonoid

derivatives, isolated from *Gingko biloba* have been shown to increase blood flow and reduce neuronal oxidative metabolism (Picq *et al.,* 1991).

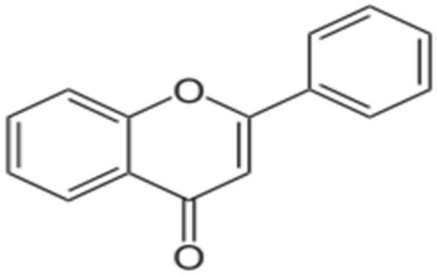


Fig 2.1 Molecular structure of the flavone backbone (2-phenyl-1, 4-benzopyrone)

Report from researches indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological "response modifiers". *In vitro* studies have also showed that flavonoids also have anti-allergic, anti-inflammatory (Spencer-Jeremy, 2008), anti-microbial (Cushnie and Lamd, 2005; 2011), anti-cancer (Sousa *et al.,* 2007) and anti-diarrheal activities (Schuier *et al.,* 2007). Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant (Aqil *et al.,* 2006)

2.6.2 Triterpenoids

Triterpenoids are non-steroidal secondary metabolites, which have been isolated from *A. cordifolia* plant. The physiological function of triterpenoids is generally believed to be a chemical defense against pathogens and herbivores (Reed, 1995). Throughout the plant and animal kingdoms, terpenoids are known to have a wide range of functions. They can act as defensive substances in plants (allomones) and animals to deter herbivores. Plant hormones are often derivatives of terpenoids, such as cytokinins, gibberellins and abscisic acid. It is therefore expected that terpenoids should act against certain pathogens causing human and animal diseases (Mahato and Sen 1997), although medicinal use of this class of compounds is rather limited,

possibly due to their hydrophobic nature, recent work in this regard indicated their great potential as drugs. Moreover, despite the remarkable diversity already known to exist, new variants continue to emerge (Mahato *et al.,* 1997).

Terpenoids are active against bacteria (Taylor *et al.,* 1996), fungi (Suresh *et al.,* 1997) and viruses (Xu *et al.,* 1996). Their mechanism of action is not fully understood. Capsaicin, a constituent of chili peppers, is bactericidal to *Helicobacter pylori,* although possibly detrimental to the human gastric mucosa (Jones *et al.,* 1997). Another terpenoids called petalostemumol, isolated from the prairie clover (*Dalea sp*) showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* as well as *Candida albicans* (Cowan, 1999).

2.6.3 Glycoside

Glycosides are compounds containing a carbohydrate and a non-carbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetal linkage at carbon atom 1 to a non-carbohydrate residue or aglycone. The sugar component is called the glycone. If the carbohydrate portion is glucose, the resulting compound is a glucoside (Edwards *et al.,* 1999). Saponin glycosides are divided into two based on the chemical structure of their aglycone (sapogenins). Saponins on hydrolysis yield an aglycone known as ‘sapogenin’.

Saponin = Sugar (glycone) + Sapogenin (aglycone).

The so-called neutral saponins are derivatives of steroids with spiroketal side chains. The acid saponins possess triterpenoid structures.

2.6.4 Tannin

Tannins are widely distributed in species throughout the plant kingdom.They are found in both gymnosperms as well as angiosperms and physically located in the vacuoles or surface wax of

plants. These storage sites keep tannins active against plant predators, and from affecting plant metabolism while the plant tissue is alive; it is only after cell breakdown and death that the tannins are active in metabolic effects. They are classified as ergastic substances, i.e., non-protoplasm materials found in cells.Tannins are found in leaf, bud, seed, root, and stem tissues. Tannins are located in stem tissue, often found in the growth areas of trees, such as the secondary phloem and xylem and the layer between the cortex and epidermis. Tannins may help regulate the growth of these tissues. The most abundant polyphenols are the condensed tannins, found in virtually all families of plants, and comprising up to 50% of the dry weight of leaves (Scalbert *et al.,* 2002). There are three major classes of tannins:

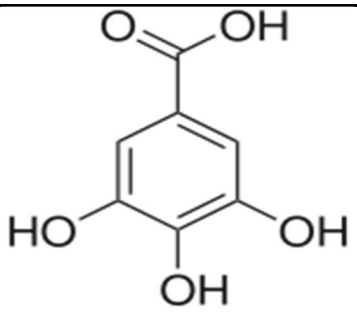
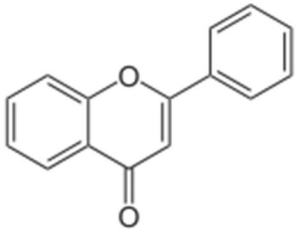
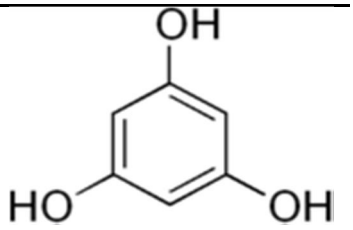
Base Unit:	 **Gallic acid**	 **Flavone**	 **Phloroglucinol**
Class/Polymer:	Hydrolyzable tannins	Non-Hydrolyzable or condensed tannins	Phlorotannins
Sources	Plants	Plants	Brown algae

Fig 2.3 Tannin groups.

The poliovirus, herpes simplex virus, and various enteric viruses are inactivated. Tannins have shown potential antiviral, (Bajaj, 1988), antibacterial (Lü *et al.*, 2004) and antiparasitic effects (Akiyama *et al.,* 2001). *Alchornea cordifolia* plant has shown promise as a potential therapeutic agent, which may be beneficial in patients with neurological disease (Adewunmi *et al.,* 2001). Souza *et al.,* (2006) discovered that the tannins isolated from the stem bark of *Alchornea cordifolia* also have anti-inflammatory and antiulcer activity in rodents, showing a strong

antioxidant property with possible therapeutic applications.Tannins are an important ingredient in the process of tanning leather. Oak bark, mimosa, chestnut and quebracho tree have traditionally been the primary source of tannery tannin, though inorganic tanning agents are also in use today and account for 90% of the world's leather production (Marion, 2006). *Pinus radiata* tannins had be investigated for the production of wood adhesives (Li, 1998). Resins made from tannins have been investigated to remove mercury and methylmercury from solution (Torres, 1999). Immobilized tannins have been tested to recover uranium from seawater (Takashi and Sakaguchia, 1987).

2.7 ANTIBACTERIAL CHEMOTHERAPEUTIC AGENTS

Chemotherapy refers to the control of any disease using drugs. However, the term chemotherapy is usually associated with the treatment of cancer by anti-tumor drugs. In the case of infectious disease, this involves the use of drugs that will kill or limit the growth of microorganisms. Until the early part of the century, no effective antibacterial chemotherapeutic drugs existed (Florey, 1945). For such a drug to be effective, it must be able to kill or limit the growth of microorganisms without severely affecting cells of the host. This is refered to as selective toxicity. For bacteria, differences in structural components (such as the cell wall, minor differences in the ribosomes and RNA polymerase) are usually what is "attacked" by the chemotherapeutic drug.

The term "antibiotic" was coined by Selman Waksman in 1942 to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Waksman, 1947). This original definition excluded naturally occurring substances that kill bacteria but are not produced by microorganisms (such as gastric juice and hydrogen peroxide) and also excluded synthetic antibacterial compounds such as the sulfonamides. In

common usage, an antibiotic is a substance or compound that kills bacteria or inhibits their growth (Davey, 2000). Antibiotics belong to the broader group of antibacterial compounds, used to treat infections caused by microorganisms. With advances in medicinal chemistry, most antibiotics are now semi synthetic, modified chemically from original compounds found in nature (Davey, 2000), as is the case with beta-lactams (which include the penicillins, produced by fungi in the genus *Penicillium*, the cephalosporins, and the carbapenems). Some antibiotics are still produced and isolated from living organisms, such as the aminoglycosides, and others have been created through purely synthetic means: the sulfonamides, the quinolones, and the oxazolidinones. In addition to this origin-based classification into natural, semisynthetic, and synthetic, antibiotics may be divided into two broad groups according to their effect on microorganisms:

Those that kill bacteria are bactericidal agent, whereas those that only impair bacterial growth are known as bacteriostatic agents. Many treatments for infections prior to the beginning of the twentieth century were based on medicinal folklore. Treatments for infection in ancient Chinese medicine using plants with antimicrobial properties were described over 2,500 years ago (Lindblad, 2008). Many other ancient cultures, including the ancient Egyptians and ancient Greeks used molds and plants to treat infections (Forrest, 1982; Wainwright, 1989). The discovery of the natural antibiotics produced by microorganisms stemmed from earlier work on the observation of antibiosis between microorganisms. Louis Pasteur observed that, if we could intervene in the antagonism observed between some bacteria, it would offer perhaps the greatest hopes for therapeutics (Kingston, 2008). Synthetic antibiotic chemotherapy as a science and the story of antibiotic development began in Germany with Paul Ehrlich, a German medical scientist in the late 1880 (Calderon *et al*., 2007). Scientific endeavours to understand the science behind

what caused these diseases, the development of synthetic antibiotic chemotherapy, and the isolation of the natural antibiotics marked milestones in antibiotic development (Foster and Raoult, 1974).

2.7.1. Mechanisms Action of Antibacterial Drugs

The mechanism of action includes:

Inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis Inhibition of metabolic pathways, interference with cell membrane integrity, inhibition of Lipoteichoic acid, inhibition of fatty acid biosynthsis, inhibition of thymidylate synthase and inhibition of DNA helicases.

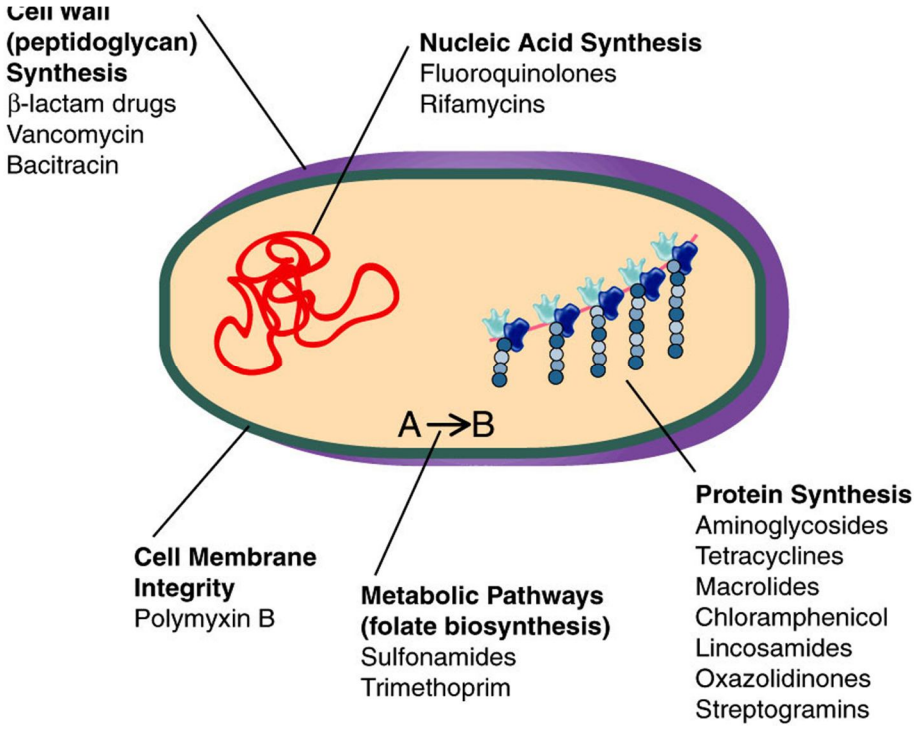


Fig 2.4 Target of antibacterial medications.

Source: Hugo and Russell (2007).

2.7.1.1 Inhibition of cell wall Sythesis.

Bacteria cell wall is unique in structure due to the presence of peptidoglycan. A number of medically useful drug interfere with cell wall biosynthesis, among these are penicillin, monobactam, carbepenem, bacitracin and vancomycin. They irreversibly inhibit transpeptidase enzyme involved in the final step of cell wall synthesis.

Figure 2.5 shows, the various part of cell wall biosynthesis that are inhibited by antimicrobial agents

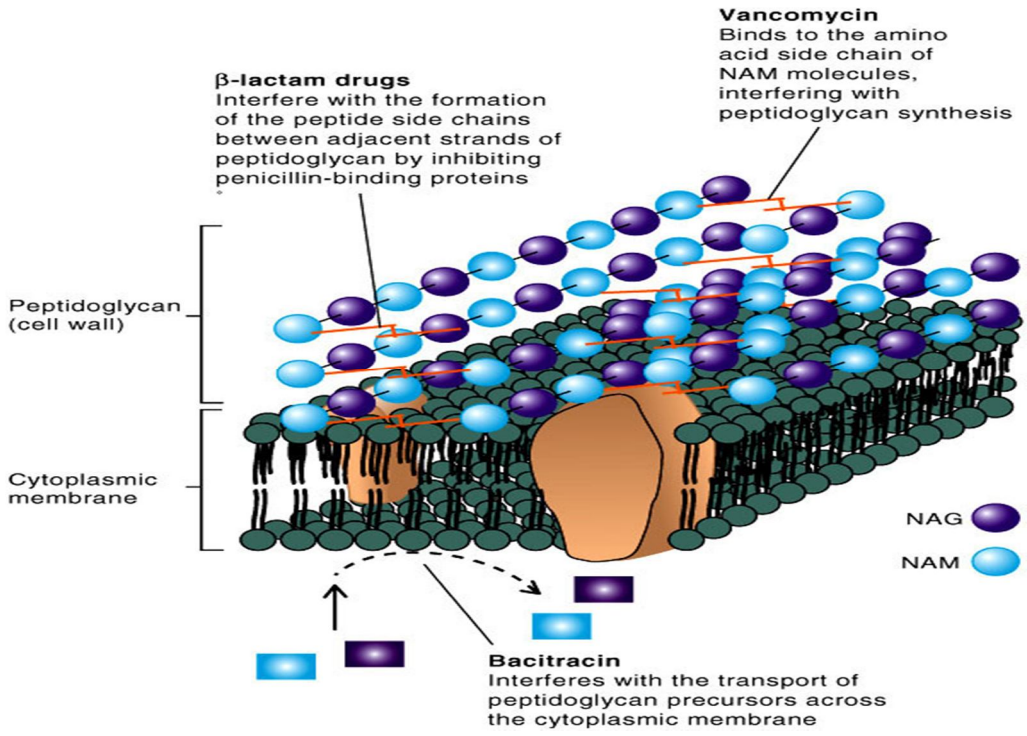


Fig 2.5 Antibacterial medications that interfere with cell wall biosynthesis.

Sources: Hugo and Russell (2007)

NAM = N-acetyl muramic acid and NAG = N- acetyl glucosamine are macropolymer that makes up the peptidoglycan cell wall of both gram positive and negative bacterial.

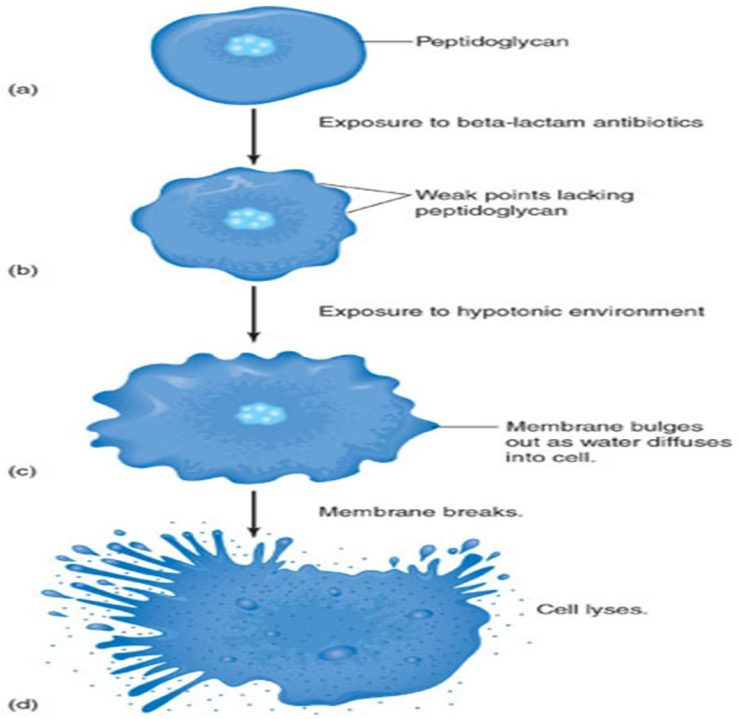


Fig 2.6 Rupture cell.

Sources: Prescott *et al.,* (2007)

As cells are formed and begin to generate a cell wall, the presence of these antibiotics disrupts cell wall integrity thus result to weak and deformed cell. Minor shifts in osmolarity can result in rupture of the cell. This group of antibiotics exerts little or no effect on bacteria that are not rapidly growing. The original penicillins were generally more effective against the gram-positive organism. They exerted little or no effect on gram-negative cells (the major exception to this was the effect that penicillin had on *Neisseria gonorrhea*, a gram-negative organism). In the case of most gram-negative organisms, the lack of activity by penicillin results from the barrier presented by the outer membrane of a gram-negative cell and from differences in the enzymes involved in peptidoglycan synthesis (Prescott *et al*., 2007). The semi synthetic penicillins that came later are able to penetrate this outer membrane and bind with the peptidoglycan forming

enzymes of the gram-negative organism as well as those of the gram-positive organism (Fraimow *et al.,* 2000). Ampicillin is slightly a modified form of penicillin but is one of the broader spectrum antibiotics on the market.

Vancomycin interferes with the synthesis of the cell wall but is not a β-lactam antibiotic; its interference with the synthesis of peptidoglycan involves blocking the action of enzymes involved in the synthesis of peptidoglycan that are different from those blocked by the beta-lactam antimicrobials (Prescott *et al*., 2007).

2.7.1.2 Inhibition of metabolic pathways

Several classes of drugs inhibit the ability of the cell to synthesize the nucleotides needed in the synthesis of nucleic acids. This is accomplished by binding to the active sites of enzymes in the pathways involved in the production of these biochemicals.

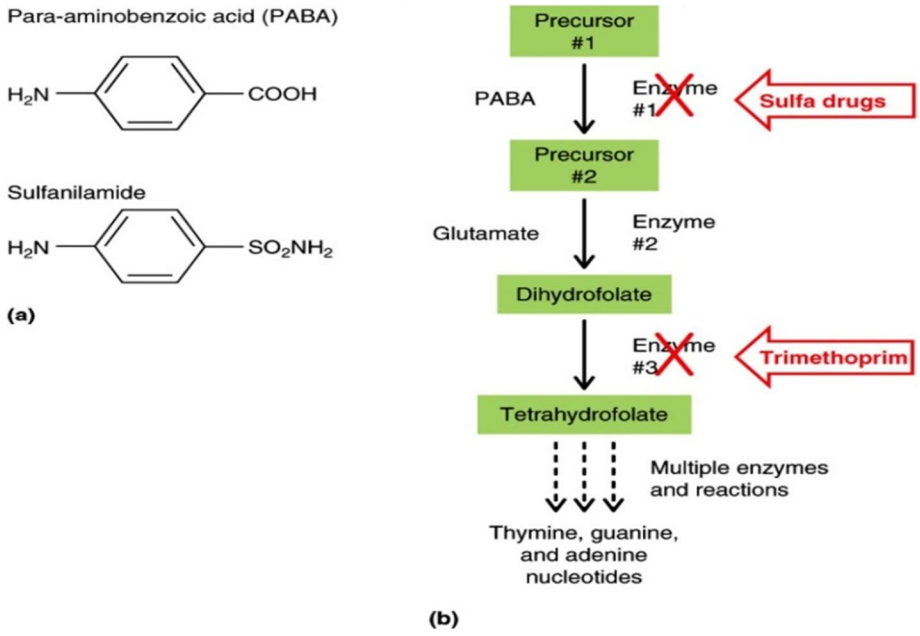


Fig 2.8 Inhibitor of folate pathway.

Sources: Prescott *et al.,* (2007)

Once in the active site they bind and do not leave. Thus, the enzyme is rendered inactive. Sulfonamides (Sulfa drugs) inhibit the enzyme, which converts para-aminobenzoic acid (PABA) into dihydrofolic acid. Dihydrofolic acid eventually will become the nitrogenous bases known as adenine and guanine. Blocking PABA conversion to dihydrofolic acid effectively halts synthesis of nucleotides (Hugo and Russell, 2004).

2.7.1.3 Inhibition of protein synthesis

All cells must be able to carry on protein synthesis, thus, all cells must have ribosomes. However, the ribosome of the procaryotic cell differs slightly from that of the eukaryotic cell. This difference is the basis of action for several antibiotics, which block translation in the procaryotic organism but have little or no effect on translation in the eukaryotic organism. These drugs include Tetracycline chloramphenicol, macrolides (erythromycin), aminoglycosides (Gentamicin).

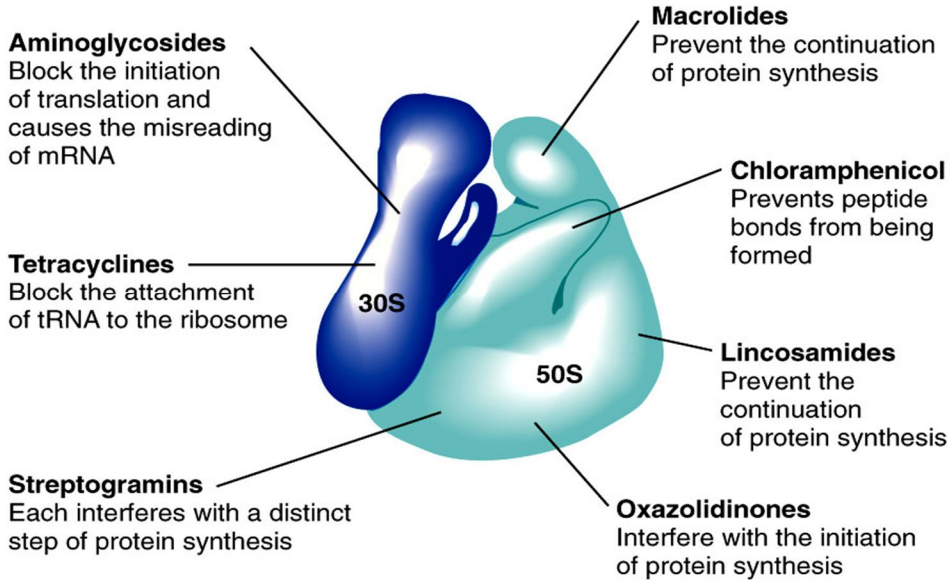


Fig 2.9 Antibacterial drugs that inhibit protein synthesis

Source: Nester *et al.,* (2004)

Aminoglycoside irreversibly binds to 30S ribosomal subunite, bocks initiation of translation and causes misreading of mRNA and eventually distortion and malfunction of ribosome. Aminoglycosides are effective against anaerobes, enterococci and streptococci. Often used in synergistic combination with β-lactam drugs, and allows aminoglycosides to enter cells that are often resistant (Hugo and Russell, 2004).

2.7.1.4 Inhibition of cell membrane synthesis

The peptide antibiotics are produce like all other peptides, through the action of ribosomes, mRNA, etc. Their mode of action involves binding to phospholipids and disrupting the integrity of the cell membrane.

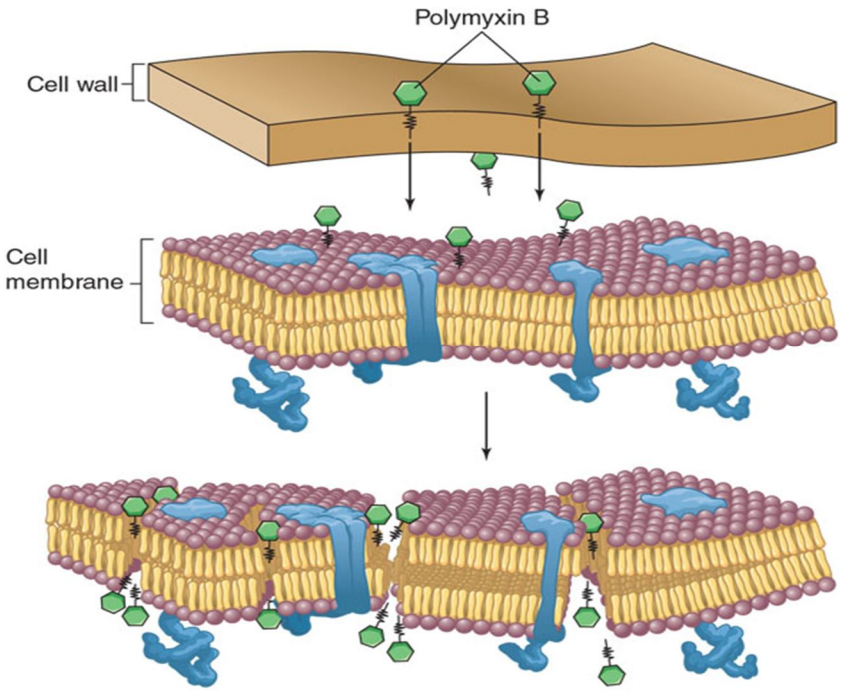


Fig 2.10 Antibacterial drug inhibiting cell membrane synthesis

Sources: Nester *et al.,* (2004).

A few antibacterial agent demage bacterial membranes. Polymyxin B, bind to the membrane of gram-negative cell and alter the permeability, leading to leakage of cellular content and eventually death of the cell. Polymyxin B, bind to eukaryotic cell, though to a lesser extent, which generally limit their use to topical applications (Nester *et al.,* 2004).

2.7.1.5 Inhibition of nucleic acid synthesis

The antibiotic rifampin (a derivative of the antibiotic rifamycin) inhibits the synthesis of mRNA. Differences in the RNA polymerase of procaryotic cells when compared with that of the eukaryotic cell allow this antimicrobial to selectively inhibit the action of procaryotic RNA polymerase, thus minimizing the toxicity of this drug. It is very effective in penetrating tissues and reaching the inside of the tubercles of tuberculosis. Consequently, it is used in controlling TB infections. Quinolones are a class of antibiotics that inhibit the function of DNA gyrase. In this way, they interfere with a cell's ability to carryout semiconservative replication. Levoquin is a commonly used quinolone (Hugo and Russell, 2004).

2.7.1.6 Inhibition of lipoteichoic acid.

Lipoteichoic acid is a major constituent of the cell wall of Gram-positive bacteria. It consists of teichoic acid, long chain of ribitol phosphate and anchored to the lipid bilayer via s glyceride. It act as regulator of autolytic enzymes (Muramidases), has an antigentic properties being able to stimulate specific immune response and is released from the bacterial cell mainly after bacteriolysis induced by lysozyme. It is presumed that synthesis of these secondary wall polymer might be essential for bacterial growth and might therefore serve as a target for antibiotic development. Daptomycin is an antibacterial drug that is active against MRSA, VRSA and VREF (Tally and Debruin, 2000). This antibiotic inhibit the formation of nucleotide-linked

sugar-peptide precursors involved in peptidoglycan biosynthesis, this is because daptomyvin inhibit the accumulation of UDP-N-acetylmuramyl pentapeptide (Nester *et al.*, 2004).

2.7.1.7 Inhibition of aminoacyl- tRNA synthetase

The aminoacyl-tRNA synthetase enzymes play crucial role in protein biosyntheseis by cataylsing the synthesis of aminoacyl-tRNAs. Once this enzyme is inhibited, protein synthsis is halted, which result in the attenution of bacterial growth under both *invitro* and infection condition (Heacock *et al.,* 2001). Mupirocin inhibit bacterial aminoacyl-tRNA synthetase by sharing structural homology with other antibiotic in clinical practice and consist of short fatty acid ester linked to monic acid, with the tail portion closely resembling the isoleucyl moiety of the isoleucyl-adenylate reaction intermediate (Anagisawa *et al.,* 1994).

2.7.1.8 Inhibition of thymidylate synthetase

Thymidylate synthetase is an enzyme used to generate thymidine monophosphate (dTMP). This is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. By means of reductive methylation, deoxyuridine monophosphate (dUMP) and N5, N10-methylene tetrahydrofolate is together used to form dTMP, yielding dihydrofolate as secondary product (Carreras and Santi, 1995). Thymidylate synthase inhibitors are chemical agents that inhibit the enzyme thymidylate synthase and have potential as an anticancer chemotherapy. Fluorouracil is an example of such inhibitor. Raltitrexed works by inhibiting dihydrofolate reductase, an enzyme used in the synthesis of tetrahydrofolate, thereby preventing the synthesis of thymidylate and fully active after polyglutamylation, by inhibiting the formation of precursor pyrimidine nucleotides and preventing the formation of DNA and RNA required for growth and survival of both normal and cancer cells

2.7.1.9 Inhibition of DNA helicases

DNA helicases are enzymes involved in the nucleoside triphosphate dependent unwinding of duplex DNA into single strands, an essential activity for replication. Replicative helicases require assistance from helicase loader to assemble at chromosomal origins of replication. Triaminotriazine I is a low micro molar inhibitor of the enzyme (Mazel *et al.,* 1994).

2.7.1.10 Inhibition of peptide deformylase

The bacterial peptide deformylase is an enzyme responsible for catalyzing the removal of the N-formyl group from N- terminal methionine following translation. This enzyme is encoded by the def – gene, which is present in all pathogenic bacteria, including Mycoplasma and Chlamydia species and which does not share a functionally equivalent gene in mammalian cells. Def- gene is an essential gene in bacterial growth and survival (Mazel *et al.,* 1994; Chang *et al.,* 2003). Blockade of bacterial peptide deformylase produces inhibition of protein synthsis similar to mechanism of tetracycline, macrolide, lincosamides, chloramphenicol and streptogramins. Atinonin and macrolactin are the only naturally occurring peptide deformylase (Chen *et al.,* 2000; Yeo *et al.,* 2006).

2.7.2 Antibiotics Used in the Study.

2.7.2.1 Ciprofloxacin

Ciprofloxacin is a synthetic antibiotic of the fluoroquinolone drug class (Kawahara, 1998; Cooper *et al.,* 2005). It is a second-generation fluoroquinolone antibacterial. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied which stops synthesis of DNA and of protein. Ciprofloxacin interacts with other drugs, herbal and natural

supplements, and a characteristic it shares with other widely used antibacterial drugs such as amoxicillin, trimethoprim, azithromycin, cephalexin, and doxycycline (Alexander *et al.,* 2004).

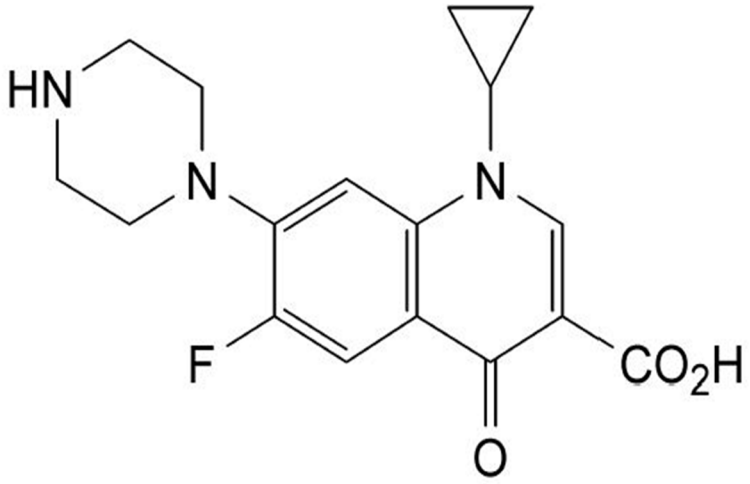


Fig 2.10 Chemical Structure of Ciprofloxacin

Ciprofloxacin is used to treat a number of infections including infections of bones and joints, endocarditis, gastroenteritis (Pankey *et al.*, 2004) due to bacterial resistance. Fluoroquinolones are no longer recommended in the USA for this indication (Fraimow *et al.*, 2000). Ciprofloxacin is not recommended for the treatment of tuberculosis (Slauch, 1995). However, it has a synergestic effect in the treatment of complicated intra-abdominal infections (in combination with metronidazole), empirical therapy for febrile neutropenic patients (in combination with piperacillin), complicated urinary tract infections and pyelonephritis due to *Escherichia coli* (Drlica *et al*., 1997) and inhalational anthrax (postexposure) (Renata, 2004).

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, (Drlica *et al*., 1997) enzymes necessary to separate bacterial DNA, thereby

inhibiting cell division. This mechanism can also affect mammalian cell replication. In particular, some congeners of this drug family (for example those that contain the C-8 fluorine) (Drlica *et al.,* 1997), display high activity not only against bacterial topoisomerases but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and *in vivo* tumor models (Sissi and Palumbo , 2003). Although quinolones are highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Quinolone-induced DNA damage was first reported in 1986 (Hussy *et al.,* 1986). Recent studies have demonstrated a correlation between mammalian cell cytotoxicity of the quinolones and the induction of micronuclei (Forsgren *et al.,* 1987; Gootz *et al.,* 1990; Lawrence *et al.,* 1993). As such, some fluoroquinolones may cause injury to the chromosome of eukaryotic cells (Elsea *et al.*, 1992; Suto *et al.,* 1992; Thomas *et al.,* 1990; Al-Soud *et al.,* 2003).

2.7.2.2. Amoxicillin

Amoxicillin is a moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is one of the most common antibiotics prescribed for children. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria. It is often combined with clavulanic acid, a β-lactamase inhibitor. This increases effectiveness by reducing its susceptibility to β-lactamase enzyme.

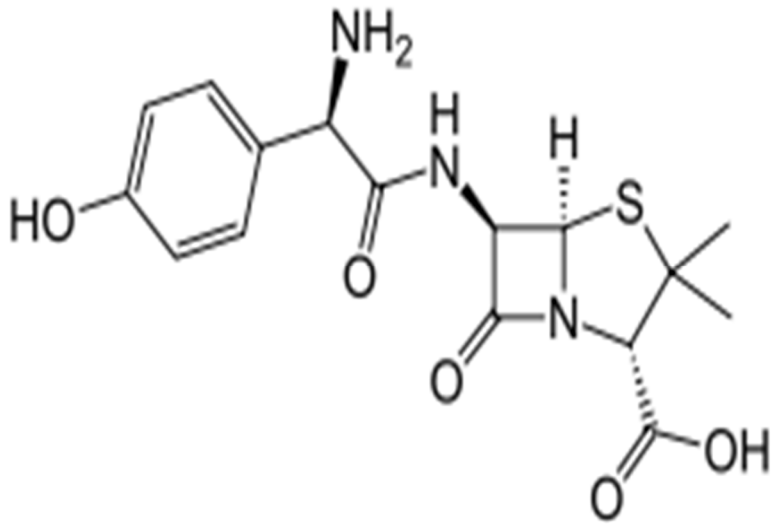


Fig 2.11 Chemical Structure of Amoxillicin

Amoxicillin is used in the treatment of a number of infections including acute otitis media, streptococcal pharyngitis, pneumonia, skin infections, urinary tract infections, *salmonella*, lyme disease, and chlamydia infections (Thornhill *et al.,* 2011). It is used to prevent bacterial endocarditis in high risk people who are having dental work done, to prevent *Streptococcus pneumococus* infections in those without a spleen, and for both the prevention and the treatment of anthrax (Thornhill *et al.,* 2011). It is also a treatment for cystic acne (Cundiff and Joe, 2007). The UK however does not recommend its use for infectious endocarditis prophylaxis (Baselt, 2008). The amoxicillin/clavulanic acid combination for more than one week has caused mild hepatitis in some patients. Young children having ingested acute overdoses of amoxicillin manifested lethargy, vomiting and renal dysfunction (Kagan, 1977).

Amoxicillin acts by inhibiting the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell walls of both Gram-positive and Gram-negative bacteria.

2.7.2.3 Gentamicin

Gentamicin is an aminoglycoside antibiotic, used to treat many types of bacterial infections particularly those caused by Gram-negative organisms (Moulds *et al.,* 2010). However, gentamicin is not used for *Neisseria gonorrhoeae*, *Neisseria meningitidis* or *Legionella pneumophila*. Gentamicin is also ototoxic and nephrotoxic, with this toxicity remaining a major problem in clinical use (Moulds *et al.,* 2010).

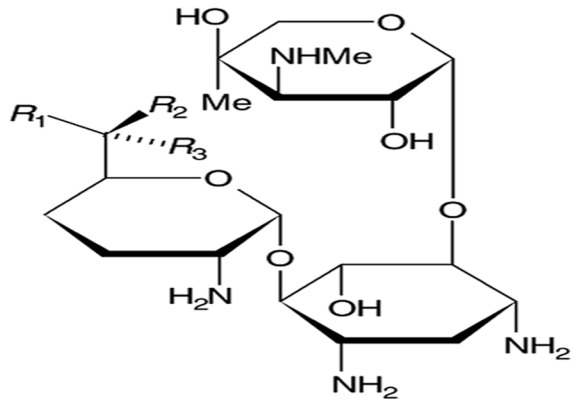


Fig 2.12 Chemical Structure of Gentamicin

It is synthesized by *Micromonospora purpurea,* a genus of Gram-positive bacteria widely present in the environment (water and soil). Like all aminoglycosides, when gentamicin is given orally, it is not systemically active. This is because it is not absorbed to any appreciable extent from the small intestine. It is administered intravenously, intramuscularly or topically to treat infections (Giannini *et al.,* 1978). Reluctance to use gentamicin for empirical therapy has led to increased use of alternative broad-spectrum antibiotics, which some experts suggest has led to the prevalence of antibiotic-resistant bacterial infections by Golden *Staph* and other so-called "superbugs" (Moulds *et al.,* 2010). Gentamicin is one of the few heat-stable antibiotics that remain active even after autoclaving, which makes it particularly useful in the preparation of

some microbiological growth media. It is used during orthopaedic surgery when high temperatures are required for the setting of cements (e.g. hip replacements) (Nikaido, 2001).

The drug (gentamicin) is taken up into sensitive bacterial cells by an active transport process within the cell. Gentamicin binds to the 30S and to some extent to the 50S, subunits of the bacterial ribosome thereby inhibiting protein synthesis and generating errors in the transcription of the genetic code.

Gentamicin has a wide range of human bacterial infections, mostly Gram-negative bacteria including *Pseudomonas*, *Proteus*, *Serratia*, and the Gram-positive *Staphylococcus* (Goljan, 2011). Gentamicin is also useful against *Yersinia pestis*, its relatives, and *Francisella tularensis* (the organism responsible for Tularemia seen often in hunters and/or trappers) (Sund *et al.,* 2001). In the mean while, *Enterobacteriaceae*, *Pseudomonas spp.*, *Enterococci*, *Staphylococcus auricolaris* and *Staphylococci* have developed resistance to Gentamicin Sulfate, USP to varying degrees (Giannini *et al.,* 1978).

It was initially used as a topical treatment for burns at the Atlanta and San Antonio burn units and was introduced into IV usage in 1971. It remains a mainstay for use in sepsis (Sund *et al.,* 2001). Gentamicin is also used in molecular biology research as an antibacterial agent in tissue and cell culture, to prevent contamination of sterile cultures.

2.8 MICROBIAL INFECTIONS

An infection is the colonization of a host organism by parasite species. Infecting parasites seek to use the host's resources to reproduce, often resulting in disease. Colloquially, infections are usually considered to be caused by microorganisms or microparasites like viruses, prions,

bacteria, and viroids, though larger organisms like macroparasites and fungi can also infect (Fleming, 1980).

Hosts normally fight infections themselves via their immune system. Infection begins when an organism successfully colonizes by entering the body, growing and multiplying. Weak, sick, and malnourished, cancer and diabetic patents have increased susceptibility to chronic or persistent infections (Fleming, 2001). Individuals with suppressed immune system are particularly susceptible to opportunistic infections. Entrance to the host generally occurs through the mucosa in orifices like the oral cavity, nose, eyes, genitalia, anus, or open wounds. Organisms, which are non-pathogenic, can become pathogenic given specific conditions, and even the most virulent organism requires certain circumstances to cause a compromising infection.

Some colonizing bacteria, such as *Corynebacteria sp.* and *Streptococci viridans*, prevent the adhesion and colonization of pathogenic bacteria and thus have a symbiotic relationship with the host, preventing infection and speeding wound healing. An example, the *Staphylococcus species* present on skin remain harmless on the skin, but, when present in a normally sterile space, such as in the capsule of a joint or the peritoneum, will multiply without resistance and create a huge burden on the host.

2.8.1 Characteristics of the Bacterial Species Used in the Study

a) *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of the Gamma Proteobacteria class of Bacteria. It is a Gram-negative, aerobic rod belonging to the bacterial family Pseudomonadaceae (Brooks *et al*., 2001).

Pseudomonas aeruginosa is a common bacterium that can cause disease in animals, including humans. *Ps.aeruginosa* is known as a hydrocarbon-using microorganism (or HUM bug), causing microbial corrosion (Mahajan-Miklos *et al.,* 1999).

Like other members of the genus, *Ps. aeruginosa* is a free-living bacterium, commonly found in soil and water (Ryan and Ray, 2004). However, it occurs regularly on the surfaces of plants and occasionally on the surfaces of animals. Members of the genus are well known to plant microbiologists because they are one of the few groups of bacteria that are true pathogens of plants.

Pseudomonas aeruginosa has been recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates (Balcht *et al*., 1994). *Pseudomonas aeruginosa* is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed (Todar, 2011).

For an opportunistic pathogen such as *Ps. aeruginosa*, the disease process begins with some alteration or circumvention of normal host defenses. The pathogenesis of *Pseudomonas* infections is multifactorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Multiple and diverse determinants of virulence are expected in the wide range of diseases caused, which include septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis (Poole *et al*., 2004).

The spread of *Ps. aeruginosa* can best be control by observing proper isolation procedures, aseptic technique, and careful cleaning and monitoring of respirators, catheters, and other instruments. Topical therapy of burn wounds with antibacterial agents such as silver sulfadiazine, coupled with surgical debridement, dramatically reduces the incidence of *Ps. aeruginosa* sepsis in burn patients (Todar, 2011).

Pseudomonas aeruginosa is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and fluoroquinolones. The combination of gentamicin and carbenicillin is frequently used to treat severe *Pseudomonas* infections. Several types of vaccines are being tested, but none is currently available for general use. Phage therapy against *Ps. aeruginosa* remains one of the most effective treatments, which can be combined with antibiotics, has no contraindications and minimal adverse effects (Sulakvelidze *et al.,* 2001; Wright *et al*., 2009). Honey may reduce colonization of many pathogens, including *Ps. aeruginosa* (Kwakman *et al.,* 2008). Probiotic prophylaxis may prevent colonization and delay onset of *Pseudomonas* infection in an ICU setting (Forestier *et al*., 2008).

2.7.2.2 *Staphylococcus aureus*

Taxonomically, the genus *Staphylococcus* is in the Bacterial family *Staphylococcaceae,* which includes three lesser known genera, *Gamella, Macrococcus* and *Salinicoccus.* The best-known of its nearby phylogenetic relatives are the members of the genus *Bacillus* in the family *Bacillaceae,* which is on the same level as the family *Staphylococcaceae* (Brooks *et al*., 2001).

Staphylococcus aureus is gram-positive, cluster-forming coccus, nonmotile, non-spore forming bacterial, forms a fairly large yellow colony on rich medium and is often hemolytic on blood agar (Ryan and Ray, 2004). Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation, which yields principally lactic acid. The bacteria are catalase-

positive and oxidase-negative. *S. aureus* can grow at a temperature range of 15 to 45 degrees and at sodium chloride concentrations as high as 15 percent. Nearly all strains of *S. aureus* produce the enzyme coagulase (Ryan and Ray, 2004). *S. aureus* should always be considered a potential pathogen (Todar, 2011).

Each year, some 500,000 patients in American hospitals contract a staphylococcal infection (Bowersox, 1999). *S. aureus* causes a variety of suppurative (pus-forming infections and toxins in humans). It was estimated that 20% of the human population are long-term carriers of *S. aureus* (Kluytmans *et al.,* 1997).

It causes superficial skin lesions such as boils, styes and furuncules; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis (Korzeniowski and Sande, 1982; Bayer *et al.,* 1998).

Staphylococcus aureus is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of super antigens into the blood stream (Todar, 2011).

The reasons why *S. aureus* is a successful pathogen are a combination of host and bacterial immuno-evasive strategies. One of these strategies is the production of carotenoid pigment staphyloxanthin, which is responsible for the characteristic golden colour of *S. aureus* colonies. This pigment acts as a virulence factor, primarily being a bacterial antioxidant which helps the microbes evade the host's immune system in the form of reactive oxygen species which the host uses to kill pathogens (Clauditz *et al.*, 2006; Liu *et al*., 2005).

Although Methicillin Resistance *Staphylococcus aureus* (MRSA) have been entrenched in hospital settings for several decades, MRSA strains have recently emerged outside the hospital becoming known as Community Associated- MRSA (CA-MRSA) or superbug strains of the organism, which now account for the majority of staphylococcal infections seen in the Emergency room or clinics. In infants, *S. aureus* infection can cause a severe disease - *staphylococcal* scalded skin syndrome (SSSS) (Curran and Al-Salihi, 1980).

A few strains are resistant to all clinically useful antibiotics except vancomycin, and vancomycin-resistant strains are increasingly reported. Methicillin resistance is widespread and most methicillin-resistant strains are also multiple drug-resistant. A plasmid associated with vancomycin resistance has been detected in *E. faecalis* which can be transferred to *S. aureus* in the laboratory (Todar, 2011).

In addition, *S. aureus* exhibits resistance to antiseptics and disinfectants, such as quaternary ammonium compounds, which may aid its survival in the hospital environment (Matthews *et al*, 1997). Aminoglycoside antibiotics, such as kanamycin, gentamicin, streptomycin, etc., were once effective against staphylococcal infections until strains evolved mechanisms to inhibit the aminoglycosides action, which occurs via protonated amine and or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit (Carter *et al*, 2000).

Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis, (Korzeniowski and Sande, 1982; Bayer *et al.,* 1998). However, its use is controversial because of the high risk of damage to the kidneys (Cosgrove *et al.,* 2009). The duration of treatment depends on the site of infection and on severity (Chambers, 2001). An important and previously unrecognized means of community-associated MRSA colonization and transmission is during sexual contact (Cook *et al., 2*007).

Staff or patients who are found to carry resistant strains of *S. aureus* may be required to undergo "eradication therapy", which may include antiseptic washes and shampoos (such as chlorhexidine) and application of topical antibiotic ointments (such as mupirocin or neomycin) to the anterior nares of the nose (Zhu, 2008).

2.7.2.3 Escherichia coli

Theodor Escherich first described *Escherichia coli* in 1885, as *Bacterium coli commune,* which he isolated from the feaces of newborns. It was later renamed *Escherichia coli,* and for many years, the bacterium was simply considered to be commensal organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants (Wakimoto *et al.,* 2004). *Escherichia coli* colonize the gastrointestinal tract of most warm-blooded animals within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth.

Escherichia coli can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts and the ecology of *E. coli* in the intestine of humans are poorly understood, despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997 (Thomas *et al.,* 1990).

Escherichia coli is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are facultative anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. A number of genera within the family are human intestinal

pathogens (e.g. *Salmonella*, *Shigella*, *and Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g. *Escherichia*, *Enterobacter*, *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans (Surette and Bonnie, 2007).

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O_2. Under anaerobic conditions, it will grow by means of fermentation, producing characteristic "mixed acids and gas" as products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats (Todar, 2011).

Escherichia coli can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Alternatively, it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism, the bacterium can survey the chemical contents in its environment in advance of synthesizing any enzymes that metabolize these compounds. It does not wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not

produce enzymes for synthesis of metabolites if they are available as nutrients in the environment (Todar, 2011).

Escherichia coli is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human gastrointestinal tract; however, it makes up a very small proportion of the total bacterial content. The anaerobic *Bacteroides* species in the bowel out number *E. coli* by at least 20:1. However, the regular presence of *E. coli* in the human intestine and faeces has led to tracking the bacterium in nature as an indicator of faecal pollution and water contamination. As such, it is taken to mean that, wherever *E. coli* is found, there may be faecal contamination by intestinal parasites of humans (Todar, 2011).

Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens. At one time serotyping was important in distinguishing the small number of strains that actually cause disease. Thus, the serotype O157:H7 (O refers to somatic antigen; H refers to flagella antigen) is uniquely responsible for causing HUS (Hemolytic Uremic Syndrome). Nowadays, particularly for diarrheagenic strains (those that cause diarrhea) pathogenic *E. coli* are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers (Todar, 2011). Pathogenic strains of *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). The diseases caused (or not caused) by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses (Todar, 2011)

As a pathogen, *E. coli* is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrheal diseases are now recognized: Enterotoxigenic *E. coli*

(ETEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), and Enteroaggregative *E. coli* (EAEC). Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

2.7.2.4 Salmonella typhimurium

Salmonella typhimurium is a pathogenic Gram-negative bacteria predominately found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides (LPS) which protect the bacteria from the environment. The LPS is made of an O-antigen, a polysaccharide core, and lipid A, which connects it to the outer membrane. Lipid A is made of two phosphorylated glucosamines, which are attached to fatty acids. These phosphate groups determine bacterial toxicity. Animals carry an enzyme that specifically removes these phosphate groups in an attempt to protect themselves from these pathogens (Tuin, 2005).

The O-antigen, being on the outermost part of the LPS complex is determines the host immune response. *Salmonella typhimurium* has the ability to undergo acetylation of this O-antigen, which changes its conformation, and makes it difficult for antibodies to recognize (Winzer and Lux 2002).

Salmonella typhimurium infect by coming in direct contact with nonphagocytic cells. This contact induces the formation of appendages on the bacterial cell surface. The appendages formed are shorter than flagella but thicker than both flagella and pili. They cause the host cytoskeleton to rearrange which allows the bacteria to enter the cell. This membrane ruffling system is due to 14 genes on the *S. typhi* chromosome (Miao and Edward, 2007).

The *inv* genes are responsible for the assemblage and emission /withdrawal of these appendages. All of the inv genes must be intact for this system to work. One of these genes, *invC,* is responsible for ATPase that provides the energy needed to complete this ruffling process (Ginocchio, 1994). *Salmonella typhimurium* causes gastroenteritis in humans and other mammals. When the bacterial cells enter epithelial cells lining the intestine they cause host cell ruffling which temporarily damages the microvilli on the surface of the cell. This causes a rush of white blood cells into the mucosa, which throws off the ratios between absorption and secretion, and leads to diarrhea.

In mice, *S. typhi* causes symptoms resembling typhoid fever in humans. Studying these bacteria in mice could eventually lead to a typhoid vaccine (McClelland and Sandersonk, 2007).

2.9. ANTIBIOTIC RESISTANCE

Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. While a spontaneous or induced genetic mutation in bacteria may confer resistance to antimicrobial drugs, genes that confer resistance can be transferred between bacteria in a horizontal fashion by conjugation, transduction, or transformation. Thus, a gene for antibiotic resistance, which had evolved via natural selection, may be shared. Evolutionary stress such as exposure to antibiotics then selects for the antibiotic resistant trait. Many antibiotic resistance genes reside on plasmids, facilitating their transfer. If a bacterium carries several resistance genes, it is called multidrug resistant (MDR) or, informally, a superbug or super bacterium. Genes for resistance to antibiotics, like the antibiotics themselves, are ancient (Li and Nikaido, 2009).

However, the increase in prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use within human medicine and veterinary medicine. Any use of

antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off. As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises (Lindblad, 2008). However, despite a push for new antibiotic therapies there has been a continued decline in the number of newly approved drugs (Kingston, 2008). Antibiotic resistance therefore poses a significant problem. The resistance mechanisms therefore depend on which specific pathways are inhibited by the drugs and the alternative ways available for those pathways that the organisms can modify to get a way around in order to survive. Antibiotic resistance can be a result of horizontal gene transfer (Ochiai *et al.,* 1959) and of unlinked point mutations in the pathogen genome at a rate of about 1 in 10^8 per chromosomal replication. The antibiotic action against the pathogen can be seen as an environmental pressure; those bacteria which have a mutation allowing them to survive will live on to reproduce. They will then pass this trait to their offspring, which will result in the evolution of a fully resistant colony.

2.9.1 Mechanisms for Acquired Resistance.

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

- Drug inactivation or modification.
- Alteration of target site.
- Alteration of metabolic pathway.
- Reduced drug accumulation.

a) Drug Inactivation or Modification:

Inactivating enzymes remain the predominant mechanism of resistance to several major classes of antimicrobial agent's e.g, enzymatic deactivation of *penicillin* G in some penicillin-

resistant bacteria through the production of β-lactamases. Resistance to β-lactamsis mediated by a wide variety β-lactamases that hydrolytically inactivate these drugs. β-Lactamases can be either plasmid or chromosomally mediated, and their expression can be constitutive or induced. Unlike those of Gram positive bacteria, β - lactamases of Gram negative organisms are confined to the periplasmic space, which may explain some of the differences in their phenotypic expression and ease of laboratory detection. Of particular importance in the hospital setting are the class I chromosomal β-lactamases of organisms, such as *Enterobacter cloacae*, that are produced in high levels after exposure to an inducing β-lactam agent (particularly to third generation cephalosporins), and the extended spectrum β-lactamases, mediating resistance to third generation cephalosporins and aztreonam (Helfand, 2003). Another rmajor class of inactivating enzymes is the family of aminoglycoside-modifying enzymes. These enzymes are widely distributed in both gram-positive and gram-negative bacteria, and usually are plasmid-mediated (Davies and Wright, 1994). Modifying or inactivating enzymes also can mediate resistance to chloramphenicol and macrolides (Davies, 1994).

b) Alteration of Target Site

Target modifications are widely used by bacteria to develop resistance to a wide variety of antimicrobial agent's e.g, alteration of PBP, the binding target site of penicillins in MRSA and other penicillin-resistant bacteria (Spratt, 1994). Some of these alterations may require as little as a single mutational event at a critical gene sequence in the primary target to create a new, functional target with reduced affinity for the antimicrobial agent. Such changes account for the relative ease of selection of rifampin-resistant mutants of *Staphylococc*i and *Streptococci* by changes in DNA polymerase or the selection of high-level streptomycin-

resistant mutants with altered ribosomes. Although some target modifications can be directly selected, others, such as development of resistance to semisynthetic penicillins in *Staphylococci,* require acquisition of novel exogenous DNA. Modification of Pencillin binding protein Penicillin bindig protein (PBP) is the primary determinant of penicillin resistance in *Streptococcus pneumoniae, Neisseria meningitidis, and Enterococcus faecium.* Modification of the genes encoding DNA gyrases and topoisomerases is the major mechanism of resistance to quinolones, and target modification is important in resistance to macrolides, tetracyclines, rifampin, and mupirocin (Spratt, 1994).

c) Reduced Drug Accumulation

The mechanisms by which intracellular concentrations of drugs are limited include decreased permeability through the outer membrane, decreased uptake through the cytoplasmic membrane, and active efflux back out across the cytoplasmic membrane and the outer membrane e.g, by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface (Li *et al.,* 2009).

Acquired outer membrane permeability changes in Gram-negative organisms previously attributed solely to alterations in outer membrane porin proteins are now also understood to be related to the up-regulation of complex multidrug efflux pumps whose expression is linked to that of outer membrane proteins, such as the MexAB-OprM system of *Pseudomonas aeruginosa* (Nikaido, 1994; Nikaido, 2001). These efflux systems are widely distributed among Gram-negative pathogens, such as *Ps. aeruginosa* and Enterobacteriaceae, and may be an important component of resistance to β-lactams, but usually result in high-level resistance only when also associated with β-lactamase production (Nikaido, 1994; Nikaido, 2001). Imipenem resistance in *Ps. aeruginosa* can be mediated by alteration of a specific porin *OprD* that is used preferentially

by this agent (Nikaido, 1994; Nikaido, 2001). Decreased outer membrane permeability through porin changes and efflux also may play a role in resistance to fluoroquinolones and aminoglycosides. Resistance mediated by decreased uptake across the metabolically active cytoplasmic membrane is best demonstrated by small-colony aminoglycoside-resistant mutants of *Staphylococci,* but this mechanism is less important than other mechanisms of aminoglycoside resistance (Davies and Wright, 1994). Active antimicrobial efflux systems play a role in resistance to many different agents, including macrolides, tetracyclines, quinolones, chloramphenicol, and β-lactams.

d) Alteration of Metabolic Pathway

Some bacteria have gone beyond simple target modification and have acquired novel systems in which the antimicrobial target is no longer necessary for survival of the organism, for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid. This is achieved by creation of new metabolic pathways to bypass the primary target. Perhaps the most elaborate examples of this are the vanA and vanB clusters that mediate resistance to glycopeptides in enterococci (Fraimow *et al.,* 2000). Target bypass also is the major mechanism of acquired resistance to folate antagonists.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment.

Refrigerator (NAPCO Model 630 Portland, Oregon, U.S.A.)

Incubator (Natural appliance: Aheinicke Company Portland, Oregon- made in U.S.A.Model-630 Serial No., 1-81-1550-1)

Autoclave (Adelphi MFG Co Ltd, Portland autoclave)

Electronic weighing balance (Top balance digital, U.S.A, Ohaus, PA313-model).

Water bath (Gallenkamp).

Oven (Baird andTatlock (London) limited, Chadwell heath Essex made in England No 4144262/27).

Evaporating dish (Gallenkamp)

3.1.2 List of Glasswares

Beakers,Test-tube, Pipettes, Petridish, Glassw Slides,Cover Slip, Mccortney Bottles, Glass Pipette, Measuringcylinder and Glass rods.

3.1.3 Reagents.

Absolute Ethanol (Sigma Aidrich- Missouri, U.S.A.)

Ethyl acetate (Sigma Aidrich- Missouri, U.S.A.)

n-butanol (Sigma Aidrich- Missouri, U.S.A.)

Hexane (Sigma Aidrich- Missouri, U.S.A.)

Tween 80 (BDH)

3.1.4 Bacteriological Media

The following under listed media used in this study were all products of Oxiod Ltd, England

Salmonella Shigella Agar SSA (Oxoid, Basingstoke, U.K)

MacConkey Agar MA (Oxoid, Basingstoke, U.K)

Eosin Methylene Blue EMB (Oxoid, Basingstoke, U.K.)

Mueller Hinton Agar MHA (Oxoid, Basingstoke, U.K.)

Nutrient Broth / Agar (Oxoid, Basingstoke, U.K.)

3.1.5 Reference Antibiotic

Ciprofloxacin tablet 500 mg (Fidson Nigerian Ltd)

Gentamicin injection 80 mg/ml (Hovid)

Amoxilllicin tablet 250 mg (Hovid)

3.1.6 Test Organisms

Clinical isolates, collected from fecal material and wound in the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika Zaria, Kaduna State, Nigeria were used.

