IN VITRO EVALUATION OF ANTIBACTERIAL ACTIVITY OF A CRUDE AQUEOUS EXTRACT OF AZADIRACHTA INDICA (NEEM) LEAVES

BY

MARY WANGARI KIHARA (HND)
Reg No: 156/6515/03

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Kihara, Mary Wangari
In Vitro evaluation of antibacterial activity
DECLARATION

I declare that this thesis is my own original work and has not been presented for a degree or any other award in any other university.

MARY WANGARI KIHARA

Signature .................................. Date 25/11/08

This thesis is submitted for examination with our approval as the university supervisors.

DR. J. N. MAKUMI
Department of Biochemistry and Biotechnology,
School of Pure and Applied Sciences,
Kenyatta University.

Signature .................................. Date 25/11/08

DR. P. M. MBUGUA
Department of Medical Physiology,
School of Medicine,
University of Nairobi.

Signature .................................. Date 18-11-2008

DR. J. N. NGERANWA
Department of Biochemistry and Biotechnology,
School of Pure and Applied Sciences,
Kenyatta University.

Signature .................................. Date 25/11/08
DEDICATION

Dedicated to my daughters Martha, Regina, Grace and Beatrice
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Neem tree is a popular medicinal plant native of India but has now spread to countries the world over including Kenya. All parts of the Neem tree, that is, the seed, leaves, bark, roots and flowers are known to exhibit antibacterial activity. Despite Neem's numerous traditional applications in treatment of infectious diseases, there is limited laboratory data to support these therapeutic claims. Genetic and environmental variability also determine the value on Neem products thus further compounding the problem. This study was carried out to evaluate an aqueous Neem leaves extract for antibacterial activity by determining the spectrum of activity, efficacy and potency of Neem leaves from coastal Kenya. The spectrum of activity was obtained by determining the susceptibility of 24 bacterial species to the extract by disk diffusion method. The efficacy and potency of the extract were assessed by comparing the Minimum Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Time-kill values of selected bacterial species to the Neem extract with those of chloramphenicol. Leaves were preferred in this study as the tree is evergreen and their use would save the trees from drying unlike when roots and bark are used. Leaves would also ensure a constant supply of the products unlike seeds which are seasonal. Out of 107 bacterial isolates, 60.7% (±10.1) were sensitive while 39.3% were resistant by disc diffusion method. Of all the gram-positive and gram-negative isolates tested, 69.7% (±9.3) and 56.8% (±10.3) were sensitive respectively at 5% confidence interval. The MIC and MBC for Staph. aureus, E. coli, Sal. typhi, Ps. euroginosa and Strep. pyogenes ranged from 1.5-6.0 mg/ml and 2-13 mg/ml compared to 0.0625-1.2 mg/ml and 0.106-4.8 mg/ml respectively for chloramphenicol. Univariate ANOVA on MIC and MBC indicated significant difference between the potency of chloramphenicol and Neem extract with chloramphenicol proving to be more potent than the Neem extract against the selected organisms except Ps. aeruginosa (p=0.001-0.008). Both however showed poor bactericidal properties; they did not eliminate a population of $10^6$ of organism on incubation for 24hrs even at concentration two times the MBC. They however, considerably suppressed growth of the organisms. The organisms that were most susceptible to the extract included; Staphylococcus aureus, Escherichia coli, Salmonella typhi and Streptococcus pyogenes and Streptococcus faecalis. Shigella, Neisseria and Haemophilus influenza species were resistant. A methicillin resistant strain of Staphylococcus aureus was resistant while an Escherichia coli isolates, which had proved resistant to a battery of antibacterial agents, was suppressed. From the results of this study it can be inferred that Neem is a low potency antibacterial agent with bacteriostatic properties and a broad spectrum of activity. It exhibits antibacterial activity worthy of consideration for use in treatment of bacterial infections. The results explain its popularity in controlling bacterial infections traditionally but also indicate necessity to perform susceptibility test for all isolates as same species showed different responses with different isolates. Neem extracts would be very useful in treatment of co-infections.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Ethnomedecinal use of plants and plant products has been practiced in various cultures for thousands of years. It has become part of inherited wisdom rather than being based on knowledge of plants bioactive chemical constituents that beneficially modulate the physiology of a sick person (Saxena, 2003). The World Health Organization estimates that about 85 per cent of population in Sub-Saharan Africa relies on traditional medicine for their primary health care needs (Chooto, 2004). Worldwide, more than 10,000 species of higher plants have been used for medicinal purposes and it is estimated that thousands of kilograms of medicinal plants and their parts are collected and used everyday by traditional healers and mothers at homes across Africa (Saxena, 2003; Chooto, 2004). Today, interest is focused on plants as likely sources of new commercial drugs. Modern analytical methods have not only revealed the enormous variety and complexity of bioactive principles of medicinal plants, but also confirmed their potential value for use as medicines or as models for synthetic drugs (Saxena, 2003).

The tea, Neem and the oregano trees are, known to be potent all-purpose healers with antiseptic and antibacterial remedies and have been in use over centuries. The tea tree (*Melaleuca alternifolia*) leaves contain terpinen-4-ol oil, a very versatile antibacterial agent. Aborigines have used tea oil for centuries while Australian Journals have documented its use since 1920 (Judith, 1920). A study published in
1995 in the Journal of Antimicrobial Chemotherapy found that a dilution of 0.5% of tea tree oil killed *Staphylococcus aureus* in test tubes (Elsom *et al.*, 1999). It is known to relieve sore throat, head congestion and phlegmy cough. A 1990 study by Lederle Laboratory and Royal Prince Alfred Hospital in Great Britain found the oil useful against acne (Bassett *et al.*, 1990). In another study documented in the Journal of Family Practice (1994), tea oil was found to be a good relief for nail fungal infections (Norine, 1998).

Oregano oil (*Origanum vulgare*) is a potent remedy for skin conditions, chronic pain, insect bites, nasty summer cold and a powerful antiseptic, antibacterial, antiparasitic, antiviral, analgesic and antifungal agent. It is also a good remedy for sunburn and fights plaque and gingivitis. In a study by the Anadoly University in Turkey and published in the Journal of Phytotherapy Research, oregano oil is claimed to be a better analgesic than ibuprofen and nearly as good as morphine in controlling chronic pain (Norine, 1998).

Since ancient times, *Azadirachta indica* has been used in India for relieving sickness and its medicinal uses are well documented in Ayurveda (Indian system of natural healing). Centuries of proven effectiveness against many diseases have given Neem an esteemed place in the Indian culture (Neem Association 17980, 1998). The Neem tree has been called “the village pharmacy” because its bark, leaves, sap, fruit, seeds, roots and trunk have many diverse uses in traditional medical practice. It to date touches the daily life of almost every Indian, from the poorest peasant who snaps
of a twig for tooth brush to wealthier individuals who purchase manufactured Neem based tooth pastes, soaps and medicines. Its reputation as a reliever of sickness has spread to far countries, in tropical Africa where it was introduced a century ago and to Latin America a decade ago. In Kenya and neighbouring countries Neem in Kiswahili language is known as “Mwarubaini” meaning the “reliever of 40 disorders” in Sanskrit it is called ”arishtha” (reliever on sickness) while in Srilanka it is known as Kohomba (Saxena, 2003).

Despite Neem’s popularity as a reliable solution to a wide range of health problems including infections, its use still remains subject to suspicion, as very little scientific evidence to support its efficacy exists. The practice of using Neem has thus remained a tradition passed from one generation to another and mainly employed when other alternatives are beyond reach (due to cost) or as a last resort to desperate situations. There is therefore need to study its antimicrobial activity and thereby standardize therapeutic regimens. Studies in India have proved Neem extract to have antibacterial, antifungal and antiviral activity but the findings cannot be directly applied to extracts obtained in Kenya as the tree, like many others, is known to differ in activity in different parts of the world due to genetic variability, soil and climatic differences (National Research Council, 1992). It is therefore necessary to evaluate locally obtained Neem extracts for their potency and efficacy. This would provide preliminary information required to standardize Neem herbal preparations and thereby make them more acceptable for therapeutic application. The leaves have been
preferred in this study for their bounty and availability year round. They ensure a constant supply of raw material for the herbal preparations while preserving the trees.

1.2.1 The Neem tree

The Neem tree (Figure 1) belongs to the family Maliaceae and was described as *Azadirachta indica* as early as 1830 by De Jussieu. It is a close cousin of the mahogany tree and is commonly known as Neem or Indian lilac (National Research

![Figure 1: A photograph showing a stump, a sprouting and a mature Neem tree at the University of Nairobi ground (adjacent to the wire fence).]
Council, 1992; Schmutterer, 1995; Kausik et al., 2002). It has its origin in India and Burma but it is in India that the tree is most widely used. Over the last century, Neem has been introduced to Africa, Fiji, Mauritius, the Caribbean and many countries of central and South America (Schmutterer, 1995). In some cases, it was probably introduced by indentured laborers from India while in other cases it was introduced by foresters. It is now grown in over 30 countries including the East African coast of Kenya, Somalia and Tanzania (from Tanga to Moshi) and also found in Zanzibar and Pemba (Arishtha Amazoia, undated).

The Neem tree is famous for its drought resistance (Schmutterer, 1995) and remains in leaf throughout except in extreme drought. It can grow in different types of soils; cotton soil, calcareous soils and high clay soils, but best grows in well-drained deep sandy soils. It is easily propagated sexually and vegetatively and sprouts vigorously on loping. All parts of the tree have some medicinal application (leaves, roots, stem bark, fruits and flowers). The tree thrives in sub-arid and sub-humid conditions at altitudes between 700-800m above sea level but is not favoured at altitude between 1000-1500m above sea level. Three distinct species are known, that is, Azadirachta siemensis, Azadirachta excelsa and Azadirachta indica. They have similar bioactive compounds but different concentrations (National Research Council, 1992; Schumutterer, 1995).
1.2.2 Neem as a medicinal plant

Many drugs in use today have their origin from plants even those now synthetically produced. Records show that the non-edible Neem oil was perhaps the oldest known medicinal oil and almost every part of the Neem tree has some medicinal use (Mitra and Patel, 1963; Saxena, 2003). The Neem has been improved by mixing its materials with other herbal medicine, and the mere presence of the tree near human dwellings is believed to materially improve human health and act as a prophylactic agent against malaria, fever and cholera. From the Sarira Sthanam, patients were even laid on Neem leaves and fanned with a belief for cure from small pox, chicken pox and syphilis (Neem Association 17980, 1998). The belief in curative properties of Neem in some populations in India is so strong that it defies explanation.

In the Aryurveda, Neem is recorded as having been used for conditions such as ulcers, inflammation, splenomegaly, alopecia, diabetes, hypertension, cancer, allergies, headache, sinusitis, leucoderma, tropical eosinophilia, epistaxis, spermatorrhea, blood morbidity, biliary afflictions and as a fertility regulator. It was also popular in treatment of dermatological, respiratory, urinary and gynecological disorders as well as general conditions such as ophthalmic care, hair care, snake bite and scorpion sting, rheumatic pain, scabies, piles and fistula and for dental hygiene. But it was even more popular in the treatment of infectious diseases ranging from bacterial, fungal, viral to parasitic infections and was commonly used on wounds as an antiseptic (Chaturvedi, 1998). Because of such diverse curative properties, it is appropriately known as the “village pharmacy” in rural India. In Kenya it has been
widely applied in treatment of typhoid, malaria, fungal infections (ring worms), wounds, scabies and even snake bites (Saxena, 2003).

