EFFECTS OF KENYAN BLACK TEA WATER SOLUBLE COMPONENTS ON THEAFLAVINS INTERACTION WITH ANTIBIOTICS AGAINST SELECTED PATHOGENIC BACTERIA

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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DEDICATION

This thesis is dedicated to my father and mother: George Tonui, Sarah Tonui, my wife Florence Bor and son Ian Cheruiyot.

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LIST OF ABBREVIATIONS

ACCoT	Ampicillin, chloramphenicol, cotrimoxazole, tetracycline
attB	Attachment site B
BHA	Butylated hydroxyanisole
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
E.coli	*Escherichia coli*
EGC	(-)-epigallocatechin
ENT	Ear, nose, throat
ESBLS	Extended-spectrum beta-lactamases
gyrA	Gyrase A
gyrB	Gyrase B
HPLC	High performance liquid chromatography
HGT	Horizontal gene transfer
KETEPA	Kenya tea packers limited
MAC	MacCkonkey agar
MBC	Maximum bactericidal concentration
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
NLF	Non-lactose fermenting
omp	Outer membrane permeability
PRSP	Penicillin-resistant *Streptococcus pneumoniae*
QRDR	Quinolone resistance determining region

RTFs	Resistance transfer factors
rRNA	Ribosomal ribonucleic acid
SCC*mec*	Staphylococcal chromosome cassette *mec*
Tet	Tetracycline
TF1	Theaflavin
TF2A	Theaflavin-3-gallate
TF2B	Theaflavin-3'-gallate
TF3	Theaflavin-3,3'-gallate
TLC	Thin layer chromatography
tRNA	Transfer ribonucleic acid
TSI	Triple sugar iron agar
UV	Ultraviolet
VISA	Vancomycin intermediate susceptible *Staphylococcus aureus*
VRE	Vancomycin resistant enterococci

Abstract

Water soluble components derived from black tea contain active antibacterial compounds, that can be utilized in combined antibiotic-herb therapy to combat bacterial resistance. This strategy is more advantageuos than using single therapy as it is more effective and has minimal side effects. However, it is not clear if the major bioactive compounds interacts with other water soluble compounds, which inturn affects their interaction with antibiotics. This research therefore aimed at comparing the antibacterial activities of hot water extract of black tea having 18µg/ml of theaflavins and 18µg/ml of isolated theaflavins and their combination with antibiotics such as ampicillin. Their combined effects with antibiotics were determined using disk diffusion and modified Checkerboard method. The chi-square test was used to test the null hypothesis, which stated that water soluble components have no effect on theaflavins interaction with antibiotics. The water soluble components of black tea extract were extracted with hot water and theaflavins in it was measured using Flavognost method. Similar amount of theaflavins were extracted using organic solvents and silica gel column chromatography. The concentrates of hot water extract and isolated theaflavins showed synergistic activity with selected antibiotics. However the level of synergism differed significantly at $P<0.05$, with isolated theaflavins having higher level. The difference in inhibitory effect between combined concentrates of hot water extract and

isolated theaflavins with MIC (10.4 µg/ml) of ampicillin against *S. typhi* was significant at (χ^2=0.56; P<0.05). The differences in inhibitory effect was also significant at (χ^2=0.699; P<0.05) between the two black tea extracts combinations with MIC (4.3 µg/ml) of norfloxacin against *P. aeruginosa*. The combination of concentrates of hot water extract and isolated theaflavins with MIC (2 µg/ml) of ciprofloxacin differed significantly in level of inhibition at (χ^2=1.98; P<0.05) against *S. aureus*. When the concentrates of the two black tea extracts were combined with MIC (5.25 µg/ml) of tetracycline, the inhibitory effect differed significantly at (χ^2=2.27; P<0.05) against *E. aeruginosa*. It was also significant at (χ^2=0.4; P<0.05) when concentrates of the two black tea extracts were combined with MIC (12 µg/ml) of chloramphenicol against *E. coli*. The differences in inhibitory effect observed were attributed to interactions within the tea infusion between water soluble components and theaflavins. Theaflavins in black tea infusion are being partially antagonized by one or more chemical components in it lowering the overall activity. However, the pattern of activity of isolated theaflavins and hot water extract of black tea were similar. This suggests that the theaflavins are the principal bioactive compounds in black tea infusion despite the existence of interaction. Isolated theaflavins and hot water extracts of black tea restored the activity of lower concentrations of antibiotics below MIC to susceptible breakpoints. The two black tea extracts together with antibiotics can be used in treatment and prevention of bacterial infections.

CHAPTER ONE

INTRODUCTION

1.1 Background

The disease causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem (Kenneth, 2008). While the development of resistant strains is inevitable, the slack ways of administering and using antibiotics in human, veterinary medicine and in agriculture has greatly exacerbated the process (Kenneth, 2008). Wound infections, gonorrhea, tuberculosis, pneumonia,

septicemia, childhood ear infections and staphylococcal infections are just a few of the diseases that have become hard to treat with antibiotics (Kenneth, 2008; Amy, 2008).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is representative of staphylococcal infections and traditionally stood for methicillin resistance, but the term increasingly refers to a multi-drug resistant group (Amy, 2008). Such bacteria often have resistance to many antibiotics traditionally used against *Staphylococcus aureus* (Amy, 2008). This resistance to methicillin is due to the presence of the *mec* gene in the bacteria which alters the site at which methicillin binds to kill the organism (Amy, 2008).

Unless antibiotic resistance problems are detected as they emerge and actions taken immediately to contain them, society could be faced with previously treatable diseases that have become again untreatable (Kenneth, 2008). The ability of bacteria to survive antibiotic therapy either by transiently tolerating antibiotics or by evolving resistance requires specific biochemical processes that may themselves be subject to intervention (Peter and Floyd, 2007). Inhibiting these processes may prolong the efficacy of current antibiotics and provide an alternative to escalating antibiotics discovery over evolution of bacterial resistance (Peter and Floyd, 2007).

Traditional methods of antibiotic discovery have failed to keep pace with the evolution of bacterial resistance, which suggests that new strategies to combat bacterial infections may be required (Peter and Floyd, 2007). Microbial development of resistance, as well as economic incentives, has resulted in research

and development in the search for new antibiotics in order to maintain a pool of effective drugs at all times (Kenneth, 2008).

Current researches have focused on strengthening the antibacterial action of the existing antibiotics through combined therapy (Nwafor *et al.,* 2003; Esimone *et al.,* 2003). The advantages of combined antibiotic therapy are broadened spectrums of antimicrobial activity, occurrence of synergistic activity and prevention of bacterial resistance development (Aurer and Planeak, 2004). Disadvantages of such treatment are elevated incidence of adverse effects (Aurer and Planeak, 2004). Alternatively, recent studies have focused on combined antibiotic-herb therapy to combat bacterial resistance (Nwafor *et al.,* 2003; Esimone *et al.,* 2003). Antibiotics are sometimes willfully or inadvertently administered concomitantly with herbs or beverages (Esimone *et al*., 2003). This portends a potential herb-drug interaction, which could be beneficial or deleterious (Esimone *et al*., 2003). One of the herbs that are widely consumed concomitantly with most drugs is tea (Esimone *et al*., 2003). Studies on tea have shown that it has some medicinal properties including antimicrobial effect against a wide range of bacteria, fungi and viruses (Sakanata *et al*., 1989; Toda *et al*., 1991).

Tea plant belongs to species *Camellia sinensis* (Higdon, 2007). The two main varieties are *Camellia sinensis var sinensis* and *Camellia sinensis var assamica* (Higdon, 2007). Tea is an infusion of the leaves of *Camellia sinensis* plant and is one of the most widely consumed beverage in the world (Higdon, 2007). Herbal teas are infusions of herbs or plants other than *Camellia sinensis*. There are

different types of tea from the leaves of *Camellia sinensis* and different processing methods produce these types of tea (Higdon, 2007). White tea is made from apical buds and immature leaves, technically called `flush' which are steamed or fired to inactivate polyphenol oxidase and then dried. Green tea is processed from withered and steamed fresh tea leaves. Semi-fermented teas (Oolong tea) is processed by macerating tea leaves and fermenting before heating to dry, to stop further biochemical changes. Fully fermented tea (black tea) is made by macerating tea leaves and then allowed to ferment completely before drying. Most black teas are rich in theaflavins and thearubigins, but relatively low in catechins (Higdon, 2007). Tea contains a number of bioactive chemicals. The phytochemical screening of tea revealed the presence of alkaloids, saponins, tannins, catechin and other polyphenols (Lai *et al*., 2001). Recent researches mainly focus on the potential health benefits of a class of compounds in tea known as flavanoids (Lai *et al*., 2001).

Flavanoids in fresh tea leaves are catechins which are a group of natural polyphenols (Lai *et al*., 2001). Catechins have four main derivatives namely (-)–epigallocatechin (EGC), (-)– epicatechin (EC), (-)–epicatechin-3-gallate (ECG) and (-)–epigallocatechin-3-gallate (EGCG) (Lai *et al*., 2001). Catechins account for 6-16% of the dry green tea leaves (Higdon, 2007). Another group of polyphenol pigments are theaflavins found in black tea (Higdon, 2007). Theaflavins are formed from polymerization of catechins due to oxidation by polyphenol oxidase at fermentation stage during the manufacture of black tea (Higdon, 2007). Theaflavins contribute to the characteristics bright orange–red color of black tea, accounting for

approximate 2g/100g of the dried water extract of black tea (Higdon, 2007). Catechins and theaflavins are believed to have a wide range of pharmaceutical benefits such as antibacterial, antihypertensive, antioxidative, hypolipidemic, antiviral and antifungal activities on their own (Hara *et al*., 1991).

Recent studies on Japanese Sencha tea (green tea) and Indian Lipton brand black tea have shown that tea extracts have got effect on efficacy of antibiotics. Most of the studies have been carried out on green tea extracts as compared to black tea extracts. A study using Indian Lipton brand black tea extracts showed synergistic activity with chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against *Salmonella typhi*, *Shigella dysenteriae*, *Yersinia enterocolitica* and *Escherichia coli* (Tiwari *et al*., 2005). Gallic acid extract from black tea showed a synergistic effect with amikacin and sulfamethoxazole tested in a dose-dependent manner against *Escherichia coli* (Tirang *et al.*, 2007).

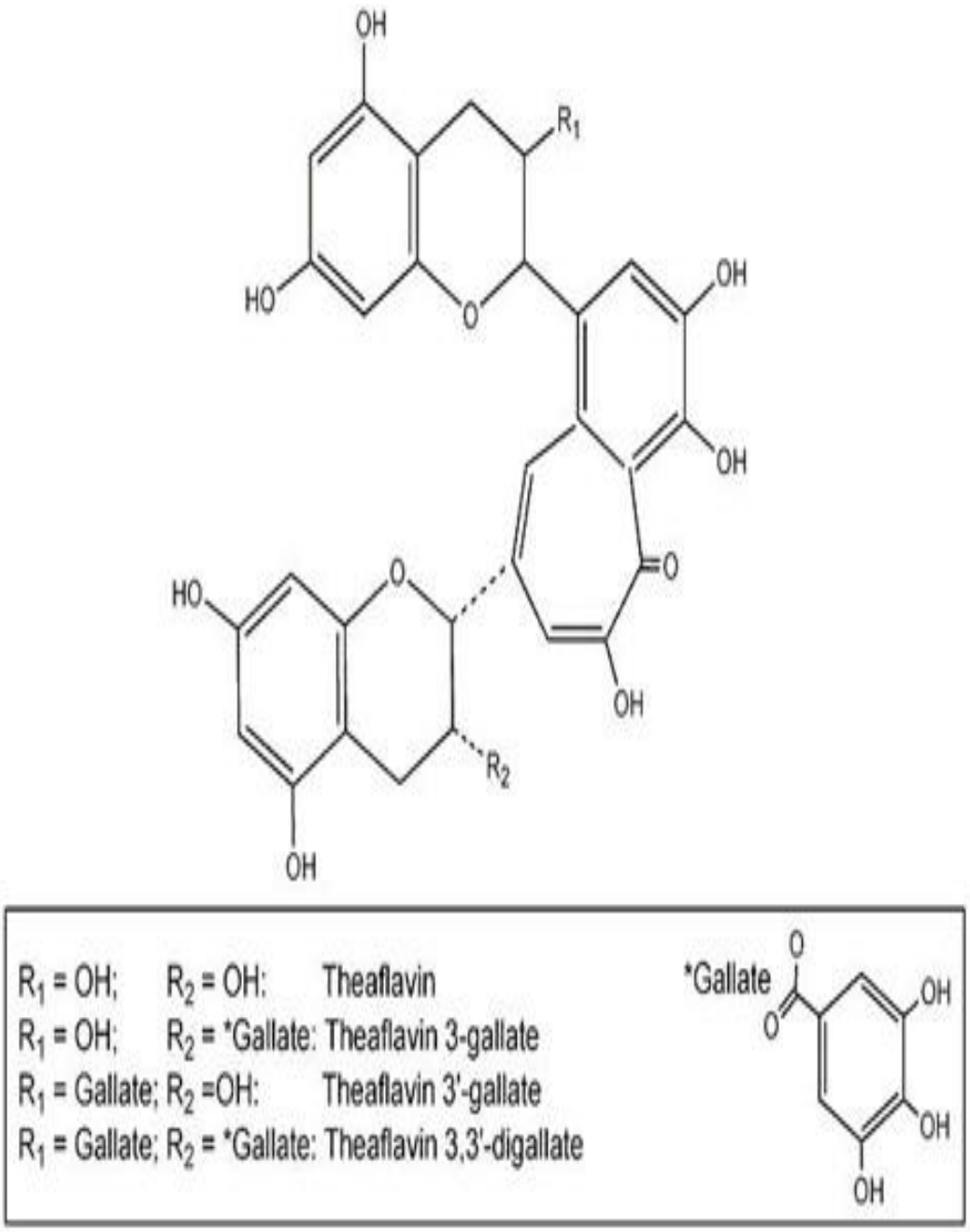

Figure 1. Chemical Structures of Theaflavins or Flavanol Dimers (Higdon, 2007).

The major theaflavins are theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3’-gallate (TF2B) and theaflavin-3, 3’-digallate (TF3).

Esimone *et al*. (2006) showed that the interactions between penicillin G and tea extracts were mainly additive against the three strains of *S. aureus*. Zhi-Qing *et al*. (2002) also observed additive, indifferent and antagonistic effects in combinations of epigallocatechin-3-gallate with 12 non-β-lactam antibiotics. These antibiotics included tetracycline, minocycline, chloramphenicol, streptomycin, gentamicin, kanamycin, erythromycin, rifampicin and ofloxacin.

A study was carried out using hot water extract of Sencha (Japanese Green Tea) and methicillin to determine the combination effect (Hara *et al*., 1991). It was found that the extract of Sencha tea is not only capable of inhibiting methicillin resistant *Staphylococcus aureus* (MRSA) but also restoring the activity of methicillin against MRSA. Hara *et al*. (1991) also observed that the extract of tea acts synergistically with methicillin against MRSA. The synergistic effect was attributed to cathecins. The Sencha group of teas has high levels of ascorbic acid (Vitamin C), tannin and most of the catechins (Goto *et al*., 1996). Other studies have indicated that Kenyan black teas have significant levels of the unoxidised flavan-3-ols (theaflavins and thearubigins) associated with human health (Owuor and Obanda, 1995).

1.2 Statement of the Problem

Combined antibiotic-herb therapy is an alternative approach to combat increasing bacterial resistance to existing antibiotics. However, there is need to determine whether the herb-drug interaction is beneficial or not. The combined effects of herb-drug may be as a result of influence by other factors. For example, interaction

of the principal bioactive compound in the herb with other chemical components in it.

1.3 Hypothesis

Water soluble components in Kenyan black tea have no effect on theaflavins interaction with common antibiotics against selected clinical isolates and standard bacterial species.

1.4 Objective

To study the effect of water soluble components in Kenyan black tea on theaflavins interaction with antibiotics in use against selected clinical isolates and standard bacteria species.

1.4.1 Specific Objectives

i) To determine antibacterial activities of hot water extract of Kenyan black tea on selected clinical isolates and standard bacteria.

ii) To determine antibacterial activities of theaflavins in Kenyan black tea on selected clinical isolates and standard bacteria.

iii) To determine the effect of hot water extract of Kenyan black tea on the efficacy of combined theaflavins with selected antibiotics.

iv) To determine synergism between theaflavins and common antibiotics.

1.5 Justification

A study in Rural Western Kenya in which bacterial causes of diarrhea were examined and found that utility of available antimicrobials for treating bacterial diarrhea is substantially limited by reduced susceptibility (Brooks *et al*., 2006). One strategy employed to overcome this bacterial resistance is the use of combination of drugs, such as β-lactams together with β-lactamase inhibitors (Hemaiswarya et al., 2008). The advantages of combined antibiotic therapy are broadened spectrums of antimicrobial activity, occurrence of synergistic activity and prevention of bacterial resistance development (Aurer and Planeak, 2004). Disadvantages of such treatment are elevated incidence of adverse effects (Aurer and Planeak, 2004). The adverse effects have led to patients not completing the prescribed dose of particular antibiotic. They end up trying several types of antibiotics sold over the counter in attempt to avoid unwanted effects. Continuous exposure of low doses of different antibiotics may be the major cause of multi-drug resistance. Therefore there is need for therapies that are effective and have minimal unwanted effects. Natural products and traditional medicines are now preferred sources of new antimicrobial agents for combined therapy. This is because of minimal side effects. However, herb-drug interaction may be beneficial or deleterious. These may be due to interaction between bioactive compounds or between bioactive and non bioactive in the same plant. These interactions within the plant extract may in turn affect the interaction of bioactive compound with antibiotics. The present study is designed to determine whether there is interaction between theaflavins and other water soluble components of Kenyan black tea. The interaction may have an effect on theaflavins

interaction with antibiotics in use against human enteropathogens. The outcome will determine the need for extraction of theaflavins or just enhancing its content during black tea processing. Also it will provide for development of technology that produces a new type of black tea that can be used for medicinal purposes. Tea is abundantly grown in Kenya and this is in line with adding value to tea by elucidating its medicinal properties.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bacterial Drug Resistance

The need to combat microbial resistance to antibiotics is an increasing global concern (Kunin, 1993; Twomey, 2002). With the popularization and rapid development of medical treatments, various kinds of antibiotics are being used in the treatment of various ailments (Herold *et al*., 1998). However, bacterial resistance to these antibiotics has emerged. It is believed that poor patient compliance such as interrupted or premature cessation of therapy and misuse or abuse of antibiotics like use of wrong antibiotic or insufficient dose, play important role in resistance development (Pillai *et al*., 2001). This form of resistance often stems from spontaneous mutations accompanied by the positive selecting pressure of the doses of antibiotics being between the minimum inhibitory concentration (MIC) and maximum bactericidal concentration (MBC) levels (Pillai *et al*., 2001).