Escherichia coli

Pseudomonas aeruginosa

Staphylococcus aureus

Salmonella typhi

3.2 METHODOLOGY

3.2.1 Plant Materials

Sample of *Alchornea cordifolia* stem barks and roots were collected from University of Benin, Benin City, Nigeria The collection of the plant was made in December 2009. These samples were authenticated by Dr. E. I. Aigbokhan of the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin City, Nigeria and identified at the herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The sample of the plant was deposited in the herbarium for reference purpose with Voucher No. 401.

3.2.2 Preparation of Crude Plant Material.

Alchornea cordifolia stem bark and roots were dried at room temperature for 10 days. The completely dried stem bark and roots were crushed to coarse powder by grinding with wooden mortar and pestle.

3.2.3 Preliminary Extraction of Plant Material

Four hundred (400) grams of each coarsely powdered stem bark and root were extracted with 2000mls of distilled water using cold maceration and left for 24 and 72 hours. The extract was filtered and evaporated to dryness on a hot water bath at 40 ^{0}C. Five hundred grams (500g) of the coarsely powdered stem bark and root were extracted with 1500 mls of 70 % ethanol by cold

maceration and left for 24 and 72 hours. The extract was filtered and evaporated to dryness on a hot water bath at 40 ^{0}C.The extracts were kept in suitable amber coloured containers until ready for use.

3.2.4 Preliminary Fractionation of Plant Extract

One hundred and eighty (180) grams of ethanol extract was suspended in 1000 mls of distilled water, using cold maceration. The extract was filtered and the filterate was partitioned in separating funnel with 750 mls of Ethyl acetate. The Ethyl acetate fraction was concentrated on a water bath. The aqueous portion was further partitioned with 750mls of n-butanol to get the n-Butanol fraction after concentration (Aliyu *et al*., 2008). The fractions were kept in suitable containers until ready for use.

3.2.5 Preparation of Test Organisms

Test organisms were aseptically purified, subcultured and grown on appropriate 10 ml agar slants and thereafter kept in the refrigerator at 2 – 8^{0}C

3.2.6 Preparation of Antibacterial Agents

These were prepared by dissolving appropriate quantities of the antibacterial agent in sterile distill water and diluted to required concentrations.

3.3 PHYTOCHEMICAL ANALYSIS

Phytochemical analysis of the stembark and root powder of *A. cordifolia* was carried out by investigating the presence or absence of the following constituents; saponin, glycosides, cardiac glycosides, anthraquinones, carbohydrates, sterols, resins, flavonoids, alkaloids.

3.3.1 Test for Resin

a) Fifteen milliliters of petroleum ether extract was prepared using 0.1 gm of the powdered stem bark and root, filtered into a test-tube. An equal volume of copper acetate solution was added and shaken vigorously then allowed to separate. The colour change was noted (Trease and Evans, 1989).

b) The powdered stem bark and root (0.5 g) was dissolved in acetic anhydride and one drop of concentrated sulphuric acid was added. The colour change was noted.

3.3.2 Test for Saponins.

(a) Frothing test

This was carried out as described by (Silver *et al.,* 1998), 0.5g of the extract was shaken with water in a test tube and frothing observed which persist for 15min, indicate the presence of saponins (Trease and Evans, 1989) .

(b) Emulsion Test

Five drops of olive oil were added to three cubic centimeter of the test extract in a test tube, the mixture were vigorously shaken, a stable emulsion indicate the presence of saponin (Silver *et al*., 1998).

3.3.3 Test for Alkaloids

Exactly 0.5g of the extract were stirred with 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate was each divided into three portions.

To the first portion, few drops of Dragendoff reagent was added and observed for the formation of orange to brownish precipitate.

To the second portion one drop of Mayer reagent were added and observed for formation of white to yellowish or cream color precipitate.

To the third portion, one drop of Wagner reagent was added to give a brown or reddished brown precipitate (Silver *et al*., 1998).

3.3.4: Test for Tannins.

A small quantity of the extract was boiled and filtered. Two drops of ferric chloride were added to the filtrate. Formation of a blue black or dirty green precipitate is taken as evidence for the presence of tannins (Trease and Evans, 1996)

3.3.5 Test for Steroids.

Lieberman- Buchard test

One milliliter (1ml) of anhydrous acetic acid was added to 1ml chloroform and cooled to 0^0C then 1 drop of concentrated sulphuric acid was added to the cooled mixture. The solution was observed for color changes (Silver *et al.,* 1998).

Salkowski test

A little quantity of the extract was dissolved in 1ml chloroform and to this,1ml of concentrated sulphuric acid was added down the test tube to form two phases. Formation of red or yellow coloration were taken as an indication for the presence of steroids (Silver *et al.*, 1998).

3.3.6 Test for Flavonoids

(a) Shinoda Test

To an alcoholic solution of the extract, three pieces of Magnesium chips was added followed by a few drop of concentrated hydrochloric acid. Appearance of an orange, pink or red to purple color indicates the presence of flavonoid (Sofowora, 1998).

(b) Sulphuric Acid Test

The sample were dissolved in concentrated sulfuric acid and the color change was observed as described by Silver *et al*., (1998)

(c) Ferric Chloride Test

Extract were boiled with water and filtered. To 2ml of the filtrate, two drops of freshly prepared ferric chloride solution was added. Formation of green, blue or violet colorations indicate the presence of phenol hydroxyl group (Sofowora, 1998).

(d) Sodium Hydroxide Tests

Two milliliters (2mls) of the extract were dissolved in 10% aqueous sodium hydroxide solution and filtered to give yellow color, a change in color from yellow to colorless on addition of dilute Hcl indicates the presence of flavonoid (Sofowora, 1998).

3.3.7 Test for Carbonhydrates

Molishch Test

The procedure described by Trease and Evans (1989) was followed . A few drop of Molisch reagent were added to the extract in a test tube. 1ml of concentrated sulphuric acid was added to flow down the side of the inclined tube, so that the acid forms a layer beneath the aqueous

solution without mixing it. The appearance of reddish brown color indicates a positive test (Trease and Evans, 1989).

Fehling's Test for Reducing Sugar

Five millimeter of Fehling's solution was added to 2ml of test extract in a test tube. The resultant mixtures were boiled for about a minute or two. The appearance of brick red precipitate indicates a positive test (Traese and Evans, 1989)

3.3.8 Test for Anthraquinones

(a) Free Anthraquinones

The extracts were shaken with 10ml of benzene, the content was filtered and 5ml of 10% ammonia solution added to the filtrate. Followed by shaking. Presence of a pink, red, or violet color in the ammoniacal layer (lower phase) indicates the presence of free anthraquinone (Sofowora, 1998).

(b) Combine Anthraquinones

For this test, the procedure described by Sofowora (1998) was adapted. The extractswere boiled with 10ml of aqueous sulphuric acid and filtered hot. The filtrate, were shaken with 5ml benzene, and the benzene layer separated. 10 % NH_4OH was added. A pink, red or violet coloration in the ammonia phase (lower phase) indicates the presence of combined Anthraquinones or anthraquinone derivative.

3.4 DETERMINATION OF ANTIBACTERIAL ACTIVITY

3.4.1 Cultivation and Standardization of test Bacterial

An Eighteen-hour nutrient broth culture was prepared from the organisms stored on nutrient agar slants. A 1:1000 dilution was prepared by serial dilution using (3x9mls) sterile normal saline which produced about 10^5-10^6 CFU/ml for the Gram-positive organisms. A 1:5000 dilution was prepared by serial dilution using 3 x 9 mls and 1 x 4 mls sterile normal saline sterile normal saline for the Gram negative organisms (Anderson, 1970).

3.4.2 Antibacterial Assay of Crude Extracts and Fractions of *Alchornea cordifolia* Stem Bark and Root.

Agar well diffusion method was used as described by (Adeniyi *et al.,* 1996) to determine the antibacterial activity of the extracts and fractions. Molten sterile nutrient agar (20 ml) was poured into sterile petri dishes and allowed to set. The sterile nutrient agar plates were flooded with 1.0 ml of the standardized inoculum (equivalent to 10^5-10^6 CFU/ML) and the excess was drained off. A sterile cork borer (No. 4) was used to bore equidistant cups into the agar plate. One drop of the molten agar was used to seal the bottom of the bored hole, so that the test agent will not sip beneath the agar. Exactly 0.1 ml of the different concentrations (100 – 0.38 mg/ml) of the extract/fraction was added to fill the bored holes. Negative control was prepared by putting 0.2 ml of pure solvent in one of bored hole. One hour pre-diffusion time was allowed, after which the plates were incubated at 37°C for 18 h. The zones of inhibition were then measured in millimeter. The above method was carried out in duplicates and the mean of the duplicate results was taken.

3.4.3 Determination of Minimum Inhibitory Concentration (M. I. C)

Two fold serial dilution of the stock solution of the extracts/fractions was made to obtain concentration between 100 - 0.38 mg/ml. A 2ml portion of each dilution was incorporated into 18 mls double strength Mueller Hinton Agar and poured into the Petri dishes. Sterile 6mm filter paper discs were aseptically placed on Mueller Hinton Agar surfaces. Standardized innoculum of the isolate was immediately added to the discs in volumes of about 20µl. A 20 µl sterile distilled water was added to the sterile paper disc as a negative control. The plates were left at ambient temperature for 15 minutes to allow excess pre- diffusion of organism prior to incubation at 37^0C for 24 hrs.

The lowest concentration that did not show any visible growth around the paper disc when compared to the control was considered as the Minimum Inhibitory Concentration (Ogbonnia *et al*., 2008).

3.4.4 Determination of Minimum Bactericidal Concentration (M. B. C)

This was determined by using sterile forceps to place the filter paper discs that did not show any growth from the MIC plates into sterile Nutrient broth containing inactivating agent 5% ^{v}/v Tween 80 (Ehinmidu, 2003). This was incubated at 37^0C for 24hrs. The Minimum Bactericidal Concentration was considered as those tubes that did not show any turbidity (Aboaba *et al*., 2006). The above methods were carried out in duplicates with the reference antibiotics (gentamicin, amoxillicin and ciprofloxcin).

3.4.5 Determination of Effect of Varying Temperature on the Antibacterial Activity of *Alchornea cordifolia* Stem Barks Extracts.

The standardized overnight culture of the test organisms (2 ml) was used to flood the Mueller – Hinton agar. The plates were allowed to dry at 37^0 C in a sterile incubator. Adopting the cup plate method, a sterile cork borer no. 4 (6 mm diameter) was used to bore hole in the agar plates. The bottoms of the well were sealed with the appropriate molten sterile agar. The extract (1g) was dissolved in 10 ml sterile distilled water, given a concentration of 100 mg/ml and subsequent concentrations from 50 – 0.38mg/ml was made. The set up was maintained at different temperatures (37 ^{0}C, 45 ^{0}C, 70 ^{0}C, and 100 ^{0}C) in water bath for 30 minutes. Thereafter, 0.1ml of the extract solution was aseptically dispensed into the holes in the agar plate and incubated 37^0C for 18 hours. This was carried out in duplicates and repeated for other temperature values.The zones of inhibition were there after measured to the nearest millimeter.

3.4.6 Determination of the Rate of Kill.

One (1.0) ml of standardized overnight culture of non susceptible bacterial and susceptible bacterial (*E. coli* and *S. aureus*) cell (10^6 CFU/ml) was added 9 ml each of 250 mg/ml and 125mg/ml of *Alchornea cordifolia* extracts and mixed thoroughly at room temperature. At different time interval (30, 60, 120, 180, 240,300 and 360 minutes) 1 ml test organism – extract admixture was taken and ten fold dilution protocol performed with sterile inactivating normal saline with 5% tween 80.These dilutions were than plated out in duplicates with sterile molten Mueller – Hinton agar supplimented with 5% tween 80. The solidified agar plates were then incubated at 37^oC for 42 hours and colonies observed were counted. Same procedure was repeated using 0.625 and 30 mg/ml of gentamicin as the standard antibacterial agent.

CHAPTER 4

RESULTS

4.1. EXTRACTION OF STEM BARK AND ROOT OF *ALCHORNEACORDIFOLIA*

Ethanol had higher percentage yield of extracts (stembark and roots) than the percentage from water yield. More yields were obtained from the stem bark than the root (Table 4.1).

Table 4.1: Percentage Yield of Extracts using Water and Ethanol as Solvents.

EXTRACTION SOLVENTS	**PERCENTAGE YIELD (%)**	
	STEM BARK	ROOT
Water	22.05	7.92
Ethanol	39.32	21.0

4.2 PHYTOCHEMICAL SCREENING OF *A. CORDIFOLIA* STEM BARK AND ROOT.

The phytochemical screening of ethanol and water extracts, ethyl acetate fraction revealed the presences of tannins, saponin, carbohydrates, sterols, flavonoids, glycosides, and anthraquinones while alkaloid was not detected (Table 4.2).

Table 4.2: Phytochemical Analysis of *A. cordifolia* Stems Bark and Root.

TEST	INFERENCE		
	Ethanol extract	Water extract	Ethylacetate fraction
STEROLS	+	+	+
FLAVONOIDS	+	+	+
TANNINS	+	+	+
SAPONIN	+	+	+
CARBOHYDRATE	+	+	+
GLYCOSIDES	+	+	+
CARDIAC GLYCOSIDES	+	+	+
RESIN	+	+	+
ANTHRAQUINONES	+	+	+

Key: + = Present

- =Absent

4.3 ANTIBACTERIAL ACTIVITY OF *A. CORDIFOLIA* STEM BARK AND ROOT.

4.3.1. Susceptability of the Test Bacterial to the Ethanol, Water Extracts and N – Butanol and Ethylactetate Fractions of *A. cordifolia*

Varying degree of inhibiting activity were exhibited by the extracts and fractions, with fractions showing higher level of activity and high level of inhibitory activity were demonstrated by extracts and fractions than the standard antibiotics (Tables 4.3 and 4.4).

Table 4.3. Diameter of Zone of Inhibition at 100 mg/ml Concentrations of Extracts of Stembark of *Alchornea cordifolia* against Test Clinical Bacterial Isolates

ORGANISMS	ZONE OF INHIBITION (mm)								
	WE	WEAF	WNBF	EE	EEAF	ENF	Cip.	Amox.	Gent.
E. coli	22.50±3.53	25.00±0.71	20.00±0.00	22.50±3.53	27.50± 3.53	22.00±353	6.00±0.00	6.00±0.00	20.00±0.00
Ps. Aeruginosa	27.50±3.53	27.00±4.24	20.00±0.00	29.00±1.41	27.50±3.53	25.00±0.00	35.00 ±0.71	6.00±0.00	26.00±5.65
S. aureus	27.50±3.53	32.50±3.53	24.50±0.71	32.50±2.23	32.50±3.53	24.50±0.71	32.50 ±3.53	18.00±2.82	29.50± 0.71
S. typhi	27.00±4.25	30.00±0.00	22.50±3.53	28.50±2.12	30.00± 0.00	22.50±3.53	39.50±0.71	20.00± 0.00	28.50±2.12

KEY:

±	Mean standard deviation.	WNBF	Water N-butanol fraction
NA	No Activity	Gent	Gentamicin (10 µg / ml)
WE	Water Water	Cipo	Ciproflaxicin (5 µg / ml)
EE	Ethanol extracts	Amox	Amoxillicin (20 µg / ml)
WEAF	Water Ethyl acetate fraction		
EEAF	Ethanol Ethyl acetate fractions		
ENBF	Ethanol N-butanol fraction		

Table 4.4: Diameter of Zone of Inhibition at 100 mg/ml Concentrations of Root Extracts of *A. cordifolia* against Test Clinical Bacterial Isolates.

ORGANISMS	ZONES OF INHIBITION(mm)				
	Water Extract	Ethanol Extract	Ciprofloxacin	Amoxicillin.	Gentamicin.
E. coli	15.00± 0.00	15.00 ± 0.00	6.00±0.00	6.00±0.00	20.00±0.00
Ps.aeruginosa	21.00 ± 0.00	20.00 ± 0.00	35.00 ±0.71	6.00±0.00	26.00±5.65
S. aureus	30.00 ± 0.00	20.00 ± 0.00	32.50 ±3.53	18.00±2.82	29.50± 0.71
S. typhi	20.00 ± 0.00	15.00 ± 0.00	39.50±0.71	20.00± 0.00	28.50±2.12

KEY: ± Mean standard deviation.

4.3.2. The Minimum Inhibitory Concentrations (M.I.C) and Minimum Bactericidal Concentrations (M.B.C) of Test Clinical Bacterial Isolates.

There were no differences in M.I.C and M. B.C values for 24 hours and 72 hours water and ethanol extract against all the test clinical bacterial isolates as displayed in table 4.5 and table 4.6 respectively.

Table 4.5: The M.I.C and M.B.C Values of Stem Bark Water Extract of *A. cordifolia* against same Test Clinical Bacterial Isolates.

ORGANISMS	CONCENTRATION (µg/ml)							
	24 Hour		72Hour		Gentamicin		Ciprofloxacin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	6.25	50.00	6.25	50.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	6.25	12.50	6.25	12.50	ND	ND	ND	ND
S. aureus	3.13	12.50	3.13	12.50	0.02	0.06	3.13	12.50
S. typhi	3.13	25.00	3.13	25.00	ND	ND	ND	ND

KEY:

ND = Not determined.

Table 4.6: The M.I.C and M.B.C Values of Stem Bark Ethanol Extract of *A. cordifolia* against same Test Clinical Bacterial Isolates.

ORGANISM	CONCENTRATION (µg/ml))							
	24 Hours		72 Hours		Gentamicin		Ciprofloxicin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	6.25	100.0	6.25	100.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	6.25	12.50	6.25	12.50	ND	ND	ND	ND
S. aureus	0.78	12.50	0.78	12.50	0.02	0.06	3.13	12.50
S. typhi	0.78	25.00	0.78	25.00	ND	ND	ND	ND

KEY:

ND = Not determined.

The M.I.C and M.B.C values of *A. cordifolia* root water and ethanol extract at 24 hours and 72 hours showed no differences in values, as shown in table 4.7 and 4.8.

Table 4.7: The M.I.C and M.B.C of Root Ethanol Extract of *A. cordifolia* against same Test Clinical Bacterial Isolates.

ORGANISMS	CONCENTRATION (µg/ml)							
	24 Hour		72 Hour		Gentamicin		Ciprofloxacin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	25.00	100.0	25.0	100.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	25.00	50.00	25.0	50.00	ND	ND	ND	ND
S. aureus	6.25	50.00	6.25	50.00	0.02	0.06	3.13	12.50
S. typhi	6.25	50.00	6.25	50.00	ND	ND	ND	ND

KEY:
ND = Not determined.

Table 4.8: The M.I.C and M.B.C of Water Extract Root of *A*. c*ordifolia* against same Test Clinical Bacterial Isolates.

ORGANISMS	CONCENTRATION (µg/ml)							
	24 Hour		72 Hour		Gentamicin		Ciprofloxacin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	25.00	100.0	25.0	100.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	25.00	50.00	25.0	50.00	ND	ND	ND	ND
S. aureus	6.25	50.00	6.25	50.00	0.02	0.06	3.13	12.50
S. typhi	6.25	50.00	6.25	50.00	ND	ND	ND	ND

KEY:

ND = Not determined.

The M.I.C and M.B.C values of n- butanol and ethyl acetate fractions of water and ethanol extract of the stem bark of *A. cordifolia* presented in tables 4.9 and 4.10 showed that the ethyl acetate fractions, were lower compared to that of n- butanol. These values were considerably lower than the nonfractionated extracts.

Table 4.9: The M.I.C and M.B.C of Ethylacetate and N-butanol Fractions of Water Stembark Extracts of *A. cordifolia*, against same Test Clinical Bacterial Isolates.

ORGANISMS	CONCENTRATION (µg/ml)							
	EAF		N – BF		Gentamicin		Ciprofloxacin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	1.53	50.00	1.53	100.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	0.39	25.00	0.78	100.00	ND	ND	ND	ND
S. aureus	0.39	25.00	0.78	100.00	0.02	0.06	3.13	12.50
S. typhi	0.39	25.00	0.78	100.00	ND	ND	ND	ND

Key: EAF = Ethyl acetate fraction
N-BF = N – Butanol fraction
ND = Not determined

Table 4.10: The M.I.C and M.B.C of Ethylacetate and N- Butanol Fractions of Ethanol Stembark Extracts of *A. cordifolia* against same Test Clinical Bacterial Isolates.

ORGANISMS	CONCENTRATION(µg/ml)							
	EAF		N – BF		Gentamicin		Ciprofloxacin	
	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C	M.I.C	M.B.C
E. coli	1.53	50.00	1.53	100.00	1.00	3.00	6400.00	204.80
Ps. aeruginosa	0.39	25.00	0.78	100.00	ND	ND	ND	ND
S. aureus	0.39	25.00	0.78	100.00	0.02	0.06	3.13	12.50
S. typhi	0.39	25.00	0.78	100.00	ND	ND	ND	ND

Key: EF = Ethyl acetate fraction
N-BF = n – Butanol fraction
ND = Not determined

4.3.3 Effect of Temperature on the Antibacterial Activity of *Alchornea cordifolia Extracts*.

The values on table 4.11 – 4.14 showed that temperature had no much effect on the activity of the plant extracts, although the zones of inhibition reduced as temperature increases.

Table 4.11 Effect of Varying Temperature on the Antibacterial Activity of *A. cordifolia E*xtracts (100 mg/ml) against *E. coli* at 37^0 C for 24 hours.

TEMPERATURE(^{0}C)	ZONE OF INHIBITION (mm)			
	STEM BARK EXTRACT		ROOT EXTRACT	
	WATER	ETHANOL	WATER	ETHANOL
25^0C	22.50±3.53	22.50±3.53	15.00±0.00	17.00±0.00
37^0 C	22.50± 3.53	22.50±3.53	19.05±0.71	22.50± 3.53
45^0C	22.00± 1.41	20.00 ± 0.00	18.00±1.41	19.50±0.00
70^0C	17.00±3.53	19.50±0.71	16.00±0.00	12.00±3.35
100^0C	12.00 ± 0.00	13.50 ± 2.12	6.00±0.00	6.00±0.00

KEY: ± Mean standard deviation

Table 4.12 Effect of Varying Temperature on the Antibacterial Activity of *A. cordifolia* Extracts (100 mg/ml) against *Ps. aeruginosa* at 37^0 C for 24 hours

TEMPERATURE (^{0}C)	ZONE OF INHIBITION (mm)			
	STEM BARK EXTRACT		ROOT EXTRACT	
	WATER	ETHANOL	WATER	ETHANOL
25^0C	27.50±3.53	29.00±1.41	25.00±0.00	20.00±0.00
37^0 C	27.00±1.41	29.00±1.41	21.00± 0.00	27.00 ± 4.24
45^0C	25.00±0.00	20.00±0.00	20.00± 0.00	20.00±0.00
70^0C	19.00± 1.41	17.50±3.53	17.00± 0.00	20.00±0.00
100^0C	17.50± 3.53	17.0 ± 1.41	6.00± 0.00	6.00±0.00

KEY: ± Mean standard deviation.

Table 4.13 Effect of Varying Temperature on the Antibacterial Activity of *A. cordifolia* Extracts (100 mg/ml) against *S. typhi* at 37^0 C for 24 hours**.**

TEMPERATURE (^{0}C)	ZONE OF INHIBITION (mm)			
	STEM BARK EXTRACT		ROOT EXTRACT	
	WATER	ETHANOL	WATER	ETHANOL
25^0C	27.00±4.25	28.00±0.00	17.00± 0.00	18.00±0.00
37^0 C	27.00±2.82	28.00±2.12	20.00± 0.00	27.00±2.82
45^0C	22.50±0.71	20.00± 0.00	20.00± 0.00	20.00±0.00
70^0C	19.00±1.41	20.00±0.00	20.00± 0.00	20.00±0.00
100^0C	19.50±0.71	20.00± 0.00	20.00± 0.00	17.00±0.00

KEY: ± Mean standard deviation.

Table 4.14 Effect of Varying Temperature on the Antibacterial Activity of *A. cordifolia* Extracts (100 mg/ml) against *S. aureus at* 37^0 C for 24 hours.

TEMPERATURE (^{0}C)	ZONE OF INHIBITION (mm)			
	STEM BARK EXTRACT		ROOT EXTRACT	
	WATER	ETHANOL	WATER	ETHANOL
25^0C	30.00±0.00	32.50±2.25	25.00±0.00	20.00±0.00
37^0 C	30.00±0.00	32.00±3.53	27.50±3.53	27.50± 3.53
45^0C	29.00±2.82	29.00±0.00	22.00±0.00	20.00±0.00
70^0C	20.00± 0.00	20.00 ±0.00	20.00±0.00	20.50±0.71
100^0C	20.00±0.00	20.00±0.00	20.00±0.00	15.00±0.00

KEY: ± Mean standard deviation.

4.3.4. Rate of Kill Studies.

The result obtained from the rate of kill experiment using *A. cordifolia* stem bark extract and gentamicin is shown in figures 4.1 – 4.2.

As contact time increases there was decreased in the number of surviving cells to the extent that at 240 minutes, there were no surviving cell population, which shows that the extract at (125.00mg/ml) exerted bactericidal activity on *S. aureus*. In comparsim with gentamicin (0.63 mg/ml), kill was exerted at 360 minutes.

The same trend was observed for the extract, at (250.00mg/ml) exerted a kill, at a contact time of 120 minutes on *E. coli*, and Gentamicin (30.00 mg/ml) demonstrated its bactericidal effect at 240 minutes contact time.

The results indicate that *A. cordifolia* stem bark pose significant biocidal activity against the test organisms, which is rapid and generally concentration dependent. Killing of cells occur as a function of time within the concentration range.

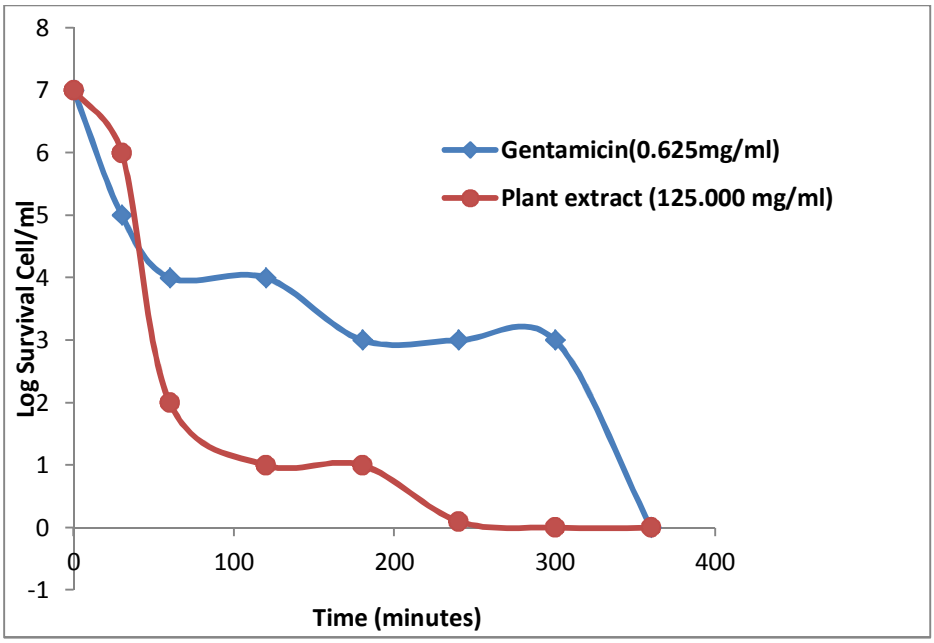


Fig 4.1: Biocidal activity of fixed concentration (0.625mg/ml) of gentamicin against *S. aureus* at different time interval

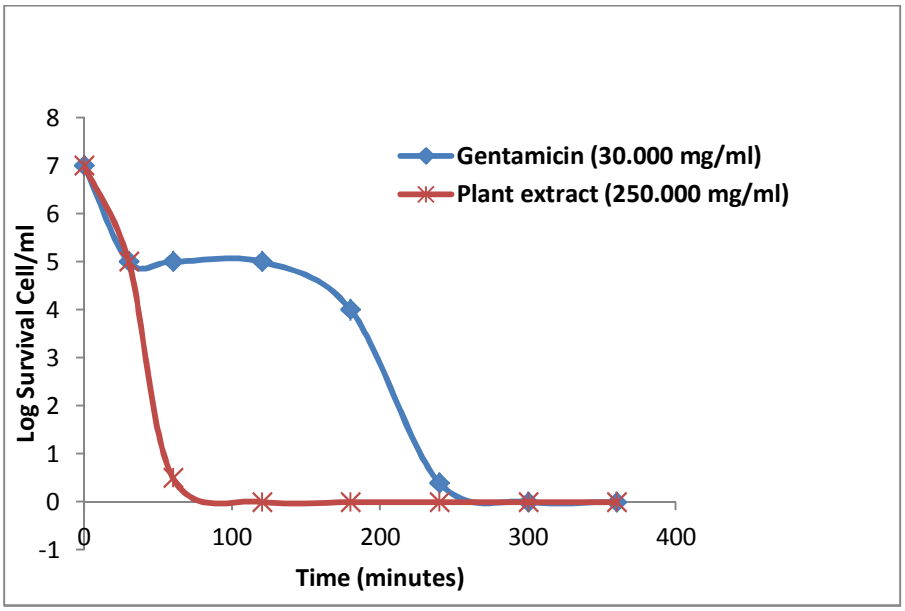


Fig 4.2: Biocidal activity of fixed concentration (30.00 mg/ml) of Gentamicin against *E. coli at* different time interval.

CHAPTER 5

DISCUSSIONS

Different solvents yielded substantial differences in the proportion of extracts. The differences in the percentage yields might be due to the polarity of the solvents: the less polar solvents such as ethyl acetate and butanol, yielding greater quantities of the extracts compared with the polar solvents such as water and ethanol.

