ASSESSMENT OF THE ANTIBACTERIAL PROPERTIES OF n-HEXANE EXTRACT OF *COCOS NUCIFERA* AND ITS INTERACTIONS WITH SOME CONVENTIONAL ANTIBIOTICS

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DECLARATION

I, the undersigned, declare that this thesis and the work contained herein being submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture, School of Science and Technology, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other university in partial or entirety for the award of any degree.

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DEDICATIONS

This thesis is dedicated to my crown (Adeorimi) and rare gem Tominiyi; our daughter Akinsewaoluwa Akinyele, you will all fulfill purpose and destiny.

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GENERAL ABSTRACT

General Abstract

Cocos nucifera belong to the family Aracaceae (palm Family). The English name is coconut and it is used extensively as medicinal remedies against infections such as urinary tract infections, gastro intestinal infections, skin and wound infections. The *in vitro* antibacterial (including anti-listerial and anti-*vibrio*) properties as well as the evaluation of the combination potentials of the plant extract with six front-line antibiotics were evaluated in this study using standard procedures.

The *in vitro* anti-listerial properties of the crude aqueous and n-Hexane extract of the husk of *Cocos nucifera* were carried out against 37 *Listeria* isolates. Twenty-nine of the test organisms were susceptible to the aqueous extract while thirty were susceptible to the n-Hexane extract both at the screening concentration of 25 mg/ml. Minimum Inhibitory Concentration (MIC) values for all the susceptible bacteria ranged between 0.6 - 5.0 mg/ml. For the aqueous extract, average log reduction in viable cell count ranged between 0.32 Log_{10} and 4.8 Log_{10} CFU/ml after 8 hours interaction in 1 × MIC and 2 × MIC. For the n-Hexane extract, the log reduction ranged between 2.4 Log_{10} and 6.2 Log_{10} CFU/ml after 8 hours interaction in 1 × MIC and 2 × MIC. The time-kill characteristics of the two extracts suggest that at higher concentration (2 × MIC) and longer duration of interaction (8 hr), more bacteria were killed.

In vitro anti-*vibrio* and antibacterial properties experiment revealed that of all the 45 *vibrio* and 25 bacteria strains that was tested, 37 were susceptible to the aqueous extract and 38 to the n-Hexane extract, while 17 were susceptible to the aqueous extract and 21 to the n-Hexane extract. Minimum Inhibitory Concentration (MIC) values for all the susceptible bacteria ranged between 0.3 - 5.0 mg/ml.

The time kill studies revealed that for the aqueous extract, average log reduction in viable cell count in time kill assay ranged between 0.12 Log_{10} and 4.2 Log_{10} CFU/ml after 8 hr interaction at 1 × MIC and 2 × MIC. For the n-Hexane extract, the log reduction ranged between 0.56 Log_{10} and 6.4 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC.

In the test for the combination interactions, the checkerboard method revealed synergy of 67% and indifferent of 33%, while the time kill assay detected synergