EVALUATION OF ANTIMICROBIAL POTENTIAL OF THE LEAF AND STEM BARK EXTRACTS OF *EUCLEA CRISPA* (THUNB.) AND ITS POSSIBLE SYNERGISM WITH STANDARD ANTIBIOTICS

BY

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DEDICATION

This study is dedicated to my mother Mrs. B Alayande and father Mr. JK Alayande for their long-standing support.

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All praises are due to God Almighty the Lord of the cosmos.

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Abstract

This study assays for preliminary phytochemical screening of leaf and stem bark extract of *Euclea crispa*, determines functional groups present in each potent fraction partitioned from the extracts, evaluates intensive antimicrobial properties of the extracts, assesses membrane attack capability of the fractions and evaluates drug-drug interaction between the most active fractions and selected antibiotics. The phytochemical screening was determined following conventional approach while functional groups were determined using FT-IR analysis. Agar-well diffusion was used for sensitivity test, agar dilution and broth-micro dilution were employed to determine minimum bacteriostatic and bactericidal concentrations, while time-kill kinetics was evaluated at different concentrations over a period of 2 h. Impact of the extracts against cell membrane was assessed via scanning electron microscopy and determination of the amount of proteins and nucleotides leakages. Evaluation of drug-drug interaction was carried out using time-kill assay at different concentrations of drugs combination against multi-drug resistant isolates.

Presence of tannins, saponins, flavonoids, cardiac glycosides, reducing sugars, steroids and absence of alkaloids were common to both extracts. Moreover, some functional groups *viz;* alkanes, alkenes, alkynes, alcohol, phenol, aldehyde, aromatics, sulfoxides, nitrile, amides and amines were also detected in the active fractions. The largest zone of inhibition (26±0.50 mm) was shown by ethyl acetate fraction of the leaf extract against *Aeromonas hydrophila* at 10 mg/ml. The lowest minimum inhibitory concentration (MIC) of 0.08 mg/ml is exhibited by the fractions partitioned into n-butanol, ethyl acetate and water against test bacterial isolates while the range of MIC against yeast is 0.31–1.25 mg/ml. Absolute mortality was achieved by n-butanol fraction against *Bacillus pumilus* and *Klebsiella pneumoniae* after 90 and 120 min respectively at 1×MIC and by n-hexane fraction at 2 × MIC. Ethyl acetate fraction achieved absolute mortality against both representative bacteria after 120 min at 2 × MIC in addition to *Escherichia coli* (1323) under similar condition. n-Hexane fraction achieve total

mortality against *Candida albicans* after 120 min at 1 × MIC. Maximum zone of inhibition (22±0.58 mm) was observed for the fractions partitioned into n-butanol and ethyl acetate from the stem bark extract at 10 mg/ml. The lowest MIC for that of n-butanol (0.31 mg/ml) is against *Enterococcus faecalis* while the lowest for that of ethyl acetate, n-hexane and water is 0.63 mg/ml against a number of test isolates. After 120 min of contact time only ethyl acetate fraction is able to eliminate both *Listeria* sp. and *Salmonella* Typhimurium at 1×MIC.

Maximum amount of proteins released by fractions of the leaf extracts from *Bacillus pumilus* (0.53±0.005 µg/ml) is by the fraction partitioned into water after 120 min of treatment at 2 × MIC while that from *Klebsiella pneumoniae* (0.57±0.001 µg/ml) is by n-butanol fraction and *C. albicans* (0.54±0.002 µg/ml) was by n-hexane fraction. Furthermore, maximum nucleotides leakage of 45.8±0.03 and 44.3±0.03 µg were obtained from *Bacillus pumilus* and *C. albicans* by n-hexane fraction at 2×MIC respectively while the maximum nucleotides leakage from *Klebsiella pneumoniae* is 40.7±0.06 µg by n-butanol fraction. On the other hand from the stem bark extracts, ethyl acetate fraction released maximum amount of proteins from *Listeria* sp. (0.625±0.004 µg/ml) and it was n-hexane fraction from *S.* Typhimurium (0.789±0.001 µg/ml) at 3 × MIC after 120 min. The maximum amount of nucleotides leakage (47.9±0.12 µg) was from *Listeria* sp. by the fraction partitioned into n-butanol at 3 × MIC after 120 min. Images of SEM reveal a level of structural damage in the membrane of test isolates which ultimately results in leakage of intracellular components. While determining possible synergism, out of 130 different combination tests between the leaf extract and antibiotics, 91.5% express synergy while 8.5% are indifferent. On the other hand, 88.5% of the same number of combination tests is synergistic between the stem bark extract and standard antibiotics with no record of antagonism in both cases.

The extracts of *E. crispa* exhibit significant antimicrobial properties which in a way confirm the plant a good source of bioactive compounds with membrane-active components and as well may serve to enhance potency of the available standard antibiotics and equally provide alternative therapy in combating infectious diseases locally.

Keywords: Microorganisms, Antibiotics, Phytochemicals, *Euclea crispa*, solvent partitioning, Time-kill kinetics, Membrane attack, SEM, Synergism

CHAPTER 1– Introduction and Literature review

1.1. Motivation

At the moment, the entire world has witnessed uncontrolled increase in the development of resistance mostly by bacterial pathogens against known powerful and broad-spectrum antibiotics. This development renders a wide array of effective antimicrobial agents useless so quickly that global public health disaster is imminent. Meanwhile, a plasmid-borne colistin resistance gene, *mcr-1* was recently isolated in china, closely followed by another one in USA from *Escherichia coli* strain cultured from urinary tract infection sample. Thus, an urgent approach is undoubtedly required; most importantly when the fact that colistin is the last resort in the line of treatments with antibiotics is taken into account (Stefanic *et al*., 2017).

Based on the foregoing, the importance of medicinal plants being the richest bio-resource of drugs and drug templates for pharmaceutical industries and as well in traditional healing practices (Ncube *et al*., 2008) cannot be overestimated. There has been a wider resurgence towards traditionally used medicinal plants with a number of initiatives actively exploring the botanical resources with the intention to augment weakened potential of the existing orthodox antibiotics (Street & Prinsloo, 2013). This campaign had gained global support due to cost effectiveness of medicinal plants, lack of major side-effects and global availability (Yamani *et al*., 2016). In a complementary effort toward the urgent need to find lasting solution to the sprouting problems of multidrug resistance by the pathogens against synthetic drugs, this study investigates comprehensive antimicrobial potential of the *Euclea crispa* leaf and stem bark extracts and equally assesses the effectiveness of its combination therapy with standard drugs against multidrug resistant pathogens. This may serves as pointer towards a source of bioactive substance of natural origin against various kinds of infectious diseases most importantly in the developing countries.

1.2. Introduction

Infectious diseases remain in the frontline causes of morbidity and mortality across the globe, notwithstanding the great level of progress made in application of science and technology in medical practices (Ayoub *et al*., 2014). Microorganisms resistant to antibiotics at present, constitutes a huge and globally acknowledged clinical challenge with high mortality rate on yearly bases (Gyles 2011; Anantaworasakul *et al*., 2017). This menace presents a pressing need for drug discovery initiatives. At least two million people are being infected by multidrug resistant bacteria in the United States, killing twenty three thousand patients annually as reported by Centre for Disease Control (CDC, 2013). Despite, antibiotics remain a conventional way of managing the outbreaks of bacterial infections even after the global adoption of vaccines dated to early 1990s which did give rise to an appreciable decrease in the prevalence of infectious diseases (Menanteau-Ledouble *et al*., 2017).

Pathogens bearing resistance genes could eventually become resistant to virtually every available and commonly prescribed antibiotic and thus could result in prolonged illness and risk of death (Yang *et al*., 2017). This may even increases the risk of spreading the resistant strains to others which may result in outbreak of the resistant strains that may in turn be expensive and difficult to eradicate (Mahlangu *et al*., 2017). For instance, over seventy percent of the hospital acquired pathogens quickly develop resistance to the antibiotics considered as their first-line treatment (Muto *et al*., 2003; Tanih *et al*., 2010), in the same way the prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is on the increase, both in the pigs and human beings coupled with the growing evidence of its transmission via food chain and the spread of the strain across Europe and beyond (Anukool & O'Neill, 2011).

Over the past decades, the increase in the bacterial strains resistant was principally driven by self-alteration of microbial genetic materials via plasmid transfer (Soltani *et al*., 2017), low permeability of

the outer-membrane in some species, expression of different efflux pumps, and or by production of drug-inactivating enzymes (Hirsch & Tam, 2010; Badamchi *et al*., 2017). For example, resistance to quinolones in Gram-negative bacteria is mainly as a result of genomic mutations which consequently prevent the antibiotic from reaching the site of action (Akasaka *et al*., 2001). *Helicobacter pylori*, a principal etiological agent of type B gastritis, peptic ulcer infection and as well been classified by World Health Organization as Class I carcinogen, has been reported with an alarming outright resistance against metronidazole (Mabeku *et al*., 2017). A report had shown that the emergence of drug resistance among *Aeromonas* sp. which is among the causal agents of gastroenteritis and extra-intestinal infections in immuno-compromised individual is not limited to the clinical strains but also environmental strains isolated from foods and natural waters (Alcaide *et al*., 2010). In the same vein, organisms such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* species impose significant threat to human health as they exhibit resistance to multiple classes of antibiotics (Mohamed *et al*., 2017). Despite the availability of antibiotics in combating *S. aureus* infections, the mortality rate associated with the infection is about 25% due to evolution of MRSA and vancomycin resistant *Staphylococcus aureus* (VRSA) (Kakarla *et el*., 2017). Persistence of these resistance trends is driving physicians towards the use of antibiotics associated with significant side effect (Li *et al*., 2017).

The use of plants in the provision of folklore remedy predates civilization in every society irrespective of its level of development at the moment (Ustue & Adamu, 2010). Mankind, in his efforts to improve on his healthcare problems, has used herbal therapies to augment the shortage in orthodox medicine which suffers inadequate effectiveness partly as a result of increasing resistance of pathogens against first-line antibiotics (Olivier *et al*., 2017). The bioactive principles in medicinal plants exposed them to human exploitation and these are basically secondary metabolites with proven significant pharmacological properties, produced during the plants metabolism as a form of defense mechanism

against invasion of pathogens, pests and other foreign bodies (Ning *et al*., 2009; Akinpelu *et al*., 2015; Anantaworasakul *et al*., 2017). Plant-derived bioactive compounds through ethno pharmacological studies have recently become of great interest owing to their versatile applications (Mendonça-Filho *et al*., 2004; Baris *et al*., 2006). Going down the line of history, medicinal plants have provided a source of inspirational clinically important novel compounds (Igbinosa *et al.,* 2009) and one of their great advantages is due to the fact that they are readily available with significantly low side effects (Wadkar *et al.,* 2008).

Consequently, when every other alternative remedy is put into consideration, phytomedicine is possibly the most popular and widely acceptable approach against infectious diseases (Nono *et al*., 2014). This traditional system of medical care has been acknowledged as one of the most convinced means of achieving global healthcare coverage (Okunlola *et al*., 2007), coupled with the fact that it can also be easily sampled for laboratory test and analysis based on their traditional use within a community (Zadra *et al*., 2013). It is no longer news that herbal therapies have taken the central role in the management of debilitating diseases in recent times in many parts of the world predominantly in developing nations (Akinloye & Khadijat, 2010).

Moreover, having known that phytochemicals provides a major source of drugs or drug templates in modern day medicine (Ahmed *et al*., 2014), the screening of plant extracts for the presence of bioactive compounds has become a crucial component of drug discovery which had led to production of many drugs that are in use at present in primary healthcare system (Shai *et al*., 2013). Bioactive compounds of plant origin could explore mechanisms of biocidal actions differently from those of conventional antibiotics, thus impact significantly on the treatment against resistant microbial strains (Ayoub *et al*., 2014). Several plant secondary metabolites such as flavonoids terpenoids, alkaloids, reducing sugars, steroids, tannins and cardiac glycosides have been deeply studied on how they are

used in modern day medicine in the treatment of diseases such as toothache, wound infections, diarrhoea, snakebite, paralysis among others (Sharma & Kaur, 2017). Phenolic compounds serve as one of the major classes of secondary metabolites in medicinal plants and flavonoids is among the ubiquitous groups of plant phenolics with broad spectrum of biological activity responsible for the variety of pharmacological potentials (Tapas *et al*., 2008; Kumar & Pandey, 2013). They are known for their antimicrobial, anti-inflammatory and antioxidant activities (Sonibare *et al*., 2016). Plant kingdom is quite indispensable in the life of man both in medicine and worldly activities. A good example among the versatile medicinal plants is *Euclea crispa* (Thunb.) a member of the family *Ebenaceae*.

1.3. Microorganisms and infectious diseases

Before microbial attack could leads to infection, the invading microorganisms need to overcome several defensive mechanisms of the host and out-compete the resident microbiota. Some of the challenges may include physical barriers, change in pH, mucus secretion by the host, and reduced oxygen tension among others (Johnson & Abramovitch, 2017). Neonates in hospitals are most vulnerable due to their underdeveloped immune system coupled with the risk of exposure to infectious diseases via contact with clinical staff members, parents, other patients and the hospital environments. This may leads to microbial colonisation followed by infection with high rate of morbidity and mortality (Dramowski *et al*., 2017). For instance, listeriosis which is a disease condition estimated as the most common cause of food-borne related deaths (Mook *et al*., 2010), is known to have resulted from *Listeria monocytogenes* infection. The occurrence of listeriosis in neonates is via congenital infection and it is associated with high fatality, in fact, it is the third most common cause of early-onset neonatal infection which symptomises as bacteraemia, meningitis and sometimes pneumonia (Chen *et al*., 2015; Sapuan *et al*., 2017). In addition, from a recently conducted systematic review of thirty neonatal outbreaks between the year 2005 and 2015, it was determined that *Klebsiella*

pneumoniae at 33%, *Serratia marcescens* at 20% and MRSA at 20%, were the most important pathogens (Birt *et al*., 2016). *Aeromonas hydrophila* is widely distributed in nature, causing zoonotic infections as a food borne pathogen (Laith & Najiah, 2013).

Moreover, among the pathogens found colonising the skin and nasal cavities of both human beings and animals as part of normal flora is *Staphylococcus aureus*. Nearly 50% of human beings are either perpetual or periodic carriers of *S. aureus* in their nasal cavity, even though the pathogen is responsible for a range of diseases in man and animals which include but not limited to skin and soft tissue infections, mastitis, severe invasive infections, toxic shock syndrome and food poisoning (Tong *et al*., 2015; Jans *et al.*, 2017). *Staphylococcus epidermidis* causes infections associated with indwelling central venous catheters, cerebrospinal fluid shunts, prosthetic heart valves and peritoneal dialysis catheters (Shankara *et al*., 2005). While *Klebsiella pneumoniae* is as well a leading pathogen causing pyogenic liver abscess (PLA) which is a potentially life-threatening disease in Asia and Western countries (Rahimian *et al*., 2004; Chen *et al*., 2007). *Enterococcus faecalis* survives extreme conditions and has been implicated in a number of life threatening ailments as well as less severe disease conditions such as obturated root canals infections with chronic apical periodontitis (Güven, 2004).

Another more prevalent human infection is the one caused by *Helicobacter pylori*, almost half of the entire global population had suffered colonization of stomach mucosa by this pathogen since its discovery as at early 1980s. This mostly manifests into chronic gastritis, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Franceschi *et al*., 2002; Makola *et al*., 2007). Epidemiological surveys have also revealed a relationship between *H. pylori* infection and diseases such as colorectal cancer, idiopathic iron deficiency anemia, idiopathic thrombocytopenic purpura and more probably cardiovascular, pancreatic and neurodegenerative diseases (Stefanic *et al*.,

2017). In the same vein, *Mycobacterium tuberculosis* remains a threat to the entire world health system. During 2014 and 2015 alone, about 9.6 and 10.4 million people respectively became victims of tuberculosis infections and almost 1.5 and 1.8 million of the affected individuals died which invariably depict tuberculosis as the leading cause of death among infectious disease (WHO, 2015; WHO, 2016). Likewise *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* are collectively responsible for an estimate of more than 1.6 million food-borne infections every year. While *Salmonella* spp. causes fever, diarrhoea and abdominal cramps shortly after the infection, Shiga toxin-producing *E. coli* has been implicated in several outbreaks where bloody diarrhoea and hemolytic uremic syndrome (HUS) appears as symptoms (Chen *et al*., 2015). It is important to note that human salmonellosis is commonly related to foods from animal origin. It may be present in the faeces and/or hide of healthy animal presented to be slaughtered for commercial purposes (Martínez-Chávez *et al*., 2015).

Furthermore, *Campylobacter* species invade epithelial cells of the gastrointestinal tract where they induce powerful inflammatory response that subsequently results into moderate and sometimes severe diarrhoea. Additionally, these pathogens have recently been associated with Crohn's disease, ulcerative colitis, septicemia and Miller Fisher syndrome (Johnson *et al*., 2017). *Campylobacter jejuni* is the leading etiological agent of bacteria related gastroenteritis across the globe and it is however confirmed endemic in Africa, Asia and Middle East most especially among young children (Kaakoush *et al*., 2015). Severe sepsis is on the list of most common causes of death among the patient under intensive care units and the mortality rate is approximately one-quarter of the victims (Loo *et al*., 2017). Tetanus is also a serious acute and extremely fatal infection resulted from neurotoxin secreted by *Clostridium tetani* which causes muscular rigidity and sudden involuntary muscular contraction (Tosun *et al*., 2017). Periodontitis is equally an important polymicrobial infectious disease ravaging the veterinary system. It is caused by complex of bacterial species that interact simultaneously with

host tissues and subsequently produces inflammatory cytokines, mediators and chemokines which eventually destroy the periodontal anatomy (Borsanelli *et al*., 2017).

Among the pathogens with life-threatening systemic infection potential is *Candida albicans* and the mortality rate is not less than 30%. Systemic *Candida* infections are common to immuno-compromised people such as low birth weight infants, HIV infected patients, organ-transplant recipients and chemotherapy patients (Kabir *et al*., 2012). *Candida albicans* is responsible for about one-third of infections associated with clinical devices in United States of America largely because of its ability to develop biofilm on medical devices and resistance to antimicrobial therapy. It accounts for a large number of fungal infections occurring in the digestive tract, muco-cuteneous tissues and skin as well as in the bloodstream (Motaung *et al*., 2015). Likewise *Cryptococcus neoformans*, an emerging yeast pathogen of man which has also been reportedly responsible for annual deaths of about six hundred thousand immuno-compromised individuals and also has the potential to colonise the airspace in the lungs thus results into pneumonia (Price *et al*., 2011). *Aspergillus fumigatus* is the causal agent of aspergillosis, a life-threatening pulmonary infection that also predominate immuno-compromised individuals. It is considered as the most prevalent airborne fungal pathogen and a major allergen (Nierman *et al.*, 2005).

1.4. Mode of action of antimicrobial agents

Invading microorganisms are equipped with different strategies to circumvent the host defense mechanisms, resulting in disease condition. Hence, disrupting pathogens' strategies to overcome the host defense system could form the basis of therapeutic approach in combating microbial infections (Staskawicz *et al*., 2001; Johnson & Abramovitch, 2017). Following the 'magic bullet' concept of Paul Erlich, an antimicrobial chemotherapist, it was believed that it is possible to treat microbial infections through specific target structures on the cell or certain physiological functions in the pathogen which is

lacking in the human host. This paved way for development of classic drugs like aminoglycosides and β-lactams, targeting protein and cell wall biosynthesis, respectively (Baron, 2010). Therefore, an effective chemotherapeutic agent must be selectively cytotoxic. A drug with selective toxicity would have a high therapeutic index and usually act against structures or pathways unique to the invading microorganism and consequently minimise the side effects (Tortora *et al*., 2004).

Antibiotics are chemical substances of natural origin or produced by chemical synthesis, with the ability to inhibit the growth or have biocidal effect on microorganisms and are used in the treatment of infectious diseases (Pelczar, 2006). The introduction of antibiotics into human therapy and veterinary practice largely impacted on both human and veterinary clinical systems (Stankevičienė & Šiugždaitė, 2016). Antibiotics development is among the most significant contributions of modern science, as its discovery completely transformed the healthcare system during the last century. The entire world witnessed a significant decline in the fatality rate associated with infectious diseases just by the introduction of these life-saving drugs into clinical practices. For instance, in the United States between 1930s and 60s the survival rate of the victims due to chronic infection of the heart valves was increased to about 95% from zero while that of spinal meningitis infection caused by *Neisseria meningitides* was increased by 88% (Sengupta & Chattopadhyay, 2012).

Antimicrobial agent can either be bactericidal or bacteriostatic in actions. In addition, some of the agents which are active against both Gram positive and Gram negative bacteria are said to have broad spectrum activity such agents include; ampicillin, rifampicin, carbenicillin, cephalosporin and streptomycin. On the other hand, those that are active against either Gram negative or Gram positive bacteria are said to have narrow spectrum. These are also include; erythromycin, penicillin, polymycin B, dapsone, bacitracin and vancomycin (Prescott *et al*., 2008). Antimicrobial agents attack microorganisms through different mechanisms and thus disrupt various molecular targets within and outside the bacteria which eventually inhibit the growth or killing the microbial cells. Common

attacking mechanisms involved in their modes of actions include: Inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, disruption of cytoplasmic membrane function and anti-metabolites

1.4.1. Inhibition of cell wall synthesis

The cell wall inhibitors are mostly members of β-lactam class of antibiotics. β-lactam ring is among the core structural features in the array of drug categories as many antibiotics containing β-lactam moiety are active against a wide range of pathogens (Ebrahimi *et al*., 2016). Members of this class inhibit peptidoglycan biosynthesis by acting on transpeptidases, carboxypeptidases, transporter and Ala-Ala dipeptide, thereby disrupting the formation of microbial cell wall (Stankevičienė & Šiugždaitė, 2016). Examples of β-lactams include: penicillin, cephalosporin, bacitracin, vancomycin, carbapenems, monobactams and aztreonam. Cephalosporins directly degenerate peptidoglycan layer of bacterial cell wall, causing the wall to collapse and eventually kill the bacteria (Tumah, 2005). Bacitracin and vancomycin interfere with the linear strands of peptidoglycan, while penicillin and cephalosporin obstruct the final cross linkage of peptidoglycans which principally interfere with the construction of the macromolecular cell wall (Pelczar, 2006).

1.4.2. Inhibition of protein synthesis

Protein biosynthesis has been a major target for antimicrobial agents. In fact some antibiotics have been identified as inhibitors of almost every step involved in translation process though with varying degrees of specificity (Wilson, 2009). Protein synthesis is a complex process involving many enzymes and most of the protein inhibitor antibiotics commonly disrupt the processes at the 30S subunit or 50S subunit of the 70S bacterial ribosome, through formation of 30S initiation complex and elongation of the process of aligning amino acids into polypeptides (LS BioFiles, 2006). A large number of antimicrobial agents have also been reportedly disrupting the assemblage of ribosomal subunits coupled with their well characterized role as inhibitors of protein biosynthesis (Champney, 2006).

Examples of protein inhibitors includes tetracycline, chloramphenicol, lincosamides, macrolides such as erythromycin, azithromycin, spiramycin and aminoglycosides such as streptomycin, gentamycin, neomycin, kanamycin (Hutchinson, 2003; Barbachyn & Ford, 2003).

Nevertheless, the mitochondrial protein synthesis machinery is in many ways similar to that of prokaryotes and as a result may be a target for antibiotics that function by binding to the bacterial ribosome (Bottger *et al*., 2001). Significant evidence has shown that bone marrow suppression often reported as a dose-dependent and reversible toxic side effect of chloramphenicol therapy in man. This is obviously caused by inhibition of mitochondrial protein synthesis (Turton *et al.,* 2002; Yoon *et al*., 2005). Almost every antibiotic that binds with ribosome is bacteriostatic in action except those in the group aminoglycoside which induce cell death (Kohanski *et al*., 2007; Wilson, 2009). This fact therefore plays down the gravity of the possible side effects as a result of ribosome-binding agents. A recent study explained how ribosome-binding kinetics of some antibiotics influence the level of bacterial resistance based on mathematical model such that reduced growth rate of microbial cells leads to more resistance to reversibly binding antibiotics whereas increased growth rate causes more resistance to irreversibly binding antibiotics (Greulich *et al*., 2015).

1.4.3. Inhibition of nucleic acid synthesis

A number of antimicrobial agents directly interfere with biosynthesis of nucleic acid by blocking synthesis of nucleotides, disrupting DNA replication and thus preventing transcription. Drugs with this mode of action have an extremely limited usefulness due to the high level of similarity between microbial and mammalian genetic materials *viz*; DNA and RNA (Talaro & Talaro 1996). This is because prokaryotes and eukaryotes do not have much difference with regards to nucleic acid synthesis mechanisms. Examples of agents exploiting this mechanism include quinolone, nofloxacin, ciprofloxacin and rifamycins such as rifampin, rifapentine, rifabutin, and rifamixin (Chopra *et al*., 2007; Ho *et al*., 2009). Ciprofloxacin and quinolone inhibit bacterial DNA gyrase and therefore

interfere with DNA biosynthesis (Tortora *et al*., 2004) while the rifamycins bind to a site on bacterial RNA polymerase adjacent to the active center thus preventing the extension of RNA chains beyond a length of 2–3 nucleotides (Sineva *et al*., 2012). Rifampicin is one of the two most powerful first-line antibiotics against tuberculosis. The side effects often result into reduced life quality of the victims, thereby prompting many of them to abandon the prescribed therapy which consequently results in increased complexity in the treatment and thus emergence of highly resistant strains of tuberculosis pathogens (Ali *et al*., 2016).

1.4.4. Disruption of cytoplasmic membrane

Certain antibiotics, especially polypeptide antibiotics, bring about changes in permeability of the plasma membrane, these changes result in the loss of important metabolites from the microbial cells. For example, polymyxin B causes disruption of the plasma membrane by binding to the phospholipid of the plasma membrane (Prescott *et al*., 2008). The potency of polypeptide antibiotics is equally attributed to their ability to cause pore generation which may results into microbial death. Binding of cationic polypeptide to the microbial cell membrane is facilitated by electrostatic attraction between positively charged amino acids in the peptide and negatively charged membrane surface, which then pave way for the hydrophobic interaction between the amphipathic domains of the peptide and phospholipid component of the cell membrane (Maturana *et al*., 2017). It is widely accepted that amphotericin B kills yeast primarily via channel-mediated membrane permeabilisation and alternatively kills yeast simply by binding ergosterol, a lipid that is vital for many aspects of yeast cell physiology (Kaitlyn *et al*., 2011).

1.4.5. Inhibition of cell metabolism (anti-metabolites)

Several antibiotics carry out their activities as anti-metabolites by blocking a functioning metabolic pathway or by competitively preventing the use of certain metabolites by key enzymes (Talaro & Talaro, 1996). For instance, sulphonamides and several other related drugs, inhibit folic acid

metabolism via competition with para-aminobenzoic acid (PABA). In many microorganisms, PABA is the substrate for an enzymatic reaction leading to the biosynthesis of folic acids, a vitamin that functions as coenzyme for the biosynthesis of purine and pyrimidine nitrogenous bases. In the presence of sulphanilamide, the enzyme that normally converts PABA to folic acid, binds with sulphanilamide instead of PABA (Pelczar, 2006). This combination prevents folic acid synthesis and thus obstruct microbial metabolism. Antibiotics with this anti-metabolite mode of action could have a high therapeutic index due to the fact that human beings do not produce folic acid. Examples of anti-metabolite drugs include trimethoprim, sulfa antibiotics such as sulphanilamide and sulphamethaxozole (Prescott *et al*., 2008).

1.5. Mechanisms of resistance by microorganisms

Rapid emergence of multidrug resistant pathogens is raising global alarm in public health sector. In the case of several severe infectious diseases, drug resistance evolves spontaneously via genetic mutations which quickly render the antibiotics ineffective (Chevereau *et al*., 2015). These traits can be passed via horizontal gene transfer between cells, leading to rapid spread of resistance determinants in bacterial populations. The strong selection pressure imposed by the antibiotics accelerates this process (Walsh, 2003; Baron, 2010). Among several processes involved in the lateral antimicrobial resistance genetic transfer, class 1 integron is the most efficient mechanism in terms of expression, recruitment, maintenance and spread of resistance genes among Gram negative clinical strains (Chamos *et al*., 2017).

In recent time, a study had shown that certain *Escherichia coli* cultures were repeatedly exposed to a high concentration of ampicillin over a period of time before the antibiotic was completely removed and growth continues in its absence. Consequently, the bacteria did not evolve resistance at any level but instead genetically modulated the duration of their lag phase to match the timing of the antibiotic

exposure. This means the antibiotic used in this regards can only be active against growing cells (Fridman *et al*., 2014; Sinova & Bollenbach, 2017). The major and best understood antibiotic resistance mechanisms, both in the clinics and laboratories, include modulation or modification of drug target, reduction or prevention of drug uptake and production of enzymes that degrade antibiotics (Holmes *et al*., 2016; Khameneh *et al*., 2016).

1.5.1. Modulation of drug target

Modification of the target is best exemplified by streptomycin and erythromycin resistance of bacteria. Both antibiotics bind with ribosome and thereby inhibit bacterial protein synthesis. Modification of the S12 protein of the 30S subunit of the ribosome makes the ribosome insensitive to streptomycin (Sengupta & Chattopadhyay, 2012). A change of one amino acid in the beta subunit of DNA-directed RNA polymerase alters binding of rifampicin. Usually the degree of resistance is related to the degree that the enzyme is changed but does not correlate strictly with enzyme inhibition. This form of resistance which exists at a low level in any microbial population develops during treatment (Denyer *et al*., 2011).

1.5.2. Decrease entry of antibiotic

A powerful strategy involved in the resistance of bacteria to tetracycline, is energy mediated efflux, which does not allow the drug to accumulate in sufficient concentration to exert its inhibitory effect. It is mediated by a trans-membrane export protein that functions as an electro-neutral anti-port system. The protein catalyses exchange of tetracycline-divalent metallic cation complex for a proton (Sengupta & Chattopadhyay, 2012). Alternatively, flushing out of drugs via efflux pumps may occur as a result of mutation in the enzyme that activates a pre-antibiotic as it is in the case of isoniazid against *Mycobacterium tuberculosis* (Martinez, 2014). Likewise, ciprofloxacin resistance in *Escherichia coli* results from mutation in the quinolone resistance-determining region of the GyrA subunit of DNA gyrase and the mutation alters regulation of efflux pumps, especially AcrAB (Vinué *et al*., 2016). On

the other hand, reduction in drug accumulation can also be achieved by blocking the entry of the drug as it happens in the absence of the imipenem transporter OprD2 which conferred resistance to *Pseudomonas aeruginosa* (Martinez, 2014).

1.5.3. Resistance by drug inactivation or destruction

Drug modification plays a significant role in rendering many therapeutically active drugs useless. For example β-lactamase, an enzyme elaborated by many Gram positive and some Gram negative bacteria, converts penicillin into penicilloic acid, which is therapeutically inactive. Likewise, chloramphenicol is converted to the therapeutically inactive compound 1, 3-diacetoxychloramphenicol by chloramphenicol acetyl transferase (CAT), produced by some resistant bacteria (Sengupta & Chattopadhyay, 2012). Some strains of *Escherichia coli* express the cat1 enzyme that inactivates chloramphenicol (Deris *et al*., 2013). The case of β-lactams class of antibiotics is a pointer toward rapid evolution of bacterial resistance. Nearly a thousand resistance-related β-lactamases that inactivate these antibiotics have been identified with a tenfold increase since before 1990 (Davies & Davies, 2010). The distribution of resistance genes, such as *Enterobacteriaceae* produced extended-spectrum β-lactamase, NDM-1, and carbapenemase produced by *Klebsiella pneumoniae*, indicates the ease with which resistance can spread (Laxminarayan *et al*., 2013).

1.6. Activities of medicinal plants against infectious diseases

The use of medicinal plants in folklore remedies is dated back several thousands of years (Chang *et al*., 2016). Books written on Ayurvedic medicine, which was developed over 3000 years ago in India, describe practices including the use of medicinal plants which subsequently formed the basis for other medical sciences on the Indian subcontinent (Pattanayak *et al*., 2010). As of today, complementary and alternative medicines largely focus on each and every part of the plants as the primary source of bioactive substances, knowing that these bioactive agents are the combinations of secondary

metabolites resulting from plant's physiological processes (Yamani *et al*., 2016). Some of the plant secondary metabolites include; tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, cardiac glycosides and other volatiles and/or essential oils. These chemical groups have been directly connected to the activities of medicinal plant against both curable and incurable diseases. Thus present medicinal plants extract as rich sources of antioxidant, antifungal and antibacterial agents of natural origin (Hossain *et al*., 2014).

Moreover, several researchers have reported that the crude extract from the plants with unique combinations of chemical components are often more effective than their chemical derivatives. This therefore led to a focus on the medicinal values of herbs and how they could best be incorporated into orthodox medical practice (Shikov *et al*., 2014). Undoubtedly, medicinal plants are the most abundant bio-resource of bioactive compounds of traditional and modern medicine, nutraceuticals and food supplements as well as chemical components for synthetic drugs. It was estimated that almost 28% of the higher plants are being used in alternative medicine and about 74% of plant-derived bioactive compounds were discovered based on the hypothesis from enthno-botanical survey on the traditional use of the plants (Ncube *et al*., 2008). In fact, plant materials remained central to traditional medical practices and as well as a good source and template for development of new drugs (Veeresham, 2012). Medicinal plants have recently gained attraction of pharmaceutical and scientific communities from which various studies have documented the therapeutic values of different bioactive compounds of plant origin in a bid to validate claims around their biological activity (Ncube *et al*., 2008). Studies on medicinal plants have further received the attention of many scientists in finding lasting solutions to the problems of multiple resistances by the pathogens to the existing synthetic antibiotics (Akinpelu *et al*., 2008).

Nearly all societies have used herbal materials as sources of remedy for their medical need and development of these herbal medicines largely depended on the local botanical flora of different communities (El-Mahmood & Ameh, 2007). The table 1.1 below shows a few of indigenous medicinal plants in Africa and their applications in folklore remedy in relation to infectious diseases.

Table1.1 List of some African indigenous medicinal plants and their application in folklore remedy

Plants	Parts	Traditional uses	References
Acacia senegal	leaf, stem bark, root	bronchitis, leprosy, antitussive, diarrhoea, gonorrhoea, haemorrhage, sore throat, typhoid, and urinary and upper respiratory tract infections	Okoro *et al*., 2011; Jain *et al*., 2012
Aloe ferox	latex	topical application on the skin, eye and mucous membrane layer	Gurib-Fakim *et al*., 2010; Mahomoodally, 2013
Agathosma betulina	leaf, stem bark	antitussive, antipyretic, kidney infection, urinary tract infections, haematuria, prostatitis, cholera, stomach ailments, bruises and antiseptic	Street & Prinsloo, 2013
Albizia gummifera	pods	stomach infections	Mahlangu *et al*., 2017
	root paste	skin infection	
	stem bark	malaria, antimicrobial	
Alchornea laxiflora	leaf	Inflammatory diseases	Akinpelu *et al*., 2015
Asparagus africanus	root	pneumonia, diarrhoea and sexually transmitted diseases	Maroyi, 2011
Asparagus suaveolens	leaf	epilepsy, livestock diseases	Olivier *et al*., 2017
Artemisia herba-alba	leaf, root	haemostatic, bronchitis, diarrhoea and other bacterial infections	Laid *et al*., 2008
Cassia occidentalis	leaf	yaws, scabies, itches, ringworm jaundice, toothache and hepatitis	Nuhu & Aliyu, 2008; Taiwo *et al*., 2013
Centella asiatica	leaf	wound healing, burns, ulcers, leprosy, tuberculosis, lupus, skin diseases, eye diseases, fever, inflammation, syphilis, epilepsy and diarrhoea	Brendler *et al*., 2010
Citrus aurantium	juice	peptic ulcers, antiseptic, anti-bilious and haemostatic	Opajobi *et al*., 2011
	leaf	sudorific, stimulant, tonic and stomachic action	
Cocos nucifera	juice of young spandex	diarrhoea, diabetes, inflammation	Naskar *et al*., 2011
Cucurbita pepo	seeds	urinary tract complications and urinary incontinence	Medjakovic *et al*., 2016
Cyclopia genistoides	leafy shoots,	urinary tract infection and stomach pain	Mahomoodally, 2013

	flowers		
Datura metel	leaf, stem bark	gonorrhoea, diarrhoea, epilepsy, catarrh, bronchitis, haemorrhoids, skin-ulcers and wounds infections	Rizwan *et al*., 2012; Hossain *et al*., 2014
Dialium guineense	leaf	gastrointestinal diseases, cholera	Akinpelu *et al*., 2011
Dietes iridioides	rhizomes	diarrhoea and dysentery	Ayoub *et al*., 2014
Dioscorea nipponica	whole plant	asthma, rheumatoid arthritis, bronchitis, and infectious disease	Cho *et al*., 2013
Dissotis thollonii	leaf, root	sinusitis and other inflammatory diseases, kidney diseases and pregnancy control	Nono *et al*., 2014
Funtumia africana	leaf	fever, inflammation, malaria, cancer, amoebic dysentery, urinary incontinence and burns	Ramadwa *et al*., 2017
Lecaniodiscus cupanioides	leaf, root, seed	fever, burns, liver abscesses, jaundice, coughs, malaria and aphrodisiac	Olowokudejo *et al*., 2008
Mentha piperita	stem bark	antimicrobial	Shalayel *et al*., 2016
Nauclea latifolia	leaf, stem bark	diarrhoea, urinary tract infections and buccal cavity infections	El-Mahmood *et al.,* 2008
Parkia biglobosa	roots, leaves	sore eyes, dental disorders, diarrhoea	Ajaiyeoba, 2002; Abioye *et al*, 2013
Pelargonium sidoides	root	acute respiratory infections	Agbabiaka *et al*., 2008
Phyllanthus amarus	whole plant	gonorrhoea, jaundiced, cough, itchiness, arthritis, otitis and skin ulcer	Adegoke *et al*., 2010
Rosa californica	leaf	poison oak dermatitis	Eluwa *et al*., 2008
	stem bark	viral infections	
	leaf and berries	infected sores, burns and wounds	
Rauwolfia vomitoria	root	tetanus, hypertension and epilepsy	Eluwa *et al*., 2008
	leaf	lice, scabies, cerebral cramps, jaundice and gastrointestinal abnormalities	Kutalek & Prinz, 2007
Tridax procumbens	leaf	liver disorders, diarrhoea and wound infection	Saritha *et al*., 2015
Umballularia californica	leaf	sore throat, chest congestion, nasal decongestion and poison oak dermatitis	Carranza *et al*., 2015

1.7. The Family Ebenaceae (Ebony family)

The family *Ebenaceae* commonly known as "ebony family" is made up of two broad genera namely; *Diospyros* and *Euclea* with approximately 600 species all together. They are highly uncommon in the temperate region while they are predominantly found across South-Eastern Asia, Madagascar, South America and tropical Africa (Wallnöfer, 2001). They are comprised of trees, shrubs or dwarf shrubs. The leaves are simple and in most cases alternate, while sometimes opposite, sub-opposite or whorled in some *Euclea* species. They are commonly evergreen while few of them are deciduous. The flowers are regular, hypogynous and unisexual and the fruits are usually berry (White, 1983; Wallnöfer, 2001).

Plants in the family *Ebenaceae* are well known for their medicinal properties and many of them have been employed in the treatment of various diseases across the globe. The largest genus of the family is *Diospyros* species with over 400 species many of which are economically important. Phytochemical screen on a number of *Diospyros* species reveals a wide variety of isolated bioactive compounds such as naphthoquinones and naphthalene derivatives which serve as the major constituents while others include; triterpenoids, tannins, coumarins, steroids and flavonoids (Mallavadhani *et al*., 1998; Grygorieva, 2013). Some of the members of *Diospyros* spp. include: *Diospyros bejaudi*, *Diospyros collinsae*, *Diospyros crumenata*, *Diospyros helferi*, *Diospyros nitida* etc. (Chheng *et al*., 2016). *Diospyros collinsae* is an important natural source for betulinic acid, two stilbenoidic compounds and four of triterpenoids were reportedly isolated from its stem bark. The isolated triterpenoids include friedelin, lupeol, betulin, and betulinic acid, all of which are promising antimicrobial and anticancer agents (Bumroong & Thanakijcharoenpath, 2016).

Members of *Euclea* are divided into two different groups, species with the corolla shallowly lobed at the apex and species with the corolla cleft at least halfway down or more (Retief *et al*., 2008). Examples of some of species include *E. crispa*, *E. divinorum*, *E. linearis*, *E. natalensis*, *E.*

angustifolia, *E. daphnoides*, *E. schimperi*, *E. undulata*, *Euclea coriacea*, *E. sekhukhuniensis* etc. (Retief *et al*., 2008; Kose *et al*., 2015).

Euclea coriacea stem bark is used for constipation, stomach pains, and purgative and gall sicknesses by the people of Lesotho (Kose *et al*., 2015). *Euclea natalensis* are specifically common on the eastern coast of Africa. People of Africa have demonstrated the antimicrobial potential of the roots of *E. natalensis* is used in the treatment of tooth decay. *In vitro* assessment of the twig of this species revealed sufficient antibacterial inhibitory potentials against virulent periodontopathic bacteria in addition with its low toxicity in healthy tissues and significant anti-inflammatory potential (Sales-Peres *et al*., 2012). Having understood the common use of *E. undulata*, *E. divinorum* and *D. lycioides* among the people of Zimbabwe as chewing sticks in the traditional healing of dental caries, Mbanga *et al*. (2013) further validated this claim with commendable bioactivities via *in vitro* assessment of the extracts from this plant against different strains of multidrug resistant *Streptococcus mutans* isolated from carious teeth. Roots extract of *Euclea divinorum* was also reported to have demonstrated marked activity against cancer cells (Al-Fatimi *et al*., 2005).

1.7.1. *Euclea crispa* subsp. *crispa*

Euclea crispa (Thunb.) is a small tree with smaller branches presenting a dense crown with a height of between 2 to 6 m and a spread of 2 to 4 m. It is evergreen, indigenous to Southern Africa, widely distributed from Eastern Cape, up the KwaZulu Natal coast and often in rocky areas across the Republic of South Africa. *E. crispa* is a drought and frost resistant plant and it survive both in full sun and partly shady conditions. The sweet scent of its flower attracts bees, while the seeds are visited by birds of diverse species. In addition, black rhinoceros brows its leaves and bark (Stoll, 2010).

Euclea crispa and other Euclea species are extensively utilised in the traditional medicine to combat a wide range of diseases which include wound infections, gonorrhea, leprosy, scabies and dysentery

(Khan and Rwekika 1992, Magama *et al*., 2003). Infusion of the root bark of *E. crispa* is used in the treatment of measles and melanomas (Pretorius *et al*., 2003). In the same way the decoction from the root of *E. crispa* is in use traditionally as antitussive by the people of Nhema community in Zimbabwe (Mayori, 2011) and also as psychoactive agent in South African healing tradition. While in the tradition of Lesotho people, the decoction from the bark of *E. crispa* is used as a purgative for constipation and for gall sickness in livestock when mixed with *Rumex lanceolatus* (Sobiecki, 2006). The leaf decoction of *E. crispa* is also in use as treatment for painful menstruation and the extract from the root is taken against epilepsy by the people of Zimbabwe (Sobiecki, 2006).

In vitro antimicrobial activity of *E. crispa* leaf extracts against human pathogens such as *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Streptococcus pneumoniae* was reported by Magama *et al*. (2003). Some of the bioactive phytochemicals that have been isolated from the leaf extract of this plant include; essential oils, bitter principles and saponins, terpenoid derivatives, alkaloids and phenolic compounds. The isolated flavonoids are catechin, epicatechin, gallocatechin, hyperoside and quercitrin (Pretorius *et al*., 2003). Natalenone and 3-oxo-oleanolic acid are another two bioactive compounds isolated from the methanolic extract of *E. crispa* and they were shown to inhibit the production of Amyloid β-Peptide from HeLa cells stably expressing Swedish mutant (Alzheimer's disease) form of amyloid precursor protein (Kwon *et al*., 2011). Lastly, the people of Eastern Free State of South Africa are popular with the application of the extract from this plant in their traditional healing practices, most especially the leaf extract in the treatment of diarrhoea.

Fig 1.1 A typical *Euclea crispa* (Thunb.) tree

1.8. The scope and objectives of this work

With the above as background, it is clear that the incidence of resistance to the existing antibiotics by microorganisms necessitates increased effort in development of new drugs for the treatment of microbial infections. Infections due to multidrug resistance pathogens are difficult to manage due to relatively limited choice of antimicrobial agents. Both the literature and ethno-botanical records indicate a general consensus on the use of potent antimicrobial medicinal plants to provide cheaper drugs that may complement existing supplies from orthodox medicine. Traditionally, herbal or alternative medicine is extensively practiced in the prevention and treatment of various ailments and it has regained public attention in recent years, most especially in the developing countries because it is freely available.

This study is therefore designed to investigate antimicrobial potentials of the leaf and stem bark extracts of *Euclea crispa* on some bacterial and yeast isolates that are associated with human diseases. The specific objectives of this study are to:

(a) Extract, fractionate and determine chemical groups that may be present in the leaf and stem bark extracts of *E. crispa* (Chapter II)

(b) Evaluate antimicrobial property and time-kill kinetics of the leaf extracts of *E. crispa* (Chapter III)

(c) Evaluate antimicrobial property and time-kill kinetics of the stem bark extracts of *E. crispa* (Chapter VI)

(d) Investigate membrane attack as a probable mechanism of biocidal action of the leaf and stem bark extract extracts of *E. crispa* (Chapter V)

(e) Evaluate probable effects of combination therapy between the most potent fractions from both the leaf and stem bark extracts of *E. crispa* and standard antibiotics against resistant strains of bacteria isolates (Chapter VI).

Reference

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CHAPTER 2–Extraction and chemical groups screening of the leaf and stem bark samples of *Euclea crispa*.

Abstract

The leaf and the stem bark samples of *Euclea crispa* were extracted and partitioned into different fractions. The samples were extracted in methanol/distilled water (3/2 v/v) and the fractionation was carried out via solvent partitioning system. Preliminary qualitative phytochemical screenings of the extracts were determined following standard procedures while FT-IR analysis was used to determine the functional groups present in each of the fractions. Tannins, saponins, flavonoids, cardiac glycosides, reducing sugars and steroids were detected in both extracts except the reducing sugars that is absent in the stem bark extract in addition with alkaloids which is lacking in both extracts. Moreover certain functional groups *viz;* alkanes, alkenes, alkynes, alcohol, phenol, aldehyde, aromatics, ether, sulfoxides, nitrile, amides and amines were also detected in the active fractions. Most of these phytochemicals are of proven pharmacological potentials, hence should be responsible for the expected bioactivity of the *E. crispa* leaf and stem bark extracts.

2.1. Introduction

Several scientific studies have ascribed pharmacological potentials of medicinal plant extracts to their inherent phytochemicals. Plants produce a diverse array of secondary metabolites which are bioactive principles, thus makes them an indispensable rich source of different therapeutic agents (Fakoya *et al*., 2014). These bioactive components are non-nutritional, naturally occurring substances employed for medicinal purposes (Okarter *et al*., 2009). For instance, tannins have been reported to have anti-inflammatory, antiseptic and anti-haemorrhagic potentials (Ukoha *et al*., 2011) while alkaloids are commonly in use against malarial fever, bacterial infection and as well as inducing analgesia (Okwu & Emenike, 2006). The efficacy of flavonoids, a common group among polyphenolic compounds, has been demonstrated against malignant cells, inflammation and microbial infections (Cushnie & Lamb,

2011), just as the phenolic compounds which are crucial bioactive principles in plants are exhibiting several functional pharmacological properties (Ahmed *et al*., 2014).

Earlier phytochemical screening of the leaf extract of *E. crispa* by Pretorius *et al*. (2003) reveals the presence of essential oils, bitter principles, phenolics and saponins. This study is therefore aimed at determining preliminary phytochemical screening as well as the functional groups which may likely be present in each of the fractions partitioned from the extracts and also compare the constituents from both the leaf and stem bark extracts of *E. crispa*.

2.2. Materials and Methods

2.2.1. Collection of plant sample

Fresh leaves and stem bark samples of *E. crispa* (Thunb.) family: *Ebenaceae*, were collected during the month of April 2015 at Puthaditjhaba area, Maluti-A-Phofung Municipality, Free State Province, South Africa and identified by Prof. Rodney Moffet. The plant samples were authenticated at University of the Free State herbarium with herbarium collection of Taylor and Van Wyk, 1994 with reference number: 6404000-400. It was then oven-dried (40 ℃) until constant weight, ground into fine powder and stored in an air tight container for further use.

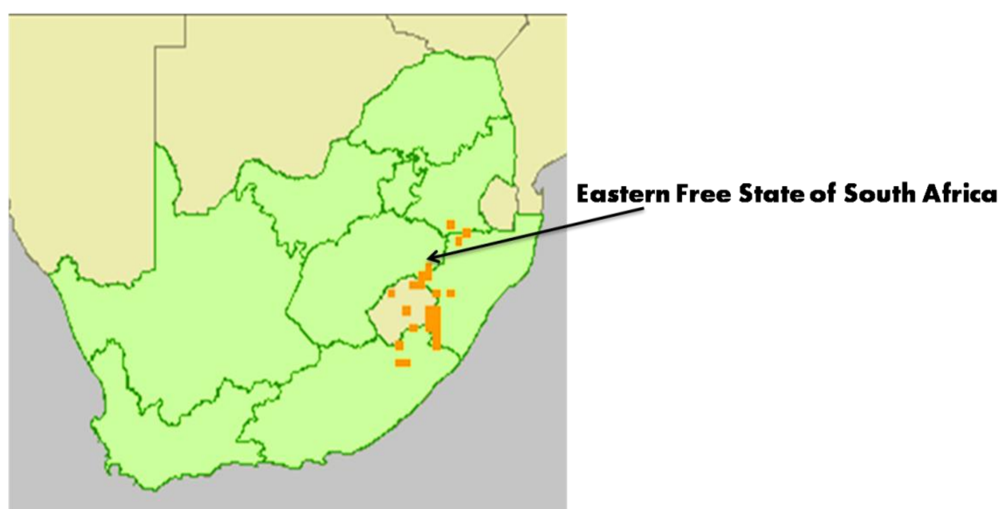

Fig 2.1: Map of South Africa showing the region where the plant samples were collected

2.2.2. Extraction of the plant samples

The ground leaves (600 g) and stem bark (400 g) samples were soaked and extracted separately in methanol/sterile distilled water (3:2, v/v) for four days, with regular agitation at intervals. The supernatants collected were filtered and the filtrates were concentrated *in vacuo* and lyophilized. The yield obtained was 0.2 g/g (leaf extract) and 0.18 g/g (stem bark extract) of the plant material.

2.2.3. Phytochemicals screening

Small portion of the freeze dried extract was subjected to qualitative phytochemical screening following standard protocol for detection of alkaloids, cardiac glycosides, flavonoids, reducing sugars, saponins, steroids and tannins (Sofowora, 1993; Harborne, 1998; Trease & Evans, 2002).

2.2.3.1. Test for tannins:

The powdered extract (1.0 g) was dissolved in sterile distilled water (10 ml) and filtered (Whatman number 1 filter paper). Two drops of 10% ferric chloride was added to the filtrate. Blue colouration afterwards signifies presence of tannins.

2.2.3.2. Test for saponins:

The powdered extract (2.0 g) was added into of 20 ml distilled water, boiled in a water bath and then filtered. Approximately 10 ml of the filtrate was mixed with 5 ml distilled water and vigorously shaken to form a stable persistent froth. The froth was mixed with few drops of olive oil, shaken vigorously and observed for the formation of emulsions which indicates the presence of saponins.

2.2.3.3. Test for steroids:

The powdered extract (0.5 g) was dissolved in 3 ml chloroform and filtered. Concentrated sulfuric acid (98%) was carefully added to the filtrate to form a lower layer. A reddish brown colouration was taken as positive for steroids.

2.2.3.4. Test for cardiac glycosides:

The powdered extract (0.5 g) was dissolved in 2 ml glacial acetic acid containing 1 drop of 1% ferric chloride and concentrated sulfuric acid was added to the solution to form a lower layer. A brown ring at the interface indicates the presence of a deoxy-sugar, a characteristic of cardiac glycosides.

2.2.3.5. Test for alkaloids:

Powdered extract (0.5 g) was dissolved in 5 ml of 1% hydrochloric acid over a steam bath and filtered. The filtrate (1 ml) was then treated with few drops of Dragendorff's reagent (precipitating agent). Observation of turbidity or precipitation was taken to indicate presence of alkaloids. Another 1 ml from the filtrate was also treated with few drops of Mayer's reagent and formation of precipitate was as well taken as presence of alkaloid.

2.2.3.6. Test for reducing sugar:

Fehling's solution I and II (1 ml each) was added to 2 ml of the aqueous solution of the plant extract. The mixture was heated in a boiling water bath for 3 min. Formation of brick red colouration signifies presence of reducing sugars.

2.2.3.7. Test for flavonoids:

Powdered extract (0.5 g) was added to 5 ml of ethyl acetate and heated in a steam bath for 3 min. This was filtered and 4 ml of the filtrate was thoroughly mixed with 1 ml of dilute ammonia solution. Formation of yellow colouration indicates the presence of flavonoids.

2.2.4. Solvent partitioning of the leaf and stem bark extracts

Exactly 110 and 60 g of the leaf and stem bark extracts was dissolved in 250 and 200 ml of sterile distilled water respectively and then partitioned into n-hexane, chloroform, ethyl acetate, n-butanol and aqueous fractions in order of the solvents polarity starting with n-hexane (4 x 200 ml). The resulting n-hexane fraction was concentrated *in vacuo* and lyophilized. The resultant aqueous phase was re-concentrated *in vacuo* and further extracted with chloroform (4 x 200 ml), ethyl acetate (4 x

200 ml) and n-butanol (5 x 200 ml) using similar procedure. The fractions collected together with the remaining aqueous fraction were also concentrated, lyophilized, kept in air-tight containers and put in a freezer for further use. The respective yields of the fractions of leaf extract were 8.20, 4.25, 21.84, 27.72 and 40.32 g, while that of the stem bark extracts were 15.70, 0.80, 4.00, 12.60 and 23.50 g. The extraction and fractionation flow chart of the leaf and stem bark extracts is presented in figure 2.2 below.

2.2.5. Determination of functional groups present in each fraction

The functional groups present in each of the leaf and stem bark fractions were assessed via Fourier transform infrared spectrometry (FT-IR) analysis. Spectra of each of the fractions were obtained on a FT-IR spectrometer (PerkinElmer, precisely) with Attenuated total reflection (ATR) attachment (PIKE, miRonle).

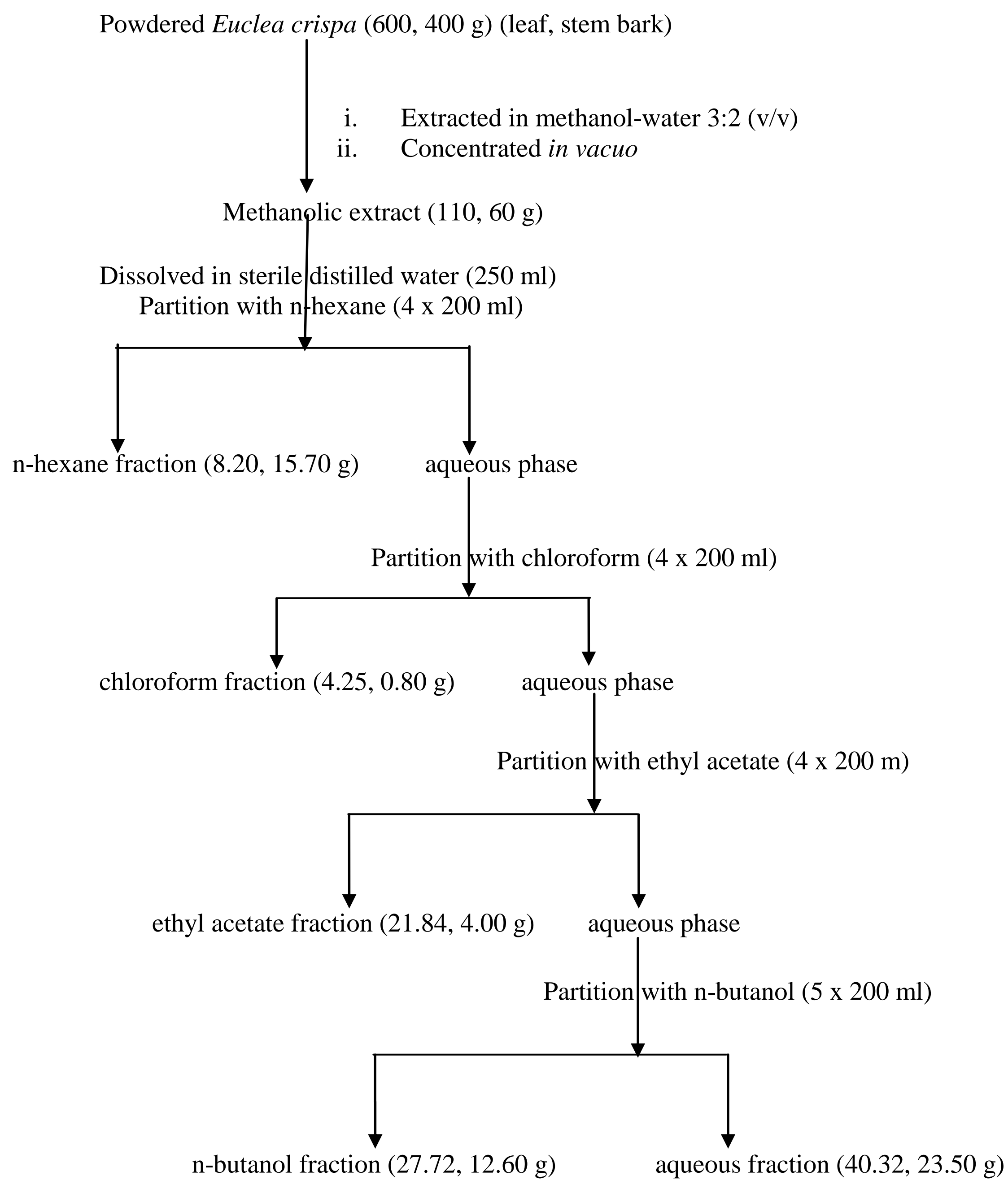

Figure 2.2: Extraction and fractionation flow chart of the *Euclea crispa* leaf and stem bark extracts

2.3. Results

2.3.1. Preliminary phytochemical screening

Qualitative phytochemical screening performed on the *E. crispa* leaf and stem bark extracts before partitioning into fractions reveals the presence of cardiac glycosides, flavonoids, reducing sugars, saponins, steroids and tannins (Table 2.1). While alkaloids are evidently absent from both extracts, tannins and cardiac glycosides are found in trace amount from the leaf extract. Whereas, only the steroids is found in trace amount in the stem bark extract. The relative abundance was determined based on the intensity of the colour change during qualitative analysis.

Table 2.1: Phytochemicals screening of the *Euclea crispa* leaf and stem bark extracts

Chemical test	**Leaf**	**Stem bark**
Alkaloids	–	–
Tannins	±	+++
Saponins	+++	+++
Flavonoids	+++	+++
Cardiac glycosides	±	+++
Reducing sugars	+++	–
Steroids	+++	±

Key: +++ = Abundant, – = Absent, ± = Trace

2.3.2. FT-IR Analysis

The components of the plant extracts were separated based on their different degrees of polarity using a solvent partitioning system. This gave rise to five different fractions from each extract. The spectra from the FT-IR analysis of the fractions of both the leaf and stem bark extracts are shown in figure 2.3 and 2.4 respectively. This indicates the presence of certain functional groups *viz;* alkanes, alkenes, alkynes, alcohol, phenol, aldehyde, aromatics, ether, sulfoxides, nitrile, amides and amines (Table 2.2 & 2.3).

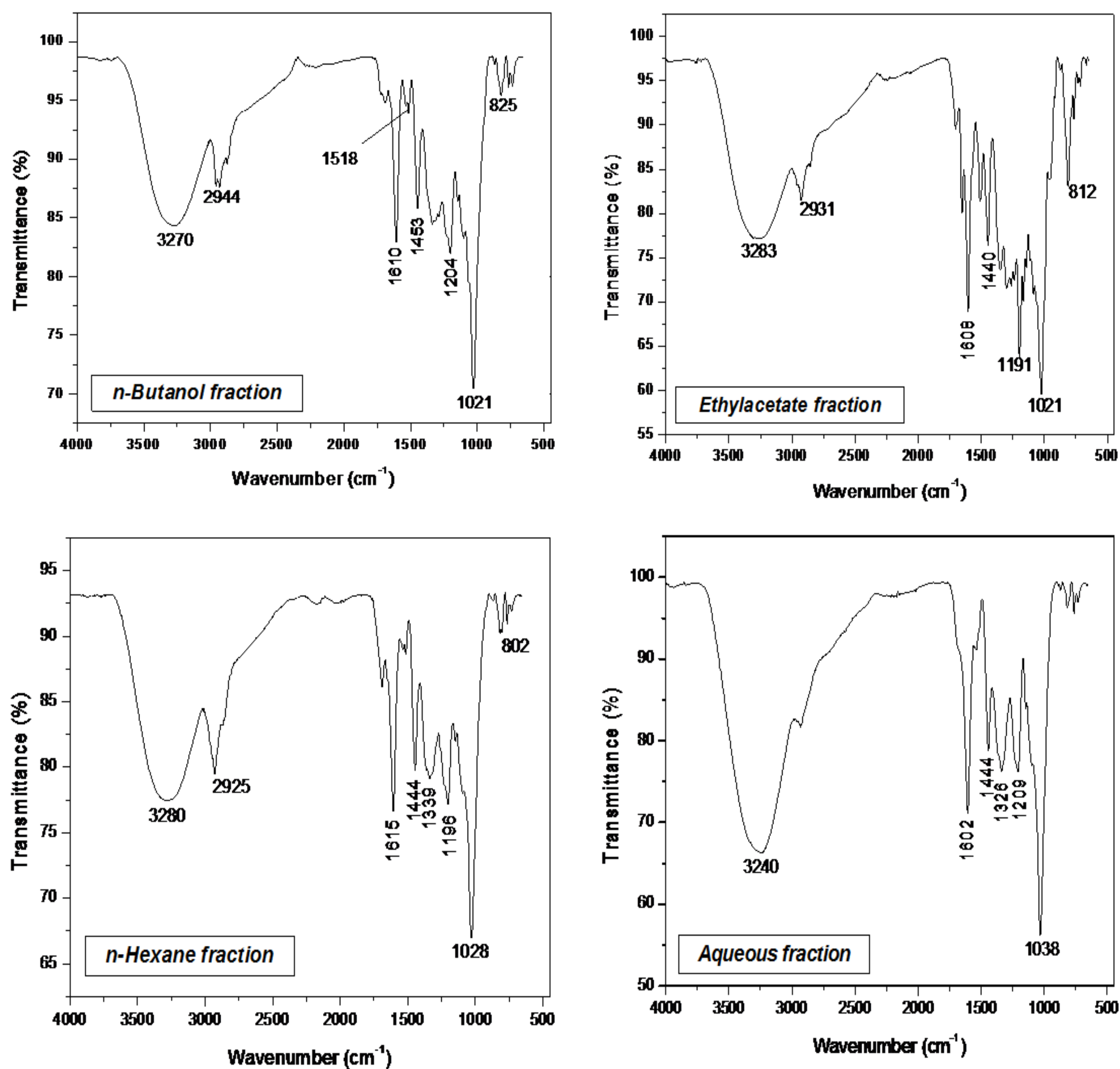

Figure 2.3: FT-IR Spectra of the n-butanol, ethyl acetate, n-hexane and aqueous fractions of *Euclea crispa* leaf extract

Table 2.2: Chemical group profiling of the potent fractions of the *Euclea crispa* leaf extract

Functional groups	Functional groups description	BUT	ETH	HEX	AQU
		Absorption frequency (cm^{-1})			
Alcohol, Phenol	C–O–H bending	1453	1440	1444	1444
	O–H stretch	3270	3283	3280	3240
Alkanes	C–H stretch	3270	3283	3280	3240
Alkenes	═C–H out of plane bend	825	812	802	–
	C═C stretch	1610	1600	1615	1602
Alkynes	≡C-H stretch	3270	3283	3280	3240
Sulfoxides	S═O stretch	1021	1021	1028	1038
Aromatics	C═C stretch	1610	1608	1615	1602
	C═C stretch	1518	–	–	–
Aldehyde	–CHO stretch	2944	2931	2925	–
Amide (1^0, 2^0)	N–H bending	1610	1600	1615	1602
	N–H stretch	3270	3283	3280	3240
Amines	N–H (1^0) bending	1610	1608	1615	1602
	C–N stretch	1204	–	1196	1209
Ethers	C–O stretch	1204	1296	1196	1209

Keys: BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction and AQU = Aqueous fraction

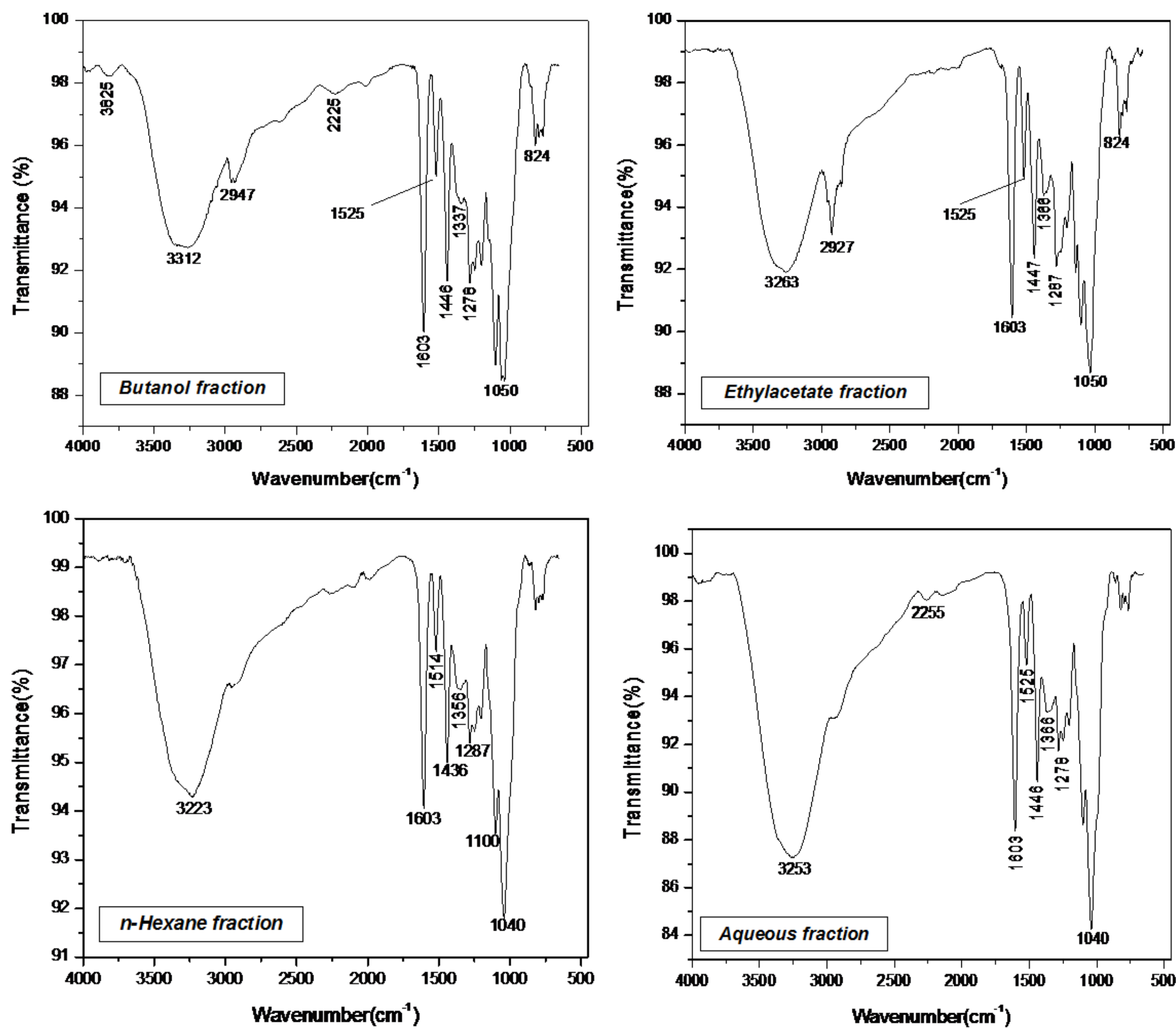

Figure 2.4: FT-IR Spectra of the n-butanol, ethyl acetate, n-hexane and aqueous fractions of *E. crispa* stem bark extract

Table 2.3: Chemical group profiling of the potent fractions of the *Euclea crispa* stem bark extract

Functional groups	Functional groups description	BUT	ETH	HEX	AQU
		Absorption frequency (cm^{-1})			
Alcohol, Phenol	C–O stretch	1278	1287	1287	1278
	C–O–H bending	1337	1366	1356	1366
	O–H stretch	3312	3263	3223	3253
Alkanes	C–H stretch	2947	2927	2949	–
	CH_2 bending	1446	1447	1436	1436
Alkenes	═C–H out of plane bend	824	824	–	–
	C═C stretch	1603	1603	1603	1603
Sulfoxides	S═O stretch	1050	1050	1040	1040
Aromatics	═C–H out of plane bend	824	824	–	–
	C═C stretch	1603	1603	1603	1603
	C═C stretch	1525	1525	1514	1525
Aromatic ketones	C–CO–C coupled stretching and bending	1278	1287	1287	1278
Nitrile	–C≡N stretch	2225	–	–	2255
Amines	N–H bending	1525	1525	1514	1525

Keys: BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction and AQU = Aqueous fraction

2.4. Discussion and Conclusion

The preliminary phytochemicals screening indicates high degree of similarity in the chemical group components of both the leaf and stem bark extracts. Although the reducing sugars which are abundantly present in the leaf extract are absent in the stem bark extract in addition with alkaloid that is lacking in both extracts. The level of similarity expressed is expected as the plant samples are both from the same mother plant. Knowing that phytochemicals are the major source of therapeutic agents or drug templates employed in modern day medicine (Ahmed *et al*., 2014), most of the phytochemicals detected from *E. crispa* extracts have proven pharmacological potential from several

studies (Gurtovenko & Anwar, 2007; Tapas *et al*., 2008; Campos *et al*., 2009; Ukoha *et al*., 2011; He *et al*., 2012). This further buttresses the hypothesis around medicinal importance of this plant which was previously informed by its application in folklore remedy.

For instance, saponins as part of detected phytochemicals are produced as secondary metabolites in plants in response to fungal or bacterial attacks, thus present them as natural antimicrobial agent (Okwu & Emenike, 2006). Tannins have also been reported to have anti-inflammatory, antiseptic and antihaemorrhagic properties (Ukoha *et al*., 2011). Abundance of phenolic compounds in anti-inflammatory constituents is believed to be an effective means by which plants self-improve resistance to pathogens (Choi *et al*., 2017). Moreso, flavonoids are widely known for decades as compounds of plant origin with broad-spectrum of biological activity and a number of flavonoids isolated from different plants have been proven to possess bacteriostatic and fungistatic activities towards several human pathogens (Tapas, 2008). Thiosulfinates, a derivative of sulfoxides from the genus *Allium* plants, are known for antimicrobial activities against bacteria and fungi (Anufrieva *et al*., 2015). Allicin, the most well-known antimicrobial and antitumor component of garlic, accounts for about 70% of all thiosulfinates formed by the β-elimination reaction of alliin and the antimicrobial potential of allicin and other thiosulfinates result from their ability to oxidize the sulfhydryl groups of proteins or enzymes in bacterial cells, meanwhile animal cells are partly protected due to presence of glutathione (Shadkchan *et al*., 2004; Anufrieva *et al*., 2015). Likewise the heterocyclic nitro-compounds such as 5-nitroimidazoles, 5-nitrofurans and nitroalkene derivatives are characterized by broad spectra antimicrobial properties against bacteria, fungi and parasites (Boguszewska-Czubara *et al*., 2016). As both the preliminary chemical and functional groups screening reveals the presence of the above mentioned phytochemicals, hence an intensive investigation of the plant for its antimicrobial potential was deemed necessary.

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CHAPTER 3 – Evaluation of antimicrobial property of *Euclea crispa* (Thunb.) leaf extract

(Part of this chapter has been published in Asian Pacific Journal of Tropical Medicine 2017; 10(4): 390–399)

Abstract

This study evaluates the antimicrobial properties of the *Euclea crispa* leaf extract and fractions partitioned from the extract as well as their time-kill kinetics. Antimicrobial properties were evaluated via susceptibility test, determination of minimum bacteriostatic and bactericidal concentrations while the time-kill kinetics of the potent fractions was determined over a period of 2 hours interaction with the *Bacillus pumilus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans* as representative isolates. The largest zone of inhibition (26±0.50 mm) is obtained by ethyl acetate fraction against *Aeromonas hydrophila* at 10 mg/ml. The lowest minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the leaf extract are 0.31 and 1.25 mg/ml respectively against the test isolates. The lowest MIC (0.08 mg/ml) is exhibited by n-butanol, ethyl acetate and aqueous fractions against test *E. coli* isolates while all the fractions exhibit MIC values between 0.31and 1.25 mg/ml against susceptible yeast isolates. n-Butanol fraction achieved absolute mortality against *Bacillus pumilus* and *Klebsiella pneumoniae* after 90 and 120 min contact time respectively at 1 × MIC. Total mortality also achieved by n-hexane fraction against *B. pumilus* and *K. pneumoniae* after 90 and 120 min respectively at 2 × MIC. Ethyl acetate fraction achieves absolute mortality against the three representative bacterial isolates after 120 min at 2 × MIC. n-Hexane fraction is able to achieve total mortality against *Candida albicans* after 120 min at 1 × MIC. This study therefore suggests the leaves of *E. crispa* a source of readily available bioactive compounds of natural origin.

3.1. Introduction

Several plant extracts have exhibited potentials against various infectious agents and thus are found useful as therapeutic agents in folkloric remedies. Circulation of multidrug resistant (MDR) pathogens presents a major pitfall in combating infectious diseases and therefore results in global medical predicament with high rate of morbidity and mortality. It has been reported from different studies that MDR is caused by prolonged abuse of antibiotics both in the clinical practices and in agricultural feeds (Mohammed *et al*., 2016). In addition to significant increment in the costs and side effects of newer drugs, resistance to antibiotics is a limiting factor in the war against infectious diseases. As resistant strains of bacteria continue to increase, there is no significantly different newer drug to remedy this problem (Jasmine *et al*., 2011).

Plant derived bioactive compounds are widely in use in most pharmaceutical industries due to their therapeutic efficacy and there are several indications from ethno-botanical records pointing to the fact that potent medicinal plants may be a source of affordable drugs that may be readily available across varying societal classes (Alayande & Ashafa, 2017). About 80% of the people living in developing countries rely directly on infusion or poultices of plants in traditional medicine for their medical remedies (Doughari, 2010). *Euclea crispa* (family *Ebenaceae*) (*E.crispa*) is one of the most common trees in South Africa. *Euclea* species are extensively used traditionally against wide range of ailments such as gonorrhoea, leprosy, scabies, diarrhoea and wound infections (Magama *et al*., 2003). Hot water extracts of the root of this plant are used as antitussive and the infusion from the roots is used in the treatment of leprosy by the people of Nhema communal area, Zimbabwe (Maroyi, 2011a & b). It has previously been established by Pretorious *et al*. (2003) that leaf extracts of *E. crispa* possess growth inhibiting potential against both bacteria and fungi. In this study, we investigate comprehensive antimicrobial potential and time-kill kinetics of the *E. crispa* leaf extracts.

3.2. Materials and Methods

3.2.1. Microorganisms

Microbial isolates used in this study include typed strains as well as locally isolated pathogens (LIPs). The LIPs which are comprise of both clinical and environmental isolates were collected from the culture collection of microbiology division, department of Microbial, Biochemical and Food Biotechnology, University of the Free State. The isolates were maintained on nutrient agar (bacteria) and yeast malt agar (yeast) medium. The strains are listed in Table 3.1.

Table 3.1: The list of microorganisms used in the study

Gram positive bacterial isolates	**Gram negative bacterial isolates**	**Different strains of *Escherichia coli* (Gram-ve)**	**Campylobacter spp. (Gram-ve)**	**Yeast isolates**
Bacillus pumilus (ATCC 14884)	*Aeromonas hydrophila*	*E. coli* (ATCC839)	*C. coli* (ATCC 33559)	*Candida albicans* (CBS8758)
Enterococcus faecalis	*Acinetobacter calcoaceticus anitratus*	*E. coli* B 98	*C. coli* (ATCC 43478)	*Candida albicans* (Ho314)
Listeria sp.	*Enterobacter faecalis*	*E. coli* B771	*C. jejuni* (ATCC 49943)	*Candida albicans* (Ho315)
Staphylococcus aureus (ATCC 6538)	*Klebsiella pneumoniae* (ATCC 13047)	*E. coli* B 841	*C. jejuni* (ATCC 29428)	*Candida albicans* (Ho316)
Staphylococcus aureus (OK 2a)	*Salmonella* Typhi	*E. coli* 1080	*C. jejuni* (NCTC 11322)	*Candida albicans* (Ho317)
Staphylococcus aureus (OK 2b)	*Salmonella* Typhimurium	*E. coli* 1304	*C. jejuni* (NCTC 11351)	*Candida albicans* (Ho318)
	Shigella flexneri	*E. coli* 1323		*Candida albicans*
	Shigella sonnei (ATCC 29930)	*E. coli* B1634		*Candida rugosa*
	Plesiomonas shigelloides	*E. coli* WCD1		*Cryptococcus neoformans*
	Proteus vulgaris (ATCC 6830)			*Trichophyton mucoides*
	Proteus vulgaris (CSIR 0030)			
	Pseudomonas aeruginosa			

3.2.2. Susceptibility testing

Agar-well diffusion method as previously described (Russell & Furr, 1997; Akinpelu *et al*., 2015; EUCAST, 2016) was employed to determine which of the selected isolates was sensitive to the plant extracts using Mueller-Hinton agar (MHA) (Oxoid, UK), a lose agar designed to allows free and proper diffusion of antimicrobial agents into the agar matrix. Exactly 0.1 ml of 24 h old standard inoculums (0.5 McFarland) was inoculated into molten MHA for the bacteria and Potato dextrose agar (Oxoid, UK) (PDA) for the yeast. This was poured into Petri dishes and allowed to set before wells were bored into the agar medium using a sterile cork borer (6 mm). The wells were carefully filled up with prepared solution of the extract at a concentration of 10 mg/ml. The plates were allowed to stand on the laboratory bench for about 2 h before incubated at 37 ℃ and 25 ℃ for the bacterial and yeast isolates respectively for 24 h, after which the plates were observed for the zones of inhibition. The *campylobacter* spp. were inoculated on Mueller-Hinton agar (MHA) with 5% sheep blood and incubated under micro-aerophilic condition (CLSI, 2010) using anaerobic jar (Oxoid, UK). The susceptibility of the isolates to the extracts were compared with that of ketoconazole, nystatin, streptomycin (1 mg/ml) and tetracycline (0.1 mg/ml) purchased from Sigma Aldrich. Sterile distilled water and 10% methanol were used as control and the experiment was carried out in triplicates.

3.2.3. Determination of the minimum inhibitory concentrations (MICs)

The MICs of the potent fractions and that of the standard drugs used were determined by agar dilution methods following standard procedure (EUCAST, 2000; CLSI M07 A10, 2015). Two-fold dilution of the extract was prepared in sterile distilled water and 2 ml of different concentrations of the aliquot was added to 18 ml of sterile molten Nutrient agar (Oxoid, UK) and PDA for the bacteria and yeasts respectively to give final concentrations ranging from 0.08 to 10.0 mg/ml. The mixture was poured into sterile Petri dishes and allowed to set. Surfaces of the media were allowed

to dry before streaking with 24 h old standard inoculums and then incubated at 37 ℃and 25 ℃ respectively for 48 h. The plates were subsequently examined for the presence or absence of growth. The MIC was taken as the lowest concentration that inhibits the growth of the isolates. Sterile agar medium plate without the extract served as control. The experiment was carried out in triplicate.

3.2.4. Determination of minimum bactericidal/fungicidal concentrations (MBCs/MFCs)

This was determined as described by Akinpelu *et al*. (2015). Samples were picked from the line of streaks with no visible growth on the MICs result plates and sub-cultured onto freshly prepared Nutrient agar plates and PDA plates respectively for the bacterial and yeast isolates. The plates were incubated at 37 ℃ (bacteria) and 25 ℃ (yeast) for 48 h. The MBCs/MFCs were taken as the lowest concentration of the extracts that did not show any visible growth on the new set of Agar plates.

3.2.5. Determination of killing rate

The killing rate by the potent fractions was determined as described by Odenholt *et al.* (2004) with slight modifications. This was carried out against *Bacillus pumilus* (ATCC 14884), *Klebsiella pneumoniae* (ATCC 13047), *Escherichia coli* (1323) and *Candida albicans* (Ho316) as representative isolates. Nutrient broth cultures (24 h) of the isolates were standardized and viable counts were determined. Then 5 ml of the known cell density of the microbial suspension was added to 45 ml of different concentrations of the extracts relative to the MIC. The resulting suspensions were mixed and held at room temperature while the killing rate was determined over 2-h period. A volume of 0.5 ml was taken from each suspension at intervals and transferred into 4.5 ml Nutrient broth recovery medium containing 3% Tween80. This was serially diluted in sterile physiological saline (0.9% NaCl) and 0.1 ml of the final dilution was plated out onto Nutrient agar (bacteria) and PDA (yeast), incubated at 37 ℃ and 25 ℃ respectively for 48 h. The control

experiment was set up without inclusion of extracts. Viable counts were made in triplicate and a decrease in the number of colony forming units indicates killing by the extracts.

3.3. Results

3.3.1. Antimicrobial susceptibility

The antimicrobial susceptibility profile indicates that all the test bacterial isolates were sensitive to both the extracts and fractions at a concentration of 20 mg/ml and 10 mg/ml respectively with varying degrees of activities, except in the case of *Campylobacter* spp. which none of it is sensitive to the chloroform fraction (Table 3.2 and 3.3). The zones of inhibition expressed by the leaf extract range between 20±0.00 and 23±0.58 mm against test bacterial and fungal isolates. Appreciably wide zones of inhibition (≥ 20 mm) were expressed by the fraction partitioned into ethyl acetate against 78% of the isolates, closely followed by that of n-butanol against 63% of the bacterial isolates (Table 3.2 - 3.4) Minimum inhibitory concentrations (MICs) exhibited by the leaf extract and that of potent fractions are shown in Table 3.5 to 3.7. The lowest MIC (0.31 mg/ml) exhibited by the leaf extract is against a number of *E. coli* strains, while the lowest MIC of 0.08 mg/ml is equally exhibited by the fractions partitioned into n-butanol, ethyl acetate and water all against *E. coli* isolates. The n-hexane fraction appeared to be most active against *Campylobacter* spp. with MIC value of 0.31 mg/ml against 66% of the isolates. The lowest concentration (0.31 mg/ml) with cidal effect on the test isolates is expressed by the ethyl acetate fraction against 70% of the *E. coli* strains, while the general range of the MBC by all the partioned fractions is between 0.31 and 2.5 mg/ml (Table 3.8 - 3.10).

Table 3.2: The sensitivity patterns of zones of inhibition of *Euclea crispa* leaf extract, fractions and standard drugs against test bacterial isolates

Bacterial isolates	**Zones of inhibition (mm) ****								
	***E. crispa* (20 mg/ml)**	**BUT (10 mg/ml)**	**ETH (10 mg/ml)**	**HEX (10 mg/ml)**	**AQU (10 mg/ml)**	**CHL (10mg/ml)**	**STP (1 mg/ml)**	**TET (0.1 mg/ml)**	**MET (10%)**
Aeromonas hydrophila	20±0.58	16±0.00	26±0.50	16±1.00	16±1.15	16±0.76	25±0.28	25±0.00	0
Acinetobacter calcoaceticus anitratus	22±0.28	20±1.20	20±0.76	20±1.20	18±0.00	18±0.58	25±0.00	26±1.00	0
Bacillus pumilus (ATCC 14884)	23±1.00	20±1.00	22±0.50	16±0.50	22±0.58	16±0.76	25±0.76	28±0.00	0
Enterobacter faecalis	23±0.58	18±0.00	20±0.50	16±0.58	18±0.28	15±0.00	25±0.58	28±0.50	0
Enterococcus faecalis	20±0.76	20±0.58	20±0.00	16±1.20	18±0.00	18±1.00	26±0.28	26±0.28	0
Klebsiella pneumoniae (ATCC 13047)	20±0.50	18±0.58	18±1.15	16±0.00	18±0.76	16±0.00	26±0.00	24±0.00	0
Klebsiella pneumoniae	22±0.00	18±0.00	16±0.50	16±0.76	16±1.00	16±1.15	23±0.58	27±0.76	0
Listeria sp.	22±0.00	22±0.50	22±1.15	18±1.15	22±0.50	16±0.58	29±1.00	27±0.58	0
Shigella sonnei (ATCC 29930)	20±0.00	20±0.28	20±1.00	16±0.76	18±0.58	16±0.58	27±1.00	25±0.28	0
Shigella flexneri	20±0.58	20±0.50	20±0.00	16±0.58	20±0.00	16±0.50	23±1.15	26±0.50	0
Salmonella Typhimurium	20±0.58	21±0.58	18±0.00	18±0.50	16±0.00	16±1.15	25±1.00	25±0.28	0
Salmonella Typhi	22±0.10	18±1.00	18±1.00	16±0.76	16±0.28	16±0.00	26±0.00	27±0.50	0
Staphylococcus aureus (ATCC 6538)	20±1.00	18±1.15	20±0.00	18±1.15	18±1.15	16±1.00	26±1.00	24±1.15	0
Staph. aureus (OK 2a)	21±0.00	16±0.00	20±0.50	18±1.00	16±0.50	17±0.28	25±0.00	25±0.00	0
Staph. aureus (OK2b)	22±0.00	20±1.15	18±0.00	18±1.00	16±0.28	15±0.50	24±0.00	26±1.15	0
Plesiomonas shigelloides	22±0.58	16±0.50	16±0.50	18±1.00	16±0.00	16±0.58	24±0.00	26±0.00	0
Proteus vulgaris (CSIR 0030)	23±1.15	16±0.50	18±0.58	16±0.28	16±1.00	18±0.50	29±1.15	26±0.58	0
Proteus vulgaris	20±1.15	20±1.20	18±0.58	18±0.50	18±0.00	16±0.00	26±1.15	25±0.00	0
Pseudomonas aeruginosa	21±0.28	16±0.00	16±0.28	17±0.00	16±1.15	15±0.76	26±1.00	25±1.00	0

Key: ATCC = American type culture collection, CSIR = Council for scientific and industrial research, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, MET = methanol, 0 = Not sensitive, mm** = Mean of three replicates

Table 3.3: Sensitivity patterns of zones of inhibition exhibited by the leaf extract of *Euclea crispa*, its fractions and standard antibiotics against *Campylobacter* spp. and strains of *Escherichia coli*

Bacterial isolates	**Zones of inhibition (mm) ****								
	***E. crispa* (20 mg/ml)**	**BUT (10 mg/ml)**	**ETH (10 mg/ml)**	**NHEX (10 g/ml)**	**AQU (10 mg/ml)**	**CHL (10 mg/ml)**	**STREP (1 mg/ml)**	**TET (0.1 mg/ml)**	**MET (10%)**
Campylobacter coli (ATCC 33559)	18±0.29	20±0.28	20±0.00	20±0.00	14±0.00	0	26±0.58	25±0.58	0
C. coli (ATCC 43478)	20±0.50	20±0.58	20±1.00	18±0.58	14±0.50	0	27±1.00	26±0.00	0
C. jejuni (ATCC 49943)	22±0.00	22±0.00	20±0.58	18±0.00	16±0.00	0	26±0.00	28±1.00	0
C. jejuni (ATCC 29428)	21±0.58	22±0.58	22±0.00	19±1.04	16±0.58	0	26±0.76	29±0.00	0
C. jejuni (NCTC 11322)	20±0.00	22±0.50	22±0.00	18±0.00	16±0.00	0	27±0.58	25±1.00	0
C. jejuni (NCTC 11351)	18±0.58	20±0.00	20±0.58	18±0.58	16±1.00	0	26±0.00	28±1.15	0
Escherichia coli (ATCC839)	20±0.00	20±0.00	20±1.15	18±0.28	20±0.00	18±1.00	27±0.00	26±0.58	0
E. coli 1304	18±0.00	22±1.00	22±0.00	16±0.50	16±1.15	18±0.00	25±1.15	25±0.50	0
E. coli 1080	19±0.58	20±1.15	20±1.00	18±0.00	20±0.58	18±0.00	25±0.00	27±0.00	0
E. coli B1634	20±0.00	18±0.50	22±0.58	18±1.15	22±0.00	16±0.50	27±0.58	25±0.58	0
E. coli B 98	18±1.00	18±0.00	20±0.58	17±1.00	16±1.00	18±0.00	28±0.00	29±1.15	0
E. coli B 841	20±1.00	20±0.00	20±0.00	18±1.00	21±0.00	16±0.58	27±1.00	28±0.00	0
E. coli B771	20±1.15	18±1.00	21±1.15	18±0.50	16±0.00	17±0.28	26±0.58	25±1.00	0
E. coli 1323	18±1.15	20±0.00	22±0.00	18±1.00	18±0.58	16±0.00	27±0.00	27±0.50	0
E. coli WCD1	21±0.58	22±0.50	20±1.00	20±0.00	19±1.15	18±0.58	28±0.28	27±0.58	0

KEY: ATCC = American type culture collection, NCTC = National collection of type cultures, BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline, MET = methanol, 0 = Not sensitive, (mm) ** = Mean of three replicates.

Table 3.4: The sensitivity patterns of zones of inhibition of *Euclea crispa* leaf extract, fractions and standard drugs against test yeast isolates

Fungal Isolates	Zones of inhibition (mm) **								
	E. crispa **(20 mg/ml)**	**BUT (10 mg/ml)**	**ETY (10 mg/ml)**	**HEX (10 mg/ml)**	**AQU (10 mg/ml)**	**CHL (10 mg/ml)**	**NYS (1 mg/ml)**	**KET (1 mg/ml)**	**MET (10%)**
Candida albicans	22±0.00	20±1.00	20±1.15	20±0.50	20±1.00	0	18±0.00	22±0.00	0
Candida albicans (CBS8758)	0	0	0	0	0	0	24±0.58	18±1.00	0
Candida albicans (Ho314)	0	0	20±0.76	0	0	0	23±0.28	18±0.58	0
Candida albicans (Ho315)	20±0.58	0	16±0.58	0	0	0	24±1.00	23±0.58	0
Candida albicans (Ho316)	21±0.76	21±1.15	22±0.50	19±1.00	19±0.58	0	22±1.15	21±0.76	0
Candida albicans (Ho317)	0	0	0	0	0	0	24±0.76	18±0.58	0
Candida albicans (Ho318)	18±1.00	18±0.76	18±1.00	16±0.58	0	0	22±1.00	0	0
Candida rugose	23±0.50	22±0.00	22±0.58	20±0.50	20±1.15	18±1.00	20±0.50	22±0.58	0
Cryptococcus neoformans	20±0.58	20±0.50	20±0.58	20±0.00	18±0.76	0	22±0.58	20±0.50	0
Trichophyton mucoides	20±1.15	18±0.58	20±0.00	18±0.28	18±0.00	0	18±0.00	19±0.76	0

Key: CBS = Cetraal bureau voor Schimmel cultures, The Netherlands, Ho = Strain numbers for locally isolated strains held in the UNESCO MIRCEN Yeast Culture collection at the University of the Free State, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, AQU = Aqueous fraction, CHL = Chloroform fraction, NYS = Nystatin, KET = Ketoconazole, MET = methanol, 0 = Not sensitive, mm** = Mean of three replicates

Table 3.5: The minimum inhibitory concentrations (MICs) of *Euclea crispa* leaf extract, fractions and standard drugs against test bacterial isolates

Bacterial isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Aeromonas hydrophila	1.25	0.63	0.63	0.63	1.25	2.50	0.063	0.013
Acinetobacter calcoaceticus anitratus	0.63	0.63	0.31	0.63	2.50	0.63	0.063	0.025
Bacillus pumilis (ATCC 14884)	1.25	0.63	0.16	0.63	1.25	0.31	0.016	0.013
Enterobacter faecalis	1.25	0.63	0.31	0.63	2.50	0.63	0.016	0.013
Enterococcus faecalis	1.25	0.63	0.63	0.63	2.50	0.63	0.016	0.013
Klebsiella pneumoniae (ATCC 13047)	1.25	0.31	0.31	0.63	1.25	1.25	0.016	0.013
Klebsiella pneumoniae	1.25	1.25	0.63	0.63	2.50	1.25	0.063	0.013
Listeria sp.	1.25	0.31	0.31	0.63	1.25	0.63	0.008	0.013
Shigella sonnei (ATCC 29930)	1.25	0.63	0.31	1.25	1.25	1.25	0.016	0.013
Shigella flexneri	2.50	0.63	0.63	0.63	1.25	0.63	0.031	0.025
Salmonella Typhimurium	2.50	0.63	0.63	0.63	1.25	1.25	0.016	0.025
Salmonella Typhi	1.25	0.63	0.31	0.63	1.25	1.25	0.016	0.013
Staphylococcus aureus (ATCC 6538)	0.63	0.63	0.31	0.63	1.25	0.63	0.016	0.013
Staph. aureus (OK 2a)	2.50	0.63	0.31	1.25	2.50	1.25	0.063	0.006
Staph. aureus (OK2b)	2.50	0.63	0.31	0.63	2.50	2.50	0.016	0.025
Plesiomonas shigelloides	1.25	0.63	0.31	0.31	2.50	1.25	0.016	0.025
Proteus vulgaris (CSIR 0030)	1.25	0.63	0.31	0.63	1.25	0.63	0.016	0.013
Proteus vulgaris (ATCC 6830)	1.25	1.25	0.63	1.25	1.25	1.25	0.125	0.025
Pseudomonas aeruginosa	1.25	0.16	0.63	0.63	1.25	0.63	0.016	0.013

Key: ATCC = American type culture collection, CSIR = Council for scientific and industrial research, EC = *E. crispa* extract, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP= Streptomycin, TET= Tetracycline

Table 3.6: The minimum inhibitory concentrations (MICs) of the leaf extract of *Euclea crispa,* its fractions and standard antibiotics against *Campylobacter* spp. and strains of *Escherichia coli*

Bacterial isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Campylobacter coli (ATCC 33559)	1.25	0.63	0.63	0.31	2.50	ND	0.063	0.025
C. coli (ATCC 43478)	1.25	0.63	0.31	0.31	1.25	ND	0.063	0.013
C. jejuni (ATCC 49943)	0.63	0.31	0.63	0.31	2.50	ND	0.031	0.013
C. jejuni (ATCC 29428)	0.63	0.31	0.31	0.31	1.25	ND	0.016	0.006
C. jejuni (NCTC 11322)	1.25	0.31	0.63	0.63	1.25	ND	0.016	0.025
C. jejuni (NCTC 11351)	1.25	0.63	0.63	0.63	2.50	ND	0.008	0.013
Escherichia coli (ATCC839)	1.25	1.25	0.63	1.25	2.50	1.25	0.016	0.013
E. coli 1304	1.25	0.63	0.08	1.25	1.25	0.16	0.125	0.100
E. coli 1080	0.63	0.63	0.31	1.25	0.63	0.63	0.031	0.050
E. coli B1634	0.63	0.31	0.16	0.31	0.08	0.31	0.031	0.050
E. coli B 98	0.63	0.63	0.31	0.63	1.25	0.63	0.008	0.006
E. coli B 841	0.31	0.16	0.16	0.63	0.08	0.63	0.004	0.100
E. coli B771	0.31	0.31	0.16	0.31	2.50	0.31	0.250	0.100
E. coli 1323	0.63	0.16	0.08	0.31	0.16	0.63	0.004	0.050
E. coli WCD1	0.31	0.08	0.16	0.16	0.16	0.63	0.004	0.006

KEY: ATCC = American type culture collection, NCTC = National collection of type cultures, BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX= n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline, ND = Not determined.
*ND because the test isolates were not sensitive to the bioactive agent, hence no further test is required

Table 3.7: The minimum inhibitory concentrations (MICs) of *Euclea crispa* leaf extract, fractions and standard drugs against test yeast isolates

Fungal isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**NYS (mg/ml)**	**KET (mg/ml)**
Candida albicans	1.25	0.63	0.63	0.63	1.25	ND	0.25	0.13
Candida albicans (CBS8758)	ND	ND	ND	ND	ND	ND	0.13	0.25
Candida albicans (Ho314)	ND	ND	0.63	ND	ND	ND	0.25	0.25
Candida albicans (Ho315)	0.63	ND	1.25	ND	ND	ND	0.13	0.13
Candida albicans (Ho316)	0.63	0.63	0.31	0.63	1.25	ND	0.25	0.25
Candida albicans (Ho317)	ND	ND	ND	ND	ND	ND	0.13	0.25
Candida albicans (Ho318)	1.25	1.25	0.63	1.25	ND	ND	0.13	ND
Candida rugosa	1.25	0.31	0.31	0.63	0.63	1.25	0.13	0.13
Cryptococcus neoformans	1.25	0.63	0.63	0.63	1.25	ND	0.13	0.13
Trichophyton mucoides	1.25	0.31	0.31	0.63	1.25	ND	0.25	0.25

Key: CBS = Cetraal bureau voor Schimmel cultures, The Netherlands, Ho = Strain numbers for locally isolated strains held in the UNESCO MIRCEN Yeast Culture collection at the University of the Free State, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, AQU = Aqueous fraction, CHL = Chloroform fraction, NYS = Nystatin, KET = Ketoconazole, MET = methanol, 0 = Not sensitive, mm** = Mean of three replicates
*ND because the test isolates were not sensitive to the bioactive agent, hence no further test is required

Table 3.8: The minimum bactericidal concentrations (MBCs) of *Euclea crispa* leaf extract, fractions and standard drugs exhibited against test bacterial isolates

Bacterial isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Acinetobacter calcoaceticus anitratus	1.25	1.25	0.63	1.25	2.50	1.25	0.063	0.025
Aeromonas hydrophila	5.00	1.25	1.25	1.25	2.50	2.50	0.125	0.025
Bacillus pumilis (ATCC 14884)	1.25	1.25	0.63	1.25	1.25	1.25	0.031	0.025
Enterobacter faecalis	2.50	1.25	0.63	1.25	2.50	1.25	0.031	0.025
Enterococcus faecalis	2.50	1.25	1.25	1.25	5.00	1.25	0.031	0.025
Klebsiella pneumoniae (ATCC 13047)	2.50	1.25	0.63	1.25	2.50	2.50	0.031	0.025
Klebsiella pneumoniae	2.50	2.50	1.25	1.25	2.50	2.50	0.125	0.025
Listeria sp.	2.50	1.25	0.63	1.25	2.50	1.25	0.031	0.025
Shigella sonnei (ATCC 29930)	2.50	1.25	0.63	1.25	2.50	2.50	0.031	0.025
Shigella flexneri	2.50	1.25	1.25	1.25	2.50	1.25	0.063	0.025
Salmonella Typhimurium	2.50	1.25	1.25	1.25	2.50	2.50	0.031	0.050
Salmonella Typhi	2.50	1.25	0.63	1.25	2.50	2.50	0.031	0.025
Staphylococcus aureus (ATCC 6538)	1.25	1.25	0.63	1.25	2.50	1.25	0.031	0.025
Staphylococcus aureus (OK 2a)	5.00	1.25	0.63	2.50	5.00	2.50	0.063	0.013
Staphylococcus aureus (OK2b)	5.00	1.25	0.63	1.25	2.50	2.50	0.031	0.025
Plesiomonas shigelloides	2.50	1.25	0.63	0.63	2.50	2.50	0.031	0.025
Proteus vulgaris (CSIR 0030)	2.50	1.25	0.63	1.25	2.50	1.25	0.031	0.025
Proteus vulgaris	2.50	1.25	0.63	1.25	2.50	2.50	0.125	0.050
Pseudomonas aeruginosa	2.50	0.63	1.25	1.25	2.50	1.25	0.031	0.025

Key: ATCC =American type culture collection, CSIR = Council for scientific and industrial research, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline

Table 3.9: The minimum bactericidal concentrations (MBCs) of the leaf extract of *Euclea crispa*, its fractions and standard antibiotics against *Campylobacter* spp. and strains of *Escherichia coli*

Bacterial isolates	**E. crispa (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Campylobacter coli (ATCC 33559)	5.00	1.25	1.25	1.25	5.00	ND	0.125	0.050
C. coli (ATCC 43478)	2.50	1.25	0.63	1.25	2.50	ND	0.125	0.025
C. jejuni (ATCC 49943)	1.25	0.63	1.25	0.63	5.00	ND	0.063	0.025
C. jejuni (ATCC 29428)	1.25	0.63	0.63	0.63	2.50	ND	0.063	0.025
C. jejuni (NCTC 11322)	2.50	0.63	1.25	1.25	2.50	ND	0.031	0.050
C. jejuni (NCTC 11351)	5.00	0.63	0.63	1.25	2.50	ND	0.031	0.025
Escherichia coli (ATCC839)	2.50	1.25	1.25	2.50	2.50	2.50	0.031	0.025
E. coli 1304	2.50	1.25	0.31	2.50	2.50	0.63	0.125	0.100
E. coli 1080	1.25	1.25	0.63	2.50	1.25	1.25	0.063	0.100
E. coli B1634	1.25	0.63	0.31	0.63	0.63	1.25	0.063	0.013
E. coli B 98	1.25	1.25	0.63	1.25	1.25	1.25	0.031	0.100
E. coli B 841	1.25	0.31	0.31	0.63	0.31	1.25	0.016	0.100
E. coli B771	1.25	0.63	0.31	0.63	2.50	0.63	0.250	0.100
E. coli 1323	1.25	0.31	0.31	0.63	0.63	0.63	0.016	0.050
E. coli WCD1	1.25	0.31	0.31	0.31	0.31	1.25	0.016	0.013

KEY: ATCC = American type culture collection, NCTC = National collection of type cultures, BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline, ND = Not determined.