The percentage yield of stembark with water extract (22.05 %) was less than the ethanol extract (39.32%), the stembark of both solvents extracts were more than the root extract (21.0 %). This is because more of the chemical components over time are deposited on the plant stembark unlike the root, which always have contact with water in the soil and equally as a medium of mineral up take and absorption. The ethanol extracts had higher yield than the water extract, which is due to solvent polarity. The fractions eluted with 100 % ethylacetate had more yield and activity than hexane. The n-butanol had more yield than the ethyl acetate and less active when compared, this is an indication that the ethylacetate was able to elute more of the active ingredient than n butanol and hexane solvents.

Irrespective of the type of solvent, all the extracts and fractions contained carbohydrates, cardiac glycosides, saponins, tannins and flavonoids and resins. These secondary metabolites are generally found as components of plants (Ogungbamila and Samuelsson, 1990). It also confirms findings made by (Abdullahi *et al.,* 2003). The secondary metabolites have been variously reported to possess appreciable inhibitory activities against various organisms (Cushnie and Lamd, 2005). The presence of these phytocompounds have be comfirmed to have antibacterial activity (Ali- Emmanuel *et al.,* 2003; Fasola, 2000; Lewis and Ausubel, 2006; Adeshoken *et al.,* 2007; Oyeleke *et al*, 2008; Udobi *et al.,* 2008). Many workers have reported the presence of

plant's chemicals found in *A. cordifolia*, these include steroid and cardiac glycosides, which is useful in the treatment of disease associated with the heart (Trease and Evans, 2000). Presence of tannin in all the extracts suggested the ability of *A. cordifolia* to play the role as antidiarrhea and antihaemoorrhagic agents (Asqwith and Butler, 1986). Tannins have been reported to hasten the healing of wounds and inflamed mucous membrane (Stary, 1998). Saponnin though are hemolytic on red cell are harmless when taken orally and they have beneficial properties of lowering cholesterol level in the body (Price *et al.,* 1987). Glycosides and flavonoids are known to inhibit tumor growth and protect against gastrointestinal infection (El-Mahmood, 2009).

The combined effect of tannin and flavonoids in ethylacetate fraction of the plant accounts for its high activity. Saponins, flavonoids and tannins are well known to possess antimicrobial activities (Akiyama *et al.,* 2001). Flavonoids have also been reported to be synthesized by plants in response to microbial infection and have been shown to have antibacterial activities (Kujumgiev *et al.,* 1999). Tannins and flavonoids in plants are known to form complexes with peptidoglycans sterols and other cell wall components resulting in cell leakage and death (Silver *et al.,* 1998). MacFoy and Sama, (1990) attributed the effectiveness of the fresh juice of *A. cordifolia* in the treatment of diarrhoea to the combined action of these secondary metabolites. The higher efficacy of the ethyl acetate fraction might be attributed to the higher proportion of some of these secondary metabolites in this extract, as judged by the higher percentage of yield. Adeshina *et al.,* (2010) also reported similar activity. The study demonstrated that different extracts exhibited different levels of activities, which were also dependent on the nature of the test organisms. Generally, Gram-positive bacteria showed more susceptibility to the extracts and fractions than the Gram-negative bacteria. The Gram-positive bacterial were more susceptible to reference antibiotic and test plant extract than their Gram-negative counterpart for instance the M.I.C of

the plant extract against *S.aureus* was 3.13 mg/ml while *Pseudomonas aeruginosa* was 6.25 mg/ml. The water extract and the ethyl acetate fraction exerted highest activity against *S. aureus*. While good inhibitory activities were exerted against *Pseudomonas aeruginosa* especially by the ethyl acetate fraction, the Enterobacteriaceae isolates were not as susceptible to the activities of the water and ethanol extracts. The differences in the susceptibilities of the isolates to the plant extracts can be related to the cell wall composition of the organisms. Gram-positive bacteria have a cell wall of peptidoglycan with techoic acid in between; therefore, they are more susceptible than Gram-negative bacteria that have their cell wall surrounded by lipopolysaccharides and lipoproteins, which prevent penetration of antibiotics through their cell wall. *Staphylococcus aureus* was more susceptible to the extracts especially the stem bark at a very low concentration of 0.78 mg/ml. This is of great importance as it has been reported that this organism has developed resistance to many antibiotic, which sometime makes its clinical management difficulty (Adewunmi *et al.,* 2001). High activity of antibacterial agent at low concentration is very essential for chemotherapeutic purposes due to toxicity in the patient system.

Generally, the degree of the test plants extracts antibacterial activity varies from test organisms to another, thus larger zones of inhibition were produced by the susceptible organisms than the resistant isolates. However, these activities are concentration dependent, irrespective of the test organism involved. This variation in level of the activity among the extracts could be due to the difference in solubility of the active ingredient in each solvent on one hand and to the constitutional or structural variability of the tested organisms on the other hand. Generally, the ethyl acetate fraction had a lower M.I.C values (0.38 mg/ml -100 mg/ml) and M.B.C values (12.5 mg/ml - 100 mg/ml) against isolates compared with other fractions, which were lower than

the figures reported (Tona *et al.,* 2000). The M.I.C and M.B.C values were generally lower for the stembark extract against the test isolates compared to those of the root extract. When biocidal concentrations were considered, *A. cordifolia* extract M.B.C were relatively higher than the standard antibiotic agent investigated in this study. This observation is a common occurance when one is dealing with crude extract having a lot of constituent that play little or no role in the antibacterial activity of the crude extract (Adewunmi *et al.,* 2001).

Furthermore, these impurities may even antagonize the antibacterial activity of the active constituent. The result of the M. I. C and M. B. C of *A. cordifolia* extract against Gram-negative organisms such as *Ps. aeruginosa* and *S.typhi* are the same. This suggested that the antibacterial activity of *A. cordifolia* against Gram-negative bacteria is the same contrary to standard antibiotic activity against the same set of Gram-negative bacteria isolates. The result of this extract had shown activity against Gram positive and negative bacteria, indicating a broad spectrum of activity. *Alchornea cordifolia* has been widely reported to have a wide spectrum of antibacterial activity (Pesewu *et al.,* 2008).

It was noticed that increase in temperature had no effect on the potency of the extracts, against the test bacterial isolates. This can be as a result of temperature not having effect on the active substance present in the crude extracts.

It was observed from the result that after 60 minutes contact time 250 mg/ml of crude extract of *A. cordifolia* stem bark extract effected 100 % kill of 10^6 cells / ml of resistant *E. coli* while 3µg/ml of gentamicin effected 100 % kill of the same organism after 180 minutes contact time.

The result of biocidal activity of *A. cordifolia* stembark extract and gentamicin indicated that they have significant antibacterial activity against resistant *E. coli*. The bactericidal activity of the test extract was rapid from the onset and generally concentration dependent. Garret and

Brown (1964) reported that there was no single concentration of an antibacterial agent at which cells in a suspension will be killed sponetanously.

Killing of cells occur chieftly as a function of time within a range of concentration and these possibly explain the increased lethal activity of higher concentration of these antibacterial agent above the minimum biocidal concentration.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

- The stembark and root extract and fractions contained steroids, flavonoids, tannins, glycosides, saponins,anthraquinones and resins
- *Alchornea cordifolia* ethanol and water extracts obtained from Benin, Edo State, Nigeria was found to posses' antibacterial action against *S. aureus, Ps. aeruginosa, E. coli,* and *S. typhi* isolates.
- The extract has shown a consistent and concentration dependent inhibition of bacterial isolates, although higher concentrations were required to produce zones of inhibition when compared to the reference antibiotic.
- The crude extracts had a stable antibacterial activity at lower temperatures and decreased in zones of inhibition as temperature increases.
- This is the first step in integration of our traditional medical practice into modern medicine as, scientific evidence that justified the use of the plant as an antibacterial agent has been established.

6.2 RECOMMENDATION

- The findings of this study have shown the need for further investigation to establish the economic viability of exploiting *A. cordifolia* plant to address health problems.
- Toxicology test for safety is required with further purification for economical purposes.
- The full potential of *A. cordifolia* plant would be realized by isolating the bioactive component, which could be a novel antibacterial therapy.

- The various principles in the extracts especially the ethyl acetate may be isolated and their specific activities as well as their mechanism of action determined.
- Steps should be put in place to encourage cultivation and conservation of plant generally.

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APPENDIX 1

Table 1: Phytochemical Analysis of *A. cordifolia.*

TEST	APPEARANCE	INFERENCE		
		EtOH	H_2O	EtO Ac
1.STEROLS a. Lieberman-Buchard	Production of blue Green, Red or Orange color that change with time	+	+	+
b. Salkowski test	Formation of red or yellow coloration was obtained	+	+	+
2.FLAVONOIDS a. Shinoda test	Appearance of an orange, pink or red to purple color was obtained	+	+	+
b. Sulphuric acid	Color change was observed	+	+	+
c. Ferric chloride	Green, Blue or violet coloration indicate the presence of phenolic hydroxyl group	+	+	+
d. Sodium Hydroxide	A change in color from yellow to colorless on addition of dilute HCl	+	+	+
3. ALKALOID a. Dragendoff reagent	Formation of yellow to brown precipitate	-	-	-
b. Mayer's reagent	Formation of white to yellowish or Green color precipitate	-	-	-
c. Wagner's reagent	Formation of brown or reddish brown precipitate No precipitate occurred in ethyl acetate extract.	-	-	-
4.TANNINS a. Lead sub-acetate	Formation of light brown precipitate.	+	+	+
b. Bromine water	A blue-black precipitate was observed	+	+	+

c. Ferric Chloride	Blue mixture was obtained in ethanol and ethyl acetate. Dirty green coloration was obtained in water extract.	+	+	+
d.FerricAmmonium citrate test	A brown mixture was obtained	+	+	+
5.SAPONIN a. Frothing test	Frothing which persisted for 15 minutes	+	+	+
b. Emulsion test or Olive oil test	Formation of stable emulsion	+	+	+
c. Fehling's test	Bluish-green precipitate observed in ethanol and water extract. No color change was observed in ethyl acetate.	+	+	+
d. Haemolytic test	Formation of haemolysis was seen in ethanol and water but not visible change/signs of haemolysis in ethyl acetate portion	+	+	+
6.CARBOHYDRATE TEST a. Molisch test	Appearance of reddish color is observed	+	+	+
b. Fehling's test for reducing sugar	Appearance of brick red precipitate	+	+	+
7.GLYCOSIDES TEST	A brick-red precipitate indicate the presence of glycosides in the test extracts	+	+	+
8.CARDIAC GLYCOSIDES		+	+	+
9. RESIN	The presence of green precipitate is a positive test	+	+	+
10ANTHRAQUINONES	Presence of pink, red or violet colour in the ammoniacal layer is observed	+	+	+

a. Free anthraquinones				
b.Combined Anthraquinones	A pink, red colouration in the ammonia phase (lower phase) indicate the presence of combined anthraquinones or anthraquinone derivative	+	+	+

Table 2: Diameter of zone of inhibition of different concentrations of extracts of stembark of *A. cordifolia* against *E.coli*

CONCENTRATIONS	**ZONE OF INHIBITION (mm)**					
(mg/ml)	WE	WEAF	WNBF	EE	EEAF	ENBF
100.0	22.5±3.53	25.0 ± 7.07	20.0±0.00	22.5±3.53	27.5± 3.53	22.5±3.53
50.0	22.5±3.53	20.0 ± 0.00	17.5±3.53	22.0±4.24	20.5±0.707	17.5±3.53
25.0	20.0±0.00	15.5±0.707	10.0±0.00	13.5±2.12	17.5± 3.53	12.5±3.53
12.50	12.5±3.53	9.5 ± 0.707	8.5 ± 2.12	10.0±1.41	15.5±0.707	8.5 ± 2.12
6.25	11.5±3.53	8.5 ± 2.12	NA	9.0±1.41	9.5±0.707	NA
3.125	NA	7.5 ± 2.12	NA	NA	8.5 ± 2.12	NA
1.5256	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA
0.38	NA	NA	NA	NA	NA	NA

KEY = ± Mean standard deviation

NA= No Activity

WE=Water extracts; WEAF= Ethyl acetate fractions of water extract

WNB= N-butanol fractions of water extract, EE= Ethanol extracts

EEAF =Ethyl acetate fractions of ethanol;ENBF= N-butanol fractions of ethanol

Table 3:Diameter of zone of inhibition of different concentrations of extracts of stembark of *A. cordifolia*against*Ps.aeruginosa.*

CONC.(mg/ml)	**ZONE OF INHIBITION (mm)**					
	WE	WEAF	WNBF	EE	EEAF	ENBF
100.0	27.5± 3.53	27.0± 4.24	20.0± 0.00	29.0± 1.41	27.5± 3.53	25.0±0.00
50.0	25.0± 3.53	22.5± 3.53	19.5± 0.71	27.5± 3.53	24.5± 0.71	22.5±3.53
25.0	22.5± 3.53	21.5± 2.12	12.5± 3.53	22.0± 4.25	23.0± 2.82	22.5±3.53
12.50	19.0± 1.41	13.0± 4.24	8.0 ± 2.82	17.5± 3.53	18.5± 3.53	20.0±0.00
6.25	17.5± 3.53	10.0± 0.00	NA	15.0± 0.00	16.0 ±1.41	19.5±0.71
3.125	10 ± 4.24	9.5 ± 0.71	NA	10.0± 0.00	9.5± 0.707	NA
1.5256	NA	NA	NA	8.5 ± 2.12	NA	NA
0.78	NA	NA	NA	NA	NA	NA
0.38	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation.

NA= No Activity

WE=water extracts;

WEAF= Ethyl acetate fractions of water extract

WNB= N-butanol fractions of water extract,

EE=Ethanol extracts

EEAF = Ethyl acetate fractions of ethanol extract;

ENBF= N-butanol fractions of ethanol extracts.

Table 4:Diameter of zone of inhibition of different concentrations of stembark extracts of*Alchornea cordifolia*against*S. aureus*

CONC.(mg/ml)	ZONE OF INHIBITION (mm)					
	WE	WEAF	WNBF	EE	EEAF	ENBF
100.0	27.5± 3.53	32.5 ± 3.53	24.5±0.707	32.5± 2.23	32.5 ±3.53	24.5± 0.707
50.0	27.0± 4.24	27.5 ± 3.53	22.5 ± 3.53	27.5± 3.53	27.5 ± 3.53	23.5 ± 2.12
25.0	24.0± 1.41	22.5 ± 3.53	21.5 ± 2.12	27.5± 3.53	25.5±0.707	21.5 ± 2.12
12.50	23.5± 2.12	22.5 ± 3.53	20.0 ± 0.00	22.5± 3.53	24.0 ± 1.41	20.0 ± 0.00
6.25	20.0± 0.00	23.5 ± 2.12	9.5 ± 0.707	22.0±4.24	22.5 ± 3.53	19.0 ± 1.41
3.125	19.5±0.707	22.0 ± 2.12	NA	16.0± 2.82	22.0 ± 2.82	15.0±4.24
1.5256	13.5 ± 2.25	21.5 ± 2.12	NA	9.5± 0.707	17.5 ± 3.53	NA
0.78	NA	17.5 ± 3.53	NA	NA	17.0 ± 4.24	NA
0.38	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation.