1.2.3 Chemical composition of Neem

Chemical investigation on Neem products was extensively undertaken in the middle of the twentieth century (Kausik et al., 2002). Siddqui (1942) produced an early report on the isolation of nimbin, a limonoid. Lavi et al. (1960) at the Weizmann Institute of Science (Israel) isolated melianone and melianol from Miazedarach (Persian Lilac) and epoxyazadrodion, azadiradione and azadirone together with epoxylactones, gedunin and 7-deacetylgedunin from neem oil. To date, more than 135 compounds have been isolated from different parts of Neem and several reviews have also been published on the chemistry and structural diversity of these compounds (Koul et al., 1990; Kraus et al., 1995).

The compounds have been divided into two major classes; isoprenoids and others (Kausik et al., 2002). Recently four Neem limonoids; gedunin, dihydrogedunin, nimbinin and nimbolide from leaves have reportedly been found to possess minimum inhibitory concentrations (IC\textsubscript{50}) of 0.72 and 1.74 μg/ml against malaria parasite Plasmodium falciparum in vitro (Philipson, 1995). Nimbidin a major crude bitter principle extracted from the oil of seed kernels has demonstrated several biological activities. From this crude extract, compounds such as tetrannortripenes including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic (Fig 1.1) have been isolated (Siddiqui, 1942). Nimbidin possess anti-inflammatory, antifungal, antimalarial,
spermicidal, antipyretic, antiarthritic, hypoglycaemic, antibacterial and diuretic activity (Bahargva et al., 1970; Pillai et al., 1981; Rochanakii et al., 1985; Pillai et al., 1991). Gedunin also from seed oil has been shown to have antifungal and antimalarial activities (Rao et al., 1977; Khalid et al., 1989). Azadirachtin isolated from Neem seed have strong antifeedant and antimalarial activity (Butterworth et al., 1968; Jones et al., 1994). Mohmoodinin from seed oil is antibacterial (Devakumar and SukhDev, 1996). Tannins from the bark contain gallic acid, gallocatechin and epicatechin compounds known to inhibit oxidative burst during inflammation (Vander Nat et al., 1991). Three tricyclic diterpenoids: margolone, margolonone and isomargolonone isolated from bark have antibacterial affect against Klebsiella, Staphylococcus and Serratia species (Ara et al., 1989). Sulfur containing compounds such as cyclic trisulphide and tetra sulphide isolated from matured Neem leaves have antifungal activity against Trichophyton mentagrophytes (Pant et al., 1986). Gla, GIla and GIIIa are polysaccharides from Melia azadirachta (syn. Azadirachta indica) bark and have been found to have anti-inflammatory and anti-tumor effects (Fujiwara et al., 1982; Fujiwara et al., 1984).
Figure 2: Some structures of bioactive Neem compounds

Source: Current Science Vol, 82. No 11. 10 June 2002
Figure 3: More structures of bioactive Neem compounds

Source: Current Science Vol, 82. No 11. 10 June 2002
Major concentrations of the active compounds are found in the seed oil although some are also found in the leaves and bark in lesser amounts. Historically, Neem leaves and barks have been the primary source of Neem ingredients in medicinal preparations because of their availability throughout the year and the ease of extracting the compounds (Neem Association 17980, 1998).

Most of the bitter active compounds are soluble in alcohol and water. Tinctures of 50-80 percent alcohol capture the majority of the medicinal compounds as well as tea using hot but not boiling water (Neem Association 17980, 1998). Excessive heat is thought to damage some of the compounds. The complex molecular structure of bioactive Neem compounds (Fig 2 and 3) would preclude their chemical synthesis economically in the near future. Consequently, the pharmaceutical industry would have to rely on the use of raw Neem materials. This could be a boom for developing countries in the tropics where neem thrives (Arishtha Amazoia, undated).

1.2.4 Antimicrobial uses of Neem extracts

The number of pharmacological studies on Neem has been somewhat limited. In those studies that have been carried out, the general conclusion is that neem not only kills some infectious organisms directly but also boosts the immune response when administered (Neem Association 17980, 1998). This increases the body’s ability to fight bacterial, viral and fungal infections. In recent years scientific evidence has emerged in support of valuable antifungal, antibacterial and antiviral properties Neem, as well as a wide range of other health benefits (Neem Association 17980, 1998).
Neem oil has been shown to suppress several species of pathogenic bacteria including *Staphylococcus aureus* (Schneider, 1986) which causes pus forming boils, abscesses, secondary infection in wounds, burns, peritonitis, cystitis and meningitis. It has also been shown to suppress *Salmonella typhosa* (Patel and Trivedi, 1962), which causes typhoid, food poisoning, blood poisoning and gastroenteritis. Deoiled Neem cake is popular in rural Kenya for treatment of typhoid and diarrheal diseases (Saxena, 2003). In a study in India, Neem bark extract was found to have strong antibiotic action against a human strain of *Mycobacterium tuberculosis* (Chopra et al., 1952; Saxena, 2003).

In laboratory tests, Neem bark extract also inhibited three of four pyogenic strains of *Staphylococcus aureus var. haemophilus* (Lorenz, 1976) confirming the value of the use of Neem twigs as tooth brushes in the Indian sub-continent. Bark extract was also found to be as effective as many synthetic anti-leprosy drugs against *Mycobacterium leprae* associated with leprosy (Subramanian and Lakshmanan, 1993). Oil from leaves, seeds and bark possess a wide spectrum of antibacterial action against Gram-positive and Gram-negative microorganisms including *M. tuberculosis* and streptomycin resistant strains (Kausik et al., 2002). Neem has been shown to inhibit *Vibrio cholerae, Klebsiella pneumoniae, M. tuberculosis* and *Mycobacterium pyogenes in vitro* (Satyavati et al., 1976). In one study, Neem oil was shown to suppress several species of pathogenic bacteria including *Staphylococcus aureus* and *Salmonella* but had no antibacterial effect against certain strains of the above bacteria and none
against *Citrobacter, E. coli, Enterobacter, Klebsiella pneumoniae, Proteus mirabilis, Proteus morgasi, Pseudomonas aeruginosa, pseudomonas EOI* and *Streptococcus faecalis* (Kausik *et al.*, 2002).

Traditionally Neem leaf decoction and topical applications of Neem oil were used to treat sexually transmitted diseases, for example, gonorrhea, syphilis and vaginal infections. These practices continue to date as an alternative to modern antibiotic treatments. Other studies have indicated activity against *Treponema pallidum* and gonorrhea infections (Bhandari and Mukerji, 1959; Vietmeger, 1992).

A study to determine the kill kinetic of Neem indicated that a stem bark extract killed all bacterial cells of *Staph. aureus* at a concentration of 1mg/ml in 8 h. A higher concentration of 2 mg/ml had the same effect in 6 h. Both *E. coli* and *P. aeruginosa*, which had previously shown an MIC of 8 mg/ml, were not killed by the extracts in 24 hours at 2, 4 and 8 mg/ml (Okemo *et al.*, 2001).

Anecdotal information on antiviral activity of Neem has existed in India for quite some time and smallpox, chicken pox and warts were traditionally treated using Neem leaf (National Research Council, 1992). Rao *et al.* (1969) reported that crude Neem leaf extracts absorbed the smallpox and chicken pox viruses preventing them from entering uninfected cells. Unfortunately, no antiviral effects were seen once the infection was established within the cell thus Neem was effective in prevention, but not as a cure. In United States, aqueous Neem leaf extract was shown to have a low to

Neem is very effective at attacking the common cold viruses soon after the infection begins (Rai and Seth, 1992). It also has interferon-like activity against the viruses that can counter the infection once it sets. Encouraging preliminary results indicate effective action against Chlamydia, vaginal warts, herpes and AIDS (Neem Association 17980, 1998). A laboratory study showed toxicity to cultures of 14 common fungi (Khan and Wassilew, 1987) including: Trichophyton, Epidermophyton, Microsporum, Trichosporon, Geotrichum and Candida species. It is popular in treating ringworm (Tinea species) in children in rural Kenya (Saxena, 2003). Neem seed and leave extracts are also effective against malarial and trypanosomal parasites and kill external parasites for example lice (Saxena, 2003).

1.2.5 Other uses

Apart from being a popular remedy for infectious diseases, Neem extracts are used to treat a wide range of other human pathological and environmental problems. It is also a good source of industrial and domestic raw materials and has been used in birth control (Kausik et al., 2002). Neem extracts have been shown to have immunostimulatory, sedative, antihypertensive, and antiarhythmic properties (Pillai and Santhakumari, 1984b). In India and Pakistan it is a popular blood purifier (Chattopadhyay et al., 1992) and is known for its antiulcer effect (Garg et al., 1991).
Neem leaves extracts have been proved useful in treatment of diabetes (El-Hawany and Kholief, 1990), arthritis (Vandere Nat et al., 1991) and tumors (Vohora, 1986). Neem has a proven ability to prevent pregnancy (Juneja and William, 1993). Some Neem compounds show early promise as the long sought oral birth control for men (Rair et al., 1988). Neem extracts, which are biodegradable, are proving to be one of the most effective natural, non-toxic methods of controlling insects on food and ornamental crops (Saxena, 1993). Being a hardy tree it is ideal for reforestation programs and for rehabilitating degraded, semiarid and arid lands and coastal areas (Saxena, 1993).

Neem wood is hard and relatively heavy and is used for making carts and tools. The wood is used in as fence posts, poles for house construction, furniture, and even icons in some parts of India (Vicmeyer, 1992). Neem grows fast and is a good source of fuels and its charcoal has a high calorific value (Arishtha Amazoia, undated: Saxena, 1993). The bark is used for making ropes while the leaves are fed to goats and camels when other feed is scarce. Flowers provide nectar, which is collected by honeybees and converted into Neem honey (Sontakke and Rath, 1988).

1.2.6 Clinical studies

Clinical studies with dried Neem leaf extract indicated its effectiveness to cure ringworm, eczema and scabies on topical application within 3-4 days in acute stage or a fortnight in chronic case (Kausik et al., 2002). Application of Neem oil on the hair
has been shown to kill head lice. Frequent application of Neem oil, following surgical removal of papilloma below the earlobe of a teenage girl, miraculously prevented the regrowth of the disfiguring tumor, which would have otherwise required repeated surgery (Saxena, 2003). Application of a paste made from Neem leaves and turmeric curcumalonga, in 4:1 proportion to the skin reportedly cured 9 percent of the patients with scabies (Charles and Charles, 1992).

In Kenya, bathing with water boiled with Neem leaves and then applying Neem oil on affected parts cured severe cases of scabies in children (Saxena, 2003). In another trial, ringworm treatment that had failed to respond to treatment with salicylic acid and benzoic acid cleared up in two to three days after using Neem extract on the affected areas (Singh et al., 1979). Neem had also proved to be highly effective in treating chronic disorders like psoriasis, acne, itching, dandruff and warts (Rajaseklbaram et al., 1980; Singh et al., 1979). In Germany a study involving 70 patients with pyorrhea in varying stages showed significant improvement after just 5 to 10 treatments with Neem based toothpaste and mouth wash (Zeppenfeldt, undated).

1.2.7 Toxicology of Neem

Perhaps the most important quality of Neem is that its products appear to have little or no toxicity to warm blooded animals (Kausik et al., 2002). Neem is the main part of diet for birds and bats in Ghana’s Accra Plains (Arishtha Amazoia, undated). Neem extracts show no mutagenicity in the Ames test, a bacterial reverse mutation assay which evaluate a substance’s genotoxicity by determining its ability to induce reverse
mutations at selected loci in several bacterial strains (Ames and Durston, 1973).