Table 3.10: The minimum fungicidal concentrations (MFCs) of *Euclea crispa* leaf extract, fractions and standard drugs exhibited against test yeast isolates

Fungal Isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**CHL (mg/ml)**	**NYS (mg/ml)**	**KET (mg/ml)**
Candida albicans	2.50	1.25	0.63	2.50	2.50	ND	0.25	0.25
Candida albicans (CBS8758)	ND	ND	ND	ND	ND	ND	0.25	0.50
Candida albicans (Ho314)	ND	ND	0.63	ND	ND	ND	0.50	0.50
Candida albicans (Ho315)	2.50	ND	1.25	ND	ND	ND	0.25	0.25
Candida albicans (Ho316)	1.25	0.63	0.63	1.25	2.50	ND	0.25	0.50
Candida albicans (Ho317)	ND	ND	ND	ND	ND	ND	0.25	0.50
Candida albicans (Ho318)	2.50	2.50	1.25	2.50	ND	ND	0.25	ND
Candida rugosa	2.50	0.63	0.63	1.25	1.25	2.50	0.25	0.25
Cryptococcus neoformans	2.50	0.63	0.63	1.25	2.50	ND	0.25	0.25
Trichophyton mucoides	5.00	0.63	0.63	0.63	2.50	ND	0.50	0.25

Key: BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, NYS = Nystatin, KET = Ketoconazole, ND = Not determined

3.3.2. Time-kill kinetics

The fraction partitioned into n-butanol is able to achieve absolute mortality rate against *Bacillus pumilus* (ATCC 14884) at a concentration of 1 × MIC after 90 min of contact time and after 120 min against *Klebsiella pneumoniae* (ATCC 13047) under the same condition. When the concentration was increased to 2 × MIC, total mortality is also achieved by the fraction partitioned into n-hexane after 90 min of contact time against *Bacillus pumilus* and after 120 min against *Klebsiella pneumoniae* while the same rate is also achieved by the fraction partitioned into ethyl acetate after 120 min of contact time against both representative bacterial isolates. The mortality rate by the fractions partitioned into water and chloroform are 96.1 and 97.5% respectively after 120 min against *Bacillus pumilus* and 95.4 and 94.9 % against *Klebsiella pneumoniae* (figures 3.1 & 3.2). Only ethyl acetate fraction is able to achieve total mortality against *E. coli* (1323) after 120 min at 2 × MIC, while the mortality rate by n-butanol, n-hexane, chloroform and aqueous fractions are 98, 94.6, 83.7 and 91.8% respectively (figure 3.3). Against *Candida albicans* (Ho316), the fraction partitioned into n-hexane is able to achieve total mortality after 120 min of contact time at a concentration of 1 × MIC while the mortality rate by those partitioned into n-butanol, ethyl acetate and water are 97.2, 97.9 and 95.7%, respectively under similar condition. When the concentration was increased to 2 × MIC all the fractions achieve absolute mortality except the aqueous fraction with 96.5% after 120 min of contact time (figure 3.4).

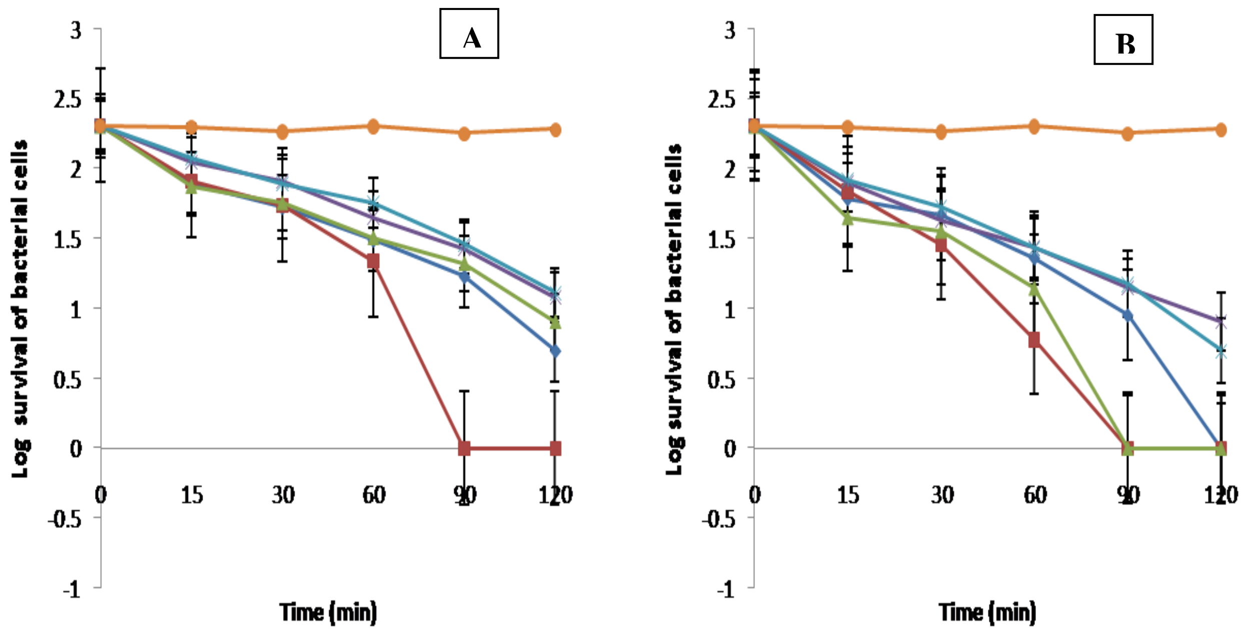

Fig. 3.1 The extent and the rate of killing of *Bacillus pumilus* by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ), water ( ) and chloroform fractions ( ) at 1 × MIC (A) and 2 × MIC (B). Control ( )

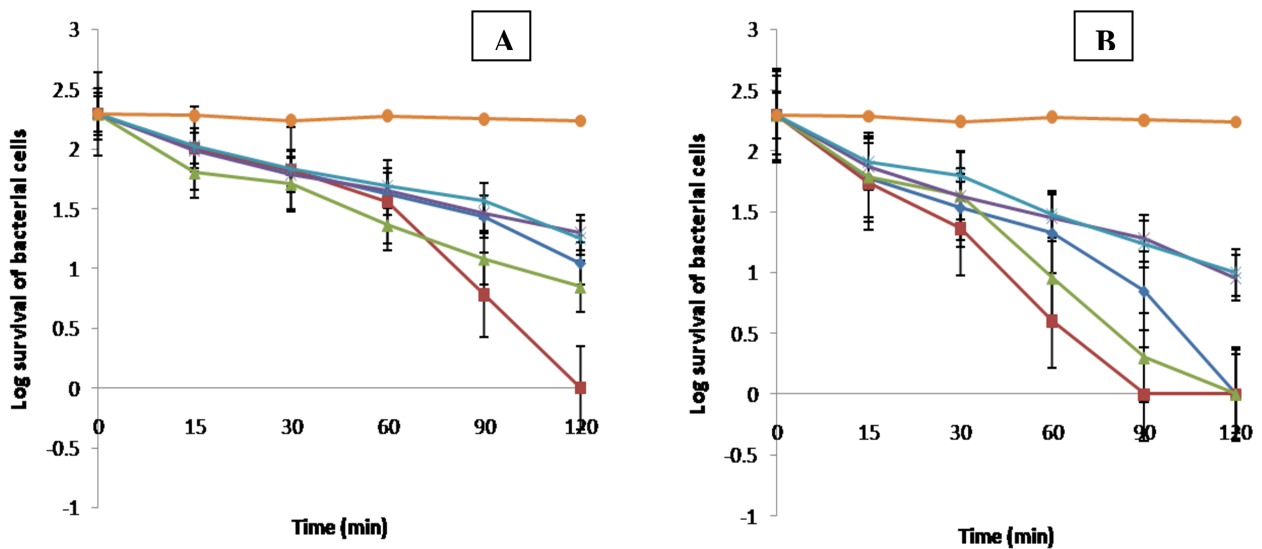

Fig. 3.2 The extent and the rate of killing of *Klebsiella pneumoniae* by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ), water ( ) and chloroform fractions ( ) at 1 × MIC (A) and 2 × MIC (B). Control ( )

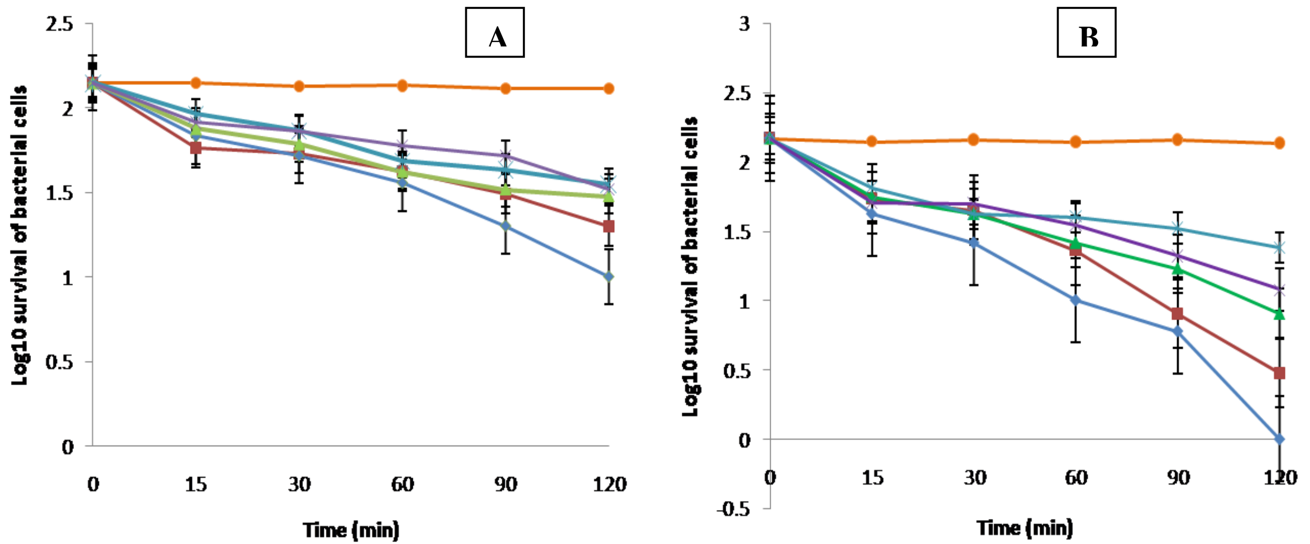

Fig. 3.3 The extent and the rate of killing of *E. coli* (1323) by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ), water ( ) and chloroform fractions ( ) at 1 × MIC (A) and 2 × MIC (B). Control ( )

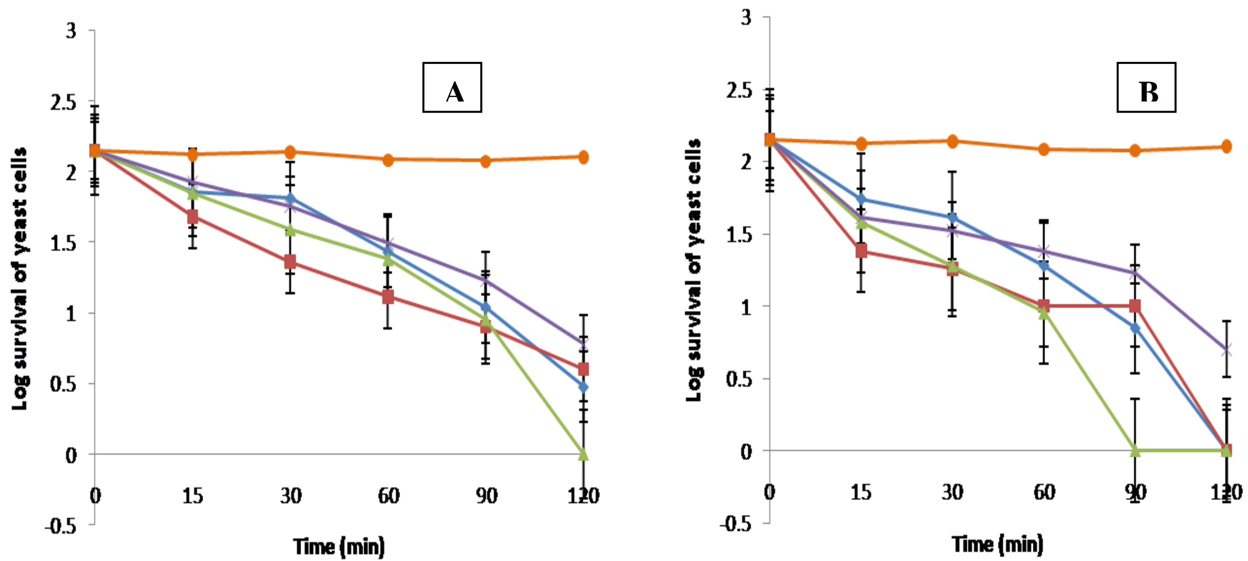

Fig. 3.4 The extent and the rate of killing of *Candida albicans* by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) at 1 × MIC (A) and 2 × MIC (B). Control ( )

3.4. Discussion and Conclusion

The leaf extract and the fractions demonstrated credible antimicrobial potentials with broad spectrum. *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria* sp. are among the susceptible pathogens. *Staphylococcus aureus* has been implicated in bacteraemia associated with high morbidity and mortality, often results into infective endocarditis (Ralph, 2009). Pathogenicity of *E. faecalis* ranges from life threatening ailments to less severe disease conditions (Güven, 2004) while *Listeria* sp. is a causative agent of listeriosis with meningoencephalitis and septicaemia as clinical symptoms (El-Shenawy *et al*., 2011). The significant activity shown by the extracts against test pathogens is as a result of the bioactive principles that were determined in the extract. Hence, leaf extracts of *E. crispa* could be a good source of readily available drugs against the aforementioned ailments in folklore remedy. The growth of *E. coli,* a common causative agent of diarrhoea and urinary tract infections (Akinpelu *et al*., 2015), and species of *Salmonella* and *Shigella* is also significantly inhibited by the extracts. This pathogens are prominent diarrhoea causing bacterial isolates, thus validates traditional use of the leaf of *E. crispa* as antidiarrhoeal agent by the people of Eastern Free State, South Africa. In addition to *Campylobacter* spp.which are most sensitive to the n-Hexane fraction, they are etiological agent of Campylobacteriosis commonly associated with eating raw or undercooked poultry. This pathogen is one of the most common types of bacteria causing diarrhoea with estimated 2.4 million people each year in the United States (CDPH, 2013). Meanwhile, antifungal capability of the plant extract was also investigated. *Cryptococcus neoformans,* an emerging yeast pathogen of man has been reportedly responsible for annual deaths of about six hundred thousand immuno-compromised individuals (Price *et al*., 2011). *Candida albicans,* a notorious pathogenic yeast, due to its inherent resistance to antimicrobial therapy accounts for a large number of fungal infections in the skin, digestive tract and bloodstream (Motaung *et al*., 2015). The susceptibility of these pathogens to the leaf extracts further affirm its efficacy in the management of infectious diseases by the people of Lesotho as earlier reported by Moteetee & Van Wyk (2011).

All fractions from the leaf extract exhibited notable antimicrobial activities against some of the test bacterial and fungal isolates with low MIC values which compared favorably with standard antimicrobial drugs used as positive control. The significantly low MIC values (0.16 and 0.08 mg/ml) was exhibited by the ethyl acetate and n-butanol fractions against *Bacillus pumilus* and *Pseudomonas aeruginosa* thus, suggesting the solvents as probable choice in extraction of most active compounds from this plant. Also the MIC value of 0.08 mg/ml against *E. coli* by the aqueous fraction further affirm the used of aqueous infusion in the treatment of diarrhoea by the people of Eastern Free State. This may be an advantage in the production of antimicrobial compounds of natural origin to combat emerging cases of multidrug resistant microbial pathogens. On a general note, ethyl acetate fraction has shown the best activity with the highest MIC values of ≤ 0.63 mg/ml against all test isolates. This corroborates with the finds of Magama *et al*. (2003) where zones of inhibition by different fractions of *E. crispa* were observed against certain human pathogens, however, it has been concluded from this study that ethyl acetate, n-butanol and n-hexane fractions are generally the most active fractions after considering the rate at which each of the potent fractions was able to bring about depression in the number of survival cells after treatment. Considering the relative degree of potency exhibited by individual fractions, bioactive principles from the leaf of *E. crispa* can be extracted both by polar and fairly polar solvents.

The leaf extracts of *E. crispa* demonstrate credible antimicrobial potential against wider range of infectious diseases which maybe applicable in the alternative therapy practices and possibly in the pharmaceutical industries. This also suggests the plant as a source of affordable and readily available therapy for diarrhoeal victims most especially in developing countries.

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CHAPTER4–Evaluation of antimicrobial property of the *Euclea crispa* (Thunb.) stem bark extracts

Abstract

The present study evaluates biocidal effect of *Euclea crispa* stem bark extracts via susceptibility test, determination minimum inhibitory concentrations (MICs) and time-kill kinetics of the potent fractions. Susceptibility of the microbial cells was determined via agar well diffusion, while the MICs was determined using broth micro-dilution method and the time-kill kinetics was evaluated at different concentrations over a 2-h period. The zones of inhibition exhibited by the stem bark extract at a concentration of 20 mg/ml range between 15±1.00 and 22±0.00 mm. Maximum zone of inhibition (22±0.58 mm) was observed for n-butanol and ethyl acetate fractions at 10 mg/ml, while the minimum (14±0.00 mm) was for aqueous fraction. The lowest MIC exhibited by the extract was 1.25 mg/ml against *Aeromonas hydrophila.* The lowest MIC for n-butanol (0.31 mg/ml) is against *Enterococcus faecalis* while the lowest for ethyl acetate, n-hexane and aqueous fractions is 0.63 mg/ml against a number of test isolates. The highest MIC exhibit by n-butanol, ethyl acetate and n-hexane fractions is 1.25 mg/ml while that of the aqueous fraction is 2.50 mg/ml. After 120 min of contact time, the mortality rate at 1 × MIC by n-butanol fraction is 98.9% against *Listeria* sp. and 96.1% against *S.* Typhimurium, while that of n-hexane fraction is 87.5 and 96.1% and that of aqueous fraction is 85.8 and 98.1% respectively. Under similar conditions, total elimination of both organisms is achieved by ethyl acetate fraction. The stem bark extracts of *E. crispa* is therefore considered to have exhibited significant antimicrobial potential with a general increase in the mortality rate as the time and concentration of the extracts increase.

4.1. Introduction

Infectious diseases have been reported as notorious causes of morbidity and mortality across the globe, killing almost 50,000 people per day (WHO, 2002). Rapid development of resistance to the available antimicrobial drugs by several pathogens poses significant challenges in the treatment of these diseases (Alli *et al*., 2011). Frequency of methicillin-resistant *Staphylococcus aureus* rose from 2% in 1975 to 32% in 1992 and at the moment, resistance to virtually all the therapeutically useful antibiotics have been evidenced (Sengupta & Chattopadhyay, 2012). Moreover, most of these antibiotics have been associated with adverse side effects which may include various degrees of allergic reactions, immune-suppression, and even loss of hearing (Ahmed *et al*., 1998).

Although, plant-derived bioactive compounds have recently become of great interest owing to their versatile applications both in pharmacological industries and folklore remedy (Baris *et al*., 2006). This is coupled with the fact that they are readily available with very low side effects (Wadkar *et al*., 2008). Thus, become of great importance to the health of individuals and communities due to their inherent chemical components that produce a definite physiological action in human body. Traditionally, alternative medicine is extensively been practiced in the prevention and treatment of various infectious diseases and has gained global attention in recent years most especially in the developing countries (Akinpelu *et al*., 2015). New drugs of herbal origin discovered through ethno-pharmacological studies have continued to show promising results (Ricardo *et al*., 2004), while plant extracts with medicinal properties represents a concrete alternative for the treatment of different pathological conditions (Akinyele *et al*., 2011).

Euclea crispa (Thunb.), our candidate plant for this study is from the family *Ebenaceae*. It is a small tree with average height of between 2 to 6 m. The extract from the root of *E. crispa* is drunk as cough medicine by the people of south central Zimbabwe (Maroyi, 2013). Also the extract from its leaves was confirmed and reported by Magama *et al.* (2003) to have antimicrobial activities against certain human pathogens. Sobiecki (2006) reported that infusions from the root of *E. crispa* are taken for

epilepsy by the people of Zimbabwe. In this study, we evaluate the intensive antibacterial potentials of *E. crispa* stem bark extract and its fractions.

4.2. Materials and Methods

4.2.1. **Microorganisms**

Bacterial isolates used in this study include type strains as well as locally isolated pathogens from clinical and environmental samples (Chapter 3, Table 3.1).

4.2.2. **Susceptibility testing**

This was determined via agar-well diffusion method as described by EUCAST (2016) and Akinpelu *et al*. (2015) with little modifications.

4.2.3. **Determination of the minimum inhibitory concentrations (MICs)**

The MICs was determined using micro-plate dilution method as developed by Eloff (1998) and Clinical and Laboratory Standards Institute (CLSI, M07-A10, 2015) with slight modification. Nutrient broth culture of the bacterial isolates (18-24 h) was diluted in sterile Muller Hinton broth (Sigma Aldrich, USA) and standardized. Two fold serial dilutions of the extract and fractions were prepared down the columns on the micro-plate to give a final concentration range of 0.157 to 20.00 mg/ml for the extract and 0.078 to 10.00 mg/ml for the fractions. Then 100 µl of the standard inoculums was added to the 100 µl aliquot of the extract and fractions in the wells and incubated at 37 ℃ for 24 h. An indicator, p-iodonitrotetrazolium chloride (Sigma Aldrich, USA) solution (40 µl of 0.2 mg/ml), was added to each well and further incubated at 37 ℃ for 30 min. Development of pink/red colour indicates growth of bacterial cells, while clear wells indicate total inhibition of the bacterial growth. The experiment was carried out in triplicate and sterile distilled water was used as control.

4.2.4. **Determination of killing rate**

The killing rate by the potent fractions was determined as described by Odenholt *et al.* (2004) with slight modifications. This was carried out against *Listeria* sp. representing Gram positive and *Salmonella* Typhimurium representing Gram negative isolates. Nutrient broth cultures (18-24 h) of the

isolates were standardized and viable count was determined from the standard culture. Then 5 ml of the known cell density of the bacterial suspension was added to 45 ml of different concentrations of the extract and fractions relative to the MIC. The resulting suspensions were mixed and held at room temperature while the killing rate was determined over 2-h period. Exactly 0.5 ml of each suspension was taken at appropriate time intervals and transferred into 4.5 ml Nutrient broth recovery medium containing 3% Tween80. This was shaken and serially diluted in sterile physiological saline (0.9% NaCl), after which 0.1 ml of the final dilution was plated out onto Nutrient agar and incubated at 37 ℃ for 48 h. The control experiment was set up without inclusion of extract or fractions. Viable counts were made in triplicate and a decrease in the number of colony forming units indicated killing by the extract and/or fractions.

4.3. Results

4.3.1. Sensitivity and MIC

The extract and fractions demonstrate credible activities against the test bacterial isolates used in this study. At a concentration of 10 mg/ml the zones of inhibition exhibited by the fractions partitioned into n-butanol and ethyl acetate range between 18±0.00 and 22±0.00 mm. While that of the n-hexane and aqueous fractions range between 15±0.58 and 20±0.00 mm (Table 4.1), none of the test isolates is susceptible to the fraction partitioned into chloroform. The lowest MIC exhibited by the stem bark extract is 1.25 mg/ml against *Aeromonas hydrophila*. The n-butanol fraction exhibits MICs which range between 0.31 and 1.25 mg/ml while those of ethyl acetate and n-hexane fractions are between 0.63 and 1.25 mg/ml and that of aqueous fraction range between 0.63 and 2.50 mg/ml (Table 4.2).

Table 4.1: The sensitivity patterns of zones of inhibition of *Euclea crispa* stem bark extract, fractions and standard antibacterial compounds against test bacterial isolates

Bacterial isolates	**Zones of inhibition (mm) ****								
	***Euclea crispa* (20 mg/ml)**	**BUT (10 mg/ml)**	**ETH (10 mg/ml)**	**HEX (10 mg/ml)**	**AQU (10 mg/ml)**	**CHL (10 mg/ml)**	**STREP (1 mg/ml)**	**TET (0.1 mg/ml)**	**SDW**
Aeromonas hydrophila	22±0.00	20±0.76	18±1.15	18±0.00	18±0.58	0	25±0.28	25±0.00	0
Acinetobacter calcoaceticus anitratus	15±1.00	20±0.00	22±0.00	16±0.00	16±0.58	0	25±0.00	26±1.00	0
Bacillus pumilus (ATCC 14884)	16±1.20	22±1.15	20±0.00	18±0.58	16±0.00	0	25±0.76	28±0.00	0
Escherichia coli (ATCC839)	18±0.00	18±0.58	19±0.76	16±0.76	15±0.28	0	27±0.00	24±1.00	0
Enterobacter faecalis	16±0.00	18±0.00	18±0.58	18±0.50	18±0.00	0	25±0.58	28±0.50	0
Enterococcus faecalis	20±0.58	22±0.58	22±1.00	20±1.15	20±0.00	0	26±0.28	26±0.28	0
Klebsiella pneumoniae (ATCC 13047)	20±0.28	22±1.00	20±0.58	18±0.58	18±1.00	0	26±0.00	24±0.00	0
Klebsiella pneumoniae	18±0.00	18±0.76	18±0.50	16±0.50	16±0.00	0	23±0.58	27±0.76	0
Listeria sp.	16±0.00	21±0.50	18±0.00	18±0.58	16±1.15	0	29±1.00	27±0.58	0
Shigella sonnei (ATCC 29930)	16±1.00	20±0.58	20±0.00	18±1.00	18±0.58	0	27±1.00	25±0.28	0
Shigella flexneri	20±0.76	18±0.58	18±0.76	18±0.00	14±0.00	0	23±1.15	26±0.50	0
Salmonella Typhimurium	20±0.50	20±1.15	21±0.58	18±0.58	16±1.00	0	25±1.00	25±0.28	0
Salmonella Typhi	16±0.00	19±0.58	20±0.00	18±0.00	16±1.15	0	26±0.00	27±0.50	0
Staphylococcus aureus (ATCC 6538)	18±0.58	18±0.00	18±0.50	16±0.76	17±0.58	0	26±1.00	24±1.15	0
Staph. aureus (OK 2a)	18±0.58	18±0.50	18±0.28	16±1.15	15±0.58	0	25±0.00	25±0.00	0
Staph. aureus (OK2b)	21±0.00	20±1.15	18±0.76	18±1.15	16±0.00	0	24±0.00	26±1.15	0
Plesiomonas shigelloides	16±1.15	18±0.58	22±0.58	18±1.00	16±1.00	0	24±0.00	26±0.00	0
Proteus vulgaris (CSIR 0030)	16±1.00	20±0.00	18±0.58	16±0.00	18±1.15	0	29±1.15	26±0.58	0
Proteus vulgaris	18±0.58	18±0.00	18±1.15	18±0.58	16±0.00	0	26±1.15	25±0.00	0
Pseudomonas aeruginosa	22±0.76	19±0.50	20±0.28	17±0.28	16±0.00	0	26±1.00	25±1.00	0

Key: ATCC = American type culture collection, BUT = n-Butanol fraction, ETH= Ethyl acetate fraction, HEX = n-Hexane fraction, CHL = Chloroform fraction, AQU = Aqueous fraction, SDW = Sterile distilled water, 0 = Not sensitive, mm** = Mean of three replicate

Table 4.2: The minimum inhibitory concentrations (MICs) of the *Euclea crispa* stem back extract, fractions and standard antibacterial compounds exhibited against test bacterial isolates

Bacterial isolates	***E. crispa* (mg/ml)**	**BUT (mg/ml)**	**ETH (mg/ml)**	**HEX (mg/ml)**	**AQU (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Aeromonas hydrophila	1.25	0.63	0.63	1.25	1.25	0.063	0.031
Acinetobacter calcoaceticus anitratus	5.00	0.63	0.63	1.25	1.25	0.063	0.025
Bacillus pumilus (ATCC 14884)	5.00	0.63	0.63	0.63	0.63	0.016	0.013
Escherichia coli (ATCC839)	2.50	1.25	1.25	1.25	2.50	0.016	0.013
Enterobacter faecalis	5.00	1.25	1.25	1.25	2.50	0.016	0.013
Enterococcus faecalis	5.00	0.31	0.63	0.63	0.63	0.016	0.013
Klebsiella pneumoniae (ATCC 13047)	5.00	1.25	0.63	0.63	1.25	0.016	0.013
Klebsiella pneumoniae	2.50	0.63	0.63	0.63	1.25	0.063	0.013
Listeria sp.	5.00	0.63	0.63	0.63	0.63	0.008	0.013
Shigella sonnei (ATCC 29930)	5.00	1.25	1.25	1.25	1.25	0.016	0.013
Shigella flexneri	2.50	0.63	0.63	1.25	2.50	0.031	0.025
Salmonella Typhimurium	5.00	0.63	0.63	0.63	0.63	0.016	0.025
Salmonella Typhi	5.00	0.63	0.63	0.63	0.63	0.016	0.013
Staphylococcus aureus (ATCC 6538)	5.00	0.63	0.63	1.25	2.50	0.016	0.013
Staph. aureus (OK 2a)	5.00	0.63	0.63	0.63	1.25	0.063	0.006
Staph. aureus (OK2b)	2.50	1.25	0.63	1.25	2.50	0.016	0.025
Plesiomonas shigelloides	5.00	0.63	0.63	0.63	1.25	0.016	0.025
Proteus vulgaris	5.00	1.25	1.25	1.25	1.25	0.016	0.013
Proteus vulgaris (ATCC 6830)	2.50	1.25	1.25	1.25	2.50	0.125	0.025
Pseudomonas aeruginosa	5.00	0.63	0.63	1.25	1.25	0.016	0.013

Key: ATCC = American type culture collection, CSIR = Council for scientific and industrial research, BUT = n-Butanol fraction, ETH = Ethyl acetate fraction, HEX = n-Hexane fraction, AQU = Aqueous fraction, STREP = Streptomycin, TET = Tetracycline, ND = Not determined

4.3.2. Killing rate

The extent and the killing rate by the potent fractions against *Listeria* sp. and *S.* Typhimurium are represented in figure 4.1 and 4.2 respectively. After 15 min of contact time, the percentage mortality exhibited by n-butanol, ethyl acetate, n-hexane and aqueous fractions at 1 × MIC are 54.0, 59.7, 47.2 and 38.1% respectively against *Listeria* sp., and 51.6, 41.8, 42.5 and 38% respectively against *S.* Typhimurium. Total mortality is achieved by the ethyl acetate fraction under the same concentration after 90 min of contact time with both organisms. Total mortality is also achieved by n-butanol and aqueous fractions against *Listeria* sp. when the concentration was

increased to 2 × MIC after 120 min of contact time. A common trend of increased mortality rate with increase in the contact time and concentration was observed for all the fractions.

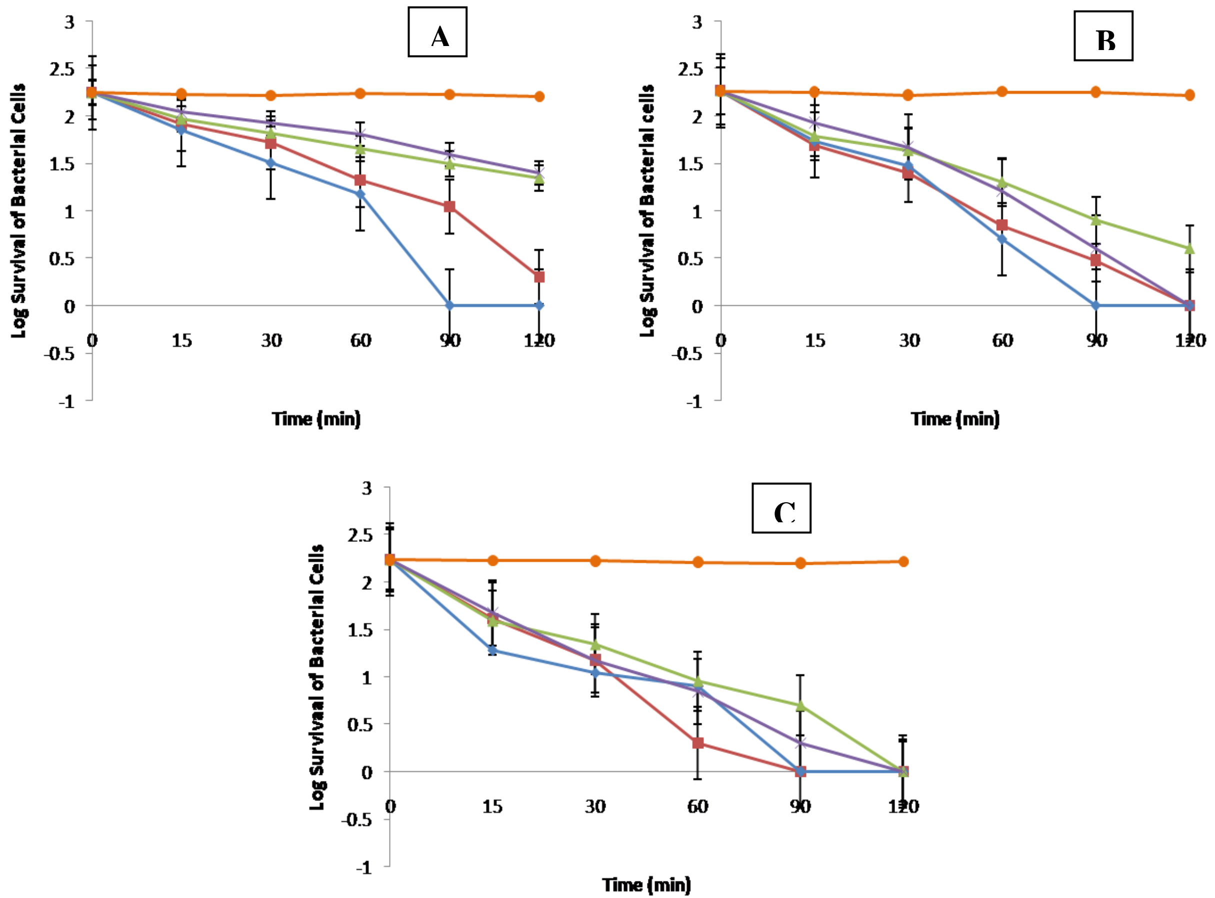

Fig.4.1 The extent and the rate of killing of *Listeria* sp.by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C). Control ( )

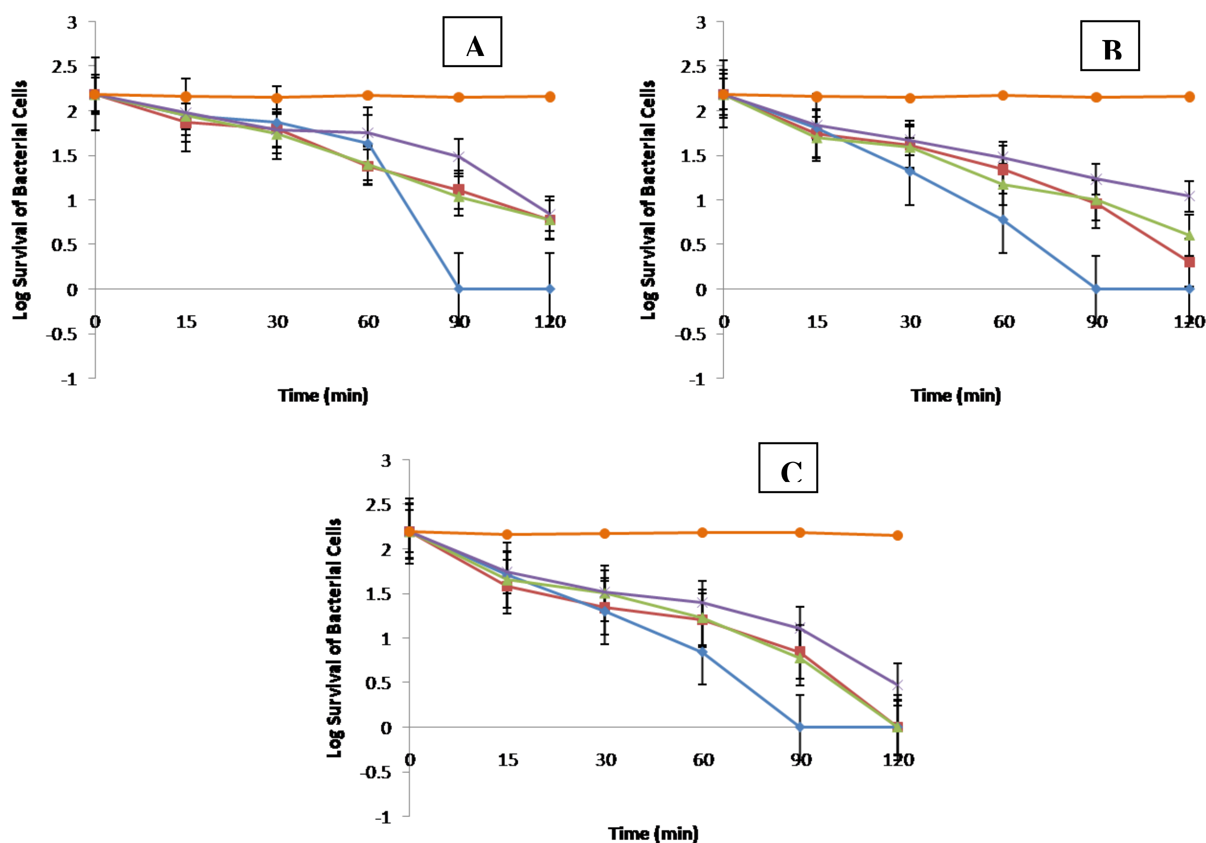

Fig. 4.2 The extent and the rate of killing of *S.* Typhimurium by fractions partitioned into n-butanol (—■—), ethyl acetate (—◆—), n-hexane (—▲—) and water (—×—) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C). Control (—●—)

4.4. Discussion and Conclusion

Evaluation of antibacterial properties of *Euclea crispa* stem bark extract reveals credible activities against the panel of Gram positive and Gram negative bacterial isolates used in this study. The widest zone of inhibition by the stem bark extract is against *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Resistance to antibiotics is a major problem with *A. hydrophila* infections. This organism is a food borne pathogen associated with zoonotic infections and widely distributed in nature (Laith & Najiah, 2013). *P. aeruginosa* is another Gram negative bacterium with elevated intrinsic and acquired resistance to different antibiotics. It has been implicated in pneumonia, urinary tract infections and bacteremia and also a causative agent for

chronic infection that leads to pulmonary damage and impaired respiration (Ochoa *et al*., 2013). The lowest MIC is exhibited by n-butanol fraction (0.31 mg/ml) against *E.* faecalis, while the lowest MIC by the ethyl acetate fraction (0.63 mg/ml) is expressed against 65% of the test bacterial isolates: including *Staphylococcus aureus, Salmonella* Typhimurium*, Shigella flexneri* and *Klebsiella pneumoniae*. The *Staphylococcus aureus* is a major cause of nosocomial infections worldwide and as well as community-acquired infections. Infections due *S. aureus* are heterogeneity and are often extremely difficult to treat (Costa *et al*., 2013). *K. pneumoniae* directly comes behind *E. coli* in the cases of nosocomial Gram negative bacteremia and urinary tract infections among catheterized patients (Vuotto *et al*., 2014) while *S.* Typhimurium is an identified causal agent of food borne salmonellosis, one of the most important bacterial zoonotic infections (Van Parys *et al*., 2012). Susceptibility of these pathogens to the plant extract indicates its efficacy in the traditional management of several infectious diseases.

The effectiveness of these extracts could be linked to the detection of certain inherent chemical groups such as hydrocarbons, nitrile, amines, phenol, sulfoxides, aromatic ketones, flavonoids and tannins. For instance, antibacterial activity has been showcased by a number of flavonoids such as Quercetin, a bioactive compound reported to have completely inhibited the growth of *S. aureus* (Tapas *et al*., 2008) and catechins that has been reported to have *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella* spp. among other bacteria (Kumar & Pandey, 2013).

The fractions of the stem bark extract present significant antimicrobial properties which compared favourably with streptomycin and tetracycline used as standard antibacterial compounds. This showcases the significance of our choice of plant in folklore remedy as it is being applied by the people of eastern Free State of South Africa and South Central Zimbabwe.

The n-butanol and ethyl acetate fractions present the best antimicrobial properties after considering the low level of MIC and the rate at which both fractions brought about depression in the viable counts of the test isolates. This therefore suggests the two fractions as potential sources of bioactive compounds of natural origin required in combating resistance of pathogens to the existing antibiotics. Therefore the stem bark of *E. crispa* could be a source of antimicrobial drugs of natural origin and this will equally go a long way in providing affordable antimicrobial drugs with probably very low side effect which is are common features with virtually all medicinal plants (Wadkar *et al.,* 2008 & Yamani *et al*., 2016).

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CHAPTER 5– Membrane attack; a probable mechanism of biocidal action of the leaf and stem bark extracts of *Euclea crispa* (Thunb.)

Abstract

Among the established mode of actions by some antimicrobial agents is cytoplasmic membrane attack which could possibly lead to leakages of certain intracellular components and eventual death of the cell. This study therefore investigates the ability of *E. crispa* leaf and stem bark extracts to compromise the integrity of microbial cytoplasmic membrane and this was carried out via determination of the amount of proteins and nucleotides released from the microbial cells after 120 min of treatment with the extracts. Scanning electron microscopy was used to determine the extent of the physical impact of the extracts on the cell envelope. In the case of the leaf extracts, the maximum amount of proteins released from *Bacillus pumilus* (0.53±0.005 µg/ml) is by the aqueous fraction after 120 min of treatment at 2 × MIC, while the maximum from *Klebsiella pneumoniae* (0.57±0.001 µg/ml) is by the n-butanol fraction and that from *C. albicans* (0.54±0.002 µg/ml) is by the n-hexane fraction. Likewise, maximum nucleotide leakage was obtained from *Bacillus pumilus* (45.8±0.03 µg) and *C. albicans* (44.3±0.03 µg) by the n-hexane fraction, while the maximum nucleotide leakage from *Klebsiella pneumoniae* (40.7±0.06 µg) is obtained by the n- butanol fraction under the same condition. On the other hand, for the stem bark extracts, ethyl acetate fraction released maximum amount of protein from *Listeria* sp. (0.63±0.004 µg/ml), while n-hexane fraction released maximum amount from *S.* Typhimurium (0.79±0.001 µg/ml) under the same concentration of 3 × MIC after 120 min of exposure. The maximum amount of nucleotide leakages (47.9±0.12 µg) from *Listeria* sp. is by n-butanol fraction at 3 × MIC after 120 min of treatment. The Images of scanning electron microscopy reveals a level of structural damage in the membrane of test isolates, hence confirms membrane disruption as one of the probable mechanism of action by the extracts of *E. crispa*.

5.1. Introduction

A number of scientific articles on the constituents and biological activity of African medicinal plants has been published but only a few studies have been done to determine probable mechanisms of their biocidal actions (Mwitari *et al*., 2013). Thus, understanding their mechanisms of biocidal action and other factors influencing their activity will go a long way in making better use of these naturally occurring, readily available biocides (Maillard, 2002). An effective chemotherapeutic agent must be selectively toxic. Such a drug with selective toxicity has a high therapeutic index and usually disrupts a structure or metabolic process unique to the pathogens with very low side effects (Tortora *et al*., 2004). Some phytochemicals have been reportedly possessing membrane-active mechanism capable of disrupting cell membrane integrity (Cho *et al*., 2013). Microbial cell membrane is an organelle with selective permeability features by which cellular internal environments are being regulated and protected. Leakages of intracellular components such as nucleotides and proteins are indicators for the identification of agents whose mode of action is associated with microbial cell membrane disruption (Hao *et al*., 2009). Polymyxin B is among conventional antibiotics that bind to the phospholipid bilayer of the plasma membrane (Prescott *et al*., 2008), likewise the amphotericin B that kills yeast primarily via channel-mediated membrane permeabilisation and alternatively by simply binding to ergosterol (Gray *et al*., 2011). This study therefore investigates cell membrane attack as a probable mechanism of biocidal action of *E. crispa* leaf and stem bark extracts.

5.2. Materials and Methods

5.2.1. Microorganisms

The representative isolates were selected based on Gram reaction of the bacterial isolates and their relatively low MIC values across the test potent fractions as observed in Chapter 3 and 4. The test isolates against the leaf extracts are *Bacillus pumilus* (ATCC 14884) (represents Gram

positive bacterial isolates), *Klebsiella pneumoniae* (ATCC 13047) (represents Gram negative bacterial isolates) and *Candida albicans* (Ho316) (represents yeast isolates). While those tested against the stem bark extracts are *Salmonella* Typhimurium (represents Gram negative) and *Listeria sp.* (represents Gram positive).

5.2.2. Analysis of scanning electron microscopy (SEM)

Sample preparation was carried out as described by Kockro *et al*. (2000) and Yang *et al*. (2015) with slight modifications. Standardized nutrient broth cultures (24 h) of the representative isolates were washed (0.9% NaCl) and 1 ml of the washed cell suspension was treated with 9 ml of the extract at a concentration of 1 × MIC over a period of 2 h. The mixture was centrifuged, cells washed three times with 0.05M phosphate buffer solution (pH 7.3) and the pellet was fixed in 2.5% (v/v) glutaraldehyde. The fixed cells were dehydrated with different concentration of ethanol. These were critical point dried using Tousimis critical point dryer (Rockville, Maryland, U.S.A.) and then mounted before coating with gold (BIO-RAD, Microscience Division Coating System, London, UK). The samples were then observed under a scanning electron microscope (JSM-7800F Extreme-resolution Analytical Field Emission SEM).

5.2.3. Determination of protein leakage

Washed cells of the representative isolates were standardized (0.5 McFarland) and exposed to various concentrations of the extracts relative to the MIC at different time intervals over a period of 2 h. Each suspension was then centrifuged at 7000 rpm and the protein concentration in the supernatant was determined (Bradford, 1976). Bradford reagent (0.4 ml) was added to1.6 ml sample (0.2 ml supernatant + 1.4 ml sterile distilled water) and optical density of the resulting solution was measured at 595 nm within 5 min. Protein quantity of each sample was determined from the equation of the best-fit linear regression obtained from the Bovine Serum Albumin (BSA) standard curve.

5.2.4. Determination of nucleotide leakage

This was determined using the method described by da Silva Jr. *et al*. (2014) and Yang *et al*. (2015) with slight modifications. Washed suspensions of the representative isolates (0.5 McFarland) were exposed to various concentrations of the fractions (relative to the MIC) at regular time intervals over a period of 2 h. Each suspension was then centrifuged at10000 rpm and the absorbance of the supernatant measured at 260 nm using a UV spectrometer. Sterile distilled water inoculated with the same inoculums was used as control.

5.3. Results

5.3.1. Scanning electron microscopy (SEM)

Scanning electron microscopy images revealing the physical impact of the potent fractions against representative isolates are shown in Figure 5.1 – 5.3 and 5.4 – 5.5 for the potent fractions of the leaf and stem bark extracts respectively. Considering the intact anatomical structure of the cell envelope of representative isolates (control), the most active fractions selected were able to cause an obvious level of disruption.

Leaf fractions

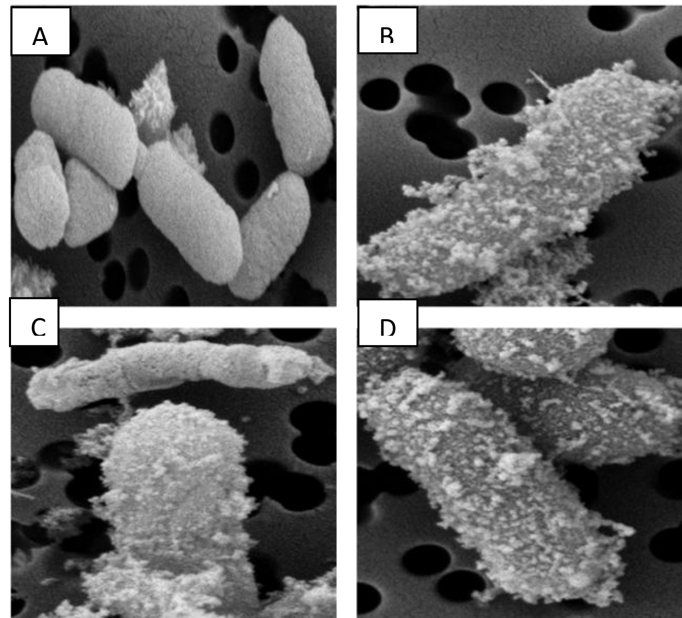

Fig. 5.1 SEM images (1µm, x15000) showing effect of fractions partitioned into ethyl acetate (B), n-butanol (C) and n-hexane (D) compared to the control (A) against *Bacillus pumilus* (ATCC 14884) at 1 × MIC after 120 min of exposure

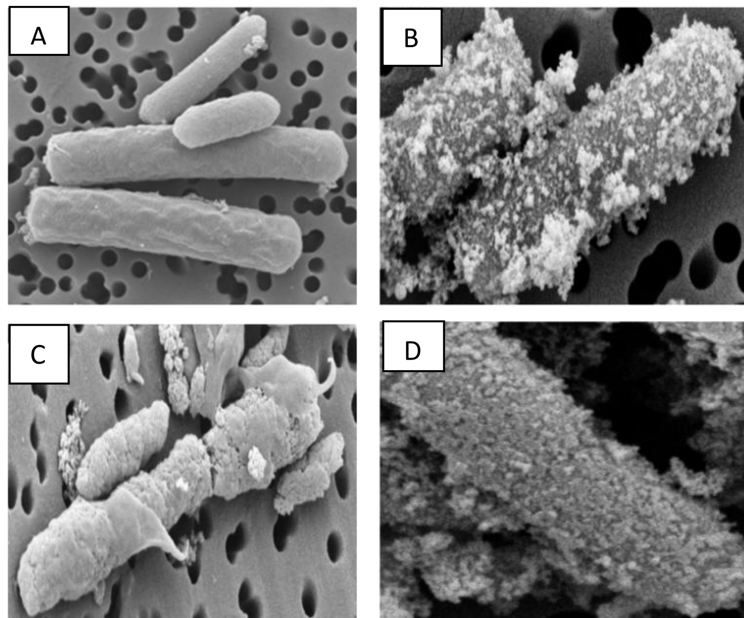

Fig. 5.2 SEM images (1µm, x15000) showing effect of fractions partitioned into ethyl acetate (B), n-butanol (C) and n-hexane (D) compared to the control (A) against *Klebsiella pneumoniae* (ATCC 13047) at 1 × MIC after 120 min of exposure

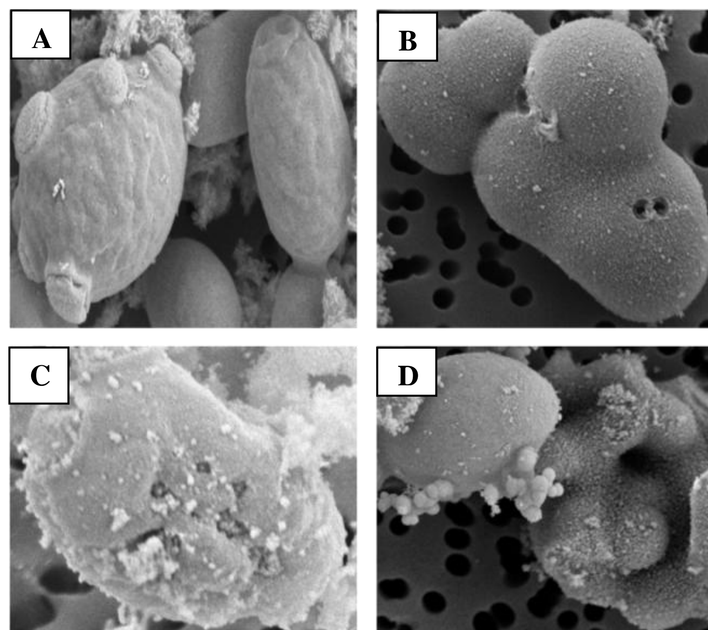

Fig. 5.3 SEM images (1µm, x15000) showing effect of fractions partitioned into ethyl acetate (B), n-butanol (C) and n-hexane (D) compared to the control (A) against *Candida albicans* (Ho316) at 1 × MIC after 120 min of exposure

Stem bark fractions

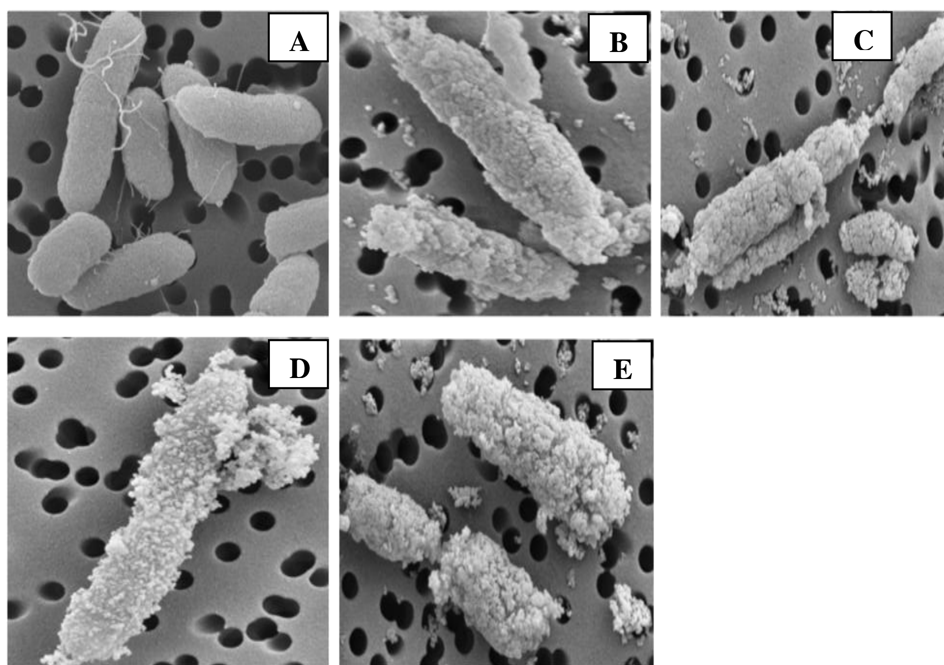

Fig. 5.4 SEM images (1µm, x15000) showing effect of fractions partitioned into n-butanol (B), ethyl acetate (C), n-hexane (D) and water (E) compared to the control (A) against *Listeria* sp. at 1 × MIC after 120 min of exposure

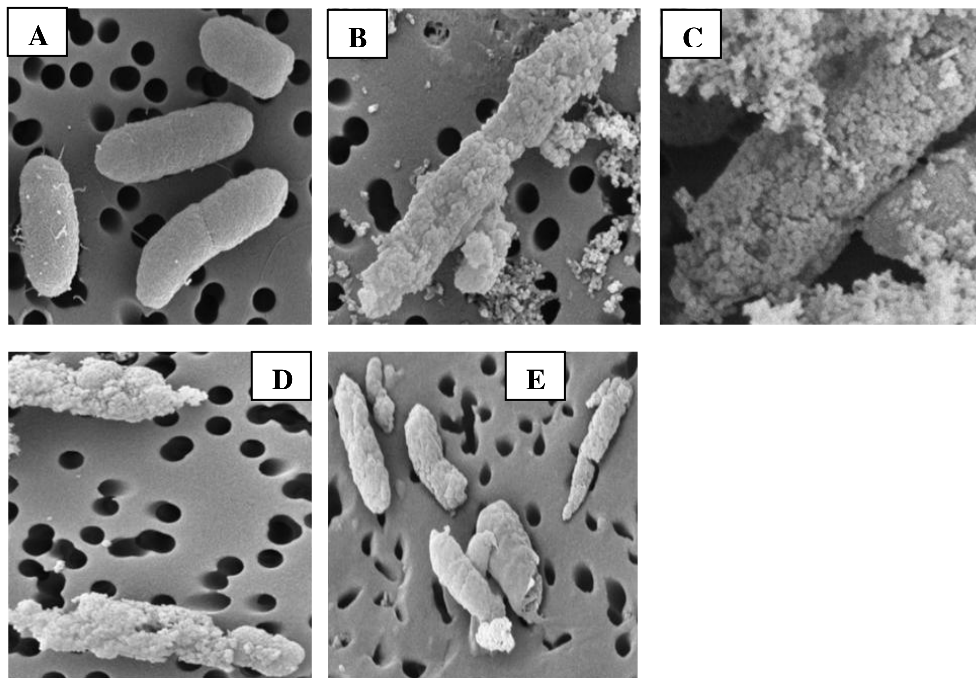

Fig. 5.5 SEM images (1µm, x15000) showing effect of fractions partitioned into n-butanol (B), ethyl acetate (C), n-hexane (D) and water (E) compared to the control (A) against *Salmonella* Typhimurium at 1 × MIC after 120 min of exposure

5.3.2. Leakages by fractions of the leaf extract

The maximum amount of proteins released from *Bacillus pumilus* (0.53±0.005 µg/ml) after 120 min of treatment, is by the fraction partitioned into water at a concentration of 2 × MIC (Figure 5.6) while the maximum from the *Klebsiella pneumoniae* (0.57±0.001 µg/ml) is by the fraction partitioned into n-butanol (Figure 5.7) and that of the *C. albicans* (0.54±0.002 µg/ml) is by the fraction partitioned into n-hexane under similar condition of 2 × MIC and 120 min of treatment (Figure 5.8). Likewise the maximum nucleotide leakage of 45.8±0.03 µg is obtained from *Bacillus pumilus* by the n-hexane fraction at 2 × MIC (Figure 5.9), while the maximum nucleotide leakage from *Klebsiella pneumoniae* is 40.7±0.06 µg by the fraction partitioned into n-butanol (Figure 5.10) at 2 × MIC and after 120 min of treatment and that from *C. albicans* (Ho316) is 44.3±0.03 µg also by n-hexane fraction under similar condition (Figure 5.11).

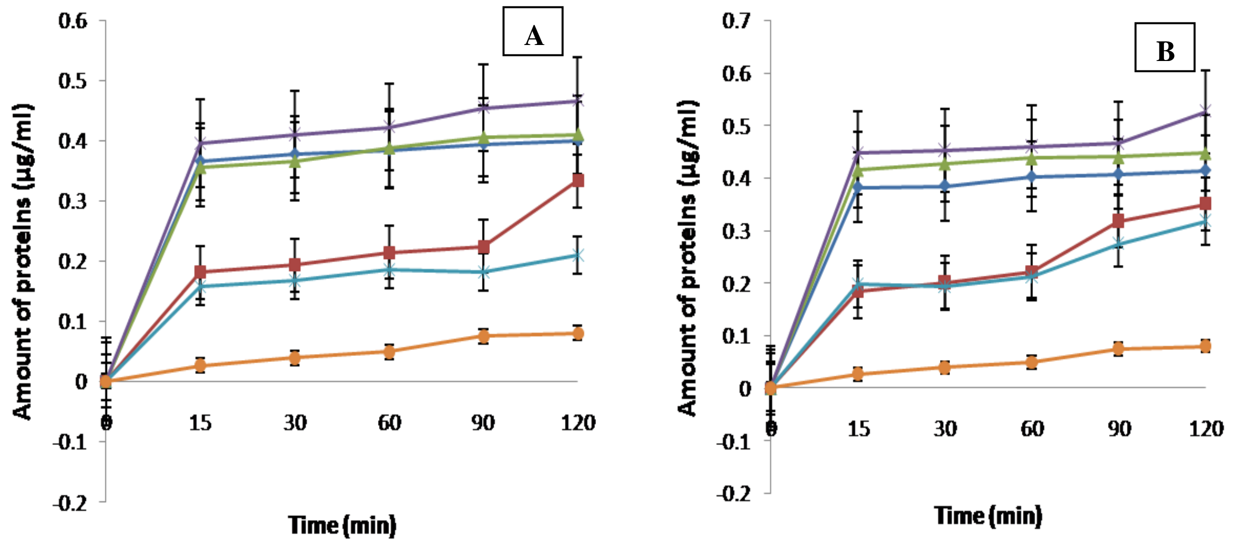

Fig. 5.6 Leakage of proteins from *Bacillus pumilus* by fractions partitioned into n-butanol (—■—), ethyl acetate (—◆—), n-hexane (—▲—), water (—×—) and chloroform (—✳—) compared to control (—●—) at 1 × MIC (A) and 2 × MIC (B)

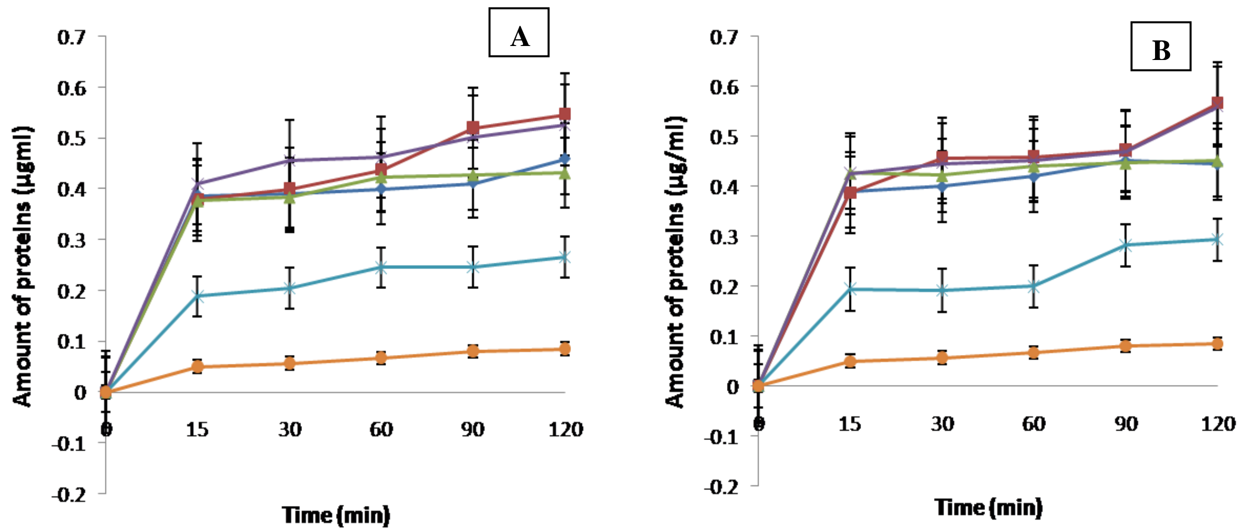

Fig. 5.7 Leakage of proteins from *Klebsiella pneumoniae* by fractions partitioned into n-butanol (—■—), ethyl acetate (—◆—), n-hexane (—▲—), water (—×—) and chloroform (—✳—) compared to control (—●—) at 1 × MIC (A) and 2 × MIC (B)

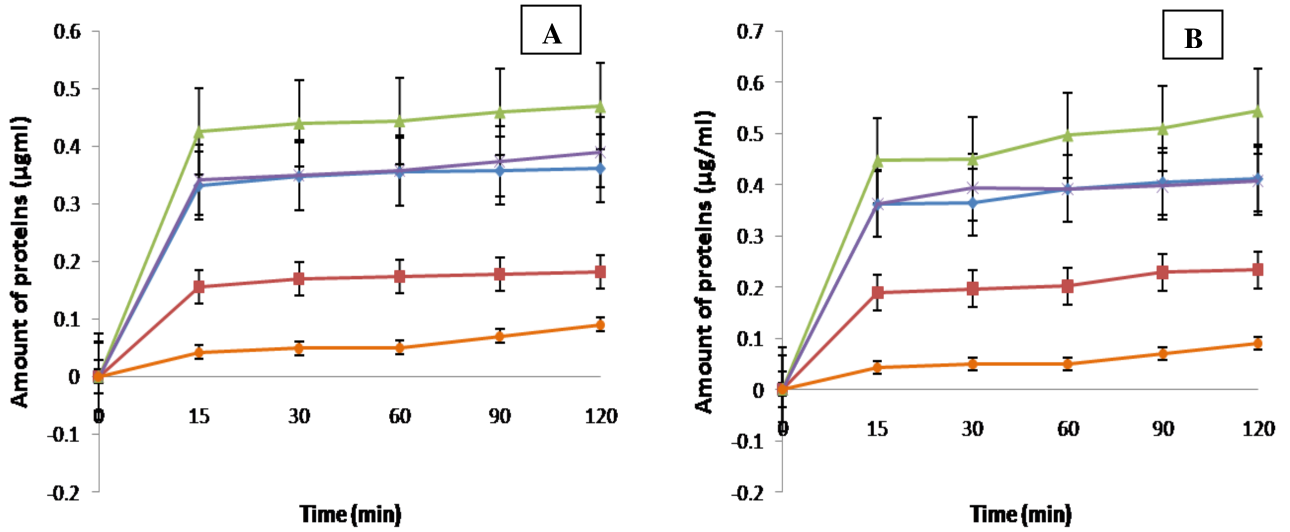

Fig. 5.8 Leakage of proteins from *Candida albicans* by fractions partitioned into n-butanol (■), ethyl acetate (◆), n-hexane (▲) and water (×) compared to control (●) at 1 × MIC (A) and 2 × MIC (B)

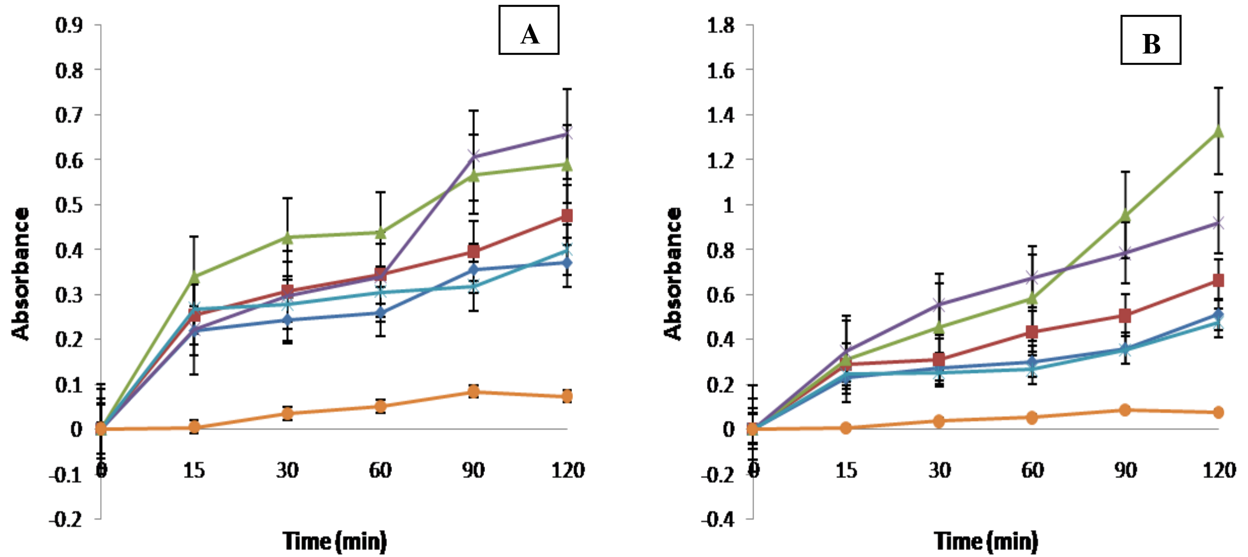

Fig. 5.9 Leakage of nucleotides from *Bacillus pumilus* by fractions partitioned into n-butanol (■), ethyl acetate (◆), n-hexane (▲), water (×) and chloroform (✱) compared to control (●) at 1 × MIC (A) and 2 × MIC (B)

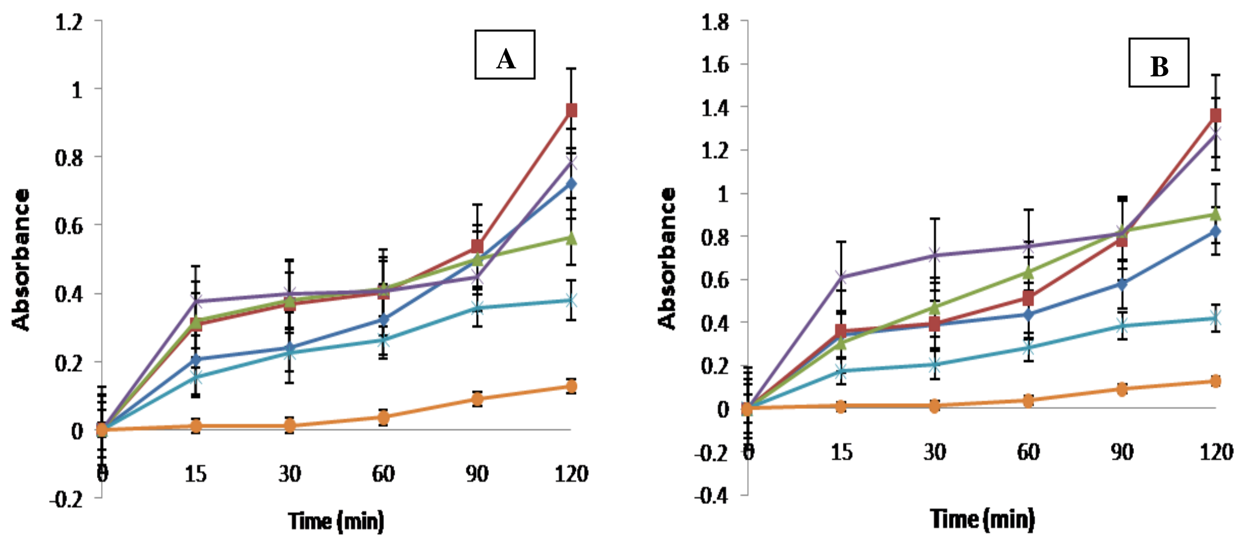

Fig. 5.10 Leakage of nucleotides from *Klebsiella pneumoniae* by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ), water ( ) and chloroform ( ) compared to control ( ) at 1 × MIC (A) and 2 × MIC (B)

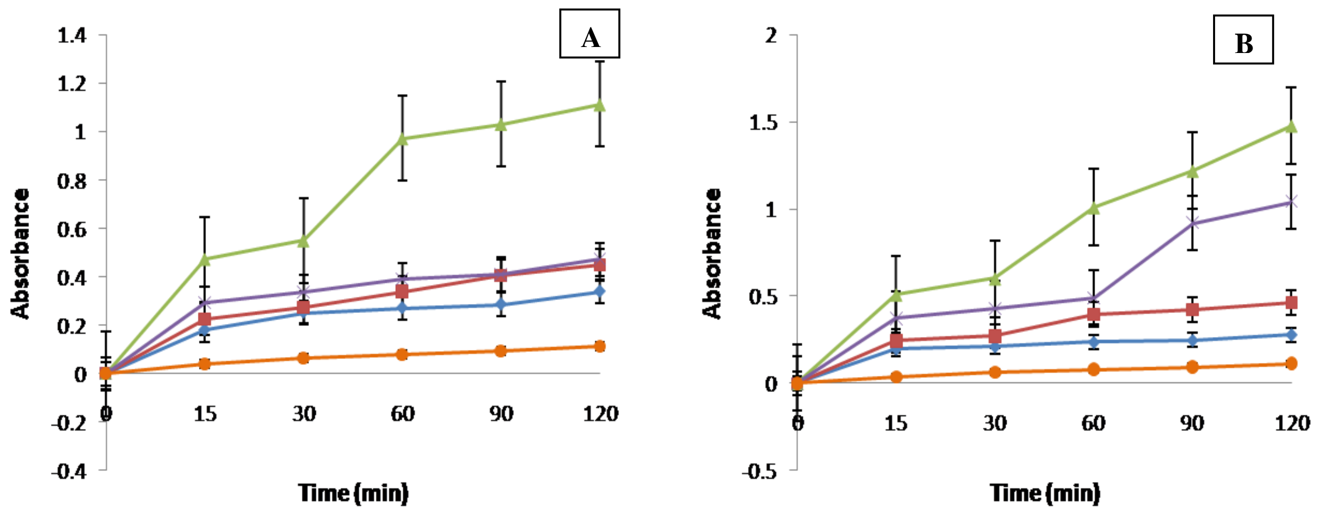

Fig. 5.11 Leakage of nucleotides from *Candida albicans* by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) compared to control ( ) at 1 × MIC (A) and 2 × MIC (B)

5.3.3. Leakages by fractions of the stem bark extract

The amount of proteins released from *Listeria* sp. and *S.* Typhimurium by the potent fractions are represented in figure 5.12 and 5.13 respectively. At a concentration of 1 × MIC, the amount of proteins released from *Listeria* sp. after 120 min of treatment with n-butanol fraction is 0.52±0.001 μg/ml and that of ethyl acetate fraction is 0.61±0.005 μg/ml. Under similar condition 0.54±0.002 and 0.49±0.002 μg/ml are the amount of proteins leaked by the n-hexane and aqueous fractions after 120 min of treatment. The amount of proteins released from *S.* Typhimurium by the n-butanol fraction after 120 min is 0.44±0.003 μg/ml and by the ethyl acetate fraction is 0.46±0.004 μg/ml. While the proteins released by the n-hexane and aqueous fractions after 120 min are 0.44±0.003 and 0.51±0.001 μg/ml respectively at the same of concentration of 1 × MIC. Slight increments were observed in the amount of proteins released from the cells when the concentration of the extracts was increased to 2 × MIC and 3 × MIC over the same period of time.

On the other hand, the amount of nucleotides released by the fractions against the representative isolates are represented in figure 5.14 and 5.15. The amount of nucleotides released from *Listeria* sp. after 120 min of contact time with the ethyl acetate fraction at 1 × MIC is 46.9±0.15 μg, followed by the n-hexane faction with 42.6±0.18 μg, then butanol and aqueous fractions with 40.4±0.12 μg and 37.6±0.09 μg respectively. On the other hand, the minimum amount of nucleotides released from *S.* Typhimurium is 32.2±0.12 μg by aqueous fraction at 1 × MIC after 15 min of treatments. While the maximum amount of nucleotides leaked out from the *S.* Typhimurium (46.0±0.15 μg) is by the ethyl acetate fraction after 120 min of exposure at 3 × MIC.

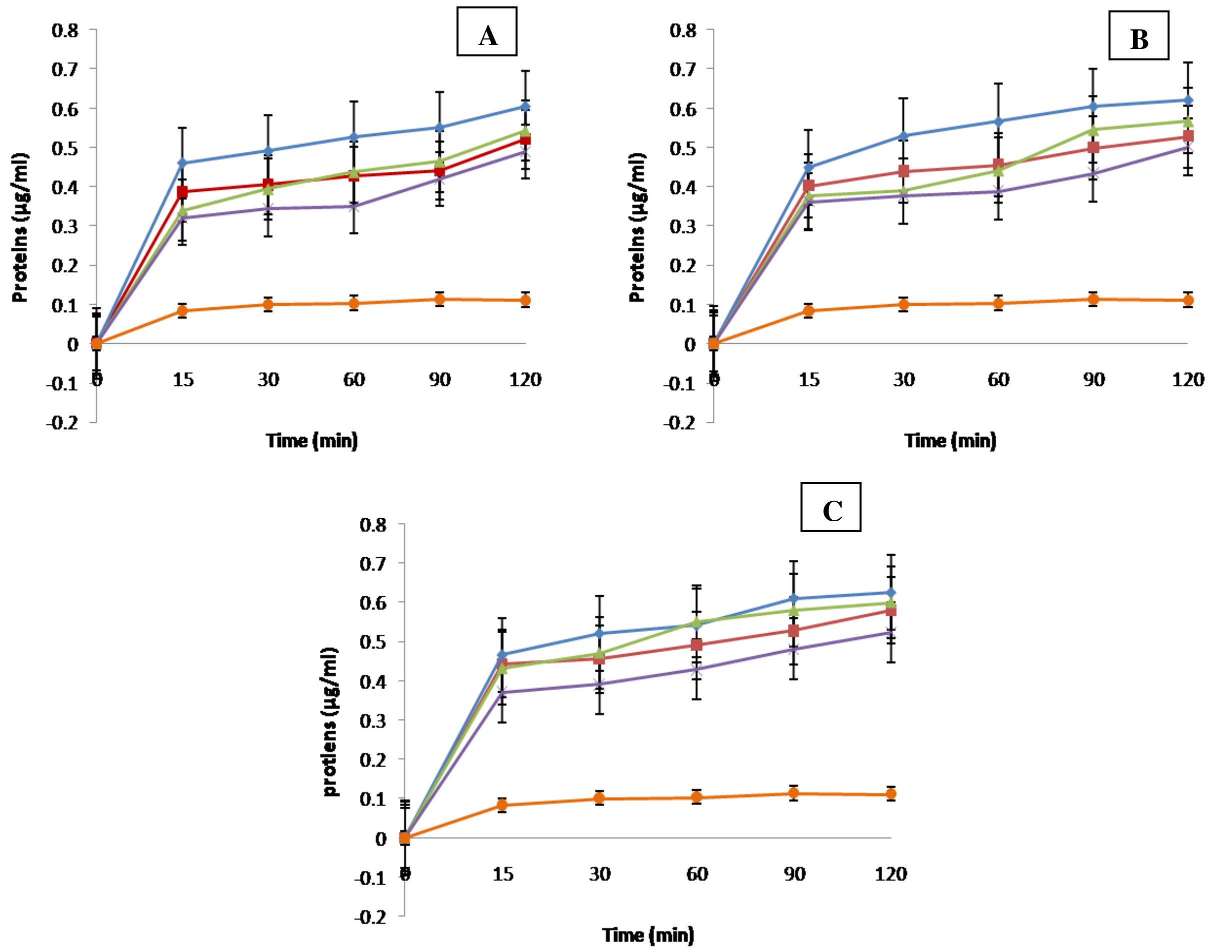

Fig. 5.12 Leakage of proteins from *Listeria* sp. by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) compared to control ( ) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C)

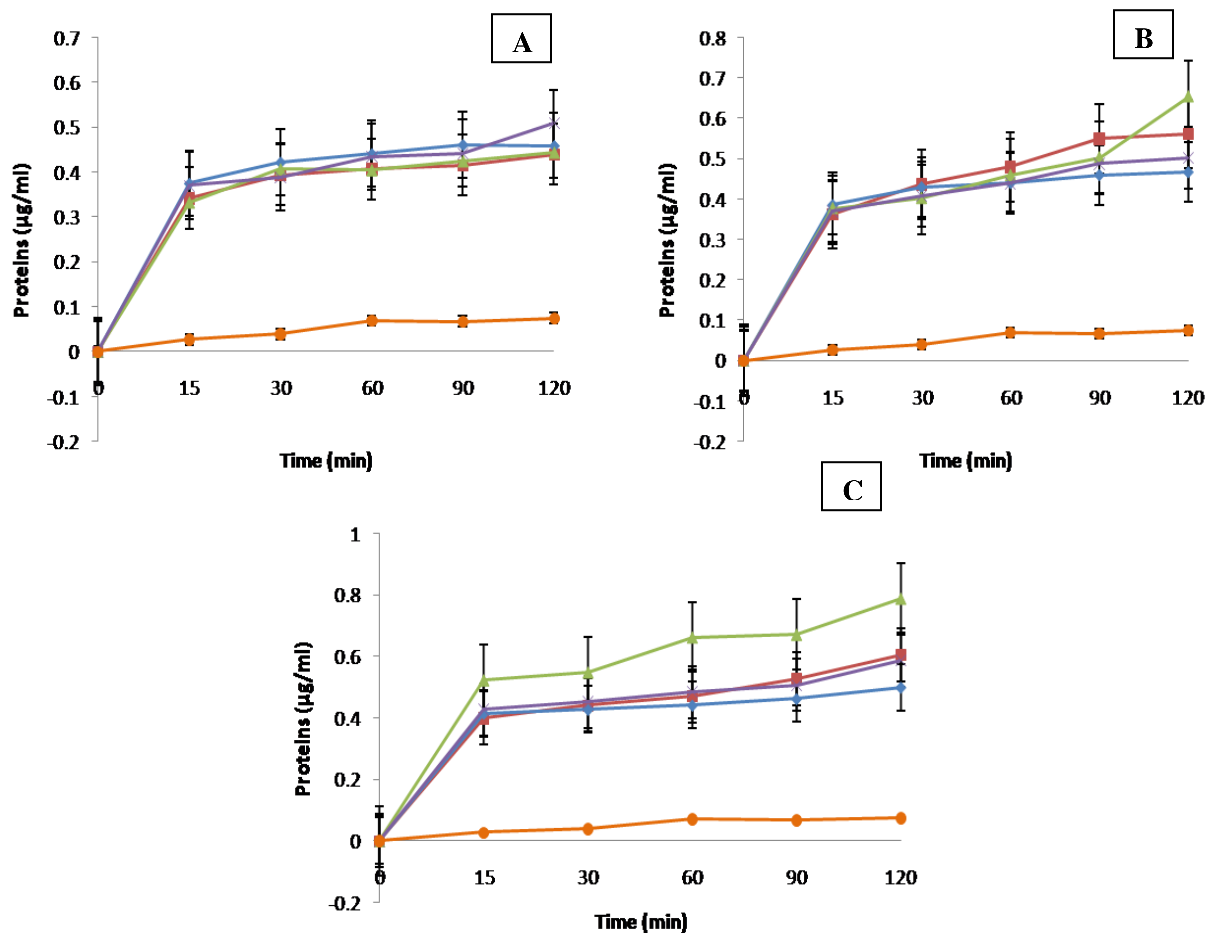

Fig. 5.13 Leakage of proteins from *Salmonella* Typhimurium by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) compared to control ( ) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C)

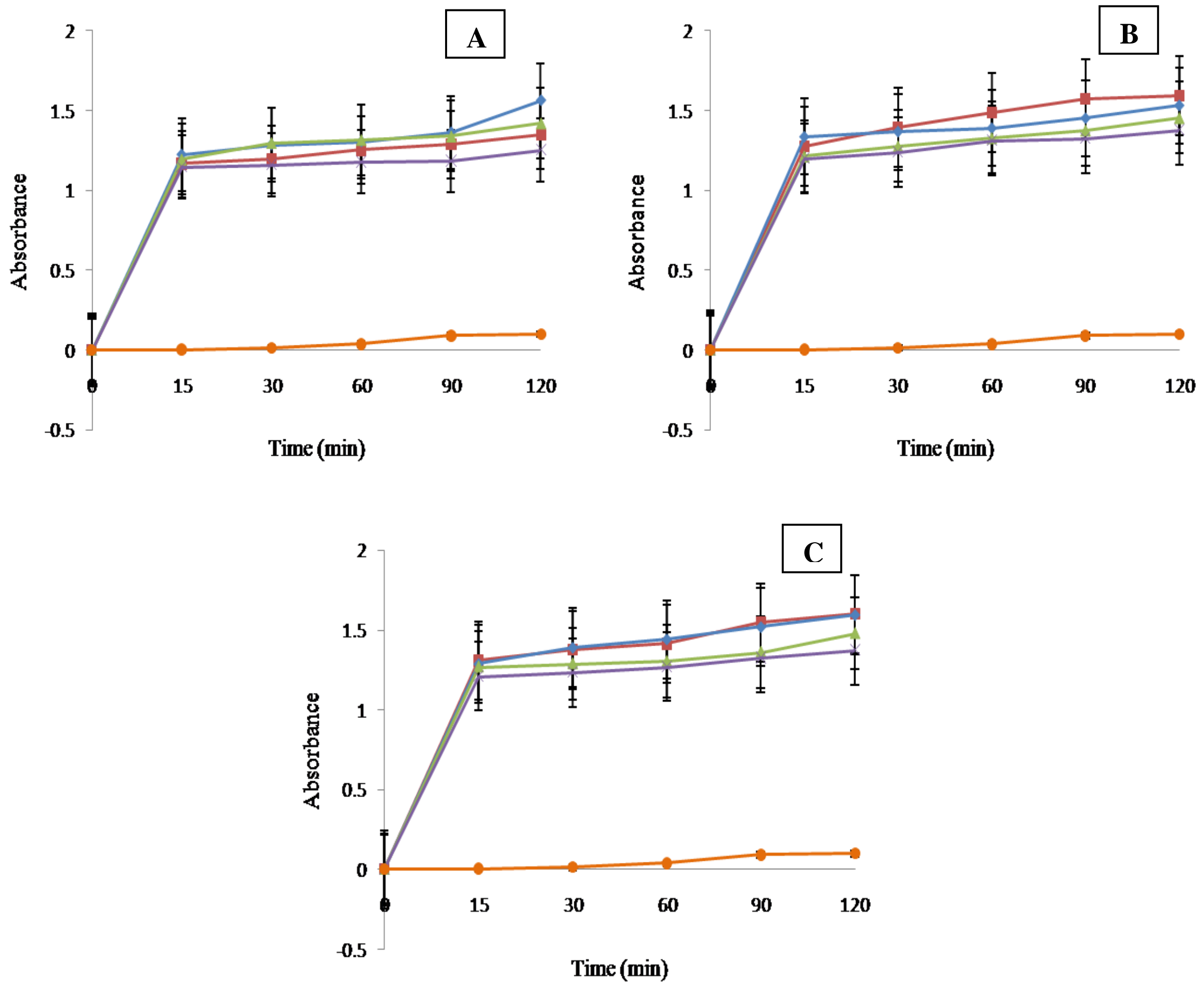

Fig. 5.14 Leakage of nucleotides from *Listeria* sp. by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) compared to control ( ) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C)

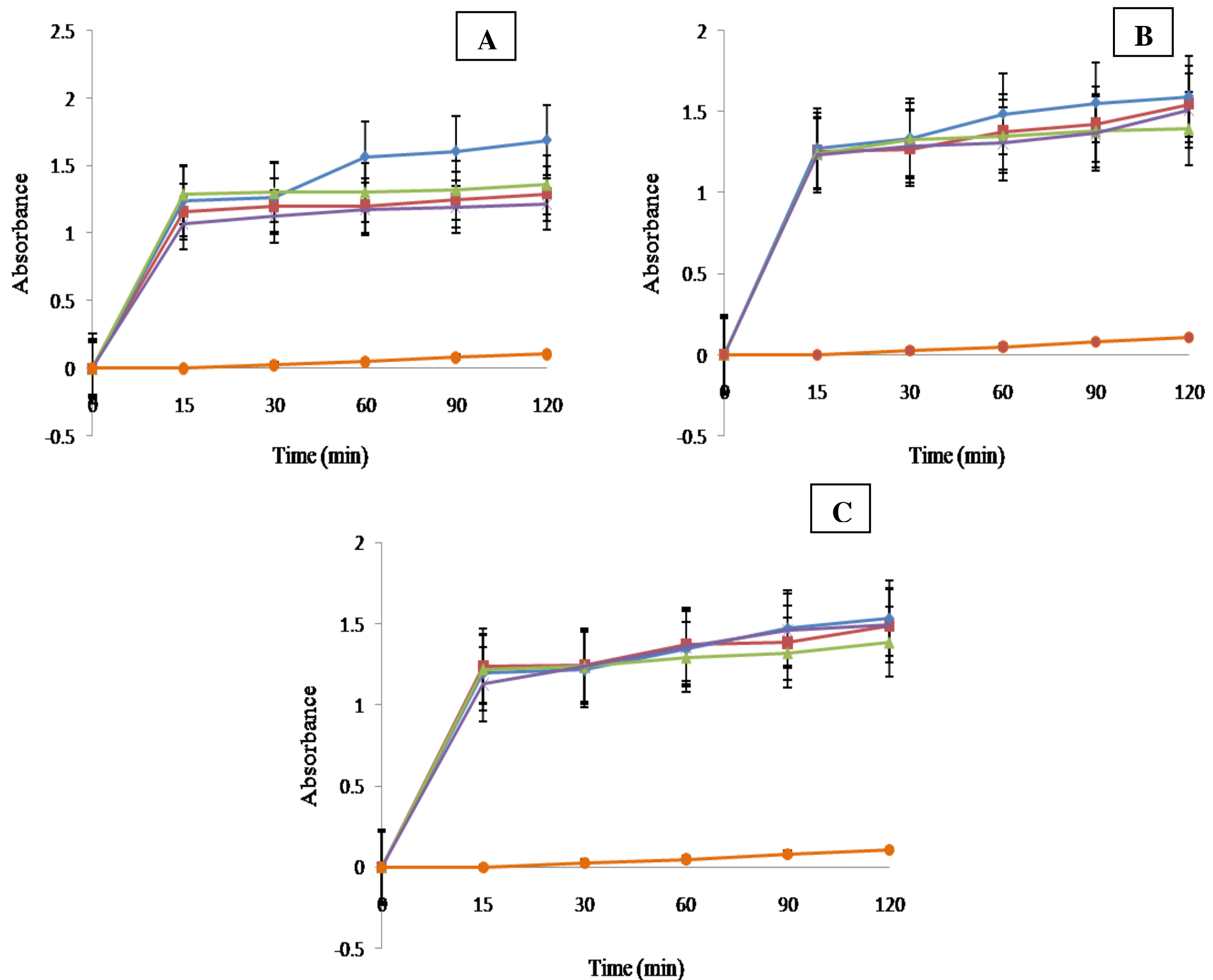

Fig. 5.15 Leakage of nucleotides from *Salmonella* Typhimurium by fractions partitioned into n-butanol ( ), ethyl acetate ( ), n-hexane ( ) and water ( ) compared to control ( ) at 1 × MIC (A), 2 × MIC (B) and 3 × MIC (C)

5.4. Discussion and Conclusion

The SEM reveals that the extracts are active against the cellular envelope, causing a level of disintegration in the physical structure of the microbial cell membrane. The intact anatomical structure of the cell membrane serves as permeability barrier between the cells and environment, hence the observed increase in the leakage of proteins and nucleic acids from the test isolates after been treated with the extracts indicate a level of compromise in the integrity of the cell membrane. The fraction partitioned into n-hexane appeared as the most potent against *C. albicans* in terms of the cell membrane attack considering the amount of intracellular

components released. Owing to the fact that biocidal effects of many active principles of plant origin are commonly associated with their ability to selectively attack microbial cell membranes (Lou *et al.*, 2011), thus the significant effect of the potent fractions against the cytoplasmic membrane of the test isolates is premised on the availability of some of the phytochemicals earlier determined from the extract (Chapter 2).

For instance, Avenacin, a well-studied saponin, has the ability to form complexes with sterols present in the fungal membrane which eventually leads to formation of pores and absolute loss of membranous selective permeability (González-Lamothe *et al*., 2009). Also from the report of Burckhardt and Thelen (1995), 1^0, 2^0 and 3^0 amines strongly depolarize cell membrane potential, while phenol compounds are well-known with the ability to attack and consequently increase membranous permeability, thereby inducing leakages of certain cellular constituents such as proteins, nucleotides and inorganic ions (Campos, 2009). Sulfoxides equally induce reduction in the rigidity and the general disruption of the membrane which in turn increases its permeability via desorption of lipid molecules and thus allowed disintegration of the bilayer structure (Gurtovenko & Anwar, 2007; He *et al*., 2012). In conclusion, the fractions partitioned into ethyl acetate, n-butanol and n-hexane are generally the most active fractions both from the leaf and stem bark extracts with that of n-hexane having edge as compared with others in terms of cell membrane attack. Membrane disruption has therefore been identified in the course of this study as one of the possible mechanisms of biocidal action of the *E. crispa* leaf and stem bark extracts.

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CHAPTER 6 – Significance of combination therapy between ethyl acetate fractions of the *Euclea crispa* (leaf and stem bark) extracts and standard antibiotics against selected drug resistant bacteria isolates

Abstract

This study evaluates the probable effect of combination therapy between the extracts of *E. crispa* and standard antibiotics against selected resistant strains of bacteria isolates. The isolates were screened via determination of minimum inhibitory concentrations (MICs) using broth micro-dilution method and the combination assessment was carried out via time-kill assay in determination of probable synergisms. The MICs by the plant extracts range from 0.310 to 0.630 mg/ml (leaf extract) and from 0.630 to 0.250 mg/ml (stem bark extract). The MIC values by erythromycin and chloramphenicol range between 0.031 and 0.250 mg/ml, while that of ampicillin is between 0.063 and 0.500 mg/ml. The range exhibited by streptomycin and tetracycline is between 0.008 and 0.250 mg/ml and 0.006 and 0.100 mg/ml respectively. Out of 130 different combination tests between the leaf extract and the antibiotics, 91.5% express synergy while 8.5% are indifferent. While on the other hand 88.5% of the same number of combination tests is synergistic between the stem bark extract and standard antibiotics with no record of antagonism in both cases. The leaf and the stem bark extracts of *E. crispa* have been confirmed to possess some bioactive principles which may serve to enhance potency of the available standard antibiotics against multidrug resistant organisms as a way of alternative therapy in combating infectious diseases.

6.1. Introduction

In recent time, efforts in development of new antibiotics are obviously do not catch up with the rate of increasing emergence of multidrug resistant (MDR) bacteria (Prescott *et al*., 2008). Development of resistance among microorganisms presents a bigger predicament because it is often not limited to a specific antibiotic, but usually extends to other member of the same class

(Chanda & Rakholiya, 2011). A known potent antibiotic at the moment could become irrelevant in anytime soon. It is therefore imperative to intensify the efforts on investigating newer drugs to combat the daily increasing occurrence of MDR organisms. Moreover in some cases, use of single antibacterial agent does not bring about the desired outcomes, thus a combination of drugs could express their synergy which may eventually outweigh that of the individual performance (Chanda & Rakholiya, 2011). Several scientific investigations have come to the conclusion that two different therapeutic agents used in combination may induce synergistic or antagonistic interactions. Aiyegoro *et al*. (2011) reported that the efficacy of antibiotics can be improved via combination with plant extracts and thus reducing the minimum inhibitory concentrations (MICs) of antibiotics against previously resistant organisms. Therefore combination of standard antibiotics with plant extracts represents an alternative approach in the treatment of microbial infection and could eventually lead to the development of new efficacious agents against infectious diseases.

Medicinal plants can either contain bioactive principles that operate in synergy with conventional antibiotics or composed of compounds with no inherent antibacterial effect but able to present the pathogen in a more susceptible way to a previously ineffective antibiotics (Betoni *et al*., 2006; Aiyegoro *et al*., 2009). The enhanced efficacy in the combined drugs could also be due to formation of certain complexes which becomes more effective in the inhibition of a previously less susceptible or resistant organisms (Chanda & Rakholiya, 2011). Chung *et al*. (2011) reported that various combinations of plant derived compounds could reduce their minimum inhibitory concentrations by half. For instance, enhanced combination therapy was revealed between ampicillin and ethanol extracts from a few selected Indian medicinal plants that are rich in alkaloids, cardiac glycosides, flavonoids, phenols and saponins (Aqil *et al*., 2005), Moreover, Aiyegoro *et al*. (2010) reported synergism between the leaf extract of *Helichrysum*

pedunculatum and various groups of antibiotics against wound infections organisms. The drug-drug interaction in terms of antibacterial activity between various plant-derived compounds increases the effectiveness of herbal extracts as compared to the isolated single compound (Wolska *et al*., 2012). Hence, in this study we evaluate possible effect of therapeutic interaction of the respective ethyl acetate fractions of *E. crispa* leaf and stem bark extract with standard antibiotics.

6.2. Materials and methods

6.2.1. Microorganisms

Owing to the fact that MIC breakpoints system are used in classification of bacteria as susceptible, intermediate or resistant to respective antibiotics, this was therefore employed in the selection of resistance isolates used in this study as defined by British society for antimicrobial chemotherapy (BSAC, 2015) and European committee on antimicrobial susceptibility testing (EUCAST, 2016). The selected isolates includes, *Escherichia coli* (ATCC839), *Protus vulgaris* (CSIR 0030), *Enterobacter faecalis*, *Shigella sonnei* (ATCC 29930), *Staphylococcus aureus* (OK2a), *Shigella flexneri, Klebsiella pneumoniae*, *Enterococcus faecalis*, *Plesiomonas shigelloides*, *Listeria sp.*, *Salmonella* Typhimurium, *E. coli* B 98, *E. coli* B1634.

6.2.2. Antibiotics

The antibiotics used in this study are Erythromycin, Tetracycline, Chloramphenicol, Ampicillin and Streptomycin purchased from Sigma Aldrich, USA.

6.2.3. Determination of the minimum inhibitory concentrations (MICs)

The MICs was determined using micro-plate dilution method as developed by Eloff (1998) and Clinical and Laboratory Standards Institute (CLSI, M07-A10, 2015) with slight modification. Nutrient broth culture of the bacterial isolates (18-24 h) was diluted in sterile Mueller Hinton broth (Sigma Aldrich, USA) and standardized (0.5 McFarland). Two fold serial dilutions of the

extract and standard antibiotics were prepared down the columns on the micro-plate to give a final concentration which ranged between 0.078 to 10.00 mg/ml (extract). Then 100 µl of the standard inoculums was added to the 100 µl aliquot of the antimicrobial agent in the wells and incubated at 37 ℃ for 24 h. An indicator, p-iodonitrotetrazolium chloride (Sigma Aldrich, USA) solution (40 µl of 0.2 mg/ml), was added to each well and further incubated at 37 ℃ for 30 min. Development of pink/red colour indicates growth of bacterial cells, while clear wells indicate total inhibition of the bacterial growth. The experiment was carried out in triplicate and sterile distilled water was used as control.

6.2.4. Extract–antibiotic combination assay

The effect of combinations of the ethyl acetate fraction of *E. crispa* extract and selected antibiotics was evaluated using the time-kill assessment as previously described by Adwan *et al*. (2008) and Aiyegoro *et al*. (2011) with slight modifications. The extract and antibiotics were incorporated into 45 ml of nutrient broth at ½ × MIC and 1 × MIC. This was then inoculated with 5 ml of 24 h old standardized inoculums (0.5 McFarland). The test flasks were incubated at 37 ℃ with shaking at 120 rpm for 24 h. After which 0.5 ml of the reaction mixture was transferred into 4.5 ml of nutrient broth recovery medium containing 3% Tween80. This was serially diluted in sterile physiological saline (0.9% NaCl) and plated out on nutrient agar in replicates of three. The plates were incubated at 37 ℃ for 24 h, and numbers of colonies were enumerated and the mean counts (CFU/ml) were determined and expressed as $\log_{10}$. The effect of the drug-drug interactions were considered synergistic if there was a decrease of $\geq 2 \log_{10}$ cfu/ml in colony counts after 24 h by the combination compared to the most active single agent (Lee *et al*., 2006), additivity or indifference was described as a $< 2 \log_{10}$ cfu/ml by the combination as compared with the most active single drug. Antagonism was defined as a ≥ 2 $\log_{10}$ CFU/ml increase in colony counts after 24 h by the combination as compared with that of

most active single agent (Sato *et al*., 2004; Lee *et al*., 2006). The nutrient broth incorporated with the extract and antibiotics but without microorganism was used as control likewise nutrient broth with microorganism but without antimicrobial agent.

6.3. Results

6.3.1. Determination of Minimum inhibitory concentrations

Determination of MIC was employed in the screening of the microorganisms selected for this study (Table 6.1). Test organisms that were resistant or at intermediate category to the test standard antibiotics were selected. The MIC values by erythromycin and chloramphenicol range between 0.031 and 0.250 mg/ml, while that exhibited by the ampicillin range between 0.063 and 0.500 mg/ml with the value of $\geq$ 0.125 mg/ml against almost 75% of the test organisms. The MIC range exhibit by the streptomycin is between 0.008 and 0.250 mg/ml with the maximum value against *E. coli* B771 while that of the tetracycline is between 0.006 and 0.100 mg/ml. The MIC by the plant extracts range from 0.310 to 0.630 mg/ml for the leaf extract and from 0.630 to 0.250 mg/ml for the stem bark extract.

6.3.2. Effect of drug-drug interaction

The effect of drug-drug interaction between the leaf extract and the standard antibiotics are shown in Tables 6.2 and 6.3 while the interaction between the stem bark extract and the standard antibiotics are shown in the Table 6.4 and 6.5. Both the ethyl acetate fraction of the leaf and stem bark extract of *E. crispa* demonstrate credible potentials to improve the efficacy of standard antibiotics with previous records of weak antimicrobial effect against particular microorganisms across Gram positive and Gram negative isolates. The highest bactericidal activity results from combination effect by the leaf extract is 2.92±0.00 depression in $\log_{10}$ CFU/ml of the total viable count against *Escherichia coli* (ATCC839) when combined with streptomycin both at concentration of ½ × MIC. All forms of combination by the leaf extract with the antibiotics were

130 tests out of which 119 express synergisms and only 11 are indifferent which amount to 91.5 and 8.5 % respectively. None of the test combination expresses antagonism. Moreover, the highest reduction in the total viable count by the stem bark extract is 2.30±0.23 $\log_{10}$ (CFU/ml) when in combination with chloramphenicol at a concentration of 1 × MIC against *Enterococcus faecalis*. Almost 83% of the combination tests at this concentration show synergy while the remaining 17% are indifferent. When the concentration was halved, about 90% of the combination tests express synergisms. Also no record of antagonism was observed at both concentrations.

Table 6.1: The minimum inhibitory concentrations (MICs) of the ethyl acetate fractions of the leaf and stem bark extracts and standard antibiotics

Bacterial isolates	**Extract (L) (mg/ml)**	**Extract (S) (mg/ml)**	**ERY (mg/ml)**	**CHL (mg/ml)**	**AMP (mg/ml)**	**STREP (mg/ml)**	**TET (mg/ml)**
Escherichia coli (ATCC839)	0.630	0.630	0.063	0.125	0.063	0.016	0.013
E. coli B1304	0.310	1.250	0.250	0.031	0.500	0.125	0.100
E. coli B 771	0.630	0.250	0.063	0.031	0.250	0.250	0.100
Enterobacter faecalis	0.310	1.250	0.125	0.063	0.125	0.016	0.013
Enterococcus faecalis	0.630	0.630	0.125	0.250	0.125	0.016	0.013
Klebsiella pneumoniae	0.630	0.630	0.063	0.063	0.250	0.063	0.013
Listeria sp.	0.310	0.630	0.250	0.125	0.250	0.008	0.013
Shigella sonnei (ATCC 29930)	0.310	1.250	0.063	0.063	0.125	0.016	0.013
Shigella flexneri	0.630	0.630	0.063	0.250	0.063	0.031	0.025
Salmonella Typhimurium	0.630	0.630	0.125	0.063	0.125	0.016	0.025
Staph. aureus (OK 2a)	0.310	0.630	0.063	0.125	0.125	0.063	0.006
Plesiomonas shigelloides	0.310	0.630	0.250	0.125	0.063	0.016	0.025
Proteus vulgaris (ATCC 6830)	0.630	1.250	0.031	0.250	0.250	0.125	0.025

Key: ERY= Erythromycin, CHL = Chloramphenicol, AMP = Ampicillin, STREP = Streptomycin, TET = Tetracycline, Extract (L) = Ethyl acetate fraction of the leaf extract, Extract(S) = Ethyl acetate fraction of the stem back extract.