The emergence of strains resistant to penicillin, streptomycin and chloramphenicol which were earlier effective drugs presents a continuing clinical challenge (Don, 2008). The eventual appearance of strains simultaneously resistant to multiple antibiotics significantly worsened the problem (Don, 2008). The latter was found to involve different resistance genes linked to each other on segments of deoxyribonucleic acid (DNA) which are able to move efficiently from one bacterial cell to another by phenomena known as horizontal gene transfer (Don, 2008).

Horizontal gene transfer (HGT) can occur by three basic mechanisms namely transformation, transduction and conjugation (Don, 2008). Transformation is a process in which a recipient cell takes up DNA from the environment, such as DNA released from a dead organism (Tami, 2008). Transduction involves the transfer of DNA from one bacterium to another through a replicating virus (Tami, 2008). Conjugation phenomena frequently involve mobile plasmids or conjugative transposons, which encode copies of themselves that are able to move from one bacterial cell to another and are widespread in the bacterial world (Clewell and Francia, 2008).

Multiple drug resistant organisms are resistant to treatment with several, often unrelated, antimicrobial agents (Kenneth, 2008). Some of the most important types of multiple drug resistant organisms that have been encountered include methicillin or oxacillin-resistant *S. aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), vancomycin-resistant enterococci (VRE), extended-spectrum beta-lactamases (ESBLs) (Kenneth, 2008). Extended-spectrum beta-lactamases (ESBLs) producing organisms are resistant to cephalosporins and monobactams while carpapenems is effective against most of them (Kenneth, 2008).

Methicillin or oxacillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are the most commonly encountered multiple drug resistant organisms in patients residing in non-hospital healthcare facilities, such as nursing homes (Kenneth, 2008). Penicillin-resistant *Streptococcus pneumoniae* (PRSP) are more common in patients seeking care in outpatient settings such as physicians' offices and clinics, especially in pediatric settings (Kenneth, 2008). Extended-

spectrum beta-lactamases (ESBLs) are most often encountered in the hospital (intensive care) setting (Kenneth, 2008). Methicillin or oxacillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) also have a significant nosocomial ecology (Kenneth, 2008).

2.1.1 Methicillin–Resistant *Staphylococcus Aureus* (MRSA)

Staphylococcus aureus is a Gram-positive cocci of uniform size, occurring characteristically in groups but also singly in pairs (Arora and Arora, 2008). They are non–motile and non capsulated (Arora and Arora, 2008). Integration of a staphylococcal chromosome cassette *mec* (SCC*mec*) element into the chromosome converts drug-sensitive *S. aureus* into the notorious hospital pathogen MRSA (Arora *et al*., 2008). Staphylococcal chromosome cassette *mec* (SCC*mec*) is site-specifically integrated into the staphylococcal chromosome at a locus known as the SCC*mec* attachment site (*attB*) (Michael *et al*., 2008). MRSA is resistant to practically all beta-lactam antibiotics (Keiichi *et al*., 2001). Staphylococcal chromosome cassette *mec* (SCC*mec*) is a novel class of mobile genetic element (Keiichi *et al*., 2001). They are composed of the *mec* gene complex encoding methicillin resistance and the ccr gene complex that encodes recombinases responsible for its mobility (Keiichi *et al*., 2001). These elements also carry various resistance genes for non-beta-lactam antibiotics (Keiichi *et al*., 2001). After acquiring a SCCmec element, methicillin-resistant *S. aureus* (MRSA) undergoes several mutational events evolving into the most difficult to treat pathogen in hospitals, against which all common antibiotics including vancomycin are

ineffective (Keiichi *et al*., 2001). Of particular concern are the vancomycin intermediate susceptible *S. aureus* (VISA) strains of methicillin-resistant *S. aureus* (Amy, 2008). These are beginning to develop resistance to vancomycin, which is currently the most effective antibiotic against methicillin-resistant *S. aureus* (Amy, 2008). This new resistance has risen because another species of bacteria, called enterococci, commonly expresses vancomycin resistance and are capable of transferring the gene for vancomycin resistance over to *S. aureus* (Amy, 2008).

Community-acquired MRSA clones have emerged as a major cause of MRSA colonization in high-risk newborns (Ulrich *et al*., 2008). Community-acquired MRSA recovery has been associated with acquisition during birth, whereas health care-associated MRSA clones seemed to be transmitted nosocomically (Ulrich *et al*., 2008). Traditionally MRSA stood for methicillin resistance but the term increasingly refers to a multi-drug resistant group (Amy, 2008). Such bacteria often have resistance to many antibiotics traditionally used against *S. aureus* (Amy, 2008).

2.1.2 *Salmonella Typhi*

Salmonella typhi is a Gram-negative, non-sporulating and capsulated bacterium (Arora and Arora, 2008). They are active motile and some strains appear mucoid (Arora and Arora, 2008). Emergence of antimicrobial resistance, in particular multi-drug resistance has greatly complicated disease management (Arora and Arora, 2008). The multi-drug resistant (MDR) *S. typhi* strains contained a transferable plasmid conferring resistance to ampicillin, chloramphenicol,

cotrimoxazole and tetracycline (Mandal *et al.*, 2005). The plasmid encoding ampicillin, chloramphenicol, cotrimoxazole and tetracycline-resistance of *Escherichia coli, Klebsiella pneumoniae* and *Proteus vulgaris* are conjugative and co-migrate with the plasmid of multi-drug resistant (MDR) *S. typhi* isolates (Mandal *et al.*, 2005). The antibiotic sensitive *S. typhi* isolates do not contain any plasmid (Mandal *et al.*, 2005). *S. typhi* strains acquire the resistant-plasmid from other enteric bacteria such as *Escherichia coli, K. pneumoniae* and *P. vulgaris* to undergo a suitable adaptation for survival in the changing antibiotic environment (Mandal *et al.*, 2005).

2.1.3 *Pseudomonas Aeruginosa*

Pseudomonas aeruginosa is a Gram-negative pathogen, versatile and opportunistic in terms of its genetics, metabolic potential and mechanisms of virulence (Kenneth, 2008). The pathogen is a distinguished and opportunistic pathogen, causing infections in patients with physical, phagocytic or immunologic defects in host defense mechanisms (Rhamphal, 2007). *P. aeruginosa* has been shown to inactivate anti-methicillin resistant *S. aureus* antibiotics as indirect pathogen (Ramphal, 2007). The co-existence of *P. aeruginosa* and *S. aureus* may make it difficult to treat staphylococcal infections (Ramphal, 2007). *P. aeruginosa* is resistant to most of the commonly used antibiotics such as chloramphenicol, rifampicin, neomycin, vancomycin and carbenicillin (Rhamphal, 2007).

Diversity of mechanisms of genetic exchange, including transformation, transduction and conjugation, help *P. aeruginosa* adapt to changing conditions by

acquiring new genetic information (Kenneth, 2008). The bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its Gram-negative outer membrane (Kenneth, 2008). Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic antibiotics (Kenneth, 2008). Since its natural habitat is the soil, living in association with the bacilli, actinomycetes and molds, it has developed resistance to a variety of their naturally occurring antibiotics (Kenneth, 2008). Moreover, *Pseudomonas aeruginosa* maintains antibiotic resistance plasmids, both resistance factors (R-factors) and resistance transfer factors (RTFs), and it is able to transfer these genes by means of the bacterial mechanisms of horizontal gene transfer (HGT), mainly transduction and conjugation (Kenneth, 2008).

Only a few antibiotics are effective against *P. aeruginosa* and these include fluoroquinolones, gentamicin and imipenem (Kenneth, 2008). However, these antibiotics are not effective against all the strains (Kenneth, 2008). The futility of treating *P. aeruginosa* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant that it cannot be treated (Kenneth, 2008). The pathogenesis of *P. aeruginosa* infections is multifactorial, as manifested by the numerous toxins or virulence factors, it produces and the variety of diseases it causes (Kenneth, 2008). *P. aeruginosa* is invasive and toxigenic and infections appear to occur in stages namely bacterial adherence, colonization, invasion and dissemination, and systemic or toxemic disease (Kenneth, 2008).

2.1.4 *Escherichia Coli*

Escherichia coli is a Gram-negative usually motile rod and minority of strains are capsulated (Arora and Arora, 2008). Enterohermorrhagic strain, a rare variety of *E. coli* produces large quantities of one or more related, potent toxins that cause severe damage to the lining of the intestine (Arora and Arora, 2008). The most frequent mechanism of resistance to quinolones in *E. coli* includes alterations in genes that encode subunits of the quinolone targets DNA gyrase (in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE*) (Yolanda *et al.,* 2003). These alterations involve mainly mutations located in the quinolone resistance-determining region (QRDR) of the *gyrA* gene and its homologous region of the *parC* gene (Yolanda *et al.,* 2003). In contrast, mutations in *gyrB* and *parE* genes are of minor importance and are rare contributors to quinolone resistance (Yolanda *et al.,* 2003). Active efflux pumps are important for intrinsic and acquired antibiotic resistance and over expression of efflux pumps affecting quinolones, tetracycline and chloramphenicol is becoming increasingly common in *E. coli* (Yolanda *et al.,* 2004).

2.1.5 *Enterobacter Aeruginosa*

Enterobacter aeruginosa is a Gram-negative, non-sporulating motile rod bacteria (Rhamphal, 2007). It is resistant to antibiotics such as imipenem (Rhamphal, 2007). In *E. aeruginosa*, multi-drug resistance involves a decrease in outer membrane permeability associated with changes in porin which is an OmpC/OmpF-like protein (De *et al.,* 2001). The G to D mutation in the putative loop three of the porin has been observed in clinical strains (De *et al.*, 2001). Given the known

importance of this loop in determining the pore properties of porins, this mutation is responsible for the novel resistance mechanism developed by this clinical strain (De *et al.*, 2001). The changes in porin channel function act as a new bacterial strategy for controlling beta-lactam diffusion through porins (De *et al.*, 2001). Sequence analysis and complementation experiments revealed that the multi-drug resistant isolate is an acrR mutant (Elizabeth and Jean-Marie, 2002). The AcrA-AcrB-TolC efflux pump contributes to multi-drug resistance in the nosocomial pathogen *E. aeruginosa* (Elizabeth and Jean-Marie, 2002).

Stephane *et al.* (2003) investigated two clinical strains of *E. aeruginosa* that exhibited phenotypes of multi-resistance to beta-lactam antibiotics, fluoroquinolones, chloramphenicol, tetracycline and kanamycin. Both strains showed a porin pattern different from that of a susceptible strain, with a drastic reduction in the amount of the major porin (Stephane *et al.*, 2003). The major porin had an apparently conserved normal structure (size and immunogenicity), together with overproduction of two known outer membrane proteins, OmpX and LamB (Stephane *et al.*, 2003). In addition, the full length O-polysaccharide phenotype was replaced by a semi-rough Ra phenotype (Stephane *et al.*, 2003). Moreover, in one isolate the intracellular accumulation of chloramphenicol was increased in the presence of the energy uncoupler carbonyl cyanide m-chlorophenylhydrazone, suggesting an energy-dependent efflux of chloramphenicol in this strain (Stephane *et al.*, 2003). The resistance strategies used by these isolates appear to be similar to that induced by stress in *E. coli* cells (Stephane *et al.*, 2003).

2.2 Selected antibiotics in current use

2.2.1 Ampicillin

It is a beta-lactam antibiotic and semi-synthetic penicillin having a wide range of activity against Gram-negative species such as *Salmonella* species and *E. coli* (Bryant, 1996). It prevents formation of peptidoglycan, an essential building block of cell membrane and hence the antibiotic prevents growth of cells (Bryant, 1996). Ampicillin is used to treat many different types of infections caused by bacteria, such as ear infections, bladder infections, pneumonia, gonorrhea and *E. coli* or *Salmonella* infection (Cerner *et al.,* 2008). Ampicillin side effects include fever, sore throat and headache with a severe blistering (Cerner *et al.,* 2008). Others are peeling, red skin rash and diarrhea that are watery or bloody (Cerner *et al.*, 2008). Headache, swollen and black tongue have also been reported (Cerner *et al.,* 2008).

2.2.2 Ciprofloxacin

Ciprofloxacin belongs to the fluoroquinolone class of antibiotics which includes levofloxacin, ofloxacin, gatifloxacin, norfloxacin, moxifloxacin and trovafloxacin (Omudhome, 2008). Ciprofloxacin stops the multiplication of bacteria by inhibiting the reproduction and repair of their genetic material (DNA) (Omudhome, 2008). Ciprofloxacin is used against enteropathogens such as *E. coli* and *E. aeruginosa* and *S. aureus* (Bryant, 1996). The most frequent side effects of ciprofloxacin include nausea, vomiting, diarrhea, abdominal pain, rash, headache and restlessness (Omudhome, 2008). Rare allergic reactions have been described, such as hives and

anaphylaxis (shock) (Omudhome, 2008). Ciprofloxacin should be used with caution in patients with central nervous system diseases such as seizures, because rare seizures have been reported in patients receiving ciprofloxacin (Omudhome, 2008).

2.2.3 Norfloxacin

Norfloxacin is an orally absorbed fluoquinolone antibiotic which is in use against *P. aeruginosa* and *E. coli* (Bryant, 1996). Its mechanism of action involves inhibition of the A subunit of the important bacterial enzyme DNA gyrase, which is essential for DNA replication (Omudhome, 2008). The side effects include nausea, headache, stomach upset, weakness, dizziness, diarrhea and drowsiness (Omudhome, 2008).

2.2.4 Tetracycline

It was the first broad spectrum oral antibiotic (Bryant, 1996). It works by inhibiting action of the prokaryotic 30S ribosome, by binding the 16S rRNA thereby blocking the aminoacyl-tRNA (Bryant, 1996). However, bacterial strains can acquire resistance against tetracycline and its derivates by encoding a resistance operon (Bryant, 1996).

Tetracyclines are characterized by their exceptional chemotherapeutic efficacy against a wide range of Gram-positive and Gram-negative bacteria, *Richettsia*, *Spirochetes* and large viruses, such as members of the Lymphogranuloma group (Rafal, 2007). The main indications for the use of tetracyclines are infections due to

E. coli, *Haemophilus influenzae* and *E. aeruginosa* (Rafal, 2007). Because of the development of strains of microorganisms resistant to the tetracyclines, these antibiotics have lost some of their usefulness (Rafal, 2007). They are no longer the drugs of first choice for treatment of *Staphylococcal*, *Streptococcal* or *Pneumococcal* infections (Rafal, 2007). The individual tetracyclines differ less in their potency than in pharmacokinetic properties such as resorption, tissue diffusion and elimination (Rafal, 2007). Common tetracycline side effects are stomach cramps, diarrhea and skin reactions to sunlight and fever (Kristi *et al.*, 2008). Some serious side effects of tetracycline include blurred vision, unusual headaches and watery diarrhea (Kristi *et al.*, 2008).

2.2.5 Chloramphenicol

It has a spectrum similar to tetracyclines, but because of toxicity, it is now not widely used (Bryant, 1996). It is effective against *S. aureus*, *Klebsiella* species and *E. coli* (Bryant, 1996). It is still used against typhoid fever and its mechanism of action involves inhibition of protein synthesis in ribosomes (Bryant, 1996). Chloramphenicol side effects include easy bruising or bleeding, persistent sore throat, fever and unusual fatigue (Omudhome, 2008). Other symptoms of an allergic reaction include rash, itching, redness, swelling or discharge (including the eye or ear area) and difficult breathing (Cerner *et al.*, 2008).

2.3 Herbal Therapy

Maria (2005) investigated the use of herbal medicines in primary health care in Maracanau, a northeast Brazilian city. Among the 226 patients interviewed, 144 (63.7%) reported previous use of herbal medicines. Among those, 131 (90.9%) observed therapeutic benefits from herbal medicines. Also 10 types of herbal medicines in the prescriptions, including syrups, dyes, capsules and ointments, for the treatment of respiratory problems, skin conditions and diabetes mellitus were identified.

In Central Province of Kenya, Njoroge and Bussmann (2006) showed that the most common diseases of ear, nose and throat (ENT) including common cold, cough, tonsillitis, otitis-media, chest pains and asthma are managed using traditional therapies. There are 36 plant families of varying habits; herbs (37.3%), shrubs (34.4%), trees (25.4%) as well as some grasses and sedges (3%) that are commonly utilized in this region (Njoroge and Bussmann, 2006). In most cases these sources are undocumented and the knowledge about them is passed orally from generation to generation, hence under threat of disappearing with current rates of modernization (Njoroge and Bussmann 2006).

2.4 Antimicrobial Activity of Flavanoids

Various plant secondary metabolites among them, flavanoids and tannins have been shown to have effects against microorganisms. Flavonoids are ubiquitous in photosynthesizing cells and are commonly found in fruits, vegetables, nuts, seeds,

stems, flowers, tea, wine, propolis and honey (Tim and Andrew, 2006). Preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases (Tim and Andrew, 2006). Flavonoids possessing antifungal, antiviral and antibacterial activity have been isolated and their structures identified (Tim and Andrew, 2006). Other high quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement (Tim and Andrew, 2006). In addition, numerous researches have sought to elucidate the antibacterial mechanisms of action of selected flavonoids (Tim and Andrew, 2006). The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase (Tim and Andrew, 2006). It has also been proposed that sophoraflavone G and (-)-epigallocatechin-3-gallate (EGCG) inhibit cytoplasmic membrane function and that licochalcones A and C inhibit energy metabolism (Tim and Andrew, 2006). Other flavonoids whose mechanisms of action have been investigated include robinetin, myricetin, apigenin, rutin, galangin, 2, 4, 2′-trihydroxy-5′-methylchalcone and lonchocarpol A (Tim and Andrew, 2006).

2.5 New Approaches to Combat Resistance

Antibiotics have been effective in treating infectious diseases, but resistance to these drugs has led to the emergence of new and the reemergence of old infectious diseases (Hemaiswarya *et al*., 2008). One strategy employed to overcome these resistance mechanisms is the use of combination of drugs, such as β-lactams together with β-lactamase inhibitors (Hemaiswarya *et al*., 2008). Several plant

extracts have exhibited synergistic activity against microorganisms (Hemaiswarya *et al*., 2008). The observed synergy and mechanism of action between natural products including flavonoids and essential oils and synthetic drugs is effective in combating bacterial, fungal and mycobacterial infections (Hemaiswarya *et al*., 2008). The mode of action of combination differs significantly than that of the same drugs acting individually; hence isolating a single component may lose its importance (Hemaiswarya *et al*., 2008).

2.5.1 Combined Antibiotic Therapy

The advantages of combined antibiotic therapy are broadened spectrums of antimicrobial activity, occurrence of synergistic activity and prevention of bacterial resistance development (Aurer and Planeak, 2004). Disadvantages of such treatment are elevated incidence of adverse effects (Aurer and Planeak, 2004).

2.5.2 Combined Antibiotic–herb Therapy

The side effects normally encountered in combined antibiotic therapy have led to shift in focus to combined antibiotic-herb therapy and the combination has been willfully or inadvertently administered (Nwafor *et al.*, 2003; Esimone *et al.*, 2003).

2.6 Antibacterial Activities of Tea Extracts

Several studies have revealed antibacterial activities of different types of tea. Friedman *et al*. (2006) evaluated the antimicrobial activities of seven green tea catechins and four black tea theaflavins, generally referred to as flavonoids, as well

as the aqueous extracts (infusions) of 36 commercial teas. The teas included black, green, oolong, white, and herbal teas against *Bacillus cereus*. The results obtained demonstrated that (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), theaflavin-3, 3'-digallate (TF3), theaflavin-3'-gallate (TF2B) and theaflavin-3-gallate (TF2A) showed antimicrobial activities at nanomolar levels. Most compounds were more active than the medicinal antibiotics, such as tetracycline and vancomycin at comparable concentrations. The bactericidal activities of the teas were accounted for by the levels of catechins and theaflavins as determined by high-pressure liquid chromatography. Also freshly prepared tea infusions were more active than day old teas.

Kim *et al.* (2004) verified antibacterial activities of water soluble extracts of green, jasmine, black, dungglre and oolong tea on foodborne pathogens. Green, jasmine and black tea suppressed growth of *S. aureus* and *Listeria monocytogenes. Streptococcus intermedius* and *Streptococcus constellatus* were also sensitive to tea extracts (Kitada *et al.,* 2006). Similar observation in aqueous extracts of teas (*Camellia sinensis*) of different types and from various sources was reported by Hamilton-miller *et al.* (1997).The aqueous extract of teas inhibited a wide range of pathogenic bacteria, including methicillin-resistant *S. aureus.* Tea extracts were bactericidal to *Staphylococci* and *Yersinia enterocolitica* at low concentrations (Hamilton-miller *et al.,* 1997). Testing of pure tea compounds and closely related chemicals suggested that the antibacterial activity of extracts of green tea can be explained by its content of (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG) (Hamilton-miller *et al.,* 1997). In

black tea extracts, theaflavin and its gallates are additional antibacterially active components (Hamilton-miller *et al.,* 1997).

Aqueous solvent extracts of black tea possess antibacterial activity, depending on the solvent used and bacterial species tested (Nihal *et al*., 2007). In black tea extracts, theaflavin-3, 3'-digallate (TF3), has been reported to have antibacterial activity against *Trichophyton mentagophytes*, *Trichophyton rubrum*, *Candida albicans* and *Cryptococcus neoformans* in a dose- and contact time-dependent manner (Okubo *et al*., 1991). Toda *et al*. (1991) verified the antibacterial activities against MRSA and food poisoning strains of *S. aureus*. Alcoholic extract of black tea inhibited all strains of *S. paratyphi* A. while only 42.19% of *S. typhi* strains were inhibited by the extract (Ciraj *et al.,* 2001).

Mbata (2006) also carried out preliminary studies to determine antibacterial activities of processed Kenyan black tea and Nigerian Lipton tea. The hot water extracts of both teas were tested for activity against six organisms namely *P. aeruginosa, S. aureus, V. cholerea, Salmonella* species, *Proteus* species and *E. coli.* The results obtained showed that 20% extract of both teas had antibacterial activities against *S. aureus, E. coli, Proteus* species and *V. cholerea*. *Salmonella* species and *P. aeruginosa* resisted.

The crude methanol and water extract of Chinese green tea (*Camellia sinensis*) exhibited antibacterial activities against *L. monocytogenes* (Mbata *et al*., 2008). The methanol residue of the tea produced larger zones of inhibition against the bacteria than the water extract (Mbata *et al*., 2008). Water soluble green tea extract

also has significant activity with bactericidal action on multi-drug resistant strains of *P. aeruginosa* (Hosseini *et a*l., 2007). Heat-treatment of green tea infusion has been found to increase its antibacterial activities (Ogura, 2006).

Green tea catechins showed a bactericidal effect against black-pigmented, Gram-negative anaerobic rods (Hirasawa *et al*., 2002). The combined use of mechanical treatment and the application of green tea catechins using a slow release local delivery system were effective in improving periodontal status (Hirasawa et al., 2002).

Twenty percent tea extract (50µl), (-)-epigallocatechin-3-gallate (EGCG) (63µg) and theaflavin-3,3'-digallate (125µg) added to one ml of culture medium each inhibited the growth of all strains of MRSA and food poisoning *S. aureus* tested (Hara *et al*., 1991). The tea extract also showed bactericidal activity against MRSA even at the same concentration as in ordinarily brewed tea (Hara *et al*., 1991). Sasaki *et al*. (2004) carried out a study to determine antibacterial activity of polyphenol components in oolong tea extract against *Streptococcus mutans.* The extract showed antibacterial activity against all of the oral *Streptococci* examined, with the highest activity against *S. mutans.* The activity was found to originate from a monomeric polyphenol-rich fraction and it was stronger than that of pure polyphenols. Moreover, some combinations of monomeric polyphenols showed the highest level of antibacterial activity. The results suggested that the antibacterial activity of oolong tea extract is caused by a synergistic effect of monomeric polyphenols, which can easily bind to proteins.

2.7 Combination Effects between Tea extracts and Antibiotics

Recent studies have shown that tea extracts have got effect on efficacy of antibiotics. Esimone *et al*. (2006) investigated the herb-drug interaction between tea (*Camellia sinensis*) extract and penicillin G against three strains of *S. aureus.* The pair combinations in an *in vitro* decimal assay for additivity test were done using three strains of *S. aureus* as test organisms. The results showed that the interactions between penicillin G and tea extracts were mainly additive against the three strains of *S. aureus*. That suggest the concomitant administration of tea and Penicillin G may not impair the antimicrobial activity of Penicillin G (Esimone *et al*., 2006).

Tiwari *et al*. (2005) in their study, attempted to describe the synergistic antimicrobial activity of tea and antibiotics against enteropathogens. Antimicrobial activity of hot water extract and organic solvent extracts of tea were studied against *S. typhi, S. dysenteriae, Y. enterocolitica* and *E. coli*. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and death rate kinetics at MBC of tea extracts in presence of sub-inhibitory concentration of antibiotic were determined. Both green tea and black tea extracts effectively inhibited the growth of *S. typhi, S. dysenteriae, Y. enterocolitica* and *E. coli* (Tiwari *et al.*, 2005). However, the growth inhibitory concentration was lower for green tea as compared to black tea extract (Tiwari *et al*., 2005). Antimicrobial activity of green tea methanol extract: water extract was better as compared to hot water extract of green tea (Tiwari *et al*., 2005). Based on death rate kinetics results, *S.*

typhi appeared to be highly sensitive and *Y. enterocolitica* the most resistant (Tiwari *et al*., 2005). Chloramphenicol and black tea extract in combination inhibited the growth of *S. dysenteriae* at 2.5µg/ml Chloramphenicol (MIC 5 µg/ml) and 5.094 mg/ml black tea extract (MIC 9.089 mg/ml) (Tiwari *et al*., 2005). The tea extract also showed synergistic activity with Chloramphenicol and other antibiotics like gentamycin, methicillin and nalidixic acid against test strains (Tiwari *et al*., 2005).

Gallic acid extract from black tea have synergistic effect with amikacin and sulfamethoxazole in a dose-dependent manner against *E. coli* (Tirang *et al.*, 2007). The microbiologic effects of both black tea and green tea extracts on certain antibiotics against *E. coli* may vary, depending on the type of the tea extract, the amount of the extract and the antibiotic being used (Tirang *et al.*, 2007).

The hot water extract of Sencha (Japanese Green Tea) tea inhibited methicillin resistant *S. aureus* (MRSA) and restored the activity of methicillin against MRSA (Yam *et al*., 1998). The extract also acted synergistically with methicillin against MRSA and the synergistic effect was attributed to catechins (Yam *et al*., 1998).

The (-)-Epicatechin-3-gallate (ECG), a constituent of an extract of green tea leaves markedly lowered the minimum inhibitory concentration (MIC) of oxacillin and other beta-lactams in strains of methicillin-resistant *S. aureus* (Shiota *et al*., 1999). The antibacterial action of (-)-epicatechin-3-gallate (ECG) plus oxacillin was a bactericidal one (Shiota *et al*., 1999).

The (-)-Epicatechin-3-gallate (ECG) sensitizes methicillin-resistant *St. aureus* (MRSA) to beta-lactam antibiotics, promotes staphylococcal cell aggregation and increases cell-wall thickness (Stapleton *et al*., 2004). The potentiation of beta-lactam activity against MRSA by (-)-epicatechin-3-gallate (ECG) was not due to decreased bacterial penicillin-binding protein (PBP2A) expression or ECG binding to peptidoglycan (Stapleton *et al*., 2004).

Compared with ampicillin, oxacillin, cefmetazole and imipenem, the combination of ampicillin and sulbactam at a constant ratio of 2:1 showed the greatest effect against 28 clinical isolates of methicillin-resistant *S. aureus* (MRSA) (Zhi-Qing *et al.*, 2001). However the minimum inhibitory concentrations (MICs) of ampicillin/sulbactam were still above the resistance breakpoint (Zhi-Qing *et al.*, 2001). When ampicillin/sulbactam was further combined with (-)-epicatechin-3-gallate (EGCg, a main constituent of tea catechins), the MIC 90 of ampicillin/ sulbactam was reduced to 4 mg/l, the susceptibility breakpoint (Zhi-Qing *et al.*, 2001). The fractional inhibitory concentration indices were between 0.19 and 0.56 in combination with 6.25 and 25 mg/l EGCg, respectively, indicating that ampicillin/sulbactam and EGCg combination may be effective against MRSA infections (Zhi-Qing *et al.*, 2001).

Zhi-Qing *et al*. (2002) observed additive, indifferent and antagonistic effects in combinations of (-)-epicatechin-3-gallate with 12 non-beta-lactam antibiotics. The test organism was MRSA. The combinations of (-)-epicatechin-3-gallate with the inhibitors of either protein or nucleic acid synthesis showed additive or indifferent

effects. The antibiotics included tetracycline, minocycline, chloramphenicol, streptomycin, gentamicin, kanamycin, erythromycin, rifampicin and ofloxacin. In contrast, (-)-epicatechin-3-gallate showed an antagonistic tendency against glycopeptide antibiotics (vancomycin, teicoplanin and polymyxin B). The common property of these antibiotics is the peptide backbone structure, suggesting a direct binding of (-)-epicatechin-3-gallate (ECG) with the antibiotics (Zhi-Qing *et al*., 2002). The results indicate that tea catechins may affect the activities of antibiotics both positively and negatively (Zhi-Qing *et al*., 2002).

(-)-Epicatechin-3-gallate (EGCG) at doses half and below its calculated MIC of 100 µg/ml, was able to reverse tetracycline resistance in *Staphylococcal* isolates expressing the specific efflux pump Tet (K) (Sudano *et al*., 2004). It appeared to improve the MICs of tetracycline for susceptible *Staphylococcal* isolates as well (Sudano *et al*., 2004). The visible effect of (-)-epicatechin-3-gallate (EGCG) was an increased accumulation of tetracycline inside bacterial cells (Sudano *et al*., 2004). The effect was likely to be due to the inhibition of pump activity and it was evident not only for Tet (K) pumps but also for efflux pumps of a different class namely Tet (B) (Sudano *et al*., 2004). That suggested a dramatic enhancement by EGCG of tetracycline activity for resistant staphylococcal isolates was caused by impairment of tetracycline efflux pump activity and increased intracellular retention of the drug (Sudano *et al*., 2004). That suggested a possible use of EGCG as an adjuvant in antibacterial therapy (Sudano *et al*., 2004).

The antibacterial activity of amoxicillin was also significantly enhanced by the presence of (-)-epicatechin-3-gallate (EGCG) against *Helicobacter pylori* (Yanagawa *et al*., 2003). The combination effect between (-)-epicatechin-3-gallate (EGCG) and other antibiotics, such as metronidazole and clarithromycin, on the antibacterial activity against clinical isolates was additive (Yanagawa *et al*., 2003). The results indicated that (-)-epicatechin-3-gallate (EGCG) may be a valuable therapeutic agent against *H. pylori* infection (Yanagawa *et al*., 2003).

Green tea showed increased antimicrobial activity against bacteria and fungi when used in combination with butylated hydroxyanisole (Simonetti *et al*., 2004). Glycolic extract taken from green tea showed only limited activity against *S. mutans* and no activity against *C. albicans* and certain strains of *E. coli* (Simonetti *et al*., 2004). Butylated hydroxyanisole (BHA) at non-inhibitory concentrations increased the microbicidal activity of green tea against *S. mutans*, non-susceptible *E. coli* and *C. albicans* (Simonetti *et al*., 2004). Green tea in combination with Butylated hydroxyanisole (BHA) reduced the hydrophobicity of *S. mutans* and greatly inhibited the formation of hyphae in *C. albicans* (Simonetti *et al*., 2004). The increased antimicrobial activity of green tea is related to an impairment of the barrier function in micro-organisms and a depletion of thiol groups (Simonetti *et al*., 2004).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Sample Materials

Processed commercial black tea packed by Kenya Tea Packers Limited (KETEPA) was purchased in Nairobi.

3.2 Preparation of Hot Water Extract of Black Tea

The black tea hot water extract was done in accordance with the method of Yam *et al.* (2002) as described by Mbata *et al.* (2006). Two grams of tea was extracted with 100ml of boiling water for 12 minutes and filtered to give a solution that contains 2g/100mL. The extract was freeze dried to powder form and stored at -4^0 C in refrigerator until use.

3.3 Measurement of Total Theaflavins Content of Black Tea

Total theaflavins in hot water extract (infusion) was measured using Flavognost method (Hilton, 1973). Briefly black tea (9g) was infused in 175ml of boiling water. The infusion temperature was kept close to boiling point for 10 minutes. The infusion was carried out in a vacuum flask with continuous mechanical shaking. The hot liquor was filtered and quickly cooled in hot water. Then, a 10ml aliquot of the filtered infusion was shaked for 10 minutes with 10ml of isobutyl methyl ketone and the two layers were allowed to separate. Next, 2ml of the aliquot of the upper layer, 4 ml of ethanol and 2 ml of flavognost reagent (2% w/v diphenylboric

acid-2-aminoethyl ester in ethanol) were mixed well in mechanical shaker. The mixture was allowed to stand for 15 minutes at room temperature and the absorbance was read in spectrophotometer at 625nm. A mixture of isobutyl methyl ketone and ethanol (1:1, v/v) was used as blank. The content of theaflavins in black tea was calculated from the following formula:

Theaflavins (μ mol/g) $=E_{625} \times 47{,}900/$ DM

Where E_{625} was optical density, 47,900 was a constant and DM the dry matter of tea sample.

3.4 Extraction of Theaflavins

Extraction of theaflavins was done using Lai *et al*. (2001) method. Briefly 250g of black tea was weighed and extracted three times using 1.875l of 70% ethanol and then filtered. After the removal of ethanol in a rotary evaporator, the remaining water solution was extracted subsequently using chloroform (0.375l), ethyl acetate (0.25l) and butanol (0.25l). The ethyl acetate extract was applied onto a silica gel column 80 (0.80 to 1.65cm i.d; silica gel 60M, 230-240 mesh). The total TF fraction was obtained when the column was eluted with a mixture of chloroform and ethyl acetate 1:1 (v/v) followed by increasing the ratio of chloroform to ethyl acetate to 4:1 (v/v). The total theaflavin fraction obtained was stored at -4^0C.

3.5 Identification of Isolated Theaflavins

The eluted fractions from the silica gel column were spotted on silica gel thin layer chromatographic plates. They were then subjected to a mobile phase (solvent mixture) having ethyl acetate-acetic acid-water at a ratio of 10:2:3. After spraying, the different compounds appeared as distinct spots at a distance from where they were spotted on the plates. The spots (compounds) that have the same relative mobility front (Rf) were combined. The absorbance of the fractions was measured at 380 and 460 nm using spectrophotometer. Theaflavins are known to have maximum absorbance at 380 nm which is specifically associated with their benzotropolone rings. The chemical (electron) shift around the benzotropolone ring which results in the bright red colour of the theaflavins is responsible for the absorbance maximum at 460 nm. The theaflavins fractions were confirmed by reacting 2ml of theaflavins solution, 4 ml of ethanol and 2 ml of flavognost reagent (2% w/v diphenylboric acid-2-aminoethyl ester in ethanol). Diphenyl boric acid ethanolamine (Flavognost reagent) reacts with the benzotropolone nucleus to form a green chromaphore with a broad absorption maximum at 625 nm.

3.6 Preparation of Tea Extracts Stock and Working Solutions

Two grams of black tea yielded 0.90 g when the water extract was freeze dried. A two-fold dilutions was made to obtain (100%, 50%, 25%, 12.5%) concentrations. Two grams of the same black tea yielded 36µg or µmol of theaflavins. A doubling dilutions of isolated theaflavins was made to obtain (100%, 50%, 25%, 12.5%) concentrations. The concentrations were stored at -4^{0}C until required.

3.7 Preparation of Antibiotic Stock and Working Solutions

The antibiotics were removed from storage (-20^{o}C) and allowed to come to room temperature. Each (250 mg) of antibiotics was weighed and dissolved in suitable solvents and diluted in appropriate diluents (Appendix IV) to make a final 100ml solution. The formula given below was used to obtain stock solutions.

$$W = \frac{1000 \times V\ (ml) \times C\ (\mu g ml^{-1})}{P\ (\mu g ml^{-1})}$$

Where:

P= potency given by manufacturer in relation to base

C=final concentration of the solution

W=weight of antibiotic in mg to be dissolved in V

V= volume required in ml (20ml)

The stock solutions of chloramphenicol, tetracycline, norfloxacin, ciprofloxacin and ampicillin were kept at -20 ^{0}C until use.

3.8 Determination of Minimum Inhibitory Concentration of Antibiotics

Doubling dilutions of stock solutions were made to obtain minimum inhibitory concentration (MIC) of each antibiotic against respective test organism using modified Bauer-Kirby method described in National Committee for Clinical Laboratory Standards (NCCLS, 2002) report. Incubation was done at 37^{o}C for 24 hours and inhibition zones were compared with those recommended by the

National Committee for Clinical Laboratory Standards (NCCLS, 2002) in Appendix III.

3.9 Preparation of Combined Concentrates of Black Tea Extracts and Antibiotics

A series of two-fold dilutions (1- ⅛) were made using MIC of each antibiotic as the starting concentration. These concentrations were then combined with concentrations of hot water extract of black tea and isolated theaflavins (100%, 50%, 25% and 12.5%). Sterile discs were soaked in these combined concentrations, air dried at room temperature and kept at -20^{o}C until use.

3.10 Test Organisms

Control and clinical isolates of *E. coli, E. aeruginosa, S. typhi, P. aeruginosa, S. aureus, E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 were obtained from the National Public Health Laboratories, Nairobi.

3.11 Identification of Bacterial Strains

Viability tests were carried out by picking the organisms from the stock using sterile loop and inoculating into 9ml of peptone water media and then incubated at 37^{o}C for 3 hours. They were then sub-cultured in sterile agar plates having Mueller Hinton Agar (MHA) and incubated at 37^{o}C for 24 hours. Well isolated colonies were picked and used for identification.

3.11.1 Biochemical Typing

Organisms from well separated colonies of similar appearance (appendix I) were transferred from each of the plating media for further testing and an identification mark on the bottom of the Petri dish corresponding to the colonies. Five non-lactose fermenting (NLF) colonies were picked and inoculated into separate tubes of Triple Sugar Iron agar (TSI), Simmon citrate and Urea agar. TSI was inoculated by stabbing the butt and then streaking the slant with a zigzag configuration. Simmon citrate was also inoculated by streaking the surface of the slant with a zigzag configuration. Urea agar was inoculated by stabbing into medium four times. The test tubes with loose caps were incubated at 37^{o}C for 24 hours. The biochemical reactions were interpreted according to biochemical reaction chart for enterobacteriaceae, aeromonas and Plesiomonas.

3.12 Preparation of Inoculants

One colony of each bacterial culture was picked and inoculated into nutrient broth and incubated at 37ºC for 24hrs. The density of a bacterial suspension in nutrient broth was adjusted to match the turbidity of the 0.5 McFarland standard which was equivalent to 1.5 x 10^5 colony forming units (CFU)/ml.

3.13 Sensitivity Test

An inoculum having 1.5 x 10^5 CFU/ml was removed using sterile cotton wrap and spread on Mueller Hinton agar plates. Impregnated discs were aseptically placed evenly on the surface of agar and pressed firmly. The plates were left for 3 hours to

allow the antibiotics to diffuse. The inoculated plates were incubated overnight at 35º C. The zones of inhibition were measured using a transparent ruler the following morning after 18 hours incubation.

3.14 Chi-Square Test

The chi-square test was used to test the null hypothesis.

The formula for calculating chi-square (χ^2) is:

$$(\chi^2)= \frac{(O-E)^2}{E}$$

Where: O is the observed results (mean inhibition zone diameter of isolated theaflavins) and E is the expected results (mean inhibition zone diameter of hot water extract of black tea).

CHAPTER FOUR

RESULTS

4.1 Comparison of inhibitory effect of isolated theaflavins and hot water extract of Kenyan black tea on *Salmonella typhi*

The inhibitory effect of hot water extract of black tea and isolated theaflavins are shown in Table 1. Isolated theaflavins showed stronger inhibitory effect as evidenced by large inhibition zones. The 100%, 50%, and 25% concentrates of the two tea extracts inhibited *S. typhi*. However, it resisted the 12.5% concentration of both extracts. Both 100% and 50% concentrations showed stronger inhibitory effect than minimum inhibitory concentration of ampicillin (10.4µg/ml).

Table 1. Antibacterial activity of hot water extract and isolated theaflavins of Kenyan black tea and MIC of ampicillin against *Salmonella typhi*

Concentrates		**Mean inhibition zone diameter in mm**		
	N	**Ampicillin**	**Hot water extract**	**Isolated theaflavins**
100%	3		14.3	16
50%	3		10.5	13.4
25%	3		7.2	8.9
12.5%	3		NI	NI
MIC of amp (10.4µg/ml)		9.4		

NI: no inhibition, amp: ampicillin, N: number of replicates

There was statistically significant difference in inhibition effect between isolated theaflavins and hot water extract of black tea based on the diameters of zones of

inhibition at (χ^2=0.94; P<0.05). However, there was similarity in pattern of activity as the inhibitory effect increased, with increasing concentration of both tea extracts.

4.2 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Pseudomonas aeruginosa*

Hot water extract of black tea showed lower inhibitory effect (smaller inhibition zone diameter) on *P. aeruginosa* as compared to isolated theaflavins. Both 100% and 50% of the hot water extract of tea inhibited *P. aeruginosa* while 25% and 12.5% concentrates did not (Table 2). Only 12.5% concentrate of isolated theaflavins failed to inhibit *P. aeruginosa*. The 100%, 50% and 25% of isolated theaflavins and 100% and 50% of hot water extract showed stronger inhibitory effect than minimum inhibitory concentration of norfloxacin (6.4µg/ml).

Table 2. Antibacterial activity of hot water extract and isolated theaflavins of Kenyan black tea and MIC of norfloxacin against *Pseudomonas aeruginosa*

Concentrates	**Mean inhibition zone diameter in mm**			
	N	**Norfloxacin**	**Hot water extract**	**Isolated theaflavins**
100%	3		11	13
50%	3		8.34	10.7
25%	3		NI	9.2
12.5%	3		NI	NI
MIC of norf (6.4µg/ml)		6.89		

NI: no inhibition, norf: norfloxacin, N: number of replicates

The difference in inhibitory effect between isolated theaflavins and hot water extract of black tea was significant at (χ^2=1.02; P<0.05). There was similarity in pattern of activity.

4.3 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Pseudomonas aeruginosa* standard (ATCC 27853)

All the concentrates of both hot water extract and isolated theaflavins inhibited *P. aeruginosa* standard (ATCC 27853) except the 12.5% dilution (Table 3). The other concentrations effectively inhibited. Isolated theaflavins had stronger inhibition than hot water extract of black tea as observed in larger inhibition zones. The 100%, 50% of both hot water extract and isolated theaflavins and 25% concentrates of isolated theaflavins also showed stronger inhibitory effect than minimum inhibitory concentration of norfloxacin (4.3µg/ml).

Table 3. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of norfloxacin against *Pseudomonas aeruginosa* standard (ATCC 27853).

Concentrates	Mean inhibition zone diameter in mm		
	N	Norfloxacin	Hot water extract	Isolated theaflavins
100%	3		14	15.68
50%	3		11.96	13.59
25%	3		8.3	9.4
12.5%	3		NI	NI
MIC of norf (4.3µg/ml)		8.5		

NI: no inhibition, norf: norfloxacin, N: number of replicates

There was significant difference between inhibitory effect of concentrates of hot water extract and isolated theaflavins of black tea at (χ^2=0.56; P<0.05). The pattern of inhibitory effect was however similar. *P. aeruginosa* standard (ATCC 27853) was more susceptible to both tea extracts (Table 3) than *P. aeruginosa* (Table 2). *P. aeruginosa* resistance to hot water extract of black tea was high, showing highly significant difference in inhibitory effect with isolated theaflavins at (χ^2=1.02; P<0.05). The difference in inhibitory effect between the tea extracts on *P. aeruginosa* standard (ATCC 27853) was significantly lower at (χ^2=0.56; P<0.05).

4.4 Comparison of inhibitory effect on hot water extract and isolated theaflavins of Kenyan black tea on *Staphylococcus aureus* standard (ATCC 25923)

Isolated theaflavins effectively inhibited *S. aureus* standard (ATCC 25923) as compared to hot water extract of black tea as shown by larger inhibition zone (Table 4). All the concentrates 100%, 50%, 25% and 12.5% of isolated theaflavins had stronger activity than minimum inhibitory concentration (1.2µg/ml) of ciprofloxacin.

Table 4. Antibacterial activity of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923)

Concentrates		Mean inhibition zone diameter in mm		
	N	Ciprofloxacin	Hot water extract	Isolated theaflavins
100%	3		17	20
50%	3		15.4	17.96
25%	3		12.9	14.5
12.5%	3		10	12.2
MIC of cipro (1.2 µg/ml)	11			

Cipro: ciprofloxacin, N: number of replicates

Only 12.5% concentration of hot water extract of black tea had lower activity than the minimum inhibitory concentration (1.2µg/ml) of ciprofloxacin. There was statistically significant difference in inhibitory effect between isolated theaflavins and hot water extract of black tea on *S. aureus* standard (ATCC 25923) at (χ^2=4.42; P<0.05).

4.5 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Staphylococcus aureus*

Hot water extract of black tea had lower inhibitory activity on *S. aureus* than isolated theaflavins (Table 5). Isolated theaflavins effectively inhibited with large zones of inhibition. All the concentrates 100%, 50%, 25% and 12.5% of isolated theaflavins showed stronger inhibitory effect than the minimum inhibitory concentration (2µg/ml) of ciprofloxacin. Only 12.5% concentrate of hot water

extract showed lower inhibitory effect than minimum inhibitory concentration (2µg/ml) of ciprofloxacin.

Table 5. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of ciprofloxacin against *Staphylococcus aureus*

Concentrates	Mean inhibition zone diameter in mm		
	N	Ciprofloxacin	Hot water extract	Isolated theaflavins
100%	3		15	19
50%	3		13.7	16.8
25%	3		11.8	14.15
12.5%	3		9.2	12.6
MIC of cipro (2 µg/ml)		9.8		

Cipro: ciprofloxacin, N: number of replicates

The inhibitory effect between isolated theaflavins and hot water extract of black tea was significant at (χ^2=1.01; P<0.05).

4.6 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Enterobacter aeruginosa*

The 12.5% concentrates of hot water extract and isolated theaflavins did not inhibit *E. aeruginosa.* However isolated theaflavins showed stronger inhibitory effect than hot water extract of black tea (Table 6). Only 25% concentration of hot water extract of tea showed lower inhibitory effect than minimum inhibitory concentration of (5.25µg/ml) of tetracycline. The 100%, 50% and 25% of isolated

theaflavins showed stronger inhibitory effect than minimum inhibitory concentration (5.25µg/ml) of tetracycline.

Table 6. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of tetracycline against *Enterobacter aeruginosa*

Concentrates	Mean inhibition zone diameter in mm			
	N	Tetracyline	Hot water extract	Isolated theaflavins
100%	3		14.2	17
50%	3		11	14
25%	3		6.9	10.3
12.5%	3		NI	NI
MIC of tet (5.25 µg/ml)		7.8		

NI: no inhibition, tet: tetracycline, N: number of replicates

The difference in level of inhibitory effect between isolated theaflavins and hot water extract of black tea on *E. aeruginosa* was highly significant at (χ^2=3.04; P<0.05).

4.7 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Escherichia coli*

The 100%, 50%, 25% and 12.5% concentrations of both black tea extracts effectively inhibited *E. coli*. However isolated theaflavins showed stronger activity with larger zones of inhibition (Table 7). All the concentrates of hot water extract and isolated theaflavins showed better inhibitory effect than the minimum inhibitory concentration of chloramphenicol.

Table 7. Antibacterial activities of varying concentrations of isolated theaflavins hot water extract of Kenyan black tea and MIC of chloramphenicol against *Escherichia coli*

Concentrates		Mean inhibition zone diameter in mm		
	N	Chloramphenicol	Hot water extract	Isolated theaflavins
100%	3		13	16
50%	3		11.6	14.4
25%	3		9.8	10.56
12.5%	3		7.2	8.4
MIC of chlo (12 µg/ml)		6.8		

Chlo: chloramphenicol, N: number of replicates

The difference in inhibitory effect between concentrates of isolated theaflavins and hot water extracts of black tea was significant at (χ^2=1.62; P<0.05). The pattern of inhibitory effect was similar as it increased with increasing concentration of both tea extracts.

4.8 Comparison of inhibitory effect of hot water extract and isolated theaflavins of Kenyan black tea on *Escherichia coli* standard (ATCC 25922)

E. coli standard (ATCC 25922) was effectively inhibited by hot water extract and isolated theaflavins of black tea (Table 8). However hot water extract of black tea showed lower inhibitory effect as shown by smaller zones of inhibition than that of isolated theaflavins. Both tea extracts have strong inhibition to *E. coli* standard (ATCC 25922) than the minimum inhibitory concentration (8.4µg/ml) of chloramphenicol.

Table 8. Antibacterial activities of varying concentrations of isolated theaflavins and hot water extract of Kenyan black tea and MIC of chloramphenicol against *Escherichia coli* standard (ATCC 25922).

Concentrates	Mean inhibition zone diameter in mm		
	N	Chloramphenicol	Hot water extract	Isolated theaflavins
100%	3		15	18
50%	3		12.8	15.2
25%	3		10.4	12.56
12.5%	3		8.35	9.4
MIC of chlo (8.4 µg/ml)	8			

Chlo: chloramphenicol, N: number of replicates

The inhibitory effect between concentrates of isolated theaflavins and hot water extracts was significant at (χ^2=1.74; P<0.05). This difference was slightly higher than that of *E. coli* (χ^2=1.62; P<0.05).

4.9 The effect of hot water extract of Kenyan black tea on the efficacy of antibiotics

The 100%, 50% and 25% concentrates of hot water extract of black tea with minimum inhibitory concentration (10.4µg/ml) of ampicillin acted synergistically against *S. typhi* as shown in Figure 3a. That was observed as the sum of activity of concentrates of hot water extract of black tea with MIC of ampicillin as presented in Table 1 did not exceed that presented in Figure 3a. The doubling dilutions of the MIC (5.2, 2.6 and 1.3 µg/ml) of ampicillin have no activities of their own. Their activities were restored when they were combined with 100%, 50% and 25% concentrates of hot water extract of black tea. That was also seen as the activities of

the combined hot water concentrates with 5.2, 2.6 and 1.3 µg/ml of ampicillin (Figure 3a) exceeded that of the concentrates of tea extract only as presented in Table 1. The restoring of activity of ampicillin clearly demonstrated synergism with hot water extract of black tea.

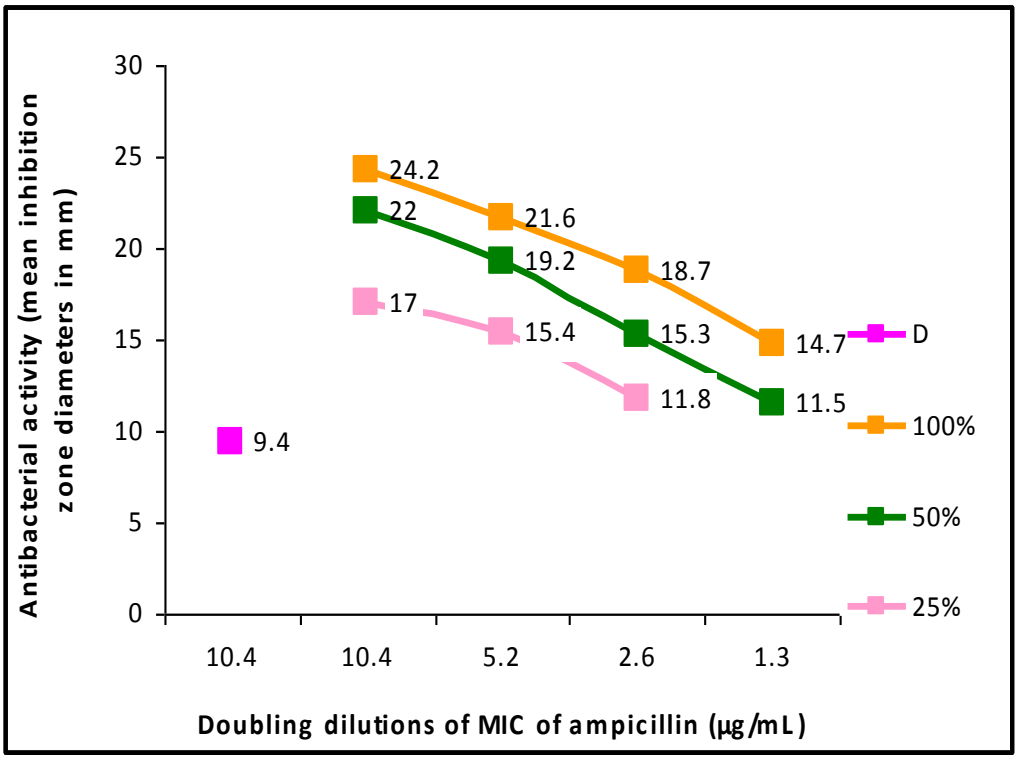

Figure 2a. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC of ampicillin against *Salmonella typhi*

D- Inhibition zone diameter of minimum inhibitory concentration (10.4 µg/ml) of ampicillin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, 12.5%- dilution of 100% to ⅛ of its original concentration, N=3.

Synergism was also observed between MIC (4.3 µg/ml) of norfloxacin, a fluoroquinolone and the 100% and 50% concentrates of hot water extract of black tea against *P. aeruginosa* (Figure 3b). That was in comparison with that of the sum

of activity of individual concentrate of hot water extract of black tea and MIC of norfloxacin as presented in Table 2. The two concentrates of tea extract were also capable of restoring the activity of the doubling dilutions (2.15 and 1.075 µg/ml) of MIC of norfloxacin against *P. aeruginosa*.

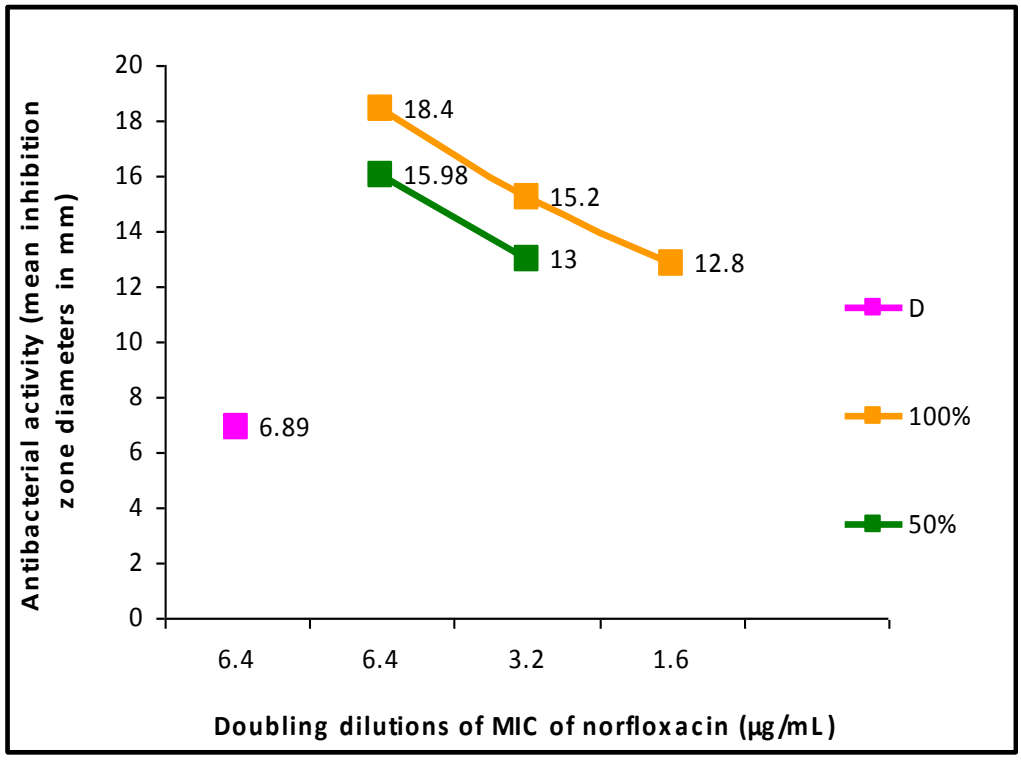

Figure 2b. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (6.4µg/ml) of norfloxacin against *Pseudomonas aeruginosa*

D- Inhibition zone diameter of minimum inhibitory concentration (6.4µg/ml) of norfloxacin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate, N=3.

The synergism of hot water extract of black tea with MIC of norfloxacin was further observed against *P. aeruginosa* (ATCC 27853). The bacteria was less resistant to the hot water extract of black tea than *P. aeruginosa*, resisting only the

12.5% concentrate (Figure 3c). Therefore, the inhibition zones of combined concentrates of hot water extract with MIC of norfloxacin against *P. aeruginosa* (ATCC 27853) were larger than those in *P. aeruginosa* cultures. This difference was also observed in activity of combined hot water extract of black tea with doubling dilutions of MIC of norfloxacin. The activities of doubling dilutions of MIC of norfloxacin were also restored.

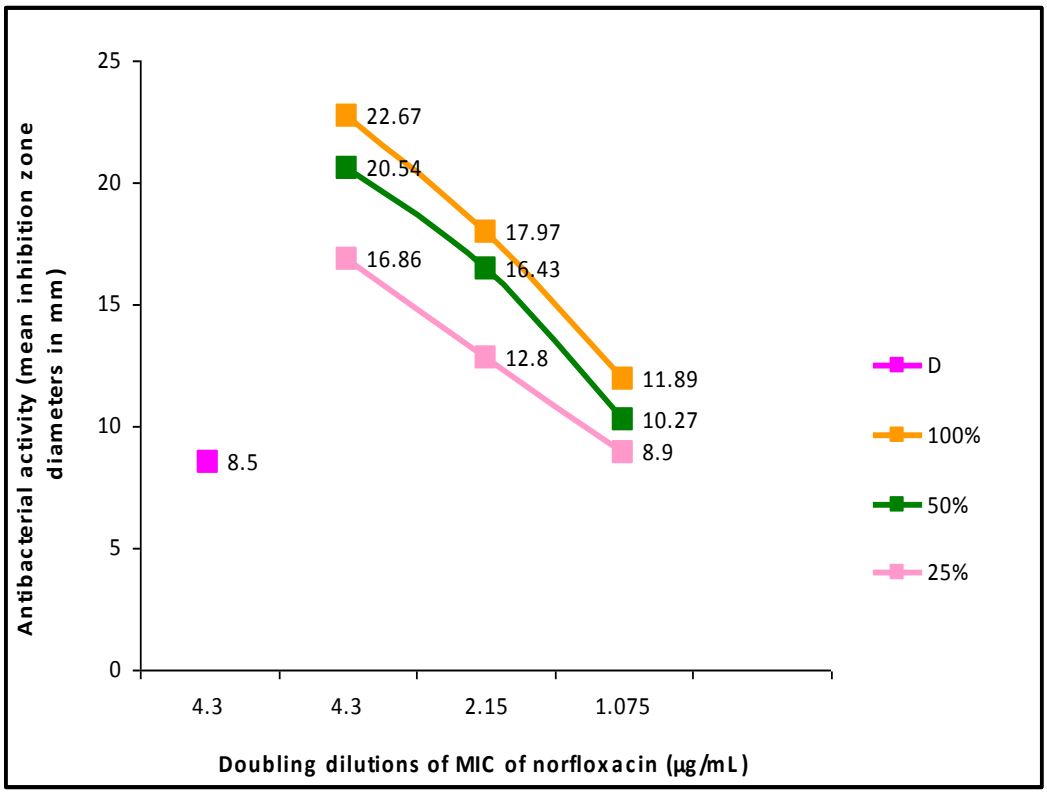

Figure 2c. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (4.3µg/ml) of norfloxacin against *Pseudomonas aeruginosa* (ATCC 27853).

D- Inhibition zone diameter of minimum inhibitory concentration (4.3µg/ml) of norfloxacin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

Synergistic effect of hot water extract of black tea with fluoroquinolone was also observed with MIC of ciprofloxacin against *S. aureus*. The *S. aureus* was very susceptible to the combined concentrates (Figure 3d). The hot water extract of black tea also restored the activity of doubling dilutions of MIC of ciprofloxacin (1, 0.5 and 0.25 µg/ml).

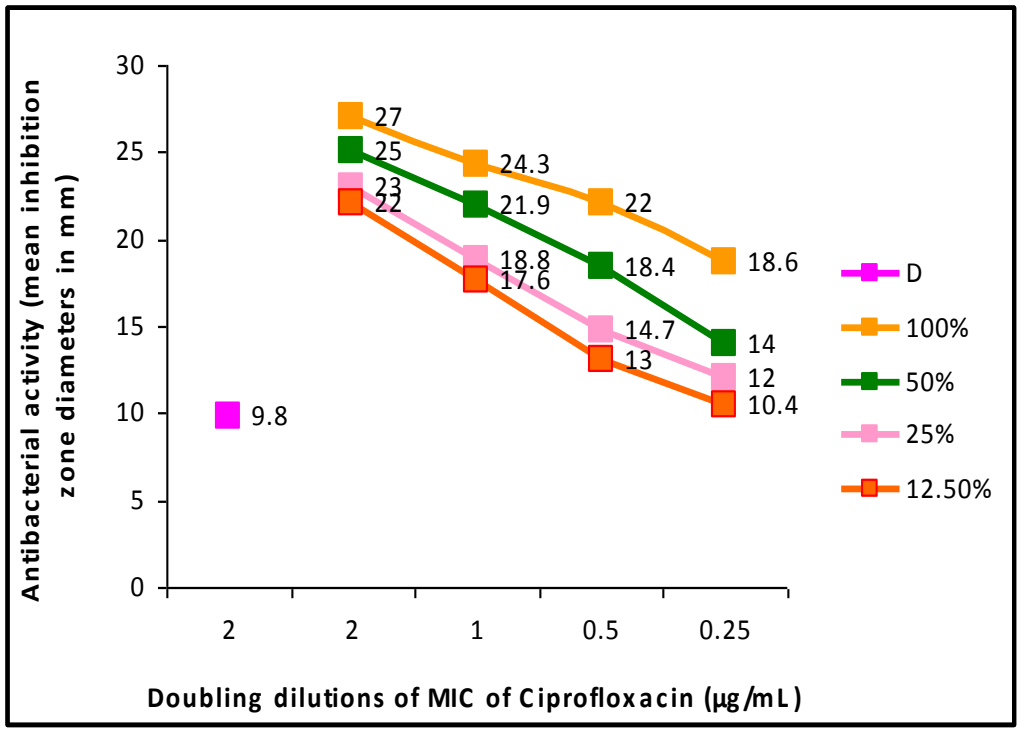

Figure 2d. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (2µg/ml) of ciprofloxacin against *Staphylococcus aureus*

D- Inhibition zone diameter of minimum inhibitory concentration (2µg/ml) of ciprofloxacin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, 12.5%- dilution of 100% to ⅛ of its original concentration, N=3.

Synergism of hot water extract of black tea with MIC of ciprofloxacin was also observed when *S. aureus* (ATCC 25923) was used as test bacteria (Figure 3e). The

susceptibility of *S. aureus* (ATCC 25923) to combined concentrates of hot water extracts with MIC and its doubling dilutions of ciprofloxacin was high as compared to that of *S. aureus* (Figure 3d).

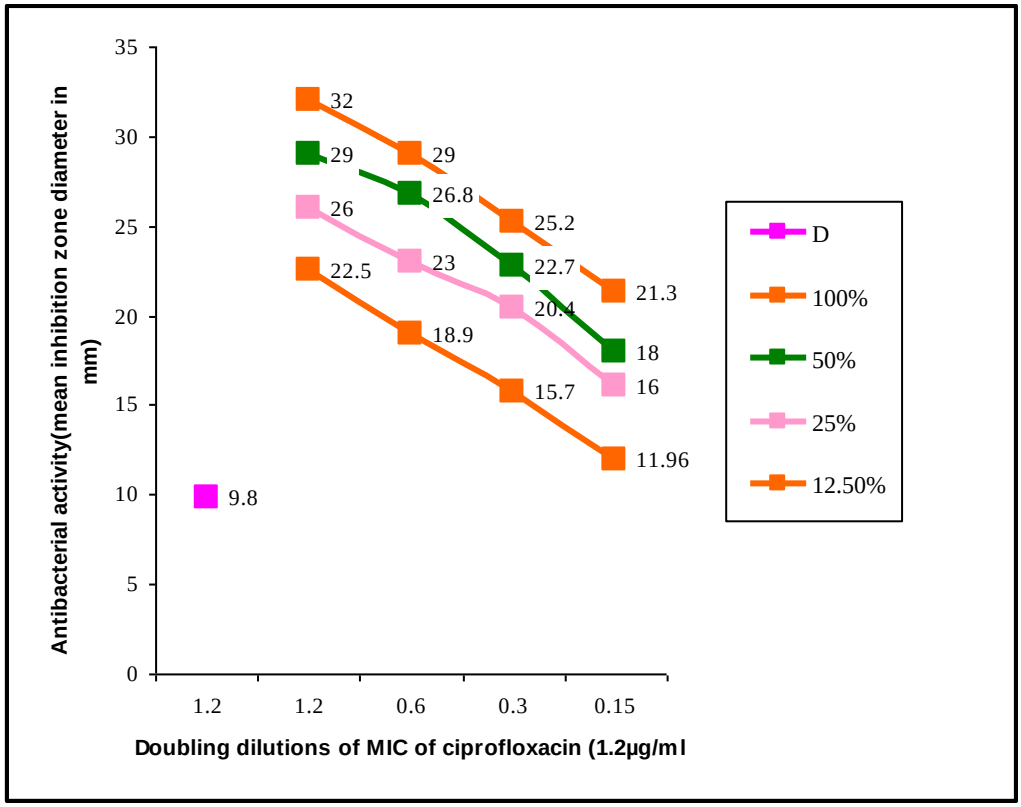

Figure 2e. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (1.2 µg/ml) of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923)

D- Inhibition zone diameter of minimum inhibitory concentration (1.2µg/ml) of ciprofloxacin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, 12.5%-dilution of 100% to ⅛ of its original concentration, N=3.

Tetracycline with hot water extract of black tea acted synergistically against *E. aeruginosa* (Figure 3f). Synergism was observed when 100%, 50% and 25% concentrates of hot water extract of black tea were combined with MIC (5.25

µg/ml) of tetracycline. Hot water extract was also capable of restoring the activity of doubling dilutions (2.625, 1.3125 and 0.656 µg/ml) of tetracycline (Figure 3f).

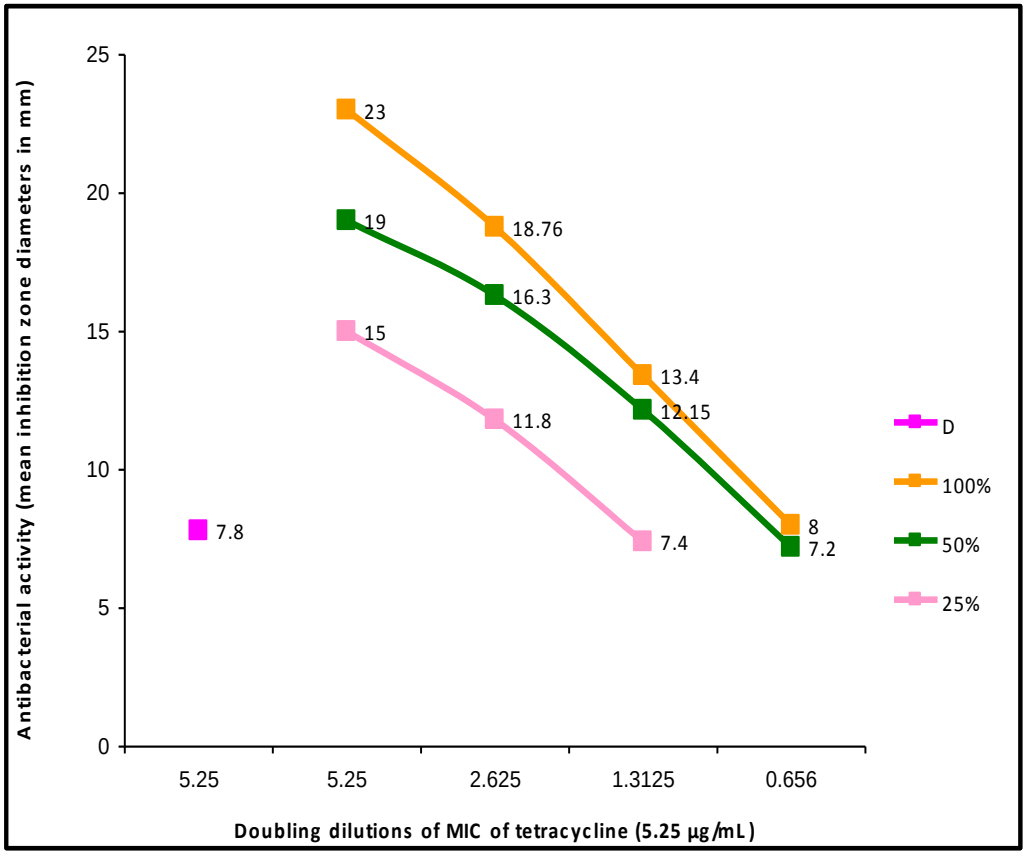

Figure 2f. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (5.25µg/ml) of tetracycline against *Enterobacter aeruginosa*

D- Inhibition zone diameter of minimum inhibitory concentration (5.25µg/ml) of tetracyline, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

The combination of MIC (12 µg/ml) of chloramphenicol with 100%, 50%, 25% and 12.5% concentrates of hot water extract of black tea also showed synergistic effect against *E. coli* (Figure 3g). *E. coli* was very susceptible to the combined concentrates of hot water extract of black tea with MIC of chloramphenicol. That

was observed in large inhibition zones around the discs having these combinations. The concentrates of hot water extract of black tea restored the activity of doubling dilutions (6, 3 and 1.5 µg/ml) of MIC of chloramphenicol.

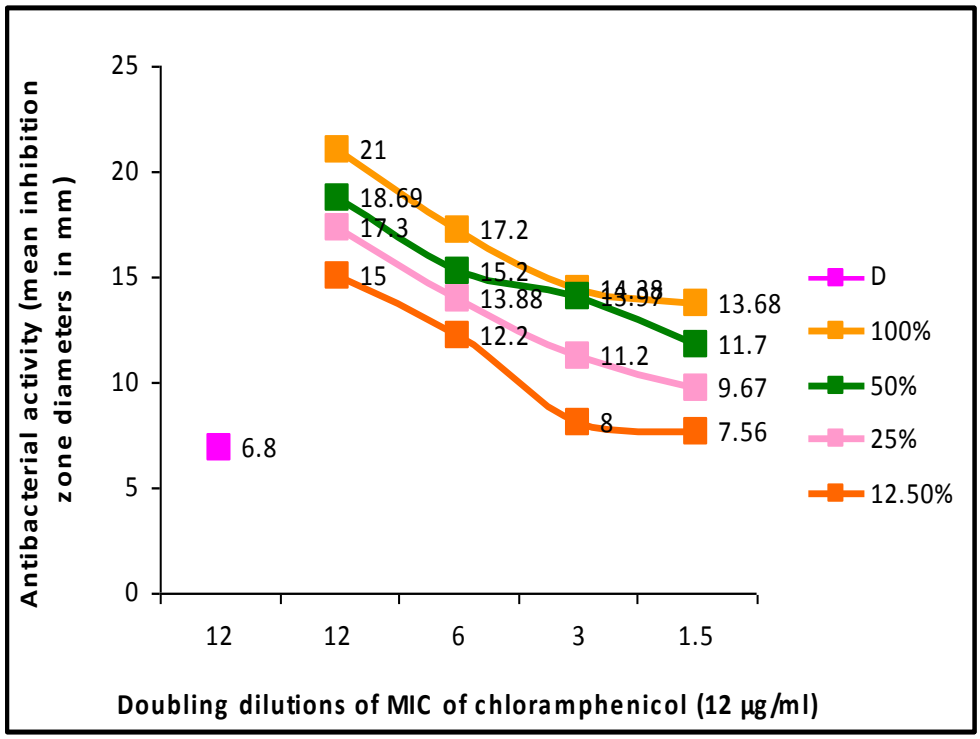

Figure 2g. Antibacterial activity of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (12µg/ml) of chloramphenicol against *Escherichia coli*

D- Inhibition zone diameter of minimum inhibitory concentration (12µg/ml) of chloramphenicol, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

Synergism of MIC of chloramphenicol with concentrates of hot water extract of black tea was also observed when *E. coli* (ATCC 25922) was used as test bacteria (Figure 3h). The MIC of chloramphenicol against *E. coli* (ATCC 25922) was 8.4 µg/ml compared to 12 µg/ml of *E. coli*. *E. coli* (ATCC 25922) was therefore more susceptible to the combined MIC of chloramphenicol with concentrates of hot

water extract of black tea as observed in large inhibition zones (Figure 3h). That was also observed in combined activity of doubling dilutions (4.2, 2.1 and 1.05 µg/ml) of MIC (8.4 µg/ml) of chloramphenicol (Figure 3h).

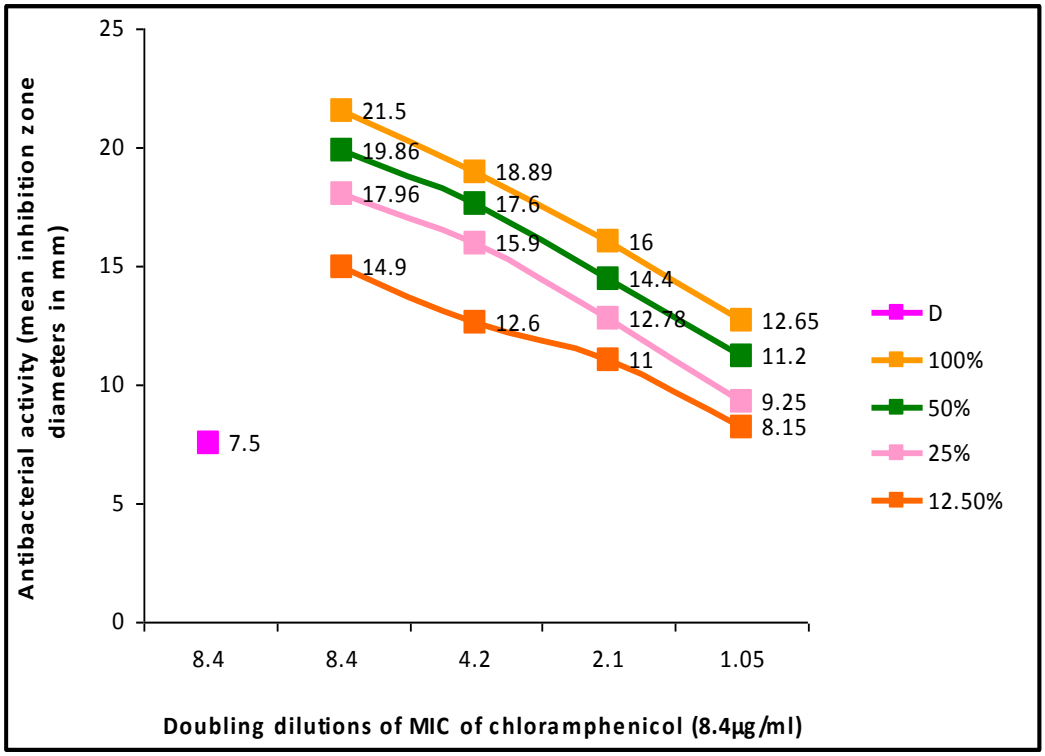

Figure 2h. Antibacterial activities of combined concentrates of hot water extracts of black tea with doubling dilutions of MIC (8.4 µg/ml) of chloramphenicol against *Escherichia coli* (ATCC 25922)

D- Inhibition zone diameter of minimum inhibitory concentration (8.4µg/ml) of chloramphenicol, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

4.10 Synergism of isolated theaflavins of Kenyan black tea with antibiotics

4.10.1 Synergistic antibacterial activities of isolated theaflavins and ampicillin

A synergistic antibacterial activity of isolated theaflavins of black tea and ampicillin against S. typhi is presented in Figure 4a below. Isolated theaflavins acted synergistically with minimum inhibitory concentration (10.4 µg/ml) of

ampicillin. Isolated theaflavins like hot water extract of black tea, restored the activity of doubling dilutions (5.2, 2.6 and 1.3 µg/ml) of MIC of ampicillin. Otherwise the combined activity of isolated theaflavins and doubling dilutions of MIC of ampicillin would have been equal to that of concentrates of isolated theaflavins alone (Table 1), if activity was not restored.

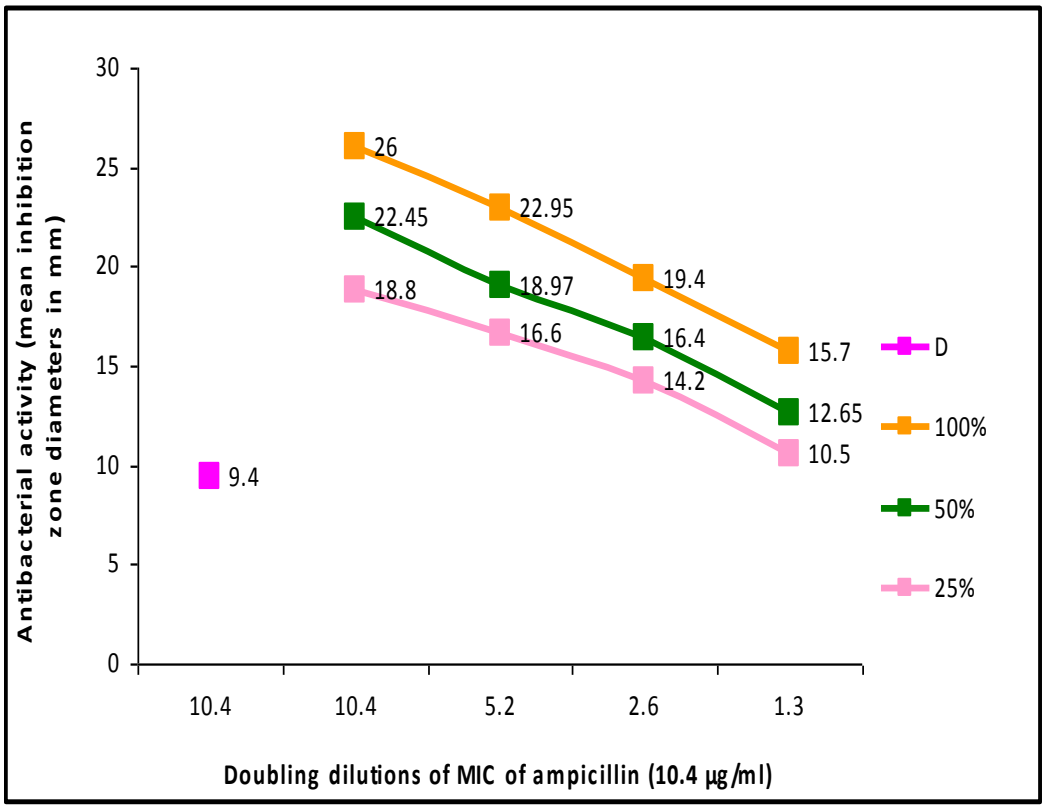

Figure 3a. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of ampicillin against *Salmonella typhi*

D- Inhibition zone diameter of minimum inhibitory concentration (10.4µg/ml) of ampicillin, 100% –undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%- dilution of 100% to ¼ of its original concentration, N=3.

4.10.2 Synergistic antibacterial activity of isolated theaflavins and norfloxacin

Isolated theaflavins showed synergistic activity with norfloxacin, a fluoroquinolone against *P. aeruginosa* (Figure 4b). Synergism was observed when 100%, 50% and 25% concentrates of isolated theaflavins were combined with MIC (6.4 µg/ml) of norfloxacin. The concentrates of isolated theaflavins also restored the activity of doubling dilutions (2.5 and 1.075 µg/ml) of norfloxacin.

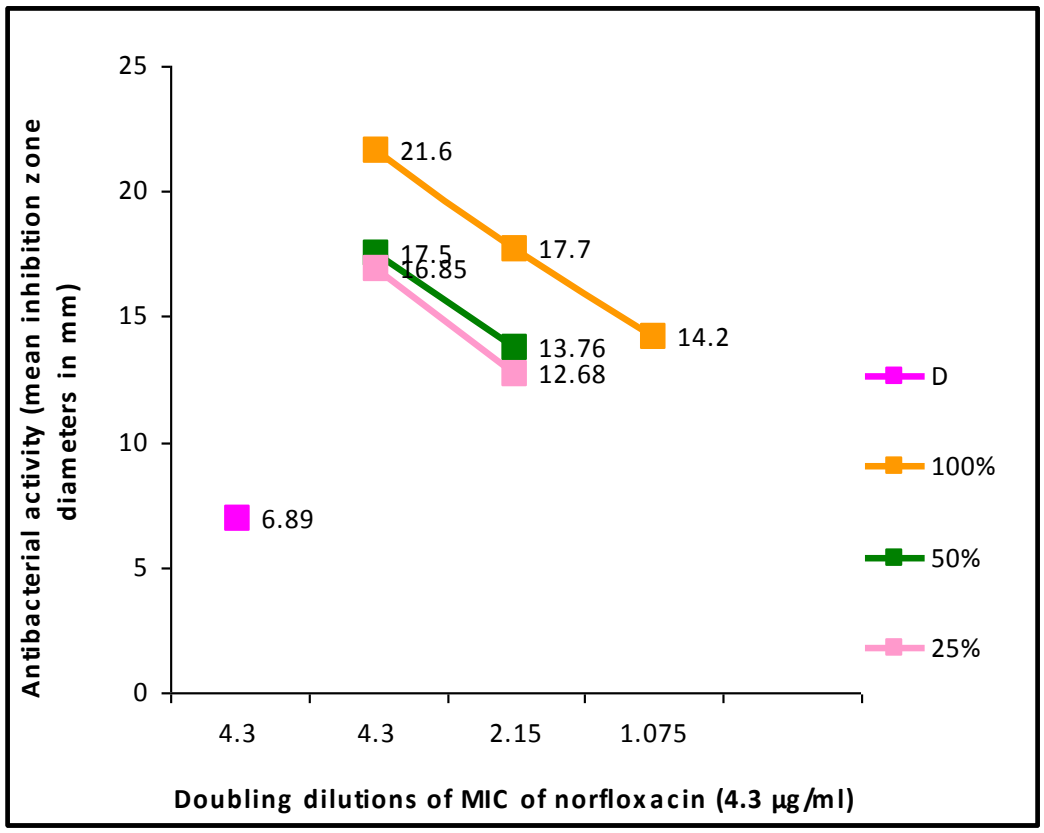

Figure 3b. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (4.3µg/ml) of norfloxacin against *Pseudomonas aeruginosa*

D- Inhibition zone diameter of minimum inhibitory concentration (4.3µg/ml) of norfloxacin, 100% –undiluted isolated, 50%-a half dilution of 100% concentrate, 25%- dilution of 100% to ¼ of its original concentration, N=3.

The synergistic antibacterial activity of isolated theaflavins and norfloxacin was also observed when *P. aeruginosa* (ATCC 27853) was used instead of *P. aeruginosa*. However the level of synergism and restored activity differed. *P. aeruginosa* (ATCC 27853) was less resistant (Figure 4c) with the MIC (4.3 µg/ml) of norfloxacin against it.

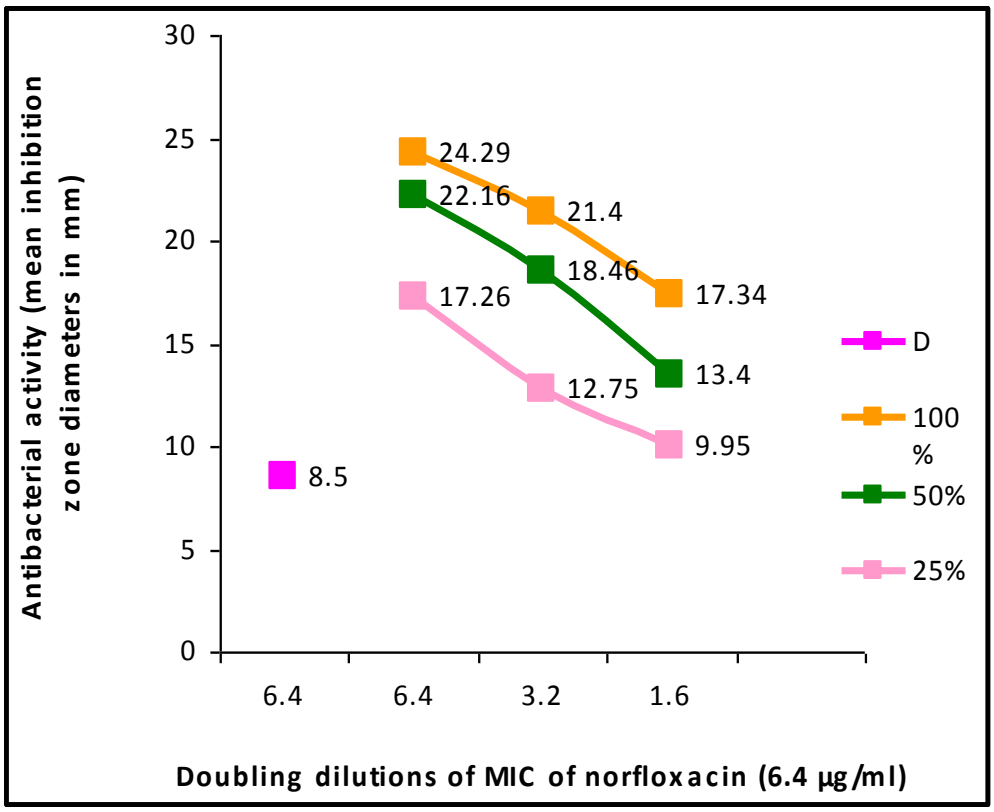

Figure 3c. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of norfloxacin against *Pseudomonas aeruginosa* (ATCC 27853)

D- Inhibition zone diameter of minimum inhibitory concentration (6.4µg/ml) of norfloxacin, 100% –undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate, 25%- dilution of 100% to ¼ of its original concentration, N=3.

4.10.3 Synergistic antibacterial activity of isolated theaflavins and ciprofloxacin

The synergistic effects between 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins and MIC (2 µg/ml) of ciprofloxacin against *S. aureus* was observed (Figure 4d).These combinations effectively inhibited *S. aureus* as shown by large inhibition zones in the cultures. Isolated theaflavins also restored the activity of doubling dilutions (1, 0.5 and 0.25 µg/ml) of ciprofloxacin.

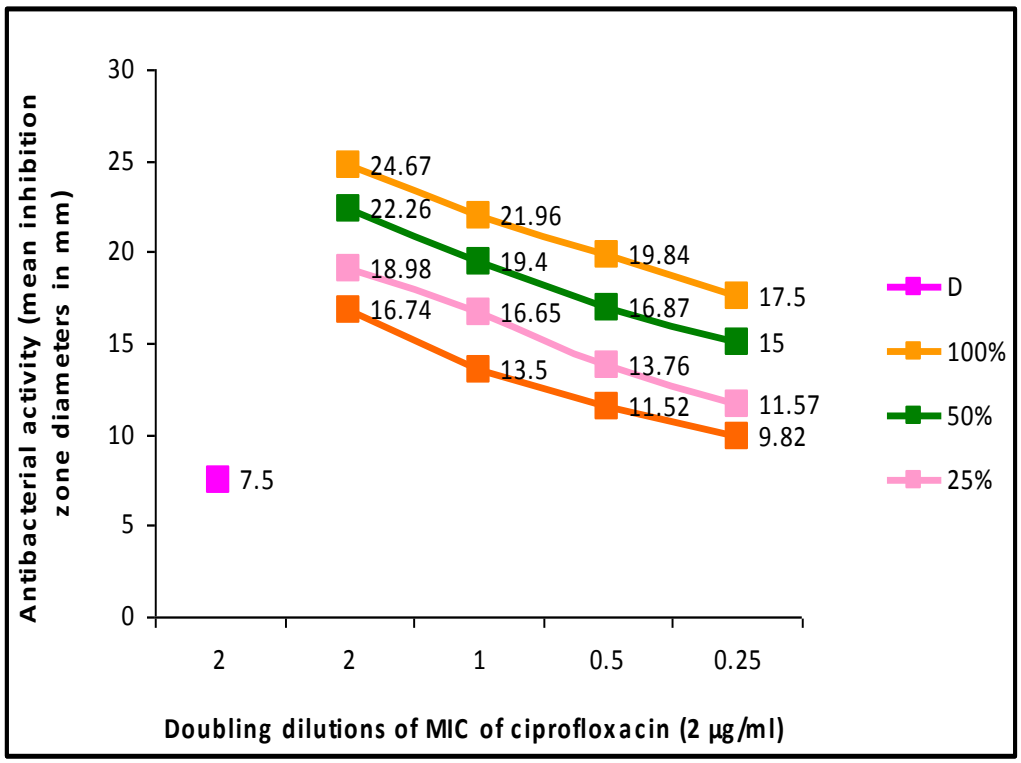

Figure 3d. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC of ciprofloxacin against *Staphylococcus aureus*

D- Inhibition zone diameter of minimum inhibitory concentration (2µg/ml) of ciprofloxacin, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, 12.5%- dilution of 100% to ⅛ of its original concentration, N=3.

The synergistic activity between MIC of ciprofloxacin and concentrates of isolated theaflavins was clearly demonstrated when *S. aureus* (ATCC 25923) was used instead of *S. aureus* (Figure 4e). That was observed in larger inhibition zones in *S. aureus* (ATCC 25923) cultures. Also the activity of doubling dilutions (0.6, 0.3 and 0.15 µg/ml) of ciprofloxacin was restored by isolated theaflavins.

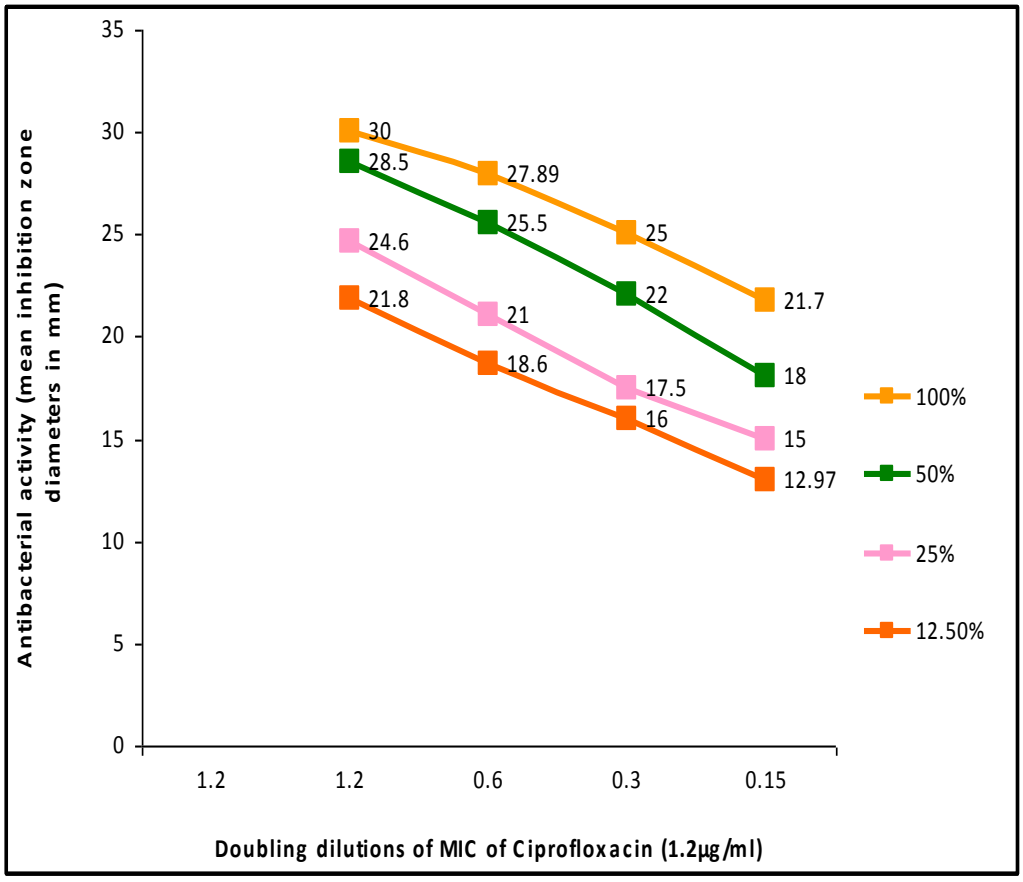

Figure 3e. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (1.2 µg/ml) of ciprofloxacin against *Staphylococcus aureus* (ATCC 25923)

D- Inhibition zone diameter of minimum inhibitory concentration (1.2µg/ml) of ciprofloxacin, 100% –undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, 12.5%- dilution of 100% to ⅛ of its original concentration, N=3.

4.10.4 Synergistic antibacterial activity of isolated theaflavins and tetracycline

The combination of isolated theaflavins concentrates (100%, 50% and 25%) and minimum inhibitory concentration (5.25 µg/ml) of tetracycline showed synergistic effect. That was observed when activity was tested on *E. aeruginosa* (Figure 4f). Isolated theaflavins concentrates restored the activity of tetracycline when combined with its doubling dilutions (2.625, 1.3125 and 0.656 µg/ml). *E. aeruginosa* has developed resistance to antibiotics. The combined formulation of tetracycline and isolated theaflavins effectively inhibited.

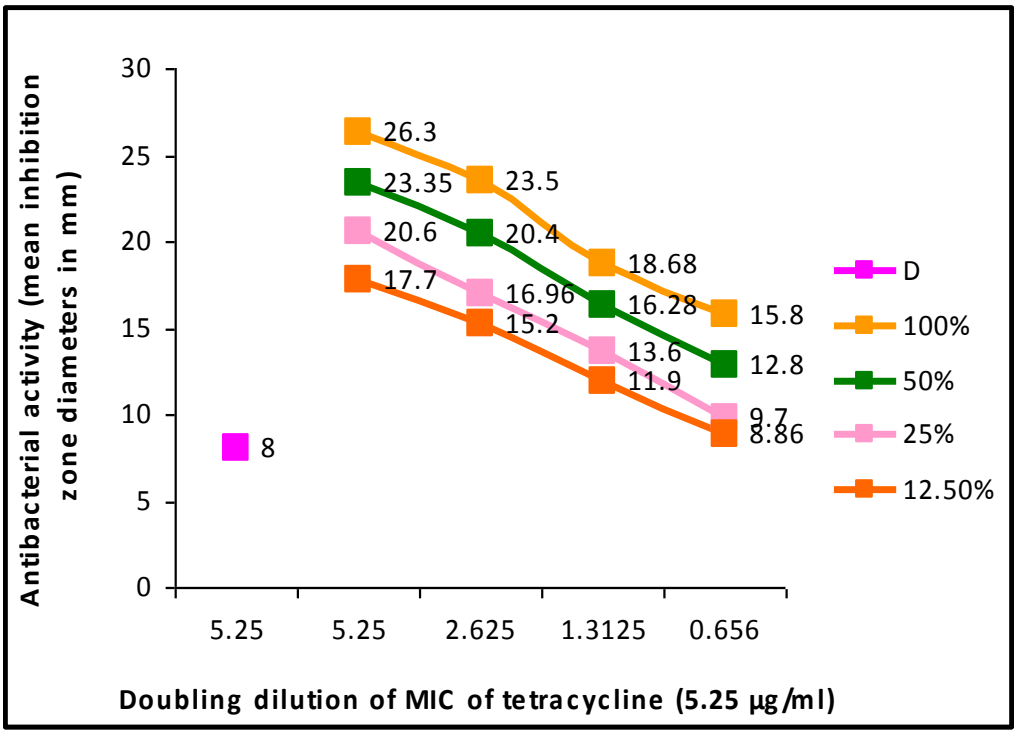

Figure 3f. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (5.25µg/ml) of tetracycline against *Enterobacter aeruginosa*

D- Inhibition zone diameter of minimum inhibitory concentration (5.25µg/ml) of tetracyline, 100% –undiluted hot water extract, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

4.10.5 Synergistic antibacterial activity of isolated theaflavins and chloramphenicol

Isolated theaflavins and chloramphenicol showed strong synergistic effect against *E. coli*. That was observed when minimum inhibitory concentration (12 µg/ml) of chloramphenicol was combined with 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins (Figure 4g). The combination effectively inhibited *E. coli* as shown by large inhibition zones. The activity of chloramphenicol was also restored when doubling dilutions (6, 3 and 1.5 µg/ml) of its MIC were combined with isolated theaflavins concentrates. The doubling dilutions have no activity against *E. coli* on their own. The resulting activity after combination with isolated theaflavins concentrates exceeded that of the concentrates only. That indicated doubling dilutions of MIC contributed to the overall activity.

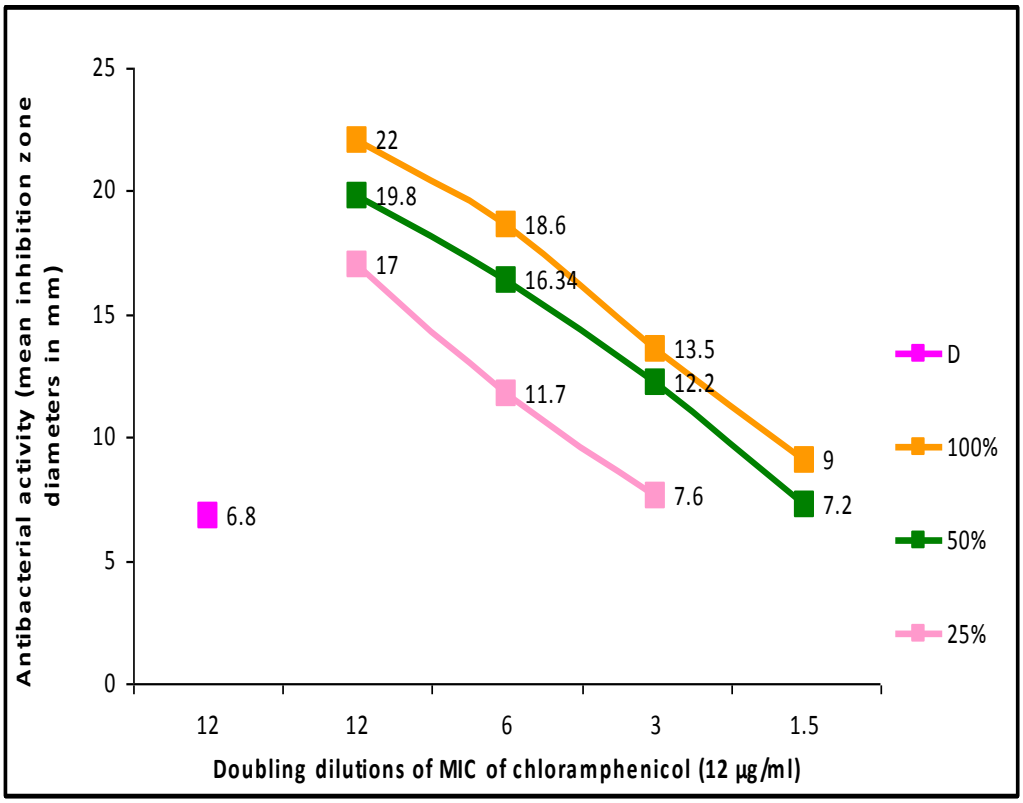

Figure 3g. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (12µg/ml) of chloramphenicol against *Escherichia coli*

D- Inhibition zone diameter of minimum inhibitory concentration (12µg/ml) of chloramphenicol, 100% –undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

Synergism was also observed when *E. coli* (ATCC 25922) was used as test bacteria

(Figure 4h).

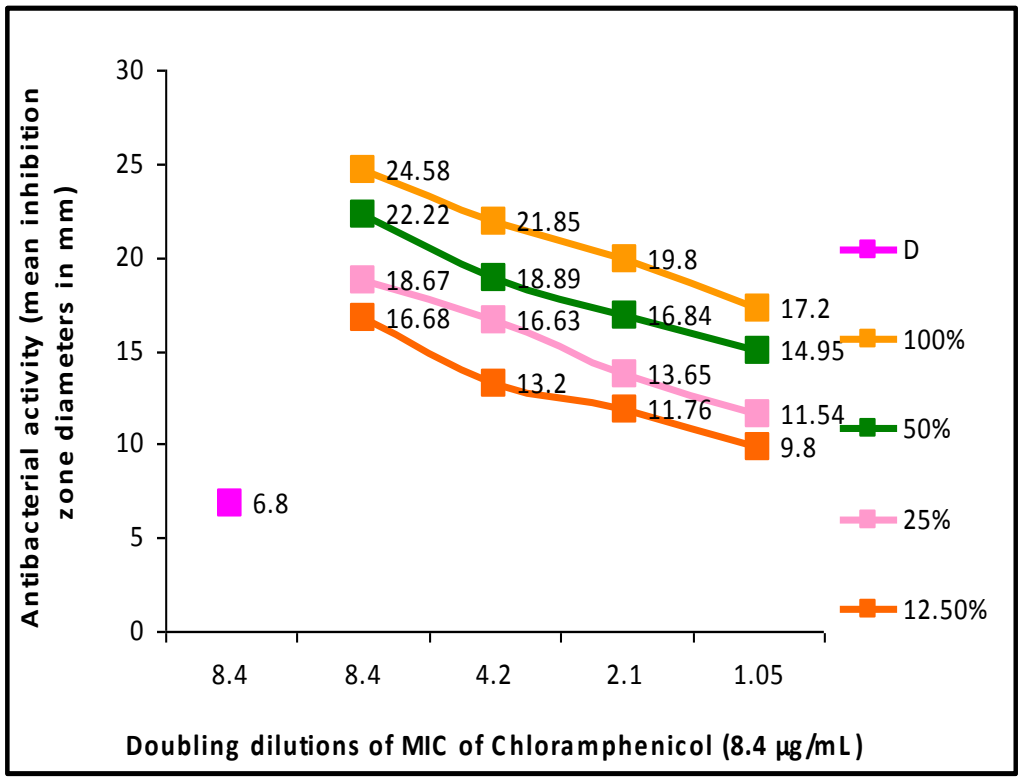

Figure 3h. Antibacterial activities of combined concentrates of isolated theaflavins of black tea with doubling dilutions of MIC (8.4 µg/ml) of chloramphenicol against *Escherichia coli* (ATCC 25922)

D- Inhibition zone diameter of minimum inhibitory concentration (8.4µg/ml) of chloramphenicol, 100% –undiluted isolated theaflavins, 50%-a half dilution of 100% concentrate , 25%- dilution of 100% to ¼ of its original concentration, N=3.

The 100%, 50%, 25% and 12.5% concentrates of isolated theaflavins were combined with MIC (8.4 µg/ml) of chloramphenicol against *E. coli* (ATCC 25922). The differences in inhibition in *E. coli* and *E. coli* (ATCC 25922) cultures was due to the differences in susceptibility.

4.11 The effect of other chemical components in black tea infusion on theaflavins interaction with antibiotics

Isolated theaflavins showed stronger inhibitory effect against the bacterial species tested. The differences in inhibitory effect was significant at ($P<0.05$). The

difference in inhibitory effect between combined concentrates of hot water extract and isolated theaflavins with MIC (10.4 µg/ml) of ampicillin against *S. typhi* was significant at (χ^2=0.56; P<0.05). There was similar observation when different bacteria species and different antibiotics were used. The differences in inhibitory effect was significant at (χ^2=0.699; P<0.05) between the two black tea extracts combinations with MIC (4.3 µg/ml) of norfloxacin against *P. aeruginosa*. It was also significant at (χ^2=0.425; P<0.05) when *P. aeruginosa* (ATCC 27853) was used as test bacteria instead of *P. aeruginosa*. The combination of concentrates of hot water extract and isolated theaflavins with MIC (2 µg/ml) of ciprofloxacin differed significantly in level of inhibition at (χ^2=1.98; P<0.05) against *S. aureus*. The difference in inhibitory effect was significant at (χ^2=0.67; P<0.05) when *S. aureus* (ATCC 25923) was used instead. When the concentrates of the two black tea extracts were combined with MIC (5.25 µg/ml) of tetracycline, the inhibitory effect differed significantly at (χ^2=2.27; P<0.05) against *E. aeruginosa*. Similar observation was also significant at (χ^2=0.4; P<0.05) when concentrates of the two black tea extracts were combined with MIC (12 µg/ml) of chloramphenicol against *E. coli*. While the difference in inhibitory effect was highly significant at (χ^2=1.039; P<0.05) when *E. coli* (ATCC 25922) was used instead.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The present *in vitro* study clearly demonstrated that hot water extract of Kenyan black tea and theaflavins isolated from the same tea exhibit synergism with ampicillin, tetracycline, chloramphenicol, ciprofloxacin and norfloxacin. The synergistic activity was observed when minimum inhibitory concentration (MIC) of each antibiotic was combined with varying concentrations of isolated theaflavins and hot water extract of black tea. The two black tea extracts also restored the activity of lower concentrations (doubling dilutions of MIC) of antibiotics to susceptible breakpoints. The low concentrations of antibiotics have no activity of their own. The restoration of activity of antibiotics confirmed synergism between antibiotics and the tea extracts. The synergism observed in Kenyan black tea concurred with other studies on different types of tea. However, most of the studies have been done on green tea extracts compared to black tea extracts.

Hot water extract of Indian Lipton brand black tea showed synergistic activity with chloramphenicol, gentamicin, methillicin and nalidixic acid against enteropathogens (Tiwari *et al.*, 2005). Growth inhibition of *Sh. dysenteriae* at low concentration of chloramphenicol (2.5 µg/ml) and tea extract (5.09 mg/ml) as compared to MIC of individual agent (chloramphenicol 5 µg/ml or black tea extract 9.09 mg/ml) further confirmed the synergistic activity (Tiwari *et al.*, 2005). Synergistic microbial growth inhibition by Indian Lipton brand black tea extract and antibiotics was attributed to the presence of dual binding sites on the bacterial

surface for antibiotic and tea extract (Tiwari *et al.*, 2005). The results were in agreement with the marked reduction in MIC of oxacillin and other beta-□lactams antibiotics as reported in presence of epicatechin gallate in methicillin-resistant *Staphylococcus aureus* (Tiwari *et al.*, 2005).

The enhanced effect of Japanese green tea on inhibitory activities of antibiotics against MRSA strains have also been observed (Hara *et al.*, 1991). The synergistic activity of hot water extract of Sencha (Japanese green tea) with methillicin against methicillin-resistant *Staphylococcus aureus* demonstrated possible benefits of tea extracts (Hara *et al.*, 1991). The extract of Sencha tea was not only capable of inhibiting methillicin-resistant *Staphylococcus aureus* but also restoring the activity of methillicin. The antibacterial activity of green tea can be explained by its content of (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) (Saroj *et al*., 1997).

The varying concentrations of hot water extract and isolated theaflavins of Kenyan black tea showed antibacterial activity against *E. coli, E. aeruginosa, S. typhi, P. aeruginosa, S. aureus, E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923). The preliminary studies of the antibacterial activities of hot water extract of processed Kenyan black tea and Nigerian Lipton tea showed inhibitory effect against *V. cholerea, E. coli, Salmonella* species, *P. aeruginosa, Proteus* species and *S. aureus* (Mbata, 2006).The Kenyan tea showed more inhibitory actions on most of the organisms tested (Mbata, 2006). The zones of inhibition produced by Kenyan tea on test organism were generally larger than

those produced by the Nigerian Lipton tea (Mbata, 2006). This could be because it contains more active ingredients (phytochemical substances) than the Nigerian tea, which resulted in a stronger inhibitory effect on the test organism (Mbata, 2006).

Theaflavins have also been reported to have antibacterial activities against *T. mentagophytes*, *T. rubrum*, *C. albicans* and *Cryp. neoformans* (Okubo *et al*., 1991). Kenyan tea clones generally produce black tea with high levels of total theaflavins (Owuor and Obanda, 1995).

Hot water extract and isolated theaflavins of Kenyan black tea differed in strength of antibacterial activities. Similar difference was observed when the two black tea extracts were combined with antibiotics. The antibacterial activities and synergistic activity with antibiotics was lower for hot water extract as compared to that of isolated theaflavins. That was despite the fact that hot water extract tested had the same amount 36 µmol (18 µmol /g) of theaflavins as isolated theaflavins (36 µmol/g). The differences in inhibitory effect observed were attributed to interactions within the tea infusion between water soluble components and theaflavins that were not isolated. Theaflavins in black tea infusion are being partially antagonized by one or more chemical components in it lowering the overall activity. The differences in inhibitory effect was significant at ($P<0.05$). However, the pattern of activity of isolated theaflavins and hot water extract (infusion) of black tea were similar. This suggested that the theaflavins that were not isolated were the principal bioactive compounds in black infusion despite the existence of interactions. This inference is in agreement with studies by Okubo *et*

al. (1991*)* that showed theaflavins as the major antibacterial compound in tea. Further, Apostolides and Weisberger (1995) significantly showed that theaflavins as the principal quality components in black tea are beneficial to human health. In addition to theaflavins, other antibacterial compounds in hot water extract of black tea are catechins, fluoride, kaempferol, quercitin and myericitin (Higdon, 2007). However the combination of these in the hot water extract in the present study does not boost the antibacterial activity of theaflavins that were not isolated to exceed that of isolated theaflavins.

These compounds isolated from other plants have been found to have antibacterial properties. Kaempferol (3,4',5,7-tetrahydroxyflavone) and quercetin (3,3',4',5,7-pentahydroxyflavone) showed the lowest minimum inhibitory concentrations (MICs) against the clinical MRSA (Lin *et al*., 2008). Tryptanthrin and kaempferol isolated from the indigo plant (*Polygonum tinctorium lour*) significantly decreased the numbers of *H. pylori* colonies in a dose-dependent manner (Mari *et al*., 2006). Querticin and kaempferol from other plants have been shown to have combined effects with antibiotics (Lin *et al*., 2008). Combinations of rifampicin and either with kaempferol or quercetin acted synergistically or partially synergistically against the clinical MRSA (Lin *et al*., 2008). Rifampicin combined with kaempferol or quercetin exhibited good beta-lactamase inhibitory effects (57.8 % and 75.8 %, respectively) against a representative isolate (Lin *et al*., 2008).

Hot water extract and isolated theaflavins of Kenyan black tea can be used to prevent development of bacterial resistance to antibiotics. Synergism and

restoration of activities of lower concentrates of ampicillin, chloramphenicol, tetracycline, norfloxacin and ciprofloxacin by the tea extracts as is shown in this study, a pointer to the value of the combination in combating bacterial resistance.

Black tea and its two polyphenols (theaflavins and thearubigins) have significant antimutagenic effects against *Salmonella* strains (Gupta *et al.,* 2002). Chunxia and Yongquan (2006) also showed that theaflavins have considerable antimutagenic effects against bacterial mutagens such as sodium azide, 4-nitro-o-phenylenediamine, cumine hydro-eroxide, 2-amino- fluorene and danthron. Development of bacterial resistance has been attributed to mutation (Grace, 2008). Errors in deoxyribonucleic acid (DNA) synthesis during replication and occasional failures in the DNA repair systems result in a spontaneous mutation (Grace, 2008). Certain antimutagenic agents have the ability to prevent development of antibiotic resistance (Pillai *et al.,* 2001). These agents include green tea catechins and other antioxidants (Pillai *et al.,* 2001). In many cases, these agents are capable of exerting these effects at doses which by themselves produce no visible effect on growth (Pillai *et al.,* 2001). These effects are exerted against resistance to antibiotics such as tetracyclines, fluoroquinolones, macrolides, beta-lactams and aminoglycosides (Pillai *et al.,* 2001).

Combined use of tea and antibiotics could be useful in fighting emerging drug resistance problem especially among enteropathogens. Multi-drug resistance by *S. typhi* was observed by Manchanda *et al.,* (2006) in their study. In the study all 56 isolates of *S. typhi* were sensitive to amoxicillin/clavulanate, gentamicin, cefixime,

cefotaxime and ceftazidime. Multidrug resistance (MDR, resistance to three drugs) was seen in 22 cases (39%) and resistance to five drugs was seen in 12 cases (21%). Only two isolates were resistant to chloramphenicol (3%). All *S. paratyphi* A. isolates were sensitive to ampicillin and chloramphenicol and resistant to nalidixic acid. According to the study by Manchanda *et al.,* (2006), treatment of enteric fever in children on the basis of current trends of antimicrobial susceptibility of *Salmonella enterica serovar typhi* and *paratyphi* A. favour use of ampicillin as a drug of choice. MIC distribution data for chloramphenicol revealed elevated MIC but still in susceptible range. Manchanda *et al.,* (2006) therefore recommended an urgent need for further clinical studies to evaluate response to chloramphenicol in such cases.

Tetracycline resistance now occurs in an increasing number of pathogenic, opportunistic, and commensal bacteria (Ian and Marilyn, 2001). The presence of tetracycline-resistant pathogens limits the use of these agents in treatment of disease (Ian and Marilyn, 2001). Tetracycline resistance is often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines (Ian and Marilyn, 2001).

Two genetically distinct classes of norfloxacin-resistant *Pseudomonas aeruginosa* PAO4009 mutants were isolated spontaneously (Hirai *et al.,* 1987). Two norfloxacin resistance genes, nfxA and nfxB, were mapped hex-9001 and leu-9005 and between pro-9031 and ilv-9023, respectively, on the *Ps. aeruginosa* PAO

chromosome (Hirai *et al.,* 1987). These findings suggested that the norfloxacin resistance mechanism in the nfxB mutant might be an alteration in outer membrane permeability to norfloxacin (Hirai *et al.,* 1987). *P. aeruginosa* has been shown to inactivate anti-methicillin resistant *S. aureus* antibiotics as indirect pathogen (Ramphal, 2007).

The combination of hot water extract and isolated theaflavins with antibiotics in this study were found to be useful *in vitro*. However, before utilizing these findings *in vivo*, there are other factors to consider. Black tea has 177-303 mg/l of caffeine (Higdon, 2007). A number of drugs can impair the metabolism of caffeine, increasing the potential for adverse effects from caffeine (Higdon, 2007). They include cimetidine (Tagamet), disulfiram (antabuse), estrogens, fluoroquinolones, antibiotics (ciprofloxacin, enoxacin and norfloxacin), fluconazole (diflucan), fluroxamine (luvox), mexilitrine (mextil), riluzol (rilutek), terbinafine (lamisil) and verapamil (calan) (Higdon, 2007). High caffeine intakes may increase the risk of toxicity of some drugs, including albuterol (alupent), cloxapine (clozaril), ephedrine, epinephrine, monoamine oxidase inhibitors, phenypropanolamine and theophyline.

Results from this study showed that ciprofloxacin and norfloxacin acts synergistically with both tea extracts *in vitro*. This benefit cannot be fully utilized *in vivo* because of the presence of caffeine in hot water extract of black tea. These antibiotics can be combined with decaffeinated black tea and isolated theaflavins.

The black tea industry will have to produce another patented tea, in which caffeine has been removed, similar to that of green tea industry.

Flavonoids in tea which includes theaflavins have been reported to bind non-heme iron, inhibiting its intestinal absorption (Higdon, 2007). Non-heme iron is the principal form of iron in plant foods, dairy products and iron supplements (Higdon, 2007). The consumption of one cup of tea with a meal has been found to decrease the absorption of non-heme iron in that meal by about 70% (Higdon, 2007). To maximize iron absorption from a meal or iron supplements, tea should not be consumed at the same time (Higdon, 2007).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i) Water soluble components in black tea reduce activity of theaflavins but does not significantly diminish their overall activity.

ii) Theaflavins and hot water extracts of black tea can be used to combat growing bacterial resistance despite the interactions.

iii) The combined formulations with antibiotics will be suitable for prevention and treatment.

iv) Both black tea extracts are capable of restoring the activities of antibiotics.

6.2 Recommendations

i) Further studies on the interaction between individual bioactive compounds in the same tea are recommended.

ii) The studies should also be extended to interactions between bioactive and non bioactive compounds.

iii) A study should be carried out to determine whether a combined therapy of chloramphenicol and flavonoids such as theaflavins could reverse the binding of non-heme iron.

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Appendix I. Protocols for materials, media and reagents preparation

These include the selection of sample materials, equipments, and the preparation of media according to approved formulation by the manufacturers and the use of well characterized test bacterial strains.

Main equipments

- Electronic kettle with capacity of 2.5 liters
- Thermos flask
- Mechanical shaker
- Suction or vacuum filter
- Spectrophotometer
- Freeze drier
- Rotavapor
- Separating funnel
- Silica gel column 80 (1.65 cm i. d)
- Thin layer chromatographic plates and sprayer
- Autoclave

Main reagents

- Analytic grade ethanol
- Analytic grade chloroform
- Analytic grade ethyl acetate
- Analytic grade butanol
- Analytic grade hexane

- Silica gel 60M, 230 -240 mesh
- Distilled water

Preparation of enrichment broth

Alkaline peptone water

The ingredients (20g) were weighed and added to the distilled water and the pH adjusted to 9.0-9.2 with concentrated sodium hydroxide (NaOH) solution. 5 ml was dispensed using a pipette into screw capped universal type bottles and then autoclaved.

The use of alkaline peptone water

The broth supports growth of bacteria and was used to rejuvenate bacteria from the stock. The broth was also used to test viability of the bacteria.

Differential media

MacConkey Agar (MAC)

MacConkey Agar was a differential medium that was used to distinguish lactose fermenters from non–lactose fermenting (NLF) bacteria. *P. aeruginosa* appear as pale coloured colonies on MAC. *E. coli* ferment lactose, producing smooth pink colonies. *Salmonellae* produced non-lactose fermenting pale coloured colonies. *S. aureus* produce smaller colonies after incubation.

Biotyping media

Triple sugar iron (TSI) agar

TSI is a carbohydrate containing screening medium used to differentiate lactose fermenters from non lactose fermenters and contains hydrogen sulphide (H_2S)

indicator. H_2S producing organisms cause the blackening of the medium in TSI. TSI contain glucose, lactose and sucrose. Organisms that ferment either lactose or sucrose produce an acid (yellow) slant while organisms that ferment neither carbohydrate will have an alkaline (red) slant. Glucose fermenters produce an acid (yellow) reaction in the butt.

Preparation

49 g of the powder was weighed and dissolved in 1 liter of distilled water and heated to boil while swirling to dissolve. 5ml of the medium were dispensed in screw capped 15×150mm tubes. The screw caps were left loose and the media were autoclaved thereafter, the slants were allowed to solidify in a manner such that the medium in the butt of each tube was about 2.5 cm and the slant was about 2.5 cm long.

Identification of organisms

P. aeruginosa is oxidase positive and produced acid only from glucose and no gas was produced. These features together with the typical pigments produced by most strains and distinctive smell of cultures were sufficient to identify the organism. Growth at 42^{o} C differentiated *Ps. aeruginosa* from less commonly *Pseudomonads*, *P. putida* and *P. fluorescens*. *S. typhi* produce small amount of H_2S. *E. coli* do not produce gas.

Simmons citrate agar

Sodium citrate is a salt of citric acid. Citrate utilization by bacteria is detected in citrate medium by the production of alkaline by-products. *E. coli* reduces nitrate to nitrite, giving a positive urine nitrite test. *S. aureus* is a coagulase and catalase positive.

Preparation

The 23g of dehydrated agar was dissolved in 1 liter of distilled water and then boiled to dissolve completely. Then 2ml were dispensed in test tubes, sterilized by autoclaving and then set to cool in slanting position.

Positive control: K. pneumonia (ATCC 13883)

Negative control: E. coli (ATCC 25922)

Mueller- Hinton Agar (MHA)

MHA is the NCCLS is the recommended medium used for standardized antimicrobial susceptibility testing of certain bacteria.

Preparation

The 38g of powder was weighed and dissolved in 1 litre of distilled water. The solution was allowed to soak for ten minutes, swirled to mix completely and then sterilized by autoclaving. After cooling to 50^{o}C, 20ml per plate was measured and dispensed into 15×100 mm sterilized Petri dishes. The depth of the agar did not exceed 4mm.

Quality control

E. coli (ATCC 25922) standard strain for antibacterial susceptibility testing was used for each new lot prepared. The pH of the medium was 7.2.

0.5 M McFarland Turbidity Standard

The 0.5 ml of 0.048 M $BaCl_2$, 1.175% W/V $BaCl_2.2H_2O$ was added to 99.5ml of 0.36 M $(NH_3)_2SO_4$ (1% V/V) and mixed. 5mls were dispensed to screwed test tubes. The turbidity of actively growing bacteria in alkaline peptone water was adjusted to correspond to that of the turbidity of 0.5 McFarland standard.

APPENDICES

Appendix II. Preparation of antibiotic stock solution was done using the formula below:

$$W = \frac{1000 \times V\ (ml) \times C\ (\mu g ml^{-1})}{P\ (\mu g ml^{-1})}$$

Where:

P= potency given by manufacturer in relation to base

V= volume required in ml (1000ml)

C=final concentration of the solution (multiple of 1000)

W=weight of antibiotic in mg to be dissolved in V

Antibiotic	W (mg)
Ampicillin	250
Tetracycline	250
Ciprofloxacin	250
Norfloxacin	250
Chloramphenicol	250

Appendix III. Acceptable limits for quality control strains used to monitor accuracy of disc diffusion testing of nonfastidious organisms (using Mueller Hinton Agar without blood or other supplements) (NCCLS 2002).

		Inhibition diameter in millimeters			
Antibiotic	**Disc Content**	***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**	***P. aerug* ATCC 27853**	***E. coli* ATCC 35218**
Ampicillin	10µg	16-22	27-35	-	6
Chloramphenicol	30µg	21-27	19-26	-	-
Ciprofloxacin	5µ	30-40	22-30	25-33	-
Norfloxacin	10µg	28-35	17-28	22-29	-

Appendix IV. Interpretive standards and equivalent breakpoints for enterobacteriae species (NCCLS, 2002)

Medium: Mueller Hinton Agar

Control: *Staphylococcus aureus* (ATCC 25923)

Escherichia coli (ATCC 25922)

Escherichia coli (ATCC 35218)

		Zone diameter (mm)				
Antibiotic	**disc content**	**R**	**I**	**S**	**R**	**S**
Ampicillin	10µg	≤16	-	≥17	≥16	≤8
Tetracycline	30µg	≤14	15-18	≥19	≤16	≤4
Ciprofloxacin	5µ	≤15	16-20	≥21	≤4	≤1
Norfloxacin	10µg	≤12	13-16	≥17	≥16	≤4
Chloramphenicol	30µg	≤12	13-17	≥18	≥32	≤8

Appendix V. Solvents and diluents for preparation of stock solution of antibiotic agents that require solvents other than water (NCCLS, 2002)

Antibiotic	Solvent	Diluent
Ampicillin	Phosphate buffer pH 8.0, 0.1 mol/L	Phosphate buffer pH 6.0, 0.1 mol/L
Chloramphenicol	95% ethanol	distilled water
Norfloxacin	½ volume of distilled water, then 0.1 mol/L NaOH dropwise to dissolve	distilled water
Ciprofloxacin	distilled water	distilled water
Tetracycline	distilled water	distilled water

Appendix VI. Analysis of theaflavins

The flavognost method is a rapid procedure producing good reproducible results (Britta and Ulrich 1989). Using the high performance liquid chromatography (HPLC) method, which is much more time consuming due to the necessity of a pre-separation by gel chromatography, one can determine four different theaflavins (theaflavin, theaflavin-3-gallate, theaflavin 3′-gallate and theaflavin 3,3′-digallate) and their relative amounts (Britta and Ulrich 1989). The difference between the results from these two methods normally increases with increasing theaflavin content (Britta and Ulrich 1989). In most cases, the values of the flavognost method are considerably higher (Britta and Ulrich 1989). Relative amounts of theaflavins are different in teas from different origins, especially those of theaflavin and theaflavin 3, 3′-digallate (Britta and Ulrich 1989).

Absorbance spectra of theaflavins

Theaflavins absorb strongly at a number of wavelengths in the UV and visible regions of electromagnetic spectrum. The absorbance at 380nm is specifically associated with benzotropolone structure and chemical electron shifts around the ring, result both in the bright red colour of the crystalline compounds (λmax 460-467nm), and the brightness of tea liquors. The flavognost method published by Hilton (1973) uses diphenylboric acid ethanolamine benzotropolone nucleus of theaflavin to form a green chromatophore with a broad absorption maximum at 625nm.

Absorption spectra λmax of the theaflavin compounds in ethanol

Compound	**λmax**
Theaflavin	270 (19200), 294 (16800), 380 (10200), 465 (3850)
Theaflavin- 3'-gallate	275 (26400), 378 (9320), 464 (3,200)
Theaflavin- 3 -gallate	275 (25200), 378 (10210), 465 (3850)
Theaflavin- 3, 3'-digallate	278 (35600), 378 (9200), 460 (3400)