Indians have been adding Neem leaves to their grain stores for centuries to control weevils. Numerous studies in the U.S and other countries have shown that the leaf and bark have very low toxicity even when taken orally (Chattopadhyay et al., 1992; Saraf et al., 1993). However, large doses of Neem leaves taken orally have caused some side effects in rats and mice, for example dermal irritation and infertility (Obasiki et al., 1985; Ibrahim et al., 1992). It appears then that Neem should not be taken in large doses for extended periods of time, which is true for almost any medicinal herb (Neem Association 17980, 1998).

1.2.8 Comparative studies

Several studies comparing Neem extracts to other drugs have been done and all prove that the Neem contains potent therapeutic principles. One study found that, oil from leaves, seeds and bark was effective against streptomycin resistant strains of bacteria (Chopra et al., 1952). Bandani (1987) showed that, alcoholic leaf and seed extracts are effective against chloroquin resistant malaria parasites. In another study, 800 units of penicillin or 5 gms streptomycin sulphate were found to be equal to 1gm of nimbidin (Singh and Sastry, 1981) while nimbidol was found to be as effective as salicylic acid against severe skin fungal infections (Narayan, 1965). A Neem extract was also found to be as effective as cortisone against psoriasis (Singh et al, 1979).

In this study chloramphenicol was used as a standard for assessing the antimicrobial potency of Neem leaves extract. Chloramphenicol was preferred due to two of its
merits that it is believed to share with Neem thus making Neem fairly comparable to it. First, it has a broad spectrum of activity against bacteria and secondly, acts as a bacteriostatic agent. Chloramphenicol an antibiotic, was first isolated by David Gottlieb from a mould known as *Streptomyces venezuelae* in 1944, but is today chemically synthesized. Its chemical modification has not been very productive. It is a broad-spectrum antimicrobial agent known to act against chlamydiae, rickettsiae and most conventional Gram-positive and Gram-negative organisms but *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are usually resistant (Lewin et al., 1982). Chloramphenicol exacts its effects against bacteria by affecting protein synthesis and resistance to this drug is mainly due to production of chloramphenicol acetyl transferases. It is poorly soluble in water and very bitter. However, it is orally very efficient and well distributed with excellent penetration into the cerebrospinal fluid. It a highly preferred drug in medical practice due to its low cost. Excretion is through urine as glucuronide conjugates and have a half-life of 2-5 hours in adults and is 50% protein bound. Its merits have however met a major draw back as it is now known to cause irreversible aplastic anemia in a small number of patients (William et al., 1965). Another choice would have been tetracycline which was originally isolated from a

![Chemical structure of chloramphenicol](image-url)
mould called *Streptomyces aureofaciens*. Resistance to tetracycline has however become common and the drug is today only used for some specific indications (Patrick et al., 2002).

<table>
<thead>
<tr>
<th>GROWTH INHIBITIONS ZONES (mm)</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥18</td>
<td>Susceptible</td>
</tr>
<tr>
<td>13-17</td>
<td>Intermediate</td>
</tr>
<tr>
<td>≤12</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

**Table 1**: Interpretation table for chloramphenicol susceptibility results.

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Susceptible</td>
</tr>
<tr>
<td>16</td>
<td>Intermediate</td>
</tr>
<tr>
<td>3</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

**Table 2**: Interpretation table for chloramphenicol MIC results.


et al., 2002). The tables above (Tables 1 and 2) shows how the susceptibility and minimum inhibition concentration (MIC) values for chloramphenicol are interpreted in practice.
1.3 Antimicrobial susceptibility tests

These are *in vitro* tests used to determine appropriate treatment for infectious diseases and entails isolation of the infectious agent and the determination of the susceptibility or resistance of the same to known antimicrobial agents used in therapy. Usually the isolated organism is tested against several drugs and a choice is made of the least toxic, least toxic to normal flora with best pharmacological characteristics and most economical of those to which the organism is susceptible. The methods used in bacteriological susceptibility testing include;

1. Disk diffusion method (qualitative)
2. Broth dilution methods (quantitative)
3. Agar dilution methods (quantitative)
4. Antimicrobial gradient strip method

These methods in addition to being used to test microbial susceptibility, have been adopted extensively in research for drug inventions in which case, products believed to have antimicrobial activity are tested not only to ascertain their activity but also to measure their efficacy and potency. In these cases, the products for example microbial or plants extracts are tested for their susceptibility against pathogens and when proved to have antimicrobial activity are developed into drugs for medical use. The methods are also useful in assessing the level of drugs in patient's blood following treatment. This way it is possible to determine whether
the required effective levels have been attained in serum and also to determine serum half-life of drugs consumed an important factor in pharmacodynamics.

For many of the bacterial susceptibility tests done a standard inoculum of bacteria must be used. This is usually done in liquid media by either microscopic counting using counting chambers, measuring optical density of a broth bacterial culture or by comparing the turbidity of broth culture to a standard that represents a known number of bacteria in suspension.

1.3.1 Disk diffusion method

This method involves the dilution of antimicrobial agent through agar medium on to which surface an organism has been inoculated. The antimicrobial agent would diffuses into the medium a in circle around the point of introduction inhibiting growth of the organism wherever the concentration of the drug is high enough as the drug diffuses along a concentration gradient. Large zones indicate more effective antimicrobial activity or greater diffusibility of drug or both. No zone indicates complete resistance. Older methods involved making tiny wells on the surface of inoculated agar and introducing the antimicrobial agent into the wells. This method was modified by Bondi (1947) by incorporating the antimicrobial agents into filter paper disks, which are then laid with a distance in between onto already inoculated agar medium. These are then incubated for enough time to allow the organism to produce macroscopic growth on the agar. The method was later standardized by Bauer (1966) and can now produce qualitative results that correlate well with
quantitative results obtained by minimum inhibitory concentrations (MIC) tests. It also allows testing of several agents or different concentrations of the same agent against one isolate at the same time.

1.3.2 Broth dilution method

In this method decreasing concentrations of the antimicrobial agent to be tested, usually made in twofold serial dilutions, are placed in tubes of a suitable broth medium that will support the growth of the test microorganism. The most commonly used broth for these tests is Muller-Hinton medium supplemented with magnesium and calcium ions. The antimicrobial agents are prepared into a concentrated solution in a suitable diluent then diluted to appropriate concentrations in broth.

A classical broth dilution susceptibility test (NCCLS M7-A2) involves adding a standard inoculum of microorganism (1.0 x10^6-10^7 colony forming units) to equal amount of each drug concentration in broth (often 1ml) and to a tube without antimicrobial agent (growth control test). This is made such as to make the right drug and organism concentrations in the final tube. An uninoculated tube of medium is included to serve as a negative growth control. After sufficient incubation (usually over night), the tubes are examined for turbidity, indicating growth of the organism. The organism will grow in the control and any other tube that does not contain enough antimicrobial agent to inhibit growth. The lowest concentration of the agent that inhibits growth of the organism, as detected by lack of visual turbidity (match the negative growth control) is designated minimum inhibitory concentration (MIC). If
the MIC of a drug can be readily attained in patient's serum by normal routes of delivery and is not toxic, the organism is said to be susceptible to the agent.

The MIC measures the ability of an antimicrobial agent to inhibit the multiplication of the organism. Thus, the organism in the inolucum may be merely inhibited and will recommence growth if the antimicrobial influence is removed. Such an agent will be regarded as bacteriostatic or inhibitory. For certain serious infections however it is important to determine the ability of the agent to actually kill the organism. This is achieved by a modification of the above broth dilution method where, when the initial microorganism suspension is being inoculated into the tubes of broth, a measured portion is removed from the growth control tube immediately after it is inoculated and this aliquot is plated on solid agar for determination of actual colony forming units (C.F.U) in the inoculum. After MIC has been determined, a known quantity (often 0.1ml) of inoculum from each of the tubes of broth that showed no visible turbidity after 22-24 hrs of incubation is subcultured on solid agar plates.

The small amount of antimicrobial agents that is carried over with this inoculum is easily removed by diffusion into the agar and the effect is negated by spreading the inoculum over a large area. The number of colonies that grow on subculture after over night incubation are counted and compared with to the number of C.F.U/s /ml in the original inoculum. The lowest concentration of the antimicrobial agent that allowed less than 0.1% of the original inoculum to survive is said to be the minimum bactericidal concentration (MBC) or the minimum lethal concentration.
1.3.3 The time-kill method

This is a broth dilution method designed to show the rate and extent of bacterial killing (kill-kinetics) of antimicrobial agents. It has been used to demonstrate the killing synergism in methicillin susceptible *Staph. aureus* (MSSA), (Tuanzon *et al.*, 1978). It is known to give better sensitivity trends to physicians than disk diffusion methods (Westh *et al.*, 1992). Okemo (2001) used this method to demonstrate the kill-kinetics of *Staph. aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. It was also used in Japan to demonstrate the susceptibility of staphylococcus and gram-negative rods to epigallocatechin (Yoshiyuki *et al.*, 2004).

The method involves the incubation of microorganisms in known concentrations of drug and then determining the CFU over a period of time usually up to 24 hrs for the incubated cultures. This helps monitor the rate of multiplication and the late or death of the microorganism on exposure to the drug. If the drug is bactericidal then it will eliminate the organism within sometime of incubation. If the drug is bacteriostatic, then it will suppress the multiplication of the bacteria but not eliminate them, while if the organism are resistant to the drug, then they will multiply at the normal growth rate.

1.4 Statement of the problem

The use of plants and plant products in disease control has persisted despite advances in modern pharmaceutical products and dominance of synthetic drugs the world over. This has been so for Neem, but where as millions of Indians swears to
the efficacy of Neem treatments, the pharmacological effects have seldom been subjected to rigorous trials and control and these claims remain unsubstantiated. There is therefore lack of credibility, awareness and laboratory data to support most of the reported therapeutic claims. This problem is further compounded by genetic and environmental variability that appears to dictate the value of Neem products since the tree is capable of growing in a wide range of environmental conditions. There is also need for continuous development of new antibacterial agents from known potential traditional herbs to keep in check the growing resistance of bacteria to common conventional drugs. This Study was aimed at shedding light into the potential of Kenyan Neem in the treatment of bacterial infections and promotes possible scientific development of antibacterial agents from it.

1.5 Hypotheses

Null hypothesis: Neem tree extract is low potency antimicrobial agent with no potential to kill bacteria even in high concentrations

Alternative hypothesis: Neem tree extract is a highly potent antibacterial agent that rapidly kills bacteria in minimal doses.

1.6 Objectives

1.6.1 General objective

To determine the antibacterial activity of an aqueous Neem leaves extract against bacterial pathogens encountered in medical practice today.
1.6.2 Specific objectives

1. To determine the susceptibility of bacterial pathogens commonly encountered in medical practice today to Neem aqueous leaf extract.

2. To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Neem leaf extracts to bacteria.

3. Determine the Time-kill (time the extract would take to kill the bacteria) of the Neem extract.

1.7 Justification

The global scenario is now changing towards the use of non-toxic plant products with traditional medicinal value especially in poor countries, where conventional drugs are commercially beyond the reach of most people. Development of therapeutic products from Neem will enhance application of centuries old knowledge of Neem through modern approaches of drug development. The products will be more acceptable to the general population when the standard and quality of medicines are verified.

This study was aimed at elucidating scientific evidence to the diverse traditional use of Neem extract in treatment of bacterial infections thereby promoting the potential of developing therapeutic antibacterial agents from it. It will provide preliminary information needed for standardization of Neem products, which largely remain crude and make them more effective and acceptable to the general population. It was also designed to tap on Neem's diverse ability to control bacterial diseases by determining the organisms, which are susceptible to the extract. This is particularly
useful in the present era of Acquired Immune Deficiency Syndrome (AIDS), which due to lowered immunity in the body result to recurrent secondary co-infections. Neem would be cheaply available to the poor who cannot afford frequent conventional treatment for these secondary infections not to forget its proved merit of boosting immune response to the same (Neem Association 17980, 1998).

This study will help to document how useful Neem could be in control of bacterial infections. It will also assist in determining the quantity of raw Neem leaves required to attain the minimum effective dose thus it is a step in evaluation of effective medical use of Neem. An aqueous extract of Neem leaves was used since in traditional practice, aqueous extracts are most common. Chloramphenicol was used as a standard for assessing the antimicrobial potency of the crude Neem leaves extract since Chloramphenicol is known to have a broad spectrum and a bacteriostatic effect against bacteria, properties that Neem is believed to exhibit. The MIC and MBC could later be used to determine the pharmacokinetics (bioavailability and degradation) of the Neem active compounds.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Neem leaves collection

Neem’s blended and dried leaves were obtained from Biop Company, a subsidiary of the International Centre for Insect Physiology and Ecology (ICIPE) in Nairobi. The leaves were harvested and collected from small-scale farmers in coastal Kenya (Malindi) by the company and dried in house at their leaf base in Malindi before transport to the factory for further processing. In the factory, the leaves were blended using a posho mill and packed in plastic bags ready for sale. The leaves are therefore a mixed blend of many trees. Older dark green leaves were harvested, as they are preferred due to their higher concentration of active compounds (my personal communication with the factory manager). The ground leaves were transported to the Biochemistry Department of the University of Nairobi where the extraction and bioassay was carried out.

2.2 Extraction

The ground leaves were dropped on the surface of hot water and the mixture was allowed to stand for thirty minutes and then filtered through Whatman filter paper No 1. to remove the extraneous materials. The filtrate was then freeze dried to obtain a fine powder which was further dried in a vacuum desiccator over anhydrous copper sulphate. About 100 g of dried leaves (ground) yielded 10 g of fine freeze dried powder. The fine powder obtained was packed in dry plastic bags in 10 g amounts and stored at room temperature in a desiccator.
2.3 Bacterial stocks

Clinical isolates and standard organisms were obtained from a routine laboratory in the Pediatrics Department of the University of Nairobi. Several isolates (at least 5 for the common pathogens) including known resistant strains of the species were used to avoid atypical mutants. These were transported in tryptic soy broth: glycerol medium (85:15v/v concentration ratio). In the laboratory they were subcultured in suitable media (Mueller Hinton agar did work for most of them but some organisms are fastidious and require enriched medium, for example, Chocolate blood agar for *Neisseria gonorrhoeae* and *Haemophilus influenzae*, while others are best identified by growth on specific medium, for example, Salmonella Shigella agar for Salmonella and Shigella species) and incubated for 24 hrs at 37°C to ascertain purity. From these subcultures, the identities of the organisms were confirmed by colonial morphology, staining by gram stain and by biochemical tests. The organisms were then subcultured into gelatin medium labeled with code numbers and stored at room temperature. The organisms included: *Staph. aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Kleb. pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexineri*, *Bacillus cereus*, *Bacillus anthracis*, *Bacillus subtilis*, *Enterobacter cloacae*, *Campylobacter jejuni*, *Citrobacter koseri*, *Morganella morganii*, *Shigella boydii*, *Staph. Albus*, *Haemophilus influenzae* and *Streptococcus viridans*. Standard organisms included *Staph. aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *Sal. typhi* ATCC 19430 and *Streptococcus faecalis* ATCC 29212.
2.4 Determination of susceptibility of bacterial isolates

The disk diffusion method (Bernard et al., 1980; Bauer et al., 1996) was used to determine the susceptibility of bacterial isolates on Muller Hinton Agar (MHA). Paper disks were obtained by cutting Whatman filter paper No. 1 with paper punch. The disks were wrapped in aluminum foil and sterilized by autoclaving at 121°C for 15 minutes. One gram of the dried extract was dissolved in 4 ml of 10% Dimethylformamide (DMFM) to make a final concentration of 250 mg/ml. The choice of 10% DMFM as the solvent was made after it was established that the extract best dissolved in that concentration allowing passed of the extract through the 0.45 μm pore microfilter. That concentration of the solvent also did not allow formation of residues when the extract was fully dissolved. A control analysis of the solvent with the bacterial isolates indicated that, the 10% DMFM had no affect on the bacteria. This solution was sterilized by passing it through 0.45 μm pore microfilter. Double dilutions of the extract were then prepared aseptically in normal saline to make concentrations ranging from 1.9 – 250 mg/ml. The 250 mg/ml Neem extract concentration was the highest concentration of the extract that could possibly pass through the filter. The lower concentrations of the extract down to 1.9 mg/ml were developed in an attempt to determine the lower cut-off concentration of the extract that could produce inhibition zones. The sterile disks were then impregnated with the various extract concentrations, which were laid separately onto sterile petridishes covered and dried in the incubator. Impregnation was done by dipping the disks into the solution and tapping on the sides of the petridish to drop excess solution.
The bacteria were grown on agar medium from which 2-3 colonies were picked and aseptically dispersed in sterile normal saline to make a suspension corresponding to Brown's opacity tube No. 5 as recommended by National Committee for Clinical Laboratory Standards (NCCLS). The suspension was further diluted to make a dilution of $5 \times 10^5 - 10^6$ organisms per milliliter after which 0.5 ml of the suspension was aseptically poured on to MHA plate and spread evenly by tilting the plates. The surface was then allowed to dry for 15 minutes after which the various disks were aseptically introduced with a distance of 1.5 cm in between. Controls for each experimental batch included disks impregnated with the diluent, that is, sterile normal saline. The cultures were then incubated at 37°C for 18-24 hrs and observed for growth inhibition zones. The diameter of the inhibition zone was recorded in millimeters and activity recorded as sensitive or resistant. All organisms that produced inhibition zones equal or greater than 7 mm were regarded susceptible.

2.5 Determination of minimum inhibition concentration (MIC)

The MIC was determined by Microtitre Dilution Method using microtitre plates (Chand, et al., 1994). About 250 mg of the powdered Neem were dissolved in 1ml of 10% dimethylformamide to make a stock solution and sterilized by passing it through a 4.5 μm membrane filter. Different extract concentrations of between 1.9 - 250 mg/ml were then made in sterile Muller Hinton broth (MHB) by double dilution. The solutions were dispensed into the wells in ascending order from the highest to the lowest concentration in 90 μl amounts. Sensitive organisms representative of gram-positive and gram-negative were selected for determination of the MIC. Those selected were
Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi. These were grown overnight on agar plates at 37°C and then 2-3 colonies of the growth were suspended in normal saline to make turbidity corresponding to Brown’s opacity tube No. 5. The suspensions were serially diluted to 1:1000 of which 10 µl of each were introduced into all the eight wells for each organism containing the extract and medium to make a final concentration of organisms of 5 x 10⁵ – 10⁶. The plates were incubated at 37°C for 18-24 hours and observed for turbidity. A control series of wells similar to that of the test, to which 10 µl of the diluent (normal saline) was added, was included in each test batch. The lowest extract concentration that showed no sign of turbidity was taken to indicate growth inhibition and so represent the MIC (NCCLS). The MIC was recorded in mg/ml.

2.6 Injectable chloramphenicol

This was purchased as chloramphenicol sodium succinate vials (1gm) a product of Flamingo Pharmaceuticals in Mumbai, India, and dissolved in MHB to make final concentrations of 0.031-8.00 mg/ml. These were dispensed on 90 µl amount in microtitre wells and organisms concurrently added as described in section 2.5 above. The wells were observed for turbidity after incubation at 37°C for 18-24 hrs. The MIC was recorded in mg/ml. This was repeated at least five times and means for each organism calculated to give the MIC for each organism for both Neem and chloramphenicol.

2.7 Determination of minimum bactericidal concentrations (MBC)
The MBCs were determined from MIC preparations. About 10 μl amounts from all wells above MIC values in each experiment were picked by micropipette and spread onto MHA plates. The plates were then incubated at 35°C for 18-25 hrs and observed for any colony growth. The lowest concentration at which no growth was obtained was recoded as the MBC in mg/ml.

2.8 Determination of Neem extracts time-kill values

This was done to determine how long bactericidal concentrations of Neem extract and chloramphenicol would take to kill a whole population of known number of microorganisms on incubation at 35°C.

Neem extract was dissolved in 10% DMF to make a concentration of 250 mg/ml and filtered through a 0.45 μm microfilter. Double dilutions of this solution were made to prepare MBC concentrations for each organism. The solutions were aseptically dispensed in 1 ml amounts into sterile test tubes (pre-sterilized while mouth covered with aluminium foil by autoclaving at 121°C for 15 minutes) in to which 10 μl amounts of the organism’s suspensions were added and the tubes incubated at 35°C. About 10 μl of the initial organism suspensions were serial diluted at 1/10, 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000 and again 10 μL amounts from these dilutions subcultured on MHA plates to determine colony forming units. From the number of colonies that were obtained from these viable counts, the number of microorganisms in the initial 10 μl suspension was calculated. Respectively, 10 μl amounts from the incubated tubes were also obtained after 4 hrs, similarly serially diluted and 10 μl amounts from the dilutions
subcultured as for the initial suspensions. Viable organisms' counts were thus obtained after 4 hrs. This was repeated at 8 hrs and then at 24 hrs. The experiment was carried out with *Staph. aureus*, *Sal. typhi*, *Strep. pyogenes*, *E. coli* and *Ps. aeruginosa*. A preparation for chloramphenicol MBC values for was included for each organism. Six control tubes were also included in each batch of tests; one containing MHB only while each of the rest 5 tubes contained MHB to which the different microorganisms were added as for the test to obtain the normal growth curve. The experiment was repeated 3 times and mean time kill values obtained for each organism.

2.9 Data analysis

All isolates with inhibition zones equal or greater than 7 mm were regarded susceptible to the extract. Susceptibility means presence of targets of antimicrobial activity within a given genera or species of bacteria which enable the antimicrobial agent to adversely affect the bacteria (Bauer, 1996). A regression analysis on the means of inhibition zones was done to demonstrate the effect of increasing Neem extract concentration on inhibition zones for each organism. For the MIC and MBC values, a regression analysis was used to analyze mean MIC and MBC for each of the selected organisms. The univariate ANOVA was used to determine the relationship between MICs and MBCs of the extract and chloramphenicol for the selected organisms. The means of colony forming units determined by time-kill method over time were plotted on graphs for each of the selected organism to demonstrate the effect of the different Neem extract and chloramphenicol concentrations on growth of the organism over time.
CHAPTER THREE

RESULTS

3.1 Susceptibility of bacterial isolates to Neem extract

The total number of isolates inhibited by the Neem extracts are shown in Table 3. Out of 107 isolates tested from different specimens 60.7% (± 10.1) were susceptible at varying degrees while 39.3% were resistant. *Sal. typhi*, *Staph. aureus*, *Ps. aeruginosa*, *Strep. pyogenes*, *Strep. faecalis*, showed greatest sensitivity with all *Strep. pyogenes*, *Strep. faecalis* species tested being susceptible while only one species among those tested for *Sal. typhi* and *Ps. aeruginosa* were resistant. *E. coli* also showed considerable sensitivity with 69.6% of the tested isolates showing sensitivity while 33.3% of the *Staph. aureus* isolates tested were resistant. Only two out of the six *Kleb. pneumoniae* isolates tested were sensitive while none of *H. influenzae* and *N. gonorrhoeae* were sensitive. The extract also suppressed growth of *B. anthracis* (Table 6). The percentage of susceptible isolates per species is shown in Figure 5. *Streptococcus pyogenes* (100%) is the most susceptible organism followed by *Sal. typhi* (83.3%), *Ps. aeruginosa* (80%) and *Staph. aureus* (75%) respectively.

A methicillin resistant staphylococcus strain was not suppressed while an *Escherichia coli* strain which had been proved resistant to gentamicin, ofloxacin, nitrofurantoin, cefactor, nalidixic acid, augmentin, cefuroxin, minocycline, tetracycline and cephalaxin was suppressed by the extract.
Table 3: Susceptibility (%) of human pathogens to Neem extract.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>SENSITIVE</th>
<th>RESISTANT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhi</em></td>
<td>83.3</td>
<td>16.7</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>69.6</td>
<td>30.4</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>80.0</td>
<td>20.0</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>33.3</td>
<td>66.7</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>shigella sonnei</em></td>
<td>42.9</td>
<td>57.1</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em></td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td><em>Shigella flexineriae</em></td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>33.3</td>
<td>66.7</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Staphylococcus albus</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><em>Citrobacter koseri</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Shegella bodyii</em></td>
<td>66.7</td>
<td>33.3</td>
</tr>
<tr>
<td><em>Streptococcus viridans</em></td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>60.7</strong></td>
<td><strong>39.3</strong></td>
</tr>
</tbody>
</table>
Figure 5: Susceptibility (%) of selected bacterial species to Neem extract.

Percentage sensitivity in Figure 5 indicates the percentage of isolates tested that were susceptible to the extract per species.

Table 4 shows the mean inhibition zones of the Neem extract against the different bacteria obtained for each organism. *Sal. typhi* recorded the biggest mean inhibition zones (11.5 mm) followed by *Ps. earuginosa* (10.8 mm) while *N. gonorrhoeae, H.*
influenza and Brucella abortus were resistant therefore recorded zero inhibition zones. The other organisms recorded inhibition zones ranging from 6-8 mm.

Table 4: The mean growth inhibition zones (mm) of Neem extract against different bacteria.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>ACTUAL RANGE</th>
<th>MEAN RANGE ±0.23 - ±0.45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella typhi</td>
<td>7.0-12.0</td>
<td>8.0-11.5</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>7.0-13.0</td>
<td>6.0-10.0</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>7.0-14.0</td>
<td>7.9-8.9</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>7.0-17.0</td>
<td>8.0-10.8</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>7.0-11.0</td>
<td>7.5-9.0</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>7.0-11.0</td>
<td>7.5-9.0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>7.0-14.0</td>
<td>8.0-10.0</td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>7.0-8.0</td>
<td>6.7-7.7</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>7.0-9.0</td>
<td>7.0-9.0</td>
</tr>
<tr>
<td>Shigella flexneriae</td>
<td>7.0-9.0</td>
<td>6.5-8.0</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>7.0-11.0</td>
<td>8.0-9.0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>7.0-12.0</td>
<td>6.5-10.5</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>7.0-10.0</td>
<td>7.7-8.7</td>
</tr>
<tr>
<td>Haemophilus influenzae*</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Bacillus subtilis*</td>
<td>6.0-7.0</td>
<td>6.0-7.0</td>
</tr>
<tr>
<td>Enterobacter cloacae*</td>
<td>7.0-8.0</td>
<td>7.0-8.0</td>
</tr>
<tr>
<td>Bacillus anthracis*</td>
<td>7.0-11</td>
<td>7.0-11</td>
</tr>
<tr>
<td>Staphylococcus albus*</td>
<td>6.0-12</td>
<td>6.0-12</td>
</tr>
<tr>
<td>Campylobacter jejuni*</td>
<td>6.0-7.0</td>
<td>6.0-7.0</td>
</tr>
<tr>
<td>Citrobacter koseri*</td>
<td>7.0-10.0</td>
<td>7.0-10.0</td>
</tr>
<tr>
<td>Morganella morganii*</td>
<td>7.0-10.0</td>
<td>7.0-10.0</td>
</tr>
<tr>
<td>Shigella bodyii</td>
<td>6.0-10.0</td>
<td>6.0-10.0</td>
</tr>
<tr>
<td>Brucella abortus*</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

- Single isolates used
Table 5: The mean growth inhibition zones (mm) of Neem extract against Standard organisms.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>INHIBITION ZONE RANGE (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staph. aureus</em> ATCC 25923</td>
<td>8.0-12.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC</td>
<td>6.0-13.0</td>
</tr>
<tr>
<td><em>Escarichia coli</em> ATCC 25922</td>
<td>7.0-9.0</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 700603</td>
<td>6.0-12.0</td>
</tr>
<tr>
<td><em>Salmonella typhi</em> ATCC 19430</td>
<td>9.0-11.0</td>
</tr>
</tbody>
</table>

The actual range on Table 4 indicates the actual range of inhibition zones determined for the organisms across all the different extract concentrations used. Mean range indicates the range obtained after calculation of mean of inhibition zones for each extract concentration used across the different isolates of the species investigated. For example, the inhibition zones determined for all *Sal. typhi* isolates with the different extract concentrations ranged between 7-12 mm while after calculating a mean for every concentration across all the isolates a range of 6-11.5 mm (± 0.23 -0.45) at 5% confidence interval was obtained.

Plates 1, 2 and 3 demonstrate the inhibition zones obtained for single isolates of *Strep. pyogenes*, *Sal. typhi* and *Sh. dysenteriae* species respectively. Plate 1 indicates that a wide inhibition zone was obtained at the highest concentration of 250 mg/ml while plates 2 and 3 shows that the widest inhibition zones were not necessarily obtained with the highest concentrations. All the plates show that, the bacteria were sensitive to the Neem extract. The inhibition zones for *Strep. pyogenes* on Plate 1 ranged between 9-11 mm, those of *Sal. typhi* ( Plate 2) ranged between 10-12 mm (Plate 2), while those of *Sh. flexineri* (Plate 3) ranged between 7-8 mm.
those of *Sal. typhi* (Plate 2) ranged between 10-12 mm (Plate 2), while those of *Sh. flexineri* (Plate 3) ranged between 7-8 mm.

**Plate 1**: Neem growth inhibition zones against *Streptococcus pyogenes*

Note: Numbers 1–8 in Plate 1 indicate the concentrations of extract in double dilutions from the highest (250 mg/ml) to the lowest (1.9 mg/ml).
Plate 2: Neem growth inhibition zones against *Salmonella typhi*.

Note: Numbers 1–8 in Plate 2 indicate the concentrations of extract in double dilutions from the highest (250 mg/ml) to the lowest (1.9 mg/ml).
Plate 3: Neem growth inhibition zones against *Shigella flexineri*

Note: Numbers 1–8 in Plate 3 indicate the concentrations of extract in double dilutions from the highest (250 mg/ml) to the lowest (1.9 mg/ml).
The susceptibility profiles for individual bacterial pathogens to Neem extract in respect to their gram reaction are shown on Table 6. From the table, most gram positive and gram-negative organisms are sensitive to the extract with sensitive percentages well.

Table 6: Susceptibility profiles of human bacterial pathogens with different gram reaction to Neem extract

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>Gram Positive</th>
<th>Gram Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SENSITIVE ISOLATES</td>
<td>RESISTANT ISOLATES</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shigella flexineriae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neisseriae gonorrhoeae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus albus</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrobacter koseri</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morganella morganii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shigella boydii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus viridans</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>PERCENTAGE</td>
<td>69.7</td>
<td>30.3</td>
</tr>
</tbody>
</table>
above 50%. However a higher percentage of sensitive organisms was obtained with Gram-positive than Gram-negative organism.

The spectrum of activity of Neem extract to bacterial pathogens is shown in figure 6. The Neem extract suppressed more than 60% of all the isolates, including 68% of Gram-positive and 58% of Gram-negative.

![Bar chart showing the percentage of sensitive and resistant bacteria](image)

**Figure 6:** Spectrum of activity of Neem extract against bacteria in percentage

Table 7 shows the mean inhibition zones for different organisms at different extract concentrations. The means of inhibition zones indicated for each concentration indicate the means of replicated results for the different isolates of the same species. The size of
in concentration after an optimum level with a range from 6-11.5 mm. In most cases, inhibition zones are narrowest with the highest concentration, that is, 250 mg/ml (6-9.5 mm) and lowest concentration, that is, 1.9 mg/ml (6-10.0 mm). In others there is barely any relationship between the size of inhibition zones and increase in extract concentration. This is indicated by the low values of adjusted $r^2$ obtained for Staph. aureus, Strep. faecalis, Sh. dysenteriae and Sh. flexineri. Note that increasing the extract concentrations does not change the response of H. influenza and N. gonorrhoeae indicating that they are very resistant even at very high extract concentrations. Sal. typhi and Ps. aeruginosa recorded highest inhibition zones.

### Table 7: The mean inhibition zones (mm) of different organisms at various concentrations of Neem extract

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NEEM EXTRACT CONCENTRATION MG/ML</th>
<th>250</th>
<th>125</th>
<th>62.5</th>
<th>31.3</th>
<th>15.6</th>
<th>7.8</th>
<th>3.9</th>
<th>1.9</th>
<th>Ad r sq*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella typhi</td>
<td>8</td>
<td>10.5</td>
<td>11.5</td>
<td>10.0</td>
<td>10.0</td>
<td>11.2</td>
<td>10.2</td>
<td>10.0</td>
<td>10.0</td>
<td>66.2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>7</td>
<td>7.5</td>
<td>8.0</td>
<td>8.3</td>
<td>10.0</td>
<td>9.0</td>
<td>6.5</td>
<td>6.0</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>7.92</td>
<td>8.6</td>
<td>8.7</td>
<td>8.9</td>
<td>8.6</td>
<td>8.5</td>
<td>8.1</td>
<td>8.3</td>
<td></td>
<td>68.3</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>8.5</td>
<td>10.5</td>
<td>10.8</td>
<td>10.3</td>
<td>10.0</td>
<td>9.0</td>
<td>8.0</td>
<td>9.0</td>
<td>9.0</td>
<td>75.8</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>7.5</td>
<td>8.5</td>
<td>8.5</td>
<td>8.7</td>
<td>8.8</td>
<td>8.8</td>
<td>9.0</td>
<td>8.3</td>
<td>8.3</td>
<td>99.7</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>8.5</td>
<td>10.5</td>
<td>8.0</td>
<td>9.0</td>
<td>10.5</td>
<td>9.5</td>
<td>10.5</td>
<td>9.0</td>
<td>9.0</td>
<td>66.0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>9.5</td>
<td>10.0</td>
<td>9.5</td>
<td>9.8</td>
<td>8.0</td>
<td>8.5</td>
<td>9.0</td>
<td>9.5</td>
<td>9.5</td>
<td>12.2</td>
</tr>
<tr>
<td>shigella sonnei</td>
<td>7</td>
<td>6.7</td>
<td>6.7</td>
<td>7.0</td>
<td>6.7</td>
<td>7.3</td>
<td>7.3</td>
<td>7.7</td>
<td>7.7</td>
<td>74.1</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>6.0</td>
<td>6.0</td>
<td>7.0</td>
<td>8.0</td>
<td>6.0</td>
<td>9.0</td>
<td>7.0</td>
<td>7.0</td>
<td></td>
<td>16.1</td>
</tr>
<tr>
<td>Shigella flexneriae</td>
<td>7.5</td>
<td>7.5</td>
<td>7.0</td>
<td>6.5</td>
<td>6.5</td>
<td>7.0</td>
<td>7.0</td>
<td>8.0</td>
<td></td>
<td>14.7</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>9</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>99.9</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>6.5</td>
<td>6.5</td>
<td>7.5</td>
<td>7.5</td>
<td>9.5</td>
<td>9.5</td>
<td>10.5</td>
<td>7.5</td>
<td></td>
<td>45.5</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Adjusted $r^2$ squared
The effect of increasing Neem extract concentrations on the size of inhibition zones is shown in Figures 7, 8 and 9 for *Ps. aeruginosa*, *Sal. typhii* and *E. coli* respectively.

**Pseudomonas aeruginosa**

![Graph showing the effect of increasing Neem extract concentration on inhibition zones against Pseudomonas aeruginosa](image)

**Figure 7** Effect of increasing Neem extract concentration on inhibition zones against *Pseudomonas aeruginosa*

The optimum activity was achieved at average concentrations (90-100 mg/ml) while less activity was obtained at the highest and lowest concentrations. It was speculated that this finding could be the result of Neem’s complex bioactive compounds forming complexes with the agar medium which hampered the diffusion of the compounds and especially worse with high concentration of the extract.
Figure 8 Effect of increasing Neem extract concentration on inhibition zones against *Salmonella typhi* with highly concentrated Neem extracts.
3.2 Minimum inhibition concentrations

Table 8 shows the univariate analysis between MIC values obtained for different organisms by Neem extract and chloramphenicol. The MICs for Neem extract and chloramphenicol differed markedly with those of Neem extract being higher than those of chloramphenicol (p< 0.05). The highest Neem extract MIC was obtained with *Strep. pyogenes* (4.26 mg/ml), while the lowest was obtained with *Staph. aureus* (1.5 mg/ml).
For chloramphenicol, the highest MIC was 0.125 mg/ml obtained for *Staph. aureus* while the lowest was 0.163 mg/ml obtained with *Strep. pyogenes*, *S. typhi* and *E. coli*.

**Table 8:** Univariate analysis of variance on mean MIC values for Neem extract and Chloramphenicol on selected organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug</th>
<th>N</th>
<th>Mean MIC</th>
<th>p-value</th>
<th>Ad $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhi</em></td>
<td>Neem</td>
<td>7</td>
<td>4.000</td>
<td>0.000</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>5</td>
<td>0.063</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>Neem</td>
<td>5</td>
<td>1.54</td>
<td>0.000</td>
<td>76.6</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>8</td>
<td>0.125</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Neem</td>
<td>5</td>
<td>6.82</td>
<td>0.011</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>10</td>
<td>0.063</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Neem</td>
<td>5</td>
<td>2.25</td>
<td>0.022</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>8</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Strep. pyogenes</em></td>
<td>Neem</td>
<td>5</td>
<td>4.26</td>
<td>0.008</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>8</td>
<td>0.063</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N- number of trials
Ad $r^2$-adjusted r squared

Table 9 shows a pair-wise comparison of the MIC between both Neem extract and chloramphenicol. The mean differences for the selected organisms range from 0.17 - 11.7 mg/ml at 5% confidence interval. The highest mean difference was recorded with *E. coli* while the lowest was recorded with *Ps. aeruginosa*. 


Table 9: Pairwise comparison of mean Minimum Inhibition concentrations for Neem and chloramphenicol on selected organisms.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NEEM MIC mg/ml</th>
<th>CHLORAMPHENICOL MIC mg/ml</th>
<th>Mean difference mg/ml</th>
<th>SE mg/ml</th>
<th>Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella typhi</td>
<td>4.68</td>
<td>.0625</td>
<td>4.62</td>
<td>0.78</td>
<td>2.82-6.41</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>1.54</td>
<td>0.125</td>
<td>1.42</td>
<td>0.22</td>
<td>0.928-1.71</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6.82</td>
<td>.0625</td>
<td>6.58</td>
<td>2.27</td>
<td>1.86-11.66</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>2.25</td>
<td>1.200</td>
<td>0.95</td>
<td>0.36</td>
<td>0.17-1.74</td>
</tr>
<tr>
<td>Strep. pyogenes</td>
<td>4.26</td>
<td>.0625</td>
<td>4.3</td>
<td>1.33</td>
<td>1.37-7.23</td>
</tr>
</tbody>
</table>

3.3 Minimum bactericidal concentrations

Table 10 shows a comparison of the MBC values for Neem and chloramphenicol.

Table 10: Univariate analysis of variance on mean MBC values for Neem extract and chloramphenicol on selected organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug</th>
<th>N</th>
<th>Mean MBC</th>
<th>p-value</th>
<th>Ad $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal. typhi</td>
<td>Neem</td>
<td>7</td>
<td>11.7</td>
<td>0.000</td>
<td>73.8</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>5</td>
<td>0.143</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>Neem</td>
<td>5</td>
<td>1.27</td>
<td>0.002</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>6</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>Neem</td>
<td>5</td>
<td>7.8</td>
<td>0.020</td>
<td>41.0</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>6</td>
<td>0.113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Neem</td>
<td>5</td>
<td>2.4</td>
<td>0.027</td>
<td>37.4</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>6</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strep. pyogenes</td>
<td>Neem</td>
<td>5</td>
<td>6.83</td>
<td>0.000</td>
<td>81.7</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>5</td>
<td>0.106</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N- number of trials

Ad $r^2$-adjusted r squared
against different organisms. Lowest MBC was observed against *S. aureus* (1.27 mg/ml) while the highest was in *Sal. typhi* (11.7 gm/ml). In comparison with chloramphenicol, the lowest MBC was observed against Strep. *pyogenes* (0.106 mg/ml) and the highest (4.8mg/ml) in *Ps. aeruginosa*. Neem extract MBC were higher than those of chloramphenicol except in the case of *Pseudomonas aeruginosa* which recorded an MBC twice that of the Neem extract (4.8 mg/ml). The p-values ranged from 0.00 - 0.027.

Table 11 shows a pair-wise comparison of the mean Neem extract and chloramphenicol MBC values. The mean differences in MBC between Neem extract and chloramphenicol ranged between 0.97 –11.56 mg/ml. *S. typhi* had the highest mean difference while *Ps. aeruginosa* had the lowest.

Table 11: Pairwise comparison of mean Minimum Bactericidal Concentrations for Neem extract and chloramphenicol on selected organisms.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>NEEM MBC mg/ml</th>
<th>CHLORAMPHENICOL MBC mg/ml</th>
<th>Mean difference mg/ml</th>
<th>Se mg/ml</th>
<th>Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhi</em></td>
<td>11.68</td>
<td>.1428</td>
<td>11.56</td>
<td>2.04</td>
<td>7.01-16.12</td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>1.90</td>
<td>0.300</td>
<td>0.97</td>
<td>0.23</td>
<td>0.46-1.48</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>7.80</td>
<td>0.113</td>
<td>7.69</td>
<td>2.73</td>
<td>1.52-13.86</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>2.4.</td>
<td>4.800</td>
<td>2.39</td>
<td>0.91</td>
<td>-4.44—0.34</td>
</tr>
<tr>
<td><em>Strep. pyogenes</em></td>
<td>6.83</td>
<td>0.106</td>
<td>6.13</td>
<td>0.956</td>
<td>3.93-8.34</td>
</tr>
</tbody>
</table>

3.4 Time-kill

The growth response of *Sal. typhi* at different concentrations of Neem and chloramphenicol is shown on Figure 10. A positive growth response was realized on
incubating *Sal. typhi* in a Neem extract concentration corresponding to the MBC (11.7 mg/ml) and double MBC with the population increasing from $10^6$/ml to about $3.5 \times 10^{10}$/ml in 24 hrs. There was no significant difference between activity of the higher

![Graph showing growth response of *Salmonella typhi*](image)

**Figure 10:** Growth response of *Salmonella typhi* incubated in medium containing different concentrations of chloramphenicol and Neem extract

concentration and the lower concentration. Similarly incubation of the organism with chloramphenicol concentration equivalent to MBC value (0.143 mg/ml) did not inactivate the bacteria and the population increased from $10^6$/ml to $10^9$/ml in 24 hrs. Comparison with a growth control experiment indicated that, growth had been suppressed as the population increased to over $10^{18}$ on incubation of the same population in drug free
medium for 24 hrs. At Neem extract concentration of 62.5 g/ml the population however
was suppressed further to a final count of $2.1 \times 10^7$/ml in 24 hrs. Thus increasing Neem
extract concentration did further suppress the bacterial multiplication but did not
eliminate them altogether.

Figure 11: Growth response of a low inoculum of *Sal. typhi* incubated in
high drug and Neem extract concentrations.

Decreasing inoculum population to below $10^3$/ml and raising the extract concentration
to 125 mg/ml did not control the growth either as the population increased to $10^4$/ml as
shown in Figure 11. This however showed a considerable suppressive activity when
compared with a control sample, which recorded a population increase of up to $10^{12}$/ml
over the same 24 hrs.

An attempt to improve the biocidal effects by increasing the concentration of
chloramphenicol to 1 mg/ml recorded a population decrease from $10^6$/ml to $10^4$/ml in 24
hrs while raising the treatment concentration to 2 mg/ml and reducing the inoculum
population to below $10^3$/ml resulted in complete inhibition of the organism as the population remained below $10^2$/ml. A growth control test run concurrently showed a population rise to $10^{12}$/ml over the same time (Figure 11).

![Graph showing growth response of *Staph. aureus*](image)

**Figure 12:** Growth response of *Staph. aureus* incubated in medium containing different concentrations of chloramphenical and Neem extract

Treating *Staph. aureus* with an MBC (1.27 mg/ml) and two times MBC Neem extract did not kill the bacteria over 24 hrs period. The population increased from $10^6$/ml to over $3.5 \times 10^{10}$ and $3.0 \times 10^{10}$/ml respectively. There was no significant difference in growth response to the two concentrations (Figure 12). An MBC concentration of chloramphenicol (0.30 mg/ml) too did not kill the organism but rather the population increased to over $2.3 \times 10^9$/ml in 24 hrs, as did 1 mg/ml strength.
Figure 13: Growth response of a low inoculum of *Staph. aureus* incubated in high drug and Neem extract concentrations.

A comparison with a growth control experiment indicated that growth had been suppressed as the population increased to over $10^{20}$ when the same number of organism was incubated for 24 hrs in drug free medium. In a Neem extract of concentration 62.5 mg/ml, the population increased to $9.0 \times 10^8$/ml while lowering the inoculum population to less than $10^3$/ml and increasing extract concentration to 125 mg/ml maintained the population below $10^3$/ml as shown in Figure 13. A control experiment run concurrently indicated an increased population to over $10^{12}$/ml. A parallel test for chloramphenicol at 2 mg/ml suppressed the organism to below $10^3$/ml.
Figure 14: Growth response of *E. coli* incubated in medium containing different concentrations of chloramphenicol and Neem extract

In case of *E. coli*, Neem extracts concentration equivalent to the MBC (7.8 mg/ml) for *Escherichia coli* and two times MBC, the population increased from $10^6$/ml to over $3.5 \times 10^{10}$/ml with no significant difference between responses to the two concentrations (Figure 14). Similarly, the population increased to $3.0 \times 10^{10}$/ml in 24 hrs in a chloramphenicol concentration equivalent to the MBC (0.113 mg/ml). In a 62.5 mg/ml Neem extract the population increased to $1.2 \times 10^9$/ml. In a 1 mg/ml chloramphenicol concentration the organism multiplied to $3.0 \times 10^9$ml in 24 hrs. A comparison with a control experiment indicated that growth had been suppressed as the population increased.
Figure 15: Growth response of a low inoculum of *E. coli* incubated in high drug and Neem extract concentrations.

to over $10^{18}$. Raising the extract concentration to 125 mg/ml and decreasing the inoculum population to below $10^3$/ml resulted to population being maintained below $10^3$/ml while in a 2 mg/ml chloramphenicol concentration at a lowered inoculum population, the population was kept below $10^2$/ml. A concurrent control experiment indicated a final count of over $10^{12}$/ml (Figure 15).

Neem extracts concentration equivalent to MBC (4.8 mg/ml) and two times MBC allowed *Ps. aeruginosa* increased from $10^6$/ml to about $3.5 \times 10^{10}$ and $3.0 \times 10^{10}$/ml
Figure 16: Growth response of *P. aeruginosa* incubated in medium containing different concentrations of chloramphenicol and Neem extract respectively in 24 hrs while in chloramphenicol at an MBC (2.4 mg/ml) concentration, the count rose to $3.5 \times 10^{11}$/ml (Figure 16). Pseudomonas was not eliminated by a Neem extract concentration of 62.5 mg/ml and a chloramphenicol concentration of 1 mg/ml with the population increasing to about $3.0 \times 10^{10}$/ml. A comparison with a growth control experiment (drug free medium) indicated that growth had been suppressed as the population increased to over $10^{20}$ in 24 hours.
Figure 17: Growth response of a low inoculum of *Ps. aeruginosa* incubated in high drug and Neem extract concentrations.

Raising the extract and chloramphenicol concentration to 125 mg/ml and 2 mg/ml respectively, and decreasing the inoculum population to below $10^3$/ml resulted in the extract maintaining the count below $10^3$/ml while in the chloramphenicol, the population rose minimally to $10^4$/ml. A control test indicated a count of $10^{12}$ in 24 hrs. From Figures 16 and 17, note that *Ps. aeruginosa* was more susceptible to Neem extract than to chloramphenicol.

Incubating *Streptococcus pyogenes* in a Neem extract MBC concentration of 6.8 mg/ml and a two times MBC concentration of 13.6 mg/ml for 24 hrs indicated that, the
organism resisted the extract with the population rising from $10^6$/ml to $2.6 \times 10^{10}$/ml and $2.0 \times 10^{10}$/ml respectively. Similarly *Streptococcus pyogenes* resisted a chloramphenicol MBC concentration of 0.106 mg/ml and multiplied to a population of $1.0 \times 10^8$/ml in 24 hrs. A growth control experiment run concurrently indicated a population of $1.0 \times 10^{16}$/ml in 24 hrs. Decreasing the organism population to below $10^3$/ml and raising the extract concentration to 0.125 mg/ml maintained the population at about $2.0 \times 10^6$/ml. On increasing the concentration of chloramphenicol to 2 mg/ml and lowering the population of organism to below $10^3$ the population was lowered to about $10^1$. A control test run concurrently indicated a population of approximately $1.0 \times 10^{12}$.
CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

The global perspective in treatment and control of diseases is slowly changing from the use of conventional drug to herbal applications due to preference of organic products but also due to their easier accesssibility and affordability. This is particularly so in the Sub-Saharan Africa where the World Health Organization (WHO) estimates that, 85% of the population relies on herbs for their primary health care needs (Chooto, 2004). Despite the prevalence of herbal extracts in informal therapy and the many years the extracts have been in use, the use of herbs remains suspicious and most people use herbs as a last resort. There is lack of comprehensive laboratory data to support claims and little information on their toxicity and thus no credibility on their use (Arishtha Amazoia, undated).

This study was carried out to evaluate Neem tree leaves for their antibacterial activity against common human bacterial pathogens. This was accomplished by determining the susceptibility of the organism to the extract and also the potency and compared with chloramphenicol, a conventional antibiotic. Chloramphenicol was used in the study as it is a known antibacterial agent with a broad spectrum of activity and also due to its bacteriostatic mode of action (William et al., 1965), merits the Neem extracts are believed to exhibit (Saxena, 2003).
In this study, extract from Neem leaves suppressed 60.7% of the isolates tested. Also of the 24 species of bacteria tested for susceptibility, the extract suppressed about 79% of them. The extract showed susceptibility against both Gram-positive and Gram-negative organisms. The Neem extract therefore exhibited a broad spectrum of activity against bacteria, which probably explains its wide popularity in treatment of infections traditionally (Chaturvedi, 1998).

While all Strep. pyogenes and Strep. faecalis isolates were sensitive, some isolates of the other organisms were resistant to the extract. For example, Sal. typhi had one of its isolate resistant while one out of five isolates of Sh. dysenteriae tested were resistant. This explains why in some studies where single isolates were used in the past, contradicting results were obtained. For example, Patel and Travedi (1962) reported resistance of Citrobacter, E. coli, Enterobacter, K. pneumoniae, Pseudomonas EOI and S. faecalis; Satyavati et al. (1976) reported that Neem inhibited K. pneumoniae while this study indicate that Neem suppressed growth of C. koseri, Enter. cloacae. Almas et al. (1999) claimed that Strep. faecalis was suppressed by Neem extract. The results confirm necessity to perform sensitivity test on all isolates irrespective of history of response to particular drugs, a practice jealously guarded by microbiologists. It enables effective treatment and surveillance of resistance development to drugs currently under use.

Neem crude extract contains numerous chemical compounds, at least 135 of which have been proved to exhibit bioactivity (Koul et al., 1990; Kraus et al., 1995). The compounds believed to be responsible for antibacterial activity is nimbidin (Neem
Association 17980, 1998; Rojanapo et al., 1985) whose content in the extract has not been determined. In this study 100 gm of dried leaves yielded approximately 10 gm of fine freeze-dried extract. From this, the MIC for selected organism ranged from 1.5-6.0 gm/ml and MBC from 2-13 mg/ml. On the other hand, the MIC for the same organisms to chloramphenicol ranged from 0.63-1.2 mg/ml and the MBC from 0.106-4.8 mg/ml.

The significant difference in the mean MIC values for chloramphenicol and Neem extract implies that the extract and chloramphenicol have significantly different potency. The results showed that chloramphenicol was more potent against bacterial pathogens than the Neem extract. However considering that chloramphenicol is a pure compound and Neem extract is crude product, then it is prudent to conclude that Neem extract has considerable effect against bacteria.

Also based on estimated marginal means, pairwise analysis of the mean MIC value for both chloramphenicol and Neem extract indicated that the mean differences are significant at 95% confidence interval. Compared per organism, the neem extract was about 70 times less potent against Salmonella typhi, about 10 times less potent against Staphylococcus aureus and 2 times less potent against Pseudomonas aeruginosa than chloramphenicol. This indicates that in pure form, Neem extract may actually be more potent against some species of bacteria than chloramphenicol.

Similarly, the mean MBC values for chloramphenicol and Neem extract against the microorganisms were significantly different with those of Neem extract being higher than those of chloramphenicol except the MBC for Ps. aeruginosa. The higher MBC value
for chloramphenicol over Neem against *Ps. aeruginosa* implies that the organism was more susceptible to Neem extract than chloramphenicol. Resistance of *Ps. aeruginosa* to chloramphenicol has been reported in other studies (Lewin and Durston, 1993)

Pairwise analysis of the mean MBC value for both chloramphenicol and Neem extract indicated that the mean differences were significant at 95% confidence interval. Neem extract contain compounds that are bacteriostatic like chloramphenicol. Chloramphenicol therefore provides a good agent for assessing the kill power of the Neem extract. Both could not eliminate bacteria even at two times the MBC values as determined by the microtitre method. However their potency was increased by increasing the concentrations with fewer number of colony forming units (CFUs) being obtained with increasing concentrations. Similarity in activity suggests that, the mode of action of the extract may be closely related to that of chloramphenicol. Bacteriostatic agents have low potency but increasing their concentrations can increase their potency. Low concentrations of bactericidal agents are known to be bacteriostatic (Bernard *et al.*, 1980), and the agents act mainly by inhibiting cell wall synthesis, inhibiting nucleic acid or protein synthesis or destroying the cell wall (Bernard *et al.*, 1980).

Bacteriostatic agents are more preferred in treatment of infections other than those requiring emergency attention because they allow the body to develop acquired immunity against the pathogens by delaying the elimination but suppressing multiplication of the same thereby giving the body a chance to fight the agents through the immune mechanism (Lawrence *et al.*, 1997)
Neem extract have been proven to boost immunity elsewhere and have been used successfully to treat acquired immunodeficiency syndrome (AIDS) by enhancing cell-mediated and lymphocytic immune responses (Sen *et al.*, 1993; Upadhyay *et al.*, 1998). The traditional success story of Neem extracts in treatment of wounds probably lies in these two merits where it would be expected to suppress multiplication of the pathogens and raise the magnitude of an immune response against the same.

The crude Neem extract suppressed most of the common bacteria known to cause nosocomial infections, such as, *Staph. aureus*, *E. coli* and especially the notorious *Ps. aeruginosa*. These are also the most common pathogens isolated from wounds in outpatients because they are naturally occurring in the environment as contaminants in water (Collee *et al.*, 1996). Neem extracts especially seed and bark extracts are popular remedies for typhoid fever caused by *Sal. typhi* (Zaxena, 2003). It is interesting to note that in this study, *Sal. typhi* obtained the highest mean inhibition zone (11.5 mm) but the highest MIC of 4.8 mg/ml. This contradiction may be explained by mobility of the organism in the liquid medium, which is highly restricted in the solid medium thus restricting multiplication. *Staph. aureus* and *Ps. aeruginosa* had the lowest Neem extract MIC values (1.5 and 1 mg/ml respectively) but the highest chloramphenicol MIC values of 0.125 and 1.2 mg/ml meaning that their growth was better inhibited by the extract than chloramphenicol.

4. 2 Conclusions

1. This study has shown that Neem leaves possess antibacterial activity against common
human pathogens and have considerable antibacterial potency and a broad
spectrum of activity. The results may therefore explain its popularity in controlling
bacterial infections traditionally. Neem extracts would be very useful in co-
infections

The extract showed high activity against the following organisms:

- *Staphylococcus aureus*
- *Escherichia coli,*
- *Salmonella typhi*
- *Streptococcus pyogenes*

2. Neem extract was not effective against *N. gonorrhoeae* and *H. influenzae* and was only
poorly effective against shigella species.

3. The aqueous Neem leaves extract demonstrated bacteriostic activity and like
chloramphenicol does not kill the bacteria but rather inhibits their multiplication.

4. 3 Recommendations

1. Although aqueous extracts would be easier to prepare at home it may be of interest to
compare these results with an alcohol (methanol or ethanol) extract. Extraction by
organic solvents may yield more potent products, as it would improve extraction of
active compounds from the leaves.