Table 6.2: Activities of drug-drug interaction between the leaf extract and standard antibiotics at 1 × MIC against test bacterial isolates

	Reduction in the viable counts [$\log_{10}$ (CFU/ml±SD)]** as compared with the two antimicrobial agents used singly				
Bacterial isolates	**ERY + Extract (L)**	**CHL + Extract (L)**	**AMP + Extract (L)**	**STREP + Extract (L)**	**TET + Extract (L)**
Escherichia coli (ATCC839)	-0.92±0.02 (S)	-0.88±0.66 (S)	-0.88±0.42 (S)	-1.64±0.00 (S)	-1.64±0.66 (S)
E. coli B1304	-1.06±0.12 (S)	-1.13±0.54 (S)	-1.22±0.24 (S)	-2.17±0.30 (S)	-0.55±0.12 (I)
E. coli B 771	-0.97±0.16 (S)	-2.25±0.00 (S)	-1.47±0.40 (S)	-1.78±0.40 (S)	-0.81±0.34 (I)
Enterobacter faecalis	-1.23±0.18 (S)	-1.28±0.42 (S)	-2.23±0.48 (S)	-2.23±0.48 (S)	-1.95±0.18 (S)
Enterococcus faecalis	-1.67±0.56 (S)	-1.31±0.13 (S)	-2.27±0.32 (S)	-1.57±0.56 (S)	-1.42±0.00 (S)
Klebsiella pneumoniae	-0.97±0.12 (S)	-2.20±0.18 (S)	-1.60±0.18 (S)	-1.24±0.42 (S)	-2.20±0.00 (S)
Listeria sp.	-1.51±0.24 (S)	-1.43±0.47 (S)	-1.10±0.17 (S)	-2.21±0.24 (S)	-1.37±0.47 (S)
Shigella sonnei (ATCC 29930)	-2.20±0.49 (S)	-1.90±0.18 (S)	-2.20±0.49 (S)	-1.36±0.49 (S)	-0.94±0.23 (I)
Shigella flexneri	-1.24±0.00 (S)	-2.28±0.46 (S)	-1.18±0.46 (S)	-1.80±0.00 (S)	-1.43±0.40 (S)
Salmonella Typhimurium	-1.22±0.17 (S)	-2.26±0.24 (S)	-1.48±0.24 (S)	-1.78±0.47 (S)	-1.26±0.05 (S)
Staph. aureus (OK 2a)	-1.23±0.24 (S)	-0.78±0.36 (I)	-1.22±0.66 (S)	-0.82±0.24 (S)	-1.18±0.42 (S)
Plesiomonas shigelloides	-2.27±0.32 (S)	-2.27±0.32 (S)	-1.42±0.55 (S)	-1.42±0.26 (S)	-1.57±0.00 (S)
Proteus vulgaris (ATCC 6830)	-0.99±0.30 (S)	-1.69±0.00 (S)	-1.09±0.06 (S)	-1.47±0.00 (S)	-1.69±0.30 (S)

Key: ERY= Erythromycin, CHL = Chloramphenicol, AMP = Ampicillin, STREP = Streptomycin, TET = Tetracycline, Extract (L) = Ethyl acetate fraction of the leaf extract, S = Synergism, I = Indifferent, ** = Triplicate

Table 6.3: Activities of drug-drug interaction between the leaf extract and standard antibiotics at ½ × MIC against test bacterial isolates

	Reduction in the viable counts (log_{10} CFU/mL±SD)** as compared with the two antimicrobial agents used singly				
Bacterial isolates	**ERY + Extract (L)**	**CHL + Extract (L)**	**AMP + Extract (L)**	**STREP + Extract (L)**	**TET + Extract (L)**
Escherichia coli (ATCC839)	-0.27±0.12 (I)	-0.31±0.19 (S)	-0.48±0.24 (S)	-2.92±0.00 (S)	-1.06±0.42 (S)
E. coli B1304	-0.77±0.30 (S)	-1.02±0.24 (S)	-0.94±0.02 (S)	-1.57±0.54 (S)	-0.38±0.12 (S)
E. coli B 771	-0.22±0.16 (I)	-1.77±0.00 (S)	-1.77±0.64 (S)	-1.95±0.00 (S)	-0.66±0.22 (S)
Enterobacter faecalis	-0.53±0.16 (I)	-1.20±0.42 (S)	-2.24±0.48 (S)	-1.94±0.71 (S)	-1.34±0.00 (S)
Enterococcus faecalis	-0.99±0.08 (S)	-1.37±0.13 (S)	-1.67±0.00 (S)	-1.32±0.08 (S)	-1.23±0.26 (S)
Klebsiella pneumoniae	-0.74±0.00 (S)	-1.50±0.18 (S)	-1.35±0.12 (S)	-0.86±0.18 (S)	-1.60±0.42 (S)
Listeria sp.	-0.89±0.00 (S)	-1.06±0.24 (S)	-0.89±0.47 (S)	-1.73±0.47 (S)	-1.21±0.24 (S)
Shigella sonnei (ATCC 29930)	-0.94±0.30 (S)	-1.90±0.49 (S)	-1.30±0.00 (S)	-1.00±0.43 (S)	-0.44±0.17 (I)
Shigella flexneri	-1.17±0.00 (S)	-2.28±0.46 (S)	-1.17±0.46 (S)	-1.32±0.28 (S)	-1.17±0.69 (S)
Salmonella Typhimurium	-0.86±0.18 (S)	-1.56±0.00 (S)	-1.14±0.00 (S)	-1.22±0.47 (S)	-1.31±0.24 (S)
Staph. aureus (OK 2a)	-0.92±0.42 (S)	-0.35±0.24 (I)	-1.14±0.36 (S)	-0.46±0.24 (I)	-1.14±0.00 (S)
Plesiomonas shigelloides	-1.37±0.32 (S)	-2.27±0.55 (S)	-1.67±0.55 (S)	-0.97±0.00 (S)	-1.32±0.26 (S)
Proteus vulgaris (ATCC 6830)	-0.53±0.12 (I)	-1.06±0.00 (S)	-0.66±0.18 (S)	-1.57±0.00 (S)	-1.32±0.30 (S)

Key: ERY= Erythromycin, CHL = Chloramphenicol, AMP = Ampicillin, STREP = Streptomycin, TET = Tetracycline, Extract (L) = Ethyl acetate fraction of the leaf extract, S = Synergism, I = Indifferent, ** = Triplicate

Table 6.4: Activities of drug-drug interaction between the stem bark extract and standard antibiotic at 1 × MIC against test bacterial isolates

	Reduction in the viable counts [$\log_{10}$ (CFU/mL±SD)] as compared with the two antimicrobial agents used singly**				
Bacterial isolates	**ERY+ Extract (S)**	**CHL + Extract (S)**	**AMP + Extract (S)**	**STREP + Extract (S)**	**TET + Extract (S)**
Escherichia coli (ATCC839)	-1.32±0.28 (S)	-1.97±0.13 (S)	-1.16±0.24 (S)	-1.43±0.00 (S)	-1.49±0.42 (S)
E. coli B1304	-0.83±0.16 (I)	-1.11±0.00 (S)	-0.72±0.24 (I)	-0.61±0.24 (I)	-1.32±1.68 (S)
E. coli B 771	-0.71±0.30 (S)	-1.41±0.06 (S)	-1.30±0.12 (S)	-1.95±0.30 (S)	-1.95±0.06 (S)
Enterobacter faecalis	-0.75±0.23 (I)	-2.30±0.23 (S)	-0.63±0.02 (I)	-1.23±0.00 (S)	-1.70±0.16 (S)
Enterococcus faecalis	-1.19±0.00 (S)	-1.24±0.30 (S)	-1.16±0.16 (S)	-1.16±0.54 (S)	-1.46±0.24 (S)
Klebsiella pneumoniae	-1.09±0.42 (S)	-1.79±0.66 (S)	-0.88±0.19 (S)	-1.36±0.24 (S)	-1.36±0.24 (S)
Listeria sp.	-0.44±0.02 (I)	-1.42±0.32 (S)	-1.20±0.11 (S)	-0.63±0.26 (I)	-0.68±0.02 (I)
Shigella sonnei (ATCC 29930)	-1.27±0.06 (S)	-1.37±0.54 (S)	-1.80±0.30 (S)	-0.99±0.06 (I)	-1.43±0.30 (S)
Shigella flexneri	-1.65±0.46 (S)	-1.08±0.46 (S)	-1.55±0.70 (S)	-1.14±0.40 (S)	-1.65±0.70 (S)
Salmonella Typhimurium	-0.35±0.46 (I)	-1.62±0.70 (S)	-0.57±0.40 (I)	-1.11±0.00 (S)	-0.97±0.22 (I)
Staph. aureus (OK 2a)	-1.61±0.08 (S)	-1.17±0.43 (S)	-1.22±0.49 (S)	-1.07±0.72 (S)	-1.61±0.30 (S)
Plesiomonas shigelloides	-1.01±0.64 (S)	-1.66±0.40 (S)	-0.98±0.22 (S)	-1.12±0.16 (S)	-0.78±0.16 (S)
Proteus vulgaris (ATCC 6830)	-1.44±0.42 (S)	-1.68±0.18 (S)	-1.44±0.00 (S)	-1.59±0.42 (S)	-1.59±0.12 (S)

Key: ERY= Erythromycin, CHL = Chloramphenicol, AMP = Ampicillin, STREP = Streptomycin, TET = Tetracycline, Extract (S) = Ethyl acetate fraction of the stem back extract, S = Synergism, I = Indifferent, ** = Triplicate

Table 6.5: Activities of drug-drug interaction between the stem bark extract and standard antibiotics at ½ × MIC against test bacterial isolates

	Reduction in the viable counts (log_{10} CFU/mL±SD)** as compared with the two antimicrobial agents used singly				
Bacterial isolates	**ERY+ Extract (S)**	**CHL + Extract (S)**	**AMP + Extract (S)**	**STREP + Extract (S)**	**TET + Extract (S)**
Escherichia coli (ATCC839)	-0.59±0.05 (S)	-0.89±0.06 (S)	-0.70±0.18 (S)	-1.16±0.48 (S)	-1.19±0.05 (S)
E. coli B1304	-0.54±0.06 (I)	-1.53±0.06 (S)	-0.49±0.18 (I)	-0.61±0.26 (S)	-0.97±0.06 (S)
E. coli B 771	-0.60±0.02 (S)	-1.47±0.12 (S)	-0.61±0.16 (I)	-0.85±0.02 (S)	-0.72±0.00 (S)
Enterobacter faecalis	-0.51±0.00 (S)	-1.18±0.24 (S)	-1.45±0.24 (S)	-0.43±0.25 (I)	-1.60±0.47 (S)
Enterococcus faecalis	-0.73±0.55 (S)	-1.28±0.02 (S)	-1.16±0.02 (S)	-0.98±0.00 (S)	-1.39±0.17 (S)
Klebsiella pneumoniae	-0.70±0.24 (S)	-1.88±0.30 (S)	-0.87±0.06 (S)	-0.79±0.30 (S)	-1.09±0.70 (S)
Listeria sp.	-0.62±0.12 (S)	-1.02±0.00 (S)	-0.92±0.42 (S)	-0.69±0.12 (S)	-0.65±0.00 (S)
Shigella sonnei (ATCC 29930)	-1.26±0.28 (S)	-1.97±0.46 (S)	-1.37±0.70 (S)	-0.66±0.02 (I)	-1.58±0.40 (I)
Shigella flexneri	-0.91±0.24 (S)	-1.48±0.24 (S)	-0.91±0.05 (S)	-0.38±0.24 (S)	-0.69±0.06 (S)
Salmonella Typhimurium	-1.04±0.30 (S)	-1.44±0.72 (S)	-1.12±0.25 (S)	-0.81±0.30 (S)	-0.82±0.49 (S)
Staph. aureus (OK 2a)	-0.78±0.12 (S)	-1.91±0.54 (S)	-0.66±0.24 (S)	-0.96±0.12 (S)	-1.10±0.18 (S)
Plesiomonas shigelloides	-0.77±0.32 (S)	-1.15±0.13 (S)	-0.85± 0.08(S)	-1.08±0.26 (S)	-1.18±0.02 (S)
Proteus vulgaris (ATCC 6830)	-0.73±0.25 (S)	-0.86±0.43 (S)	-1.99±0.49 (S)	-0.39±0.18 (I)	-1.21±0.17 (S)

Key: ERY = Erythromycin, CHL = Chloramphenicol, AMP = Ampicillin, STREP = Streptomycin, TET = Tetracycline, Extract (S) = Ethyl acetate fraction of the stem back extract, S = Synergism, I = Indifferent, ** = Triplicate

6.4. Discussion and Conclusion

The preliminary study reveals that all test standard antimicrobial agents demonstrate weak activities against certain strains of bacterial isolates and this informed the selection of the isolates for further study. A total amount of 260 combination tests were carried out between the plant extracts and the standard antibiotics each of which were compared with the more active single test between the extracts and the standard drug in order to evaluate effectiveness of the plant extract in the enhancement of antimicrobial potency of the previously over powered standard antimicrobial agents. The MICs were determined to form the basis for the reference points in order to conveniently assess the outcome of the combination therapy. Virtually all the selected isolates were either resistant or at intermediate to the test standard antibiotics. Most of the isolates are with multiple folds increment in the MIC values above the recommended breakpoint for the respective standard antimicrobial agent (EUCAST, 2016). For instance, the breakpoint for the ampicillin against *Enterococcus faecalis* is > 0.008 mg/ml to be classified as resistant and that of streptomycin is ≤ 0.512 mg/ml to be classified as low-level intrinsic resistant. The breakpoint for erythromycin and ampicillin against *Listeria* sp. is > 0.001 mg/ml to be classified as resistant. While that of erythromycin and tetracycline against *Staphylococcus aureus* is > 0.002 mg/ml to be classified as resistant. Based on the acceptable evaluation that decrease in the colony counts by ≥ 2 $\log_{10}$ CFU/ml with the combined agents as compared to the more active single agent (Lee *et al*., 2006), both the leaf and the stem bark extracts of *E. crispa* exhibited commendable level of combinational therapeutic potential with broad spectrum across the groups of organisms and classes of antibiotics. It was equally observed that the synergistic potential of the plant extracts is not concentration dependent as there was no significant difference in the combination outcome at different concentrations of the combined agent as it is obvious with individual bioactive agent. This therefore suggests *E. crispa* as a valuable source of bioactive principles with inherent ability to improve the efficacy of the available synthetic antimicrobial agents, premised on the fact that combined antibiotic therapy often produces

enhanced effect in the treatment of infectious diseases and has also been established that it does suppresses or delays the emergence of resistance among microorganisms (Aiyegoro & Okoh, 2009; Chung *et al*., 2011). More importantly that modulation of resistance in microorganisms by bioactive compounds of natural origin has been reported in several studies (Sato *et al.*, 2004; Hemaiswarya *et al*., 2008; Zuo *et al*., 2011).

The FT-IR analysis of both ethyl acetate fractions used in this study (Chapter 2) reveals a high degree of similarity among the functional groups detected, hence the closely related activities of the two extracts against the selected drug resistant isolates was not a surprise. There is no doubt about the fact that some of these phytochemicals are the agents behind the biocidal and combined therapeutic potential exhibit by these extracts. While membrane attack as a mode of biocidal action of these extracts has been unveiled during the course of this study, the mode at which the extracts interact with the standard drug to improve the efficacy is not known. One may suggest a structural modulation of the standard drug by some of the chemical groups in the extracts which may eventually allow the antimicrobial agent to circumvent the resistance mechanism of the microorganism. It is also not impossible that the impact of the extract on the drug resistant microorganisms predisposed the organisms to absolute attack by the standard drug and thereby become more susceptible.

In conclusion, the outcome of the *in vitro* combination therapy between the *E. crispa* leaf and stem bark extracts together with standard antimicrobials has proven the significant potential of the plant extract to enhance the efficacy of standard drugs against multidrug resistant bacteria isolates. This study therefore not only confirms the antimicrobial properties of this plant but also substantiate its potential to enhance the effectiveness of standard drugs against resistance organism. This will go a long way in the fight against infectious diseases as drug resistance by the etiological agents remains a stumbling block in combating the menace.

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CHAPTER 7– General discussion and Conclusion

The phytochemicals screening of *Euclea crispa* extracts indicates the presence of certain chemical groups which are also common to several medicinal plants. Most of the phytochemicals detected have also been reported in the literature with proven pharmacological properties (Campos *et al*., 2009; Ukoha *et al.*, 2011; He *et al*., 2012, Abioye *et al*., 2013). For instance, saponins are produced as secondary metabolites by plants in response to pathogens attacks (Okwu & Emenike, 2006) while flavonoids and tannins have also been reported with antiseptic and anti-inflammatory properties (Tapas, 2008; Ukoha *et al*., 2011). Likewise thiosulfinates, an enzymatic derivative of sulfoxides, are well known for antimicrobial activities against several human pathogens (Anufrieva *et al*., 2015).

The leaf extract and fractions of *E. crispa* demonstrate commendable antimicrobial potentials across the spectrum of test microorganisms. Among the susceptible bacterial isolates are *Escherichia coli*, *Campylobacter* spp., *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria* sp. The commendable activity shown by the extracts against these pathogens is largely due to the bioactive components that were detected as present in the extract. It is not a hiding fact that virulence of *S. aureus*, *E. faecalis*, *K. pneumoniae* and *Listeria* sp. among others normally results into debilitating ailment which range between life threatening and less severe conditions, coupled with their swift resistance to antibiotics (Güven, 2004; Ralph, 2009; El-Shenawy *et al*., 2011). Hence, leaf extracts of *E. crispa* could be a good source of readily available therapy against some of the infectious diseases in traditional healing system. In addition, the outstanding suppressive capability express by the leaf extract against prominent diarrhoea causing bacteria isolates among the test organisms equally validate the use of the plant by the people of Eastern Free State Province of South Africa as antidiarrhoeal agent, thus further strengthened its application in herbal medicine knowing fully-well that diarrhoea is one of the common infectious diseases globally among children under the age of five.

In the same vein, the extract and the fractions from the stem bark of *E. crispa* demonstrate commendable activities against the array of test organisms which is closely related to that observed for the leaf extract. The activity of the fractions from both samples compared favourably with the standard antibiotics used as positive controls after considering the low MIC values exhibit by some of the fractions. It is however, obvious from the bacteriostatic concentration generated against individual isolate and the killing rates by each of the potent fractions that the ethyl acetate and n-butanol fractions both from the leaf and stem bark extracts are the most active. This serves as a pointer to the fact that bioactive compounds of the extracts are more of its polar components, hence suggests the two fractions as potential solvent in the extraction of bioactive compounds of natural origin which may be useful in the ongoing combat against resistance of pathogens to the existing antibiotics.

After the antimicrobial potential of the candidate plant for this study have been verified and confirmed, this study took a step further to investigate the probable mechanism behind the biocidal effect of the extracts. Having known that among the established mode of actions by some antimicrobial agents is membrane disruption; the scanning electron microscopy reveals that the extracts are active against the cellular envelope after been exposed to the test isolates over a period of 120 min at a concentration of 1 × MIC. The observed level of disintegration in the physical structure of the microbial cell membrane is expected to degenerate into loss of certain intracellular materials based on the fact that intact anatomical structure of cell membrane serves as permeability barrier between the cells and its environment. Hence the inquiry into possible leakage of proteins and nucleic acid components from the treated cell which equally reveals a general increase in the amount of intracellular material released with increase in the contact time between cells and extract. But this seems not to be concentration dependent, because when the concentration of the extract was increased over the same given period of time, the differences in the amount of released materials are not obvious. In addition, the membrane attack ability associated with the extracts of this plant is believed to have been the function of the phytochemicals earlier determined

from the extract, owing to the fact that biocidal effects of many active principles of plant origin are commonly associated with their ability to selectively attack microbial cell membranes (Lou *et al.*, 2011). For example, Sulfoxides normally compromise the rigidity and intact structure of cell membrane which in turn increases its permeability via desorption of lipid molecules and thereby allowed lipid-bilayer structure to disintegrate (Gurtovenko & Anwar, 2007; He *et al*., 2012). It is important to mention that the n-hexane fraction appeared as the most potent against *C. albicans* in terms of the cell membrane attack considering the amount of intracellular components released. This may suggest that membrane of the yeast cells is more prone to attack by some the active non-polar/fairly polar components of the extract.

Lastly, the ethyl acetate fractions of both the leaf and stem bark extracts showcase a significant combination therapy with some selected antibiotics against a number of multidrug resistant bacteria. In some instances, medicinal plants may contains as part of its bioactive components, substance which expresses synergy with conventional antibiotics or even with no inherent antibacterial effect but able to present the pathogen in a more susceptible way to a previously ineffective antibiotics (Betoni *et al*., 2006; Aiyegoro *et al*., 2009). Virtually all isolates selected for this study were resistant to the test standard antibiotics, some with well over 500% folds increment in the MIC values above the recommended breakpoint for the respective standard antimicrobial agent (EUCAST, 2016). For instance, the breakpoint for the ampicillin against *E. faecalis* is > 0.008 mg/ml to be classified as resistant while that for the erythromycin and ampicillin against *Listeria* sp is > 0.001 mg/ml. A total amount of 260 different combination tests were assayed between the plant extracts and the standard antibiotics. Based on the acceptable evaluation that depression in the number of colony counts by $\geq 2 \log_{10}$ CFU/ml with the combined agents as compared to the more active single agent is considered as synergy (Lee *et al*., 2006), both the leaf and the stem bark extracts of *E. crispa* were considered to have exhibited commendable level of combinational therapeutic potential with broad-spectrum across the groups of

organisms and classes of antibiotics. From the different rounds of combination test by the leaf extract, 92% express synergism with no records of antagonism from the leaf and as well the stem bark extracts. This finding therefore suggests *E. crispa* as a valuable source of bioactive principles with inherent ability to improve the efficacy of the previously suppressed synthetic antibiotics by some resistant pathogens. While cell membrane disruption has been unveiled during the course of this study as a mode action employed by these extracts, mode at which the extracts interact with standard drugs to improve the efficacy is not known. It may be suggested that impact of the extract on the drug resistant strains predisposed the organisms to absolute attack by the standard drug and thereby become more susceptible.

In conclusion, data generated from the activities of the leaf and stem bark extracts of *E. crispa* prove that the plant is made up of important phytochemical components which are active against diverse array of human pathogens. Moreover, outcome of the *in vitro* combination therapy between the extracts and standard antimicrobials has proven the significant potential of the plant extracts to enhance efficacy of standard drugs against multidrug resistant bacteria isolates. This will therefore not only validate the usefulness of the plant in traditional healing system, but also serve as a pointer to the fact that *E. crispa* could be a veritable source of bioactive substance of natural origin required to combat increasing resistance of pathogens to the existing antibiotics. Generally, ethyl acetate and n-butanol fractions are the most active, closely followed by n-hexane fraction which proves to be most active against the yeast isolates. This study will therefore suggest the three solvents as probably the best choice of solvent in the extraction of most active antimicrobial compounds for the benefit of pharmaceutical industries.

The suggestion above shall be prioritised as we continue investigation on the usefulness of *E. crispa* against infectious diseases among the future studies, with a view to isolating, characterising and structurally elucidating the major bioactive compounds from the identified three most active fractions both from the leaf and stem bark extracts. As well as to further probe into other probable mechanisms of biocidal action by the active compounds *viz*; possible prevention of the pathogens from colonising

epithelia cells via inhibition of virulence factor, by examining the ability of the plant-derived compounds to suppress expression of cell adhesion gene. And lastly to carry out toxicological studies of the isolated bioactive components.

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Appendix A

Media composition

NUTRIENT AGAR (Oxoid, UK)

Formulation	**gram/ litre**
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH	7.4±0.2

NUTRIENT BROTH (Oxoid, UK)

Formulation	**gram/ litre**
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH	7.4±0.2

MUELLER HINTON AGAR (Oxoid, UK)

Formulation	gram/litre
Beef, dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH	7.3±0.1

MULLER HINTON BROTH (SIGMA ALDRICH, USA)

Formulation	gram/litre
Beef infusion solid	2.0
Casein hydrolysate	17.5
Starch	1.5
pH	7.4±0.2

POTATO DEXTROSE AGAR (Oxoid, UK)

Formulation	gram/litre
Potatoes extract	4.0
Glucose	20.0
Agar	15.0
pH	5.6±0.2

Appendix B

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Bacillus pumilus* (ATCC 14884) at 1 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CHLOROFORM			CONTROL	
	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	203±2.50	2.307
15	56.9	82±1.15	1.914	64.0	78±2.08	1.892	63.1	75±2.50	1.875	45.3	111±2.65	2.045	40.9	120±1.73	2.079	198±2.08	2.297
30	72.9	55±1.53	1.740	73.9	53±1.53	1.724	72.2	57±1.73	1.756	59.6	82±2.00	1.914	61.6	78±2.08	1.892	184±1.53	2.265
60	89.2	22±1.00	1.342	84.7	31±2.00	1.491	84.2	32±1.15	1.505	77.8	45±1.15	1.653	71.9	57±1.73	1.756	201±2.65	2.303
90	100	0	0	91.6	17±1.00	1.230	89.7	21±1.73	1.322	86.7	27±2.00	1.431	85.7	29±1.00	1.462	179±2.00	2.252
120	100	0	0	97.5	05±0.58	0.699	96.1	08±1.00	0.903	94.1	12±1.00	1.079	93.6	13±0.00	1.114	191±1.73	2.281

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Bacillus pumilus* (ATCC 14884) at 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CHLOROFORM			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	0	203±2.50	2.307	203±2.50	2.307
15	66.0	69±1.73	1.839	70.0	61±2.08	1.785	77.8	45±2.50	1.653	61.1	79±2.65	1.898	58.6	84±2.65	1.924	198±2.08	2.297
30	85.7	29±2.00	1.462	76.8	47±2.00	1.672	82.3	36±1.53	1.556	78.8	43±1.15	1.633	73.9	53±1.73	1.724	184±1.53	2.265
60	97.0	06±0.58	0.778	88.7	23±0.58	1.362	93.1	14±1.73	1.146	86.7	27±0.58	1.431	86.7	27±2.08	1.431	201±2.65	2.303
90	100	0	0	95.7	09±1.00	0.952	100	0	0	93.1	14±1.00	1.146	92.6	15±1.00	1.176	179±2.00	2.252
120	100	0	0	100	0	0	100	0	0	96.1	08±1.15	0.903	97.5	05±0.00	0.699	191±1.73	2.281

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Klebsiella pneumoniae* (ATCC 13047) 1 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CHLOROFORM			CONTROL	
	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	195±2.08	2.290
15	48.2	101±3.00	2.004	49.7	98±1.73	1.99	67.7	63±1.53	1.799	50.3	97±2.00	1.987	46.2	105±2.50	2.021	190±3.00	2.279
30	65.6	67±0.58	1.826	66.7	65±2.50	1.813	73.8	51±1.15	1.707	68.7	61±2.08	1.785	65.1	68±1.00	1.833	173±2.65	2.238
60	81.5	36±1.53	1.556	78.5	42±2.08	1.623	88.2	23±1.53	1.361	76.9	45±1.73	1.653	74.9	49±0.58	1.690	189±2.08	2.276
90	97.0	6±1.00	0.778	86.2	27±1.00	1.431	93.8	12±0.00	1.079	85.1	29±2.00	1.462	81.0	37±2.00	1.568	178±1.73	2.250
120	100	0	0	94.4	11±1.15	1.041	96.4	07±0.58	0.845	89.7	20±0.58	1.301	90.8	18±0.58	1.255	172±1.53	2.236

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Klebsiella pneumoniae* (ATCC 13047) 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CHLOROFORM			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	0	195±2.08	2.290	195±2.08	2.290
15	72.3	54±1.53	1.732	69.2	60±1.73	1.778	68.7	61±1.53	1.785	62.1	74±2.50	1.869	58.5	81±1.73	1.908	190±3.00	2.279
30	88.2	23±0.58	1.361	82.6	34±1.00	1.531	77.9	43±1.00	1.633	78.5	42±2.08	1.623	67.7	63±2.65	1.799	173±2.65	2.238
60	97.9	4±1.00	0.602	89.2	21±1.15	1.322	95.4	09±1.15	0.954	85.6	28±1.00	1.447	84.6	30±1.00	1.477	189±2.08	2.276
90	100	0	0	96.4	7±0.00	0.845	99.0	02±0.00	0.301	90.3	19±0.58	1.279	91.3	17±2.00	1.230	178±1.73	2.250
120	100	0	0	100	0	0	100	0	0	95.4	09±1.00	0.954	94.9	10±1.15	1.000	172±1.53	2.236

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Candida albicans* (Ho316) 1 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	141±1.15	2.149	0	141±1.15	2.149	0	141±1.15	2.149	0	141±1.15	2.149	141±1.15	2.149
15	66.0	48±2.08	1.681	48.9	72±1.53	1.857	49.6	71±1.53	1.851	39.7	85±2.50	1.929	132±2.65	2.121
30	83.7	23±1.00	1.361	53.9	65±2.00	1.813	72.3	39±1.15	1.591	59.6	57±1.00	1.755	138±3.00	2.139
60	90.8	13±0.58	1.114	80.9	27±1.73	1.431	83.0	24±1.00	1.380	78.0	31±1.53	1.491	121±1.73	2.083
90	94.3	8±0.00	0.903	92.2	11±1.15	1.041	93.6	09±0.58	0.954	87.9	17±0.58	1.230	119±1.15	2.076
120	97.2	4±1.00	0.602	97.9	03±0.00	0.477	100	0	0	95.7	06±0.58	0.778	127±2.00	2.104

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Candida albicans* (Ho316) 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	141±1.15	2.149	0	141±1.15	2.149	0	141±1.15	2.149	0	141±1.15	2.149	141±1.15	2.149
15	83.0	24±2.08	1.380	61.0	55±1.73	1.740	73.0	38±1.53	1.580	70.9	41±1.00	1.613	132±2.65	2.121
30	87.2	18±0.58	1.255	70.9	41±2.08	1.612	86.5	19±0.58	1.279	76.6	33±1.73	1.519	138±3.00	2.139
60	92.9	10±1.15	1.000	86.5	19±1.00	1.279	93.6	09±0.00	0.954	83.0	24±1.15	1.380	121±1.73	2.083
90	92.9	10±0.00	1.000	95.0	07±0.58	0.845	100	0	0	87.9	17±0.00	1.230	119±1.15	2.076
120	100	0	0	100	0	0	100	0	0	96.5	05±1.00	0.699	127±2.00	2.104

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Escherichia coli* (1323) at 1 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			CHLOROFORM			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	140±2.00	2.146	0	140±2.00	2.146	0	140±2.00	2.146	0	140±2.00	2.146	0	140±2.00	2.146	140±2.00	2.146
15	58.6	58±2.50	1.763	51..4	68±1.73	1.833	44.8	75±1.15	1.875	49.4	91±2.65	1.959	39.7	82±2.00	1.913	140±1.73	2.146
30	61.4	54±1.53	1.732	62.9	52±1.00	1.716	55.2	61±2.08	1.785	59.4	73±1.00	1.863	46.3	73±0.58	1.863	136±2.08	2.127
60	70.0	42±1.15	1.623	74.3	36±1.53	1.556	69.1	42±0.58	1.623	73.3	48±1.00	1.681	55.8	60±1.15	1.778	134±1.53	2.130
90	77.9	31±2.00	1.491	85.7	20±1.15	1.301	75.7	33±1.53	1.518	76.6	42±1.15	1.623	61.7	52±1.53	1.716	130±1.73	2.114
120	85.7	20±1.15	1.301	92.9	10±0.58	1.000	79.4	30±2.00	1.477	80.5	35±1.73	1.544	75.7	33±1.00	1.519	130±1.15	2.114

The extent and the killing rate by the fractions of *Euclea crispa* leaf extract against *Escherichia coli* (1323) at 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			CHLOROFORM			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	147±1.00	2.167	0	147±1.00	2.167	0	147±1.00	2.167	0	147±1.00	2.167	0	147±1.00	2.167	147±1.00	2.167
15	63.3	54±1.73	1.732	71.43	42±1.15	1.623	61.9	56±1.53	1.748	55.8	65±1.15	1.959	65.3	51±3.00	1.708	140±1.73	2.146
30	69.4	45±1.00	1.653	82.31	26±2.08	1.415	71.4	42±1.73	1.623	71.4	42±1.73	1.863	66.0	50±2.08	1.699	144±3.00	2.158
60	84.4	23±1.15	1.362	93.20	10±0.00	1.000	82.3	26±1.53	1.415	72.8	40±0.58	1.681	76.2	35±2.50	1.544	138±1.73	2.140
90	94.6	08±0.58	0.903	95.92	6±1.00	0.778	88.4	17±1.00	1.230	77.6	33±1.00	1.623	85.7	21±1.00	1.322	145±2.50	2.161
120	98.0	03±0.00	0.477	100	0	0	94.6	08±1.00	0.903	83.7	24±1.53	1.544	91.8	12±0.58	1.079	136±2.65	2.134

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Listeria* sp. at 1 x MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	176±2.50	2.246	0	176±2.50	2.246	0	176±2.50	2.246	0	176±2.50	2.246	176±2.50	2.246
15	53.98	81±2.00	1.908	59.66	71±0.73	1.851	47.16	93±2.65	1.968	38.07	109±2.00	2.037	170±2.08	2.230
30	70.45	52±1.73	1.716	81.82	32±1.53	1.505	62.50	66±1.00	1.820	52.84	83±2.08	1.919	163±2.00	2.212
60	88.07	21±1.15	1.322	91.48	15±1.00	1.176	74.43	45±1.15	1.653	64.63	64±2.00	1.806	172±1.73	2.236
90	93.75	11±0.58	1.041	100	0	0	82.39	31±1.73	1.491	77.84	39±1.00	1.591	168±1.15	2.225
120	98.86	02±0.00	0.301	100	0	0	87.50	22±1.53	1.342	85.80	25±1.73	1.398	163±1.73	2.204

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Listeria* sp. at 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	183±2.65	2.262	0	183±2.65	2.262	0	183±2.65	2.262	0	183±2.65	2.262	183±2.65	2.262
15	73.22	49±2.00	1.690	70.49	54±1.73	1.732	66.67	61±1.53	1.785	54.10	84±1.15	1.924	178±2.08	2.250
30	86.34	25±1.15	1.398	83.61	30±1.00	1.477	78.14	43±1.00	1.633	74.32	47±2.00	1.672	162±1.53	2.220
60	96.17	7±0.00	0.845	97.27	5±0.58	0.699	89.07	20±1.00	1.301	91.26	16±0.58	1.204	180±1.73	2.255
90	98.36	3±0.58	0.477	100	0	0	95.63	8±0.58	0.903	97.81	4±0.58	0.602	178±2.50	2.250
120	100	0	1.690	100	0	0	97.81	4±0.00	0.602	100	0	0	165±1.53	2.217

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Listeria* sp. at 3 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	172±2.08	2.236	0	172±2.08	2.236	0	172±2.08	2.236	0	172±2.08	2.236	172±2.08	2.236
15	76.16	41±1.00	1.613	88.95	19±0.58	1.279	77.33	39±1.15	1.591	72.67	47±1.73	1.672	170±2.50	2.230
30	91.28	15±1.00	1.176	93.61	11±1.15	1.041	87.21	22±0.58	1.342	91.28	15±0.58	1.176	167±1.15	2.223
60	98.84	2±0.00	0.301	95.35	8±0.58	0.903	94.77	9±1.00	0.954	95.93	7±0.00	0.845	162±2.08	2.210
90	100	0	0	100	0	0	97.09	5±1.00	0.699	98.84	2±0.58	0.301	158±1.53	2.199
120	100	0	0	100	0	0	100	0	0	100	0	0	165±2.00	2.217

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Salmonella* Typhimurium at 1 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	153±1.73	2.185	0	153±1.73	2.185	0	153±1.73	2.185	0	153±1.73	2.185	153±1.73	2.185
15	51.63	74±1.53	1.869	41.83	89±2.00	1.949	42.48	88±2.00	1.944	37.91	95±1.53	1.978	145±2.00	2.161
30	58.82	63±2.08	1.799	51.63	74±2.50	1.869	64.05	55±2.08	1.740	59.48	62±2.00	1.792	140±1.00	2.146
60	84.31	24±1.00	1.380	71.90	43±1.15	1.633	83.66	25±0.58	1.398	62.75	57±±1.53	1.756	150±1.15	2.176
90	91.50	13±1.15	1.114	100	0	0	92.81	11±1.00	1.041	79.74	31±1.00	1.491	142±1.53	2.152
120	96.08	6±0.00	0.778	100	0	0	96.08	6±0.00	0.778	95.42	7±0.58	0.845	145±1.15	2.161