NA= No activity

WE=water extracts;

WEAF=ethyl acetate fractionsof water extract

WNB=water N-butanol fractions,

EE= ethanol extracts

EEAF =ethanol ethyl acetate fractions;

ENBF=ethanolN-butanolfractions.

Table 5: Diameter of zone of inhibition of different concentrations of stembark extracts of *Alchornea cordifolia* against *S. typhi.*

CONC.(mg/ml)	**ZONE OF INHIBITION(mm)**					
	WE	WEAF	WNBF	EE	EEAF	ENBF
100.0	27.0± 4.25	30.0 ± 0.00	22.5 ± 3.53	28.5 ± 2.12	30.0 ± 0.00	22.5 ± 3.53
50.0	24.0± 1.41	25.0 ± 0.00	20.0 ± 0.00	27.5 ± 3.53	27.5 ± 3.53	21.5 ± 2.12
25.0	24.0±1.41	22.5 ± 3.53	17.5 ± 3.53	22.5 ± 3.53	22.5 ± 3.53	20.0 ± 0.00
12.50	22.5± 3.53	23.5 ± 2.12	15.5 ± 4.94	20.0 ± 0.00	21.5 ± 3.53	19.5± 0.71
6.25	19.5±0.71	22.5 ± 3.53	9.5 ± 0.71	9.5 ± 0.71	20.0 ± 0.00	17.0 ± 4.21
3.125	17.0± 4.24	20.0 ± 0.00	NA	10.0 ± 0.00	18.5 ± 2.82	9.0 ± 1.41
1.5256	12.5± 3.53	19.5±0.71	NA	NA	18.5± 0.707	NA
0.78	NA	15.0 ± 7.1	NA	NA	16.5 ± 2.12	NA
0.38	NA	NA	NA	NA	NA	NA

KEY = ± Mean standard deviation.

NA= No Activity

WE=Water extracts;

WEAF= Water ethyl acetate fractions

WNB=Water N-butanol fractions,

EE= Ethanol extracts

EEAF =Ethanol ethyl acetate fractions

ENBF= Ethanol N-butanol fractions.

Table 6: Diameter of zone of inhibition of different concentrations of ethanol root extracts obtained after 24 and 72 hours maceration times.

CONC. (mg/ml)	ZONE OF INHIBITION (mm)							
	24 hours EE				72hours EE			
	E.coli	*Ps. aeruginosa*	*S. typhi*	*S. aureus*	*E.coli*	*Ps. aeruginosa*	*S. typhi*	*S. aureus*
100.0	17.0± 0.00	20.0 ± 0.00	18.0± 1.41	20.0± 0.00	15.0± 0.00	20.0 ± 0.00	15.0± 0.00	20.0± 0.00
50.0	16.5±0.71	19.0 ± 1.41	17.0± 1.41	19.5± 0.71	10.0± 0.00	19.0 ± 0.00	10.0± 0.00	19.0± 1.41
25.0	15.5± 1.41	17.0 ± 0.00	17.0± 0.00	17.5± 1.41	9.0 ± 1.41	17.5 ± 2.12	9.0 ± 1.41	17.5± 2.12
12.50	15.0± 0.00	15.0 ± 1.41	16.0± 1.41	16.0± 1.41	NA	15.0 ± 0.00	NA	15.0± 0.00
6.25	7.0 ± 0.00	10.0 ± 0.00	9.0 ± 1.41	10.0± 0.00	NA	10.0±0.00	NA	10.0±0.00
3.125	NA	NA	NA	NA	NA	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY = ± Mean standard deviation.

NA= No activity.Conc. = Concentrations. EE=Ethanol Extract.

Table 7: Diameter of zone of inhibition of different concentrations of water root extracts obtained after 24 and 72 hours maceration times.

CONC	**ZONE OF INHIBITION (mm)**							
mg/ml)	24 hours WE				72hours EE			
	E. coli	*Ps.aeruginoa*	*S. typhi*	*S.aureus*	*E. coli*	*Ps. aeruginosa*	*S. typhi*	*S. aureus*
100.0	15.0± 0.00	21.0 ± 0.00	20.0± 0.00	30.0 ± 0.00	15.0±0.00	25.0 ± 0.00	17.0± 0.00	25.0 ± 0.00
50.0	14.0± 1.41	20.0 ± 0.00	20.0± 0.00	27.5 ± 3.53	14.0± 1.41	22.5 ± 3.53	14.0± 1.41	25.0 ± 0.00
25.0	13.5±0.77	18.0 ± 2.82	18.0± 0.00	25.0 ± 0.00	12.0± 0.00	18.0 ± 2.82	12.0± 0.00	18.5 ± 3.53
12.50	9.0 ± 1.41	17.0 ± 1.41	10.0± 0.00	20.0 ± 0.00	10.0± 0.00	15.0 ± 0.00	10.0± 0.00	15.0 ± 1.41
6.25	NA	14.0 ± 0.00	NA	12.5 ± 3.53	NA	10.0 ± 0.00	NA	10.0 ± 0.00
3.125	NA	NA	NA	9.0 ± 1.41	NA	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation.

NA =No Activity

WE =Water extract.

CONC. = Concentrations

Table 8: Diameter of Zone of Inhibition of Water and Ethanol Extract of Stembark of *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *E. coli*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100C
100.0	22.5± 3.53	22.0± 1.41	17.0±3.53	12±0.00	22.5±3.53	20.0±0.00	19.5±0.71	13.5 ± 2.12
50.0	20.5±0.71	20.0±0.00	13.5±2.12	10.0±0.00	22.0±4.24	17.5± 3.53	17.5±3.53	11.5± 2.12
25.0	20.0 ± 0.00	20.0± 0.00	NA	NA	19.0±1.41	16.0 ±1.41	12.0± 2.28	8.5 ± 2.12
12.5	15.0 ± 0.00	17.5± 2.82	NA	NA	13.5±2.12	14.0± 0.00	9.0±1.41	NA
6.25	10.0 ± 0.00	12.5± 3.53	NA	NA	10.0±0.00	9.5± 0.707	NA	NA
3.125	NA	NA	NA	NA	NA	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

EE=Ethanol Extracts

Table 9: Diameter of Zone of Inhibition of Water and Ethanol Extract of Stembarkof *A. cordifolia*obtained by 24 hours maceration, and heated at different temperatures on *Ps. aeruginosa*

Conc(mg/m)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	27.0±1.41	25.0±0.00	19.0±1.41	17.5 ± 3.53	29.0±1.41	20.0±0.00	20.0±0.00	17.0 ±1.41
50.0	25.0±0.00	23.5±2.12	17.5±0.71	17.5 ± 3.53	27.5±3.53	19.0±1.41	18.0±0.00	13.5 ± 2.12
25.0	24.0±1.41	23.0± 2.82	15.0± 0.00	NA	22.0±4.24	17.5± 3.53	17.5 ±3.53	9.0 ± 1.41
12.5	22.5±3.53	20.0± 0.00	13.5± 4.94	NA	17.5±3.53	14.0± 1.41	10.0± 0.00	NA
6.25	21.0±1.41	19.5±0.71	10.5±0.71	NA	15.0±0.00	9.0 ± 1.41	NA	NA
3.125	15.0±0.00	15.0±0.00	NA	NA	9.0 ± 1.41	8.0 1.41	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation.

NA = No Activity

EE=Ethanol Extracts

WE=Water= Extracts

Table 10: Diameter of Zone of Inhibition of Water and Ethanol Stembark Extract of*A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *S.typhi*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	27.0±2.82	22.5±0.71	19.0±1.41	19.5±0.71	28.0±2.12	20.0± 0.00	20.0 ± 0.00	20.0 ± 0.00
50.0	24.0±1.41	21.0± 1.41	17.5±3.53	17.0 ± 1.41	27.5±3.53	19.0± 1.41	20.5±0.71	19.0 ± 0.00
25.0	22.5±3.53	20.0± 0.00	14.0±0.71	15.0 ± 0.00	22.5±3.53	16.0± 1.41	19.0 ± 1.41	17.5 ± 3.53
12.5	20.0±0.00	19.0± 1.41	13.0±0.00	9.0 ± 1.41	20.0±0.00	12.0±0.00	16.5 ± 2.12	9.0 ± 1.41
6.25	17.5±3.53	17.0± 4.21	10.0±0.00	NA	15.0±0.00	9.0± 1.41	9.0 ± 1.41	NA
3.125	10.0±0.00	NA	NA	NA	NA	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

EE=Ethanol ExtractsWE=Water Extracts

Table 11: Diameter of Zone of Inhibition of Water and Ethanol Extract of Stembarkof *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *S. aureus*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	30.0±0.00	29.0±2.82	20.0± 0.00	20.0±0.00	32.5±3.53	22.0±0.00	22.0 ± 0.00	20.0±0.00
50.0	27.5±3.53	24.0±1.41	18.0±2.82	19.5±0.707	27.5±3.53	20.0±0.00	21.0 ± 1.41	19.0±1.41
25.0	25.0±0.00	23.0±1.41	17.5 ± 0.71	16.5± 2.12	27.5±3.53	19.0±1.41	20.0±0.00	15.0±0.00
12.5	24.0±1.41	17.5±3.53	13.5 ± 2.12	15.0± 0.00	22.5±3.53	18.5± 0.71	19.0 ± 0.00	11.0±1.41
6.25	20.0±0.00	15.0± 0.00	11.0 ± 1.41	10.0± 0.00	22.5±3.53	12.0±3.53	12.5 ± 3.53	NA
3.125	17.5±3.53	13.0±4.24	NA	NA	15.0±0.00	8.0±0.00	NA	NA
1.5256	13.5±2.12	NA	NA	NA	13.5±2.12	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

EE=Ethanol Extracts

Table 12: Diameter of Zone of Inhibition of Roots Extract of of *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *E. coli*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	19.0±0.71	18.0±1.41	16.0±0.00	NA	22.5± 3.53	19.5±0.00	12.0±3.35	NA
50.0	17.0±1.41	16.5±2.12	NA	NA	21.0± 1.41	15.0 ± 0.00	NA	NA
25.0	17.0± 0.00	15.0±0.00	NA	NA	20.± 0.71	12.0±1.41	NA	NA
12.5	15.0± 0.00	9.5±0.71	NA	NA	10.0± 0.00	9.0 ± 1.41	NA	NA
6.25	NA	NA	NA	NA	7.5± 0.71	NA	NA	NA
3.125	NA	NA	NA	NA	NA	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

EE=Ethanol Extracts

Water = Extracts

Table 13: Diameter of Zone of Inhibition of Root Extract of *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *Ps. aeruginosa.*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0 C	100^0C
100.0	21.0± 0.00	18.0±2.82	17.0± 0.00	NA	27.0 ± 4.24	20.0±0.00	20.0.0±0.00	NA
50.0	20.0± 0.00	17.5±2.12	14.5±0.71	NA	22.5 ± 3.53	18.0±2.12	18.0±0.00	NA
25.0	18.0±2.82	14.0±0.00	11.0±1.41	NA	19.5±0.707	16.5±2.12	10.0±0.00	NA
12.5	17.0± 1.41	12.0±3.53	9.0±1.41	NA	18.0 ± 0.00	15.0±0.00	NA	NA
6.25	14.5± 0.71	7.5 ± 0.71	NA	NA	15.0 ± 0.00	10.0±0.00	NA	NA
3.125	NA	NA	NA	NA	10.0 ± 0.00	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

EE=Ethanol Extract: WE=Water Extracts

Table 14: Diameter of Zone of Inhibition of Roots Extract of *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *S. typhi*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	20.0± 0.00	20.0± 0.00	20.0±0.00	20.0±0.00	27.0±2.82	20.0±0.00	20.0±0.00	17.0±0.00
50.0	18.0± 2.82	19.0± 1.41	19.0±0.71	17.0±1.41	20.0±0.00	18.5±0.00	20.0±0.00	9.0 ±1.41
25.0	17.0±0.00	19.0±0.00	15.0±0.00	15.0±0.00	19.5±0.71	17.5±3.53	18.0±0.00	7.0±0.00
12.5	15.0± 0.00	15.0± 0.00	NA	NA	15.0±0.00	12.5± 3.53	NA	NA
6.25	15.0±0.00	9.0 ± 1.41	NA	NA	12.0± 0.00	10.0± 0.00	NA	NA
3.125	9.0±0.00	NA	NA	NA	10.0±0.00	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA
	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity. EE=Ethanol Extracts

Table 15: Diameter of Zone of Inhibition of Roots Extract of *A. cordifolia* obtained by 24 hours maceration, and heated at different temperatures on *S. aureus*

Concentration(mg/ml)	ZONE OF INHIBITION (mm)							
	WE				EE			
	37^0C	45^0C	70^0C	100^0C	37^0C	45^0C	70^0C	100^0C
100.0	27.5±3.53	20.0±0.00	20.0±0.00	20.0±0.00	27.5±3.53	20.0±0.00	20.5.0±0.71	15.0±0.00
50.0	27.0±4.24	19.5±0.71	19.0±1.41	17.0±4.21	27.0±2.82	19.0±1.41	20.0±0.00	11.0±2.12
25.0	23.0±2.82	18.5±0.71	18.0±2.82	15.0±0.00	22.0±2.82	18.0±0.00	19.0±0.00	9.0±1.41
12.5	20.0±0.00	15.0±0.00	15.0±0.00	NA	20.0±0.00	17.5±3.53	NA	NA
6.25	17.0±0.71	10.5±0.00	NA	NA	15.5±0.71	12.0±0.00	NA	NA
3.125	10.0±0.00	NA	NA	NA	11.0±1.41	NA	NA	NA
1.5256	NA	NA	NA	NA	NA	NA	NA	NA
0.78	NA	NA	NA	NA	NA	NA	NA	NA
0.39	NA	NA	NA	NA	NA	NA	NA	NA

KEY: ± Mean standard deviation

NA = No Activity

WE=Water-extract

Table 16: Biocidal activity of fixed concentration (30mg/ml) of gentamicin against*E. coli*at different time interval.

E.Coli

Time(min)	Gentamicin(30mg/ml)	Plant extract(250mg/ml)
0	7	7
30	5	5
60	5	0
120	5	0
180	4	0
240	0	0
300	0	0
360	0	0

Table 17: Biocidal activity of fixed concentration (0.625mg/ml) of gentamicin against *S.aureus* at different time interval.

S.aureus

Time(min)	Gentamicin(0.625mg/ml)	Plant extract(125.000mg/ml)
0	7	7
30	5	6
60	4	2
120	4	1
180	3	1
240	3	0
300	3	0
360	0	0