2. There is also need for more research to determine the bioavailability of these
extracts to find out whether it is possible to attain the MIC levels in blood after oral
consumption.
3. The resistance of *Ps. aeruginosa* to chloramphenicol and the favourable susceptibility of the organism to Neem extract, necessitate an enquiry to the possibility of developing an antibiotic against *Ps. aeruginosa*, a notorious nosocomial infectious agent by fractionating Neem extract.

4. Although there have not been indications of serious toxicity contraindications, there is need for more toxicity studies to aid in determination of safe regimens if the extract is to be scientifically encouraged for internal use.

5. Further work to compare efficacy of the leaves extract to that of roots, seed and bark as antibacterial agent should be done.

6. It would be of interest to evaluate antibacterial activity of Neem extract from the same region in different seasons and also compare extracts from different regions in Kenya.
REFERENCES


Chooto, K. (2004). Herb have place in modern healthcare. Daily Nation September 20\textsuperscript{th} 2004


APPENDIX 1

Bacteriological media

(i) Faecal coliforms medium

Medium used for the detection and enumeration of faecal coliforms organisms

Formula

Lactose 12.5 g
Bacteriological peptone 10 g
Proteose peptone 5 g
Sodium Chloride 5 g
Yeast Extract 3 g
Bile salts 1.5 g
Aniline Blue 0.1 g
Bacteriological agar 15 g

Method

Suspend 52 g of the medium in one litre of distilled water. Dissolve until completely dissolved. Add 10 ml of rosalie acid at 1% in NaOH 0.2N. Mix well to obtain a homogenous suspension. Heat with frequent agitation till boiling. Cool to 45-50°C and pour into petri dish.

(ii) Blood Agar/Chocolate blood agar

Formula
Casein pancreatic digest 15 g
Soy peptone 5 g
Sodium Chloride 5 g
Bacteriological agar 15 g
Sterile Sheep blood 20 mls

Method
Suspend 40 gms of the medium in one litre of distilled water. Mix well. Heat with frequent agitation and boil for one minute until the medium is completely dissolved. Dispense and sterilize in autoclave at 121°C for 15 minutes. If large quantities are to be prepared sterilization time in autoclave, may be increased, but not temperature. To prepare blood plate for haemoglobin studies, add 5-10% of defibrinated sterile blood rabbit or sheep to the sterile medium, cooled to about 45°C and pour. For chocolate blood agar plates, raise the temperature to 80°C in a water bath. With frequent agitation maintain until the medium turns chocolate colour.

(iii) Salmonella Shigella Agar

Formula
Beef Extract 5 g
Peptone 5 g
Lactose 10 g
Bile Salts 8.5 g
Sodium Nitrate 8.5 g
Sodium thiosulphate  8.5 g  
Ferric Citrate  1 g  
Bacteriological agar  13.5 g  
Neutral Red  0.025 g  
Brilliant Red  0.33 mg  

Method  
Suspend 60 mg of the medium in one litre of distilled H₂O. Mix well until homogenous suspension is obtained. Heat with frequent agitation and boil for one minute. DO NOT STERILIZE IN AUTOCLAVE. Cool to 45°C and 50°C and distribute in petri plates. Allow the medium to solidify partially uncovered. This is a selective media for all the isolation of salmonella and shigella species.

(iv) Nutrient gelatin  
Formula  
Beef extract  3 g  
Peptone  5 g  
Gelatin  120 g  
Distilled Water  1000 ml  
Method  
Warm the mixture to about 50°C to dissolve completely distribute in tubes and sterilize in the autoclave for 15 minutes at 121°C.
(v) Muller Hinton Medium

Formula

Beef infusion 2 g
Casamino acids 17.5 g
Starch 1.5 g
Agar 17 g
Distilled Water 1000 ml

Method

Suspend the mixture in cold distilled water and heat to boiling to dissolve the medium completely. Distribute in flask and sterilize for 10 minutes at 116°C. Cool to 50-55°C and pour into plates.

(vi) Azide Blood Agar base

Formula

Tryptose 10 g
Beef Extract 3 g
Sodium Chloride 5 g
Sodium azide 0.2 g
Bacto-agar 15 g
Distilled Water 1000 ml

Suspend the contents in cold distilled water and heat to boiling point to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 121°C. Cool to 50-
55°C and pour into plates. Azide blood agar base is a selective medium for the isolation of streptococci.
APPENDIX 2

Biochemical tests

(i) Oxidase test

Formula

\[
\text{Dimethyl} - p - \text{Phenylenediamine hydrochloride} \quad 1 \text{ g} \\
\text{Distilled Water} \quad 100 \text{ ml}
\]

Method

Dissolve Dimethyl–p–Phenylenediamine hydrochloride in cold distilled water. Soak the solution into strips of filter paper and dry them in the incubator. Oxidase test is used to detect oxidase production by microorganisms. It is particularly useful in detection of \textit{Neisseria gonorrhoeae} colonies. On touching the colonies with the strip, the paper turns pink within a minute or two and darkens to become black. Oxidize negative colonies do not change colour.

(ii) Gram stain

Formula

\begin{align*}
\text{Gram’s Iodine} \\
\text{Iodine} & \quad 1 \text{ gm} \\
\text{Potassium Iodide} & \quad 2 \text{ gm} \\
\text{Distilled water} & \quad 300 \text{ ml}
\end{align*}

\begin{align*}
\text{Crystal Violet} \\
\text{Solution A} \\
\text{Crystal violet (90\% dye content)} & \quad 2 \text{ g}
\end{align*}
Ethyl alcohol (95%) \hspace{1cm} 20 \text{ ml}

Solution B

Ammonium oxalate \hspace{1cm} 0.8 \text{ gm}
Distilled water \hspace{1cm} 80 \text{ ml}

Mix the two solutions in the above quantities.

Safranin

Safranin 2.5% solution in 95% alcohol \hspace{1cm} 10 \text{ ml}
Distilled water \hspace{1cm} 100 \text{ ml}

Straining Procedure

1. The heat fixed smear is stained for 1 minute with ammonia oxalate crystal violet.
2. Wash with tap water
3. Flood with Gram’s iodine solution and allow standing for 1 minute.
4. Wash in tap water and blot dry.
5. Decolorize 30 seconds with gentle agitation in 95% alcohol and blot dry.
6. Counter strain 10 to 30 seconds in safranin.
7. Wash in tap water, blot dry and examine microscopically.

Gram stain is one of the most valuable and most generally applied staining methods and serves to separate bacteria into two groups. Those that retain crystal violet are said to be Gram-positive, while those that are decolorized and stain with counter stain are said to be Gram-negative.
(iii) Catalase test

Formula

Hydrogen Peroxide  10 ml
Distilled water  90 ml

Method

Mix the two label and store at room temperature.

3.0 Turbidity Standard

Formula

0.045 M Barium chlorides Solution

Barium Chloride  1.175 mg
Distilled water  100 ml

0.36N Sulphuric acid solution

Concentrated sulphuric acid  1 ml
Distilled Water  100 ml

Method

Combine solution 1 and 2

Solution 1  0.5 ml
Solution 2  99.5 ml

Distribute into test tubes closed by rubber stopper. Agitate tube well before use. Store in the dark at room temperature.
Standard opacity tube NO: 5 corresponds to

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1,895,000,000</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>1,522,000,000</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>3,527,000,000</td>
</tr>
<tr>
<td><em>Neisseriae gonorrhoeae</em></td>
<td>1,789,000,000</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1,894,000,000</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>2,289,000,000</td>
</tr>
<tr>
<td><em>Salmonella paratyphi</em></td>
<td>2,086,000,000</td>
</tr>
<tr>
<td><em>Neisseriae catarrhalis</em></td>
<td>1,806,000,000</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>5,698,000,000</td>
</tr>
</tbody>
</table>
APPENDIX 3

Bacterial identification criteria

**Staphylococcus aureus:** pin head, convex, circular colonies with entire margins, Gram-positive cocci, catalase positive and coagulase positive.

**Streptococcus pyogenes:** pin point colonies surrounded by clear halo (β-haemolysis) on blood agar, Gram-positive cocci in chains and coagulase negative.

**Streptococcus pneumoniae:** Gram-positive cocci in chains, partially haemolytic (α-haemolysis) on Blood agar, catalase negative and optochin sensitive.

**Streptococcus faecalis:** Gram-positive cocci in chains have more diffuse zones of β-haemolysis than *Streptococcus pyogenes* and the colonies are also larger.

**Streptococcus viridans:** Gram-positive cocci in chains, colonies surrounded by greenish halo on blood agar (α-haemolysis), catalase negative and optochin resistant.

**Escherichia coli:** pink, shiny, mucoid, slightly raise colonies with entire margins, Gram-negative rods, grows on Mackonkey’s, lactose fermenting producing acid, oxidase negative.

**Salmonella typhi:** Shiny convex colonies with entire margins, Gram-negative rods, oxidase negative, grows on Mackonkey’s, non-lactose fermentor producing hydrogen sulphide and or G antigens.

**Shigella species:** Gram-negative rods, oxidase negative, grow on Mackonkey’s and a non-lactose fermentor.

**Shigella dysenteriae:** non-motile and anaerogenic

**Shigella sonnei:** methyl red positive, urease negative, indole negative, ornithine decarboxylase positive.
**Pseudomonas aeruginosa:** mucoid colonies with diffusible green pigmentation, umbonate elevation and characteristic odour, β-haemolytic, Gram-negative rods, oxidase positive and grows on nutrient agar.

**Proteus species:** highly motile on agar plates (with a single bacteria capable of spreading through the surface of agar plate overnight), Gram-negative, oxidase negative, urease splitting, grows on Mackonkey’s and non-lactose fermentor.

- **Proteus vulgaris:** indole positive.
- **Proteus mirabilis:** indole negative,

**Klebsiella pneumoniae:** Gram-negative rods, non-motile encapsulated facultative anaerobe, grow on Mackonkey’s and ferment lactose to produce acid (pink colonies) and Voges-proskauer positive.

**Neisseria gonorrhoeae:** Gram-negative cocci, maltose non-fermentor, require raise carbon dioxide tension and enriched medium to grow.

**Haemophilus influenzae:** Gram-negative coccobacilli, oxidase negative, no growth on blood agar but grows on chocolate agar (requires X and V factors for growth) and does not grow on Mackonkey’s.

**Bacillus cereus:** Small, shiny compact to large, feathery spreading colonies, β-haemolytic on sheep blood, produce lecithinase but does not encapsulate.

**Bacillus anthracis:** Large, raised, white to gray, opaque irregular colonies with curved margins, Gram positive spore forming aerobic bacilli, large square ended cells in chains giving a bamboo appearance, non-motile, non-haemolytic, encapsulating on enriched medium and Voges-Proskauer positive.
**Bacillus subtilis:** large, dry, flat irregular colonies with ground-glass appearance, sporulating with lobate margins, Gram-positive rods.

**Campylobacter jejuni:** small, curved or seagull-winged faintly staining Gram-negative rods, oxidase positive, catalase positive, darting motility and curved forms on wet preparation, aerobic and do not grow on nutrient agar.

**Morganella morganii:** indole negative, methyl red negative, urea positive, citrate negative Gram-negative rods.

**Enterobacter cloacae:** Gram-negative rods, motile, indole negative, methyl red negative, Voges-Proskauer positive, citrate positive with slow urea hydrolysis.

**Staphylococcus albus:** convex, circular colonies with entire margins, Gram-positive cocci, catalase positive and coagulase negative.

**Citrobacter coseri:** straight rods, single or in pairs, motile and anaerobic.

**Brucella abotus:** Gram-negative coccobacilli, mostly single and non-motile. Identified serologically with antiserum.