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Salmonella* Typhimurium at 2 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	161±2.08	2.207	0	161±2.08	2.207	0	161±2.08	2.207	0	161±2.08	2.207	161±2.08	2.207
15	65.22	56±1.15	1.748	59.63	65±2.00	1.813	68.94	50±1.00	1.699	57.14	69±2.65	1.839	155±1.15	2.190
30	74.53	41±1.15	1.613	86.96	21±1.53	1.322	75.78	39±1.73	1.591	70.81	47±1.00	1.672	150±2.50	2.176
60	86.34	22±1.00	1.342	96.27	6±1.00	0.778	90.68	15±0.58	1.176	81.37	30±1.73	1.477	147±2.00	2.167
90	94.41	9±0.58	0.954	100	0	0	93.79	10±1.00	1.000	89.44	17±1.15	1.230	158±1.73	2.199
120	98.76	2±0.58	0.301	100	0	0	97.52	4±0.00	0.602	93.17	11±0.58	1.041	154±2.00	2.188

The extent and the killing rate by the fractions of *Euclea crispa* stem bark extract against *Salmonella* Typhimurium at 3 × MIC

Time (min)	n-BUTANOL			ETHYL ACETATE			n-HEXANE			AQUOEUS			CONTROL	
	% Killed	Mean colony survival ± SD	Log (Mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	% Killed	Mean colony survival ± SD	Log (mean colony survival)	Mean colony survival ± SD	Log (mean colony survival)
0	0	157±1.53	2.196	0	157±1.53	2.196	0	157±1.53	2.196	0	157±1.53	2.196	157±1.53	2.196
15	75.80	38±1.00	1.580	67.52	51±1.00	1.708	71.34	45±2.08	1.653	64.97	55±1.73	1.740	145±1.15	2.161
30	85.99	22±0.58	1.342	87.26	20±1.73	1.301	79.62	32±1.15	1.505	78.98	33±1.00	1.519	149±2.00	2.173
60	89.81	16±1.15	1.204	97.45	4±0.00	0.845	87.17	17±1.53	1.230	84.08	25±0.58	1.398	152±1.00	2.181
90	95.54	7±0.58	0.845	100	0	0	96.18	6±0.58	0.778	91.72	13±1.53	1.114	155±2.08	2.190
120	100	0	0	100	0	0	100	0	0	98.09	3±0.58	0.477	142±1.00	2.152

Appendix C

Leakages of protein from *Bacillus pumilus* (ATCC 14884) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time(min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.231±0.002	0.181	0.341±0.001	0.365	0.335±0.004	0.355	0.359±0.003	0.395	0.217±0.001	0.157	0.139±0.005	0.027
30	0.238±0.002	0.193	0.348±0.003	0.377	0.341±0.001	0.365	0.368±0.002	0.410	0.223±0.005	0.168	0.146±0.001	0.039
60	0.251±0.004	0.214	0.352±0.001	0.384	0.354±0.004	0.387	0.375±0.001	0.422	0.234±0.003	0.186	0.152±0.003	0.049
90	0.257±0.003	0.224	0.358±0.002	0.394	0.365±0.001	0.405	0.394±0.002	0.454	0.231±0.001	0.181	0.168±0.003	0.075
120	0.322±0.001	0.333	0.362±0.003	0.400	0.368±0.002	0.410	0.401±0.003	0.466	0.248±0.001	0.209	0.171±0.002	0.080

Leakages of protein from *Bacillus pumilus* (ATCC 14884) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time(min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.233±0.001	0.184	0.351±0.002	0.382	0.371±0.001	0.415	0.390±0.002	0.447	0.241±0.003	0.198	0.139±0.005	0.027
30	0.243±0.004	0.201	0.353±0.003	0.385	0.378±0.003	0.427	0.393±0.001	0.452	0.238±0.001	0.193	0.146±0.001	0.039
60	0.255±0.002	0.221	0.363±0.001	0.402	0.384±0.001	0.437	0.397±0.003	0.459	0.249±0.001	0.211	0.152±0.003	0.049
90	0.312±0.001	0.317	0.366±0.002	0.407	0.385±0.002	0.439	0.401±0.001	0.466	0.287±0.006	0.275	0.168±0.003	0.075
120	0.332±0.002	0.350	0.370±0.002	0.414	0.390±0.001	0.447	0.437±0.005	0.526	0.313±0.002	0.318	0.171±0.002	0.080

Leakages of protein from *Klebsiella pneumoniae* (ATCC 13047) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time(min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.349±0.004	0.379	0.354±0.002	0.387	0.348±0.006	0.377	0.368±0.001	0.410	0.235±0.002	0.188	0.153±0.005	0.050
30	0.362±0.002	0.400	0.357±0.005	0.392	0.352±0.004	0.384	0.395±0.002	0.456	0.245±0.001	0.204	0.158±0.002	0.057
60	0.384±0.005	0.437	0.362±0.002	0.400	0.376±0.002	0.424	0.399±0.001	0.462	0.269±0.003	0.245	0.163±0.003	0.067
90	0.433±0.001	0.519	0.369±0.002	0.412	0.379±0.002	0.428	0.423±0.006	0.503	0.270±0.006	0.246	0.171±0.001	0.080
120	0.449±0.001	0.546	0.397±0.003	0.459	0.381±0.001	0.432	0.437±0.001	0.526	0.281±0.004	0.265	0.174±0.001	0.085

Leakages of protein from *Klebsiella pneumoniae* (ATCC 13047) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time(min)	n-BUTANOL		ETHYLACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.354±0.002	0.387	0.355±0.004	0.388	0.378±0.002	0.427	0.377±0.006	0.425	0.239±0.000	0.194	0.153±0.005	0.050
30	0.394±0.003	0.456	0.361±0.002	0.399	0.375±0.001	0.422	0.389±0.002	0.446	0.237±0.005	0.191	0.158±0.002	0.057
60	0.397±0.002	0.459	0.373±0.000	0.419	0.386±0.003	0.441	0.392±0.005	0.451	0.242±0.002	0.199	0.163±0.003	0.067
90	0.405±0.001	0.472	0.392±0.001	0.451	0.390±0.001	0.447	0.403±0.001	0.469	0.291±0.002	0.281	0.171±0.001	0.080
120	0.461±0.001	0.566	0.388±0.001	0.444	0.393±0.001	0.452	0.457±0.001	0.559	0.298±0.004	0.293	0.174±0.001	0.085

Leakages of protein from *Candida albicans* (Ho316) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.216±0.001	0.156	0.321±0.004	0.332	0.377±0.003	0.425	0.327±0.001	0.342	0.148±0.002	0.042
30	0.224±0.003	0.169	0.331±0.002	0.348	0.386±0.002	0.440	0.332±0.001	0.350	0.152±0.004	0.049
60	0.226±0.001	0.173	0.335±0.000	0.355	0.388±0.001	0.444	0.337±0.003	0.358	0.153±0.001	0.050
90	0.229±0.002	0.178	0.337±0.001	0.358	0.397±0.001	0.459	0.346±0.001	0.374	0.165±0.003	0.070
120	0.231±0.002	0.181	0.339±0.005	0.362	0.403±0.002	0.469	0.355±0.004	0.389	0.177±0.002	0.090

Leakages of protein from *Candida albicans* (Ho316) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.236±0.002	0.189	0.339±0.001	0.362	0.389±0.006	0.446	0.340±0.001	0.363	0.148±0.002	0.042
30	0.240±0.002	0.196	0.341±0.003	0.365	0.391±0.001	0.449	0.358±0.004	0.394	0.152±0.004	0.049
60	0.243±0.001	0.201	0.357±0.002	0.392	0.419±0.002	0.496	0.357±0.002	0.392	0.153±0.001	0.050
90	0.259±0.003	0.228	0.365±0.003	0.405	0.427±0.000	0.509	0.360±0.001	0.397	0.165±0.003	0.070
120	0.262±0.001	0.233	0.369±0.004	0.412	0.447±0.002	0.543	0.366±0.002	0.407	0.177±0.002	0.090

Leakages of protein from *Listeria* sp. by the fractions of *Euclea crispa* stem bark extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.354±0.001	0.387	0.398±0.005	0.461	0.326±0.001	0.340	0.314±0.000	0.320	0.173±0.002	0.084
30	0.365±0.003	0.405	0.416±0.000	0.491	0.358±0.004	0.394	0.328±0.002	0.343	0.183±0.001	0.101
60	0.378±0.002	0.427	0.438±0.001	0.527	0.384±0.006	0.437	0.332±0.003	0.350	0.185±0.005	0.104
90	0.386±0.001	0.441	0.452±0.003	0.551	0.400±0.001	0.464	0.373±0.002	0.419	0.191±0.003	0.114
120	0.434±0.001	0.521	0.484±0.005	0.605	0.447±0.002	0.543	0.415±0.002	0.489	0.190±0.002	0.112

Leakages of protein from *Listeria* sp. by the fractions of *Euclea crispa* stem bark extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.363±0.002	0.402	0.391±0.003	0.449	0.348±0.005	0.377	0.339±0.003	0.362	0.173±0.002	0.084
30	0.385±0.003	0.439	0.439±0.001	0.529	0.355±0.001	0.389	0.348±0.002	0.377	0.183±0.001	0.101
60	0.395±0.002	0.456	0.461±0.002	0.566	0.387±0.004	0.442	0.355±0.003	0.389	0.185±0.005	0.104
90	0.421±0.001	0.499	0.484±0.001	0.605	0.449±0.001	0.546	0.382±0.001	0.434	0.191±0.003	0.114
120	0.438±0.004	0.528	0.494±0.001	0.621	0.462±0.001	0.568	0.422±0.004	0.501	0.190±0.002	0.112

Leakages of protein from *Listeria* sp. by the fractions of *Euclea crispa* stem bark extracts at 3 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.388±0.003	0.444	0.401±0.000	0.466	0.381±0.006	0.432	0.345±0.004	0.372	0.173±0.002	0.084
30	0.395±0.001	0.456	0.434±0.002	0.521	0.404±0.001	0.471	0.357±0.001	0.391	0.183±0.001	0.101
60	0.416±0.005	0.491	0.446±0.001	0.541	0.452±0.001	0.551	0.379±0.003	0.429	0.185±0.005	0.104
90	0.438±0.002	0.528	0.487±0.002	0.610	0.469±0.003	0.580	0.411±0.006	0.482	0.191±0.003	0.114
120	0.469±0.001	0.580	0.496±0.004	0.625	0.481±0.002	0.600	0.436±0.001	0.524	0.190±0.002	0.112

Leakages of protein from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.327±0.002	0.342	0.347±0.001	0.375	0.322±0.001	0.333	0.344±0.002	0.370	0.139±0.000	0.027
30	0.358±0.001	0.394	0.375±0.005	0.422	0.366±0.003	0.407	0.355±0.001	0.389	0.146±0.001	0.039
60	0.366±0.004	0.407	0.386±0.002	0.441	0.365±0.006	0.405	0.382±0.005	0.434	0.164±0.002	0.069
90	0.371±0.002	0.415	0.398±0.002	0.461	0.377±0.001	0.425	0.387±0.002	0.442	0.163±0.001	0.067
120	0.385±0.003	0.439	0.397±0.004	0.459	0.388±0.003	0.444	0.427±0.001	0.509	0.167±0.005	0.074

Leakages of protein from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.339±0.006	0.362	0.353±0.001	0.385	0.348±0.004	0.377	0.343±0.001	0.369	0.139±0.000	0.027
30	0.383±0.002	0.436	0.379±0.004	0.429	0.363±0.001	0.402	0.366±0.005	0.407	0.146±0.001	0.039
60	0.409±0.002	0.479	0.385±0.002	0.439	0.397±0.002	0.459	0.385±0.002	0.439	0.164±0.002	0.069
90	0.451±0.005	0.549	0.399±0.002	0.459	0.423±0.003	0.503	0.414±0.001	0.487	0.163±0.001	0.067
120	0.457±0.004	0.560	0.401±0.000	0.466	0.513±0.001	0.653	0.422±0.001	0.501	0.167±0.005	0.074

Leakages of protein from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 3 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)	Mean optical density±SD	Proteins quantity (µg/ml)
0	0	0	0	0	0	0	0	0	0	0
15	0.361±0.002	0.399	0.371±0.001	0.415	0.436±0.004	0.524	0.378±0.003	0.427	0.139±0.000	0.027
30	0.386±0.001	0.441	0.378±0.006	0.427	0.451±0.003	0.549	0.392±0.002	0.451	0.146±0.001	0.039
60	0.404±0.002	0.471	0.386±0.002	0.441	0.519±0.005	0.663	0.412±0.002	0.484	0.164±0.002	0.069
90	0.438±0.005	0.528	0.399±0.001	0.462	0.524±0.002	0.672	0.425±0.006	0.506	0.163±0.001	0.067
120	0.484±0.003	0.605	0.421±0.001	0.499	0.594±0.001	0.789	0.473±0.001	0.586	0.167±0.005	0.074

Appendix D

Leakages of Nucleotides from *Bacillus pumilus* (ATCC 14884) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time(min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.253±0.002	7.59	0.219±0.005	6.57	0.340±0.006	10.20	0.222±0.002	6.66	0.268±0.001	08.04	0.004±0.002	0.12
30	0.306±0.001	9.18	0.244±0.002	7.32	0.427±0.002	12.81	0.296±0.001	8.88	0.277±0.002	8.31	0.034±0.001	1.02
60	0.344±0.004	10.32	0.260±0.004	7.80	0.439±0.001	13.17	0.338±0.001	10.14	0.305±0.004	9.15	0.050±0.002	1.50
90	0.396±0.001	11.88	0.356±0.000	10.68	0.566±0.004	16.98	0.607±0.004	18.21	0.317±0.001	9.51	0.083±0.003	2.49
120	0.475±0.002	14.25	0.371±0.002	11.13	0.590±0.003	17.70	0.657±0.001	19.71	0.398±0.003	11.94	0.072±0.003	2.16

Leakages of Nucleotides from *Bacillus pumilus* (ATCC 14884) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.288±0.003	8.64	0.226±0.001	6.78	0.311±0.006	9.33	0.345±0.003	10.35	0.242±0.002	7.26	0.004±0.002	0.12
30	0.309±0.002	9.27	0.269±0.000	8.07	0.454±0.003	13.62	0.553±0.005	16.59	0.249±0.002	7.47	0.034±0.001	1.02
60	0.434±0.003	13.02	0.299±0.001	8.97	0.585±0.001	17.55	0.675±0.002	20.25	0.263±0.004	7.89	0.050±0.002	1.50
90	0.506±0.005	15.18	0.358±0.004	10.74	0.953±0.002	28.59	0.782±0.004	23.46	0.351±0.001	10.53	0.083±0.003	2.49
120	0.662±0.002	19.86	0.510±0.000	15.30	1.325±0.001	45.75	0.918±0.003	27.54	0.472±0.002	14.16	0.072±0.003	2.16

Leakages of Nucleotides from *Klebsiella pneumoniae* (ATCC 13047) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.308±0.002	9.24	0.205±0.003	6.15	0.318±0.002	9.54	0.377±0.004	11.31	0.154±0.002	4.62	0.010±0.001	0.30
30	0.368±0.000	11.04	0.240±0.001	7.20	0.380±0.005	11.40	0.398±0.002	11.94	0.227±0.001	6.81	0.013±0.002	0.39
60	0.402±0.005	12.06	0.323±0.004	9.69	0.414±0.001	12.42	0.405±0.002	12.15	0.265±0.003	7.95	0.036±0.001	1.08
90	0.536±0.004	16.08	0.498±0.002	14.94	0.501±0.001	15.03	0.449±0.006	13.47	0.360±0.002	10.80	0.090±0.003	2.70
120	0.934±0.006	28.02	0.722±0.002	21.66	0.563±0.004	16.89	0.782±0.005	23.46	0.381±0.002	11.43	0.128±0.001	3.84

Leakages of Nucleotides from *Klebsiella pneumoniae* (ATCC 13047) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CHLOROFORM		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0	0	0
15	0.360±0.003	10.80	0.341±0.002	10.23	0.307±0.001	9.21	0.608±0.003	18.24	0.173±0.000	5.19	0.010±0.001	0.30
30	0.393±0.001	11.79	0.388±0.001	11.64	0.471±0.004	14.13	0.713±0.001	21.39	0.202±0.003	6.06	0.013±0.002	0.39
60	0.511±0.005	15.33	0.436±0.003	13.08	0.634±0.002	19.02	0.753±0.004	22.59	0.285±0.002	8.55	0.036±0.001	1.08
90	0.784±0.001	23.52	0.576±0.000	17.28	0.824±0.001	24.72	0.814±0.002	24.42	0.384±0.002	11.52	0.090±0.003	2.70
120	1.357±0.002	40.71	0.822±0.002	24.66	0.904±0.005	27.12	1.272±0.003	38.16	0.421±0.006	12.63	0.128±0.001	3.84

Leakages of Nucleotides from *Candida albicans* (Ho316) by the fractions of *Euclea crispa* leaf extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	0.224±0.003	6.72	0.180±0.002	5.40	0.472±0.005	14.16	0.292±0.001	8.76	0.038±0.003	1.14
30	0.274±0.004	8.22	0.253±0.005	7.59	0.549±0.001	16.47	0.338±0.002	10.14	0.064±0.002	1.92
60	0.339±0.002	10.17	0.271±0.001	8.13	0.971±0.003	29.13	0.390±0.001	11.70	0.080±0.003	2.40
90	0.405±0.005	12.15	0.286±0.002	8.58	1.031±0.001	30.93	0.410±0.002	12.30	0.095±0.006	2.85
120	0.449±0.002	13.47	0.339±0.000	10.17	1.112±0.006	36.36	0.473±0.005	14.19	0.114±0.006	3.42

Leakages of Nucleotides from *Candida albicans* (Ho316) by the fractions of *Euclea crispa* leaf extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	0.243±0.003	7.29	0.198±0.004	5.94	0.509±0.001	15.27	0.372±0.001	11.16	0.038±0.003	1.14
30	0.271±0.001	8.13	0.211±0.001	6.33	0.601±0.006	18.03	0.430±0.005	12.90	0.064±0.002	1.92
60	0.395±0.004	11.85	0.236±0.003	7.08	1.010±0.002	30.30	0.491±0.002	14.73	0.080±0.003	2.40
90	0.422±0.001	12.66	0.247±0.003	7.41	1.220±0.002	36.60	0.921±0.001	27.63	0.095±0.006	2.85
120	0.462±0.002	13.86	0.279±0.002	8.37	1.478±0.001	44.34	1.040±0.004	31.20	0.114±0.006	3.42

Leakages of Nucleotides from *Listeria* sp. by the fractions of *Euclea crispa* stem bark extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.165±0.002	35.95	1.223±0.003	36.69	1.194±0.006	35.82	1.147±0.006	34.41	0.002±0.000	0.06
30	1.193±0.005	35.79	1.285±0.004	38.55	1.298±0.002	38.94	1.160±0.002	34.80	0.013±0.001	0.39
60	1.251±0.001	37.53	1.304±0.001	39.12	1.316±0.005	39.48	1.177±0.005	35.31	0.036±0.002	1.17
90	1.287±0.002	38.61	1.362±0.002	40.86	1.339±0.004	40.17	1.183±0.006	35.49	0.090±0.001	2.70
120	1.346±0.004	40.38	1.562±0.005	46.86	1.421±0.006	42.63	1.252±0.003	37.56	0.098±0.000	2.94

Leakages of Nucleotides from *Listeria* sp. by the fractions of *Euclea crispa* stem bark extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.274±0.003	38.22	1.336±0.004	40.08	1.214±0.005	36.42	1.201±0.003	36.03	0.002±0.000	0.06
30	1.395±0.005	41.85	1.368±0.005	41.04	1.277±0.003	38.31	1.241±0.004	37.23	0.013±0.001	0.39
60	1.486±0.004	44.58	1.390±0.006	41.70	1.329±0.004	39.87	1.313±0.003	39.39	0.036±0.002	1.17
90	1.571±0.006	47.13	1.452±0.003	43.56	1.378±0.004	41.34	1.326±0.005	39.78	0.090±0.001	2.70
120	1.591±0.004	47.73	1.532±0.005	45.96	1.455±0.006	43.65	1.378±0.002	41.34	0.098±0.000	2.94

Leakages of Nucleotides from *Listeria* sp.by the fractions of *Euclea crispa* stem bark extracts at 3 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.306±0.005	39.18	1.287±0.002	38.61	1.269±0.004	38.07	1.208±0.002	36.24	0.002±0.000	0.06
30	1.375±0.003	41.25	1.389±0.005	41.67	1.288±0.003	38.64	1.233±0.005	36.99	0.013±0.001	0.39
60	1.411±0.002	42.33	1.441±0.003	43.23	1.303±0.005	39.09	1.268±0.002	38.04	0.036±0.002	1.17
90	1.547±0.006	46.41	1.518±0.002	45.54	1.361±0.003	40.83	1.323±0.003	39.69	0.090±0.001	2.70
120	1.598±0.004	47.94	1.594±0.004	47.82	1.478±0.005	44.34	1.372±0.006	41.16	0.098±0.000	2.94

Leakages of Nucleotides from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 1 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.158±0.005	34.74	1.237±0.003	37.11	1.286±0.005	38.58	1.073±0.005	32.19	0	0
30	1.199±0.004	35.97	1.266±0.005	37.98	1.298±0.004	38.94	1.125±0.002	33.75	0	0
60	1.202±0.006	36.06	1.565±0.002	46.95	1.302±0.006	39.06	1.175±0.004	35.25	0.028±0.001	0.84
90	1.247±0.002	37.41	1.605±0.006	48.15	1.319±0.003	39.57	1.192±0.005	35.76	0.050±0.001	1.50
120	1.291±0.005	38.73	1.687±0.004	50.61	1.358±0.002	40.74	1.215±0.006	36.45	0.083±0.003	2.49

Leakages of Nucleotides from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 2 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.255±0.004	37.65	1.272±0.005	38.16	1.241±0.002	37.23	1.229±0.003	36.87	0	0
30	1.270±0.002	38.10	1.335±0.003	40.05	1.328±0.004	39.84	1.286±0.002	38.58	0	0
60	1.375±0.005	38.85	1.485±0.004	44.55	1.348±0.003	40.44	1.302±0.005	39.06	0.028±0.001	0.84
90	1.422±0.003	39.66	1.553±0.005	46.59	1.379±0.006	41.37	1.363±0.002	43.89	0.050±0.001	1.50
120	1.545±0.002	40.35	1.592±0.006	47.76	1.393±0.001	41.79	1.505±0.005	45.15	0.083±0.003	2.49

Leakages of Nucleotides from *Salmonella* Typhimurium by the fractions of *Euclea crispa* stem bark extracts at 3 × MIC

Time (min)	n-BUTANOL		ETHYL ACETATE		n-HEXANE		AQUEOUS		CONTROL	
	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)	Mean absorbance	Mass of the leaked nucleotides (µg)
0	0	0	0	0	0	0	0	0	0	0
15	1.239±0.003	37.17	1.194±0.005	35.82	1.222±0.002	36.66	1.127±0.002	33.81	0	0
30	1.245±0.006	37.35	1.215±0.002	36.45	1.237±0.004	37.11	1.234±0.001	37.02	0	0
60	1.371±0.002	41.13	1.343±0.004	40.29	1.294±0.001	38.82	1.356±0.005	40.68	0.028±0.001	0.84
90	1.382±0.004	41.46	1.472±0.002	44.16	1.323±0.003	39.69	1.457±0.004	43.71	0.050±0.001	1.50
120	1.485±0.003	41.55	1.533±0.005	45.99	1.387±0.003	41.61	1.492±0.004	44.76	0.083±0.003	2.49

APPENDIX D

Viable counts for the combination of standard drugs and the leaf extract at 1 × MIC

Bacterial isolates	Control	Extract	ERY	E+ S	CHL	C+S	AMP	A+S	STREP	S+S	TET	T+S
Escherichia coli (ATCC839)	173±2.65	101±1.73	92±2.00	21±2.52	83±2.08	23±0.58	95±1.15	19±1.00	85±1.15	4±0.00	89±1.73	4±0.58
E. coli B1304	148±2.00	83±2.89	55±2.65	13±1.53	55±1.15	11±0.58	45±1.00	9±1.15	51±1.53	0	62±3.06	42±1.53
E. coli B 771	179±2.52	119±3.06	94±1.73	19±1.73	62±3.00	1±0.00	77±2.65	6±1.00	74±1.73	3±1.00	61±2.52	28±1.15
Enterobacter faecalis	170±3.00	85±2.52	66±1.53	10±2.00	73±1.15	9±1.15	50±1.00	0	66±2.89	0	73±2.89	2±0.00
Enterococcus faecalis	185±2.08	94±2.65	73±2.89	4±0.58	82±2.65	9±1.53	61±1.15	0	58±1.53	5±0.58	66±2.65	7±0.00
Klebsiella pneumoniae	158±1.53	68±1.53	63±3.00	17±1.15	52±2.00	1±0.00	55±2.00	4±1.00	51±1.73	9±0.58	54±0.58	0
Listeria sp.	162±1.73	87±2.00	59±2.65	5±1.00	60±1.53	6±0.58	59±1.53	13±1.15	62±3.06	0	59±1.53	7±0.58
Shigella sonnei (ATCC 29930)	159±3.06	75±1.73	99±1.15	0	72±1.73	2±0.00	52±1.00	0	66±3.00	7±1.00	53±1.53	18±1.73
Shigella flexneri	191±2.89	99±3.00	85±1.00	3±0.00	64±2.89	0	76±3.00	10±1.00	83±2.65	3±0.00	60±1.73	7±1.15
Salmonella Typhimurium	184±1.73	82±2.89	68±2.65	11±1.15	50±1.00	0	61±1.53	6±1.00	55±1.53	3±0.58	48±0.58	10±1.53
Staph. aureus (OK 2a)	152±2.65	72±3.00	71±1.00	9±1.53	61±2.08	25±1.15	54±1.53	9±0.58	61±2.08	23±1.53	61±2.65	10±1.00
Plesiomonas shigelloides	185±2.08	88±2.65	61±1.53	0	83±2.65	0	60±1.53	7±0.58	54±1.73	7±1.15	55±1.73	5±0.00
Proteus vulgaris (ATCC 6830)	149±1.15	69±2.08	55±1.73	15±0.58	66±1.53	3±0.00	83±2.65	12±1.00	53±1.53	5±1.15	49±1.53	3±0.58

Viable counts for the combination of standard drugs and the leaf extract at ½ × MIC

Bacterial isolates	Control	Extract	ERY	E+ S	CHL	C+S	AMP	A+S	STREP	S+S	TET	T+S
Escherichia coli (ATCC839)	173±2.65	121±2.08	120±1.73	94±2.00	143±3.00	85±1.73	150±2.52	57±1.53	134±3.06	6±0.00	102±2.89	15±1.00
E. coli B1304	148±2.00	92±1.73	85±2.65	25±1.00	75±2.08	14±1.15	62±1.73	17±2.08	64±1.73	4±0.58	97±1.15	61±1.53
E. coli B 771	179±2.52	131±2.89	119±2.08	106±1.73	102±2.00	3±0.00	86±3.06	3±0.58	97±2.08	2±0.00	93±1.73	39±1.53
Enterobacter faecalis	170±3.00	108±3.06	74±1.73	51±2.08	126±1.73	11±1.15	53±1.73	0	82±1.53	2±0.58	87±2.52	8±0.00
Enterococcus faecalis	185±2.08	103±1.15	81±1.53	19±1.73	94±1.00	8±1.53	77±2.52	4±0.00	73±1.73	9±1.73	72±2.08	11±1.15
Klebsiella pneumoniae	158±1.53	85±1.73	98±2.08	29±1.53	71±1.73	5±1.00	81±2.08	7±1.15	69±2.65	22±1.00	66±1.73	4±0.58
Listeria sp.	162±1.73	94±1.00	75±3.06	21±1.73	87±2.65	14±1.00	74±1.73	21±0.58	88±2.89	3±0.58	88±3.06	10±1.00
Shigella sonnei (ATCC 29930)	159±3.06	101±2.89	130±1.15	18±1.53	117±1.73	2±1.00	64±1.15	8±0.00	71±3.00	16±1.15	62±1.73	58±2.08
Shigella flexneri	191±2.89	125±1.73	92±1.73	13±0.00	96±1.53	0	109±2.08	13±1.00	118±1.15	9±1.53	89±2.52	13±0.58
Salmonella Typhimurium	184±1.73	97±1.15	77±1.53	25±1.15	63±1.00	5±0.00	78±3.00	7±0.00	65±1.53	11±0.58	63±2.08	9±1.00
Staph. aureus (OK 2a)	152±2.65	104±2.52	89±2.08	16±1.00	86±3.06	68±1.53	68±1.73	11±1.15	82±2.08	53±1.53	83±1.73	11±0.00
Plesiomonas shigelloides	185±2.08	114±1.73	78±2.00	8±1.00	109±2.52	1±0.58	91±1.53	4±0.58	67±2.08	20±0.00	74±1.73	9±1.15
Proteus vulgaris (ATCC 6830)	149±1.15	107±2.52	63±1.00	44±1.53	126±1.73	13±0.00	135±3.06	32±1.73	74±1.73	4±0.00	61±3.06	7±0.58

Viable counts for the combination of standard drugs and the stem bark at 1 × MIC

Bacterial isolates	Control	Extract	ERY	E+ S	CHL	C+S	AMP	A+S	STREP	S+S	TET	T+S
Escherichia coli (ATCC839)	186±1.53	75±2.00	55±3.06	9±2.89	63±2.52	2±2.08	71±0.58	13±2.65	67±1.73	7±1.53	36±2.00	6±0.58
E. coli B1304	168±2.52	82±1.73	62±1.00	25±1.73	71±1.53	13±2.52	68±1.15	32±1.00	78±2.08	41±1.00	52±1.53	8±2.08
E. coli B 771	179±1.15	61±1.00	44±0.58	12±0.58	52±2.08	7±1.00	51±2.89	9±1.53	53±0.58	2±0.58	48±1.53	2±0.00
Enterobacter faecalis	198±2.89	93±0.58	74±2.08	35±1.53	68±0.58	1±1.53	61±0.58	49±3.06	58±2.00	11±2.89	32±0.58	4±2.00
Enterococcus faecalis	173±2.00	101±1.53	80±1.00	11±2.00	74±1.53	10±1.00	71±0.58	12±2.89	61±2.08	12±0.58	41±2.89	6±1.15
Klebsiella pneumoniae	185±2.65	81±1.15	89±2.89	15±1.00	62±3.06	3±0.58	75±2.00	24±1.73	49±1.00	8±1.53	44±0.58	8±1.53
Listeria sp.	157±2.08	75±2.65	95±0.58	64±2.00	58±2.89	6±1.00	65±1.53	10±2.65	58±2.00	37±1.15	55±2.08	33±2.00
Shigella sonnei (ATCC 29930)	188±2.00	97±2.08	61±2.89	10±1.73	66±2.65	8±0.58	77±1.00	3±0.00	49±0.58	19±1.73	48±1.53	7±1.00
Shigella flexneri	179±2.89	108±1.73	69±2.08	4±1.00	81±1.53	9±1.00	89±0.58	5±0.58	101±1.00	13±1.15	31±2.89	4±0.58
Salmonella Typhimurium	166±2.89	99±1.53	87±3.06	75±1.00	66±0.58	4±0.58	90±1.73	45±1.15	59±2.89	9±2.89	39±3.06	18±1.73
Staph. aureus (OK 2a)	164±3.06	86±1.73	69±1.15	4±2.52	56±1.53	11±1.00	77±1.15	10±0.58	130±1.53	14±1.00	48±1.00	4±1.53
Plesiomonas shigelloides	182±2.52	94±0.58	120±2.89	18±0.58	79±0.58	4±0.00	92±1.00	19±1.53	72±2.08	22±1.73	57±2.08	30±1.73
Proteus vulgaris (ATCC 6830)	193±1.53	72±2.65	62±1.00	7±0.58	63±0.58	4±1.00	53±1.73	7±1.53	43±0.58	5±0.58	49±1.73	5±1.15

Viable counts for the combination of standard drugs and the stem bark extract at ½ × MIC

Bacterial isolates	Control	Extract	ERY	E+ S	CHL	C+S	AMP	A+S	STREP	S+S	TET	T+S
Escherichia coli (ATCC839)	186±1.73	115±0.58	153±1.15	48±1.53	76±0.58	24±2.00	98±2.08	37±1.15	132±2.00	13±0.58	54±1.15	12±1.53
E. coli B1304	168±1.15	122±1.00	73±1.53	49±1.00	58±1.73	5±0.00	110±1.53	55±1.73	104±0.58	41±2.08	85±1.73	18±1.00
E. coli B 771	179±2.00	98±1.00	92±1.15	45±2.08	76±1.15	6±1.53	87±1.73	44±2.89	73±2.00	25±2.08	93±2.00	34±2.00
Enterobacter faecalis	198±1.73	131±0.58	125±2.00	62±1.73	62±3.06	13±1.00	126±1.53	7±1.00	115±1.73	74±3.06	55±3.06	5±0.58
Enterococcus faecalis	173±2.08	124±2.00	111±1.53	32±0.58	71±2.00	9±2.00	81±2.89	12±2.00	89±1.53	18±2.08	84±1.00	7±3.06
Klebsiella pneumoniae	185±1.15	94±2.00	84±2.08	37±2.00	55±2.00	2±0.58	83±1.15	25±1.00	79±2.00	30±0.58	74±2.08	15±2.89
Listeria sp.	157±1.53	103±1.00	115±2.86	38±1.15	77±1.15	15±1.53	120±1.73	19±0.58	99±0.58	32±1.15	97±3.06	35±1.53
Shigella sonnei (ATCC 29930)	188±2.89	129±3.06	87±1.73	12±1.53	104±1.00	2±0.00	88±2.00	8±0.58	72±1.53	41±3.06	30±0.58	5±1.15
Shigella flexneri	179±1.73	133±2.08	137±3.06	22±1.00	114±2.89	6±1.00	72±1.00	22±1.53	119±3.06	47±1.00	92±1.15	36±2.00
Salmonella Typhimurium	166±3.06	126±2.00	98±2.89	15±1.53	75±2.89	6±0.58	71±2.89	13±1.73	82±1.73	26±1.53	83±0.58	25±1.00
Staph. aureus (OK 2a)	164±2.00	108±2.00	87±1.73	27±1.53	59±1.53	2±0.58	120±2.00	36±1.15	95±1.73	18±1.53	55±0.58	13±3.06
Plesiomonas shigelloides	182±2.08	137±1.53	147±2.89	31±1.00	72±1.00	13±1.53	110±2.08	26±1.73	86±2.08	15±1.15	47±1.15	12±2.00
Proteus vulgaris (ATCC 6830)	193±3.06	117±1.53	97±1.15	36±1.73	86±3.06	27±1.15	108±1.53	2±0.00	93±0.58	79±1.73	41±0.58	12±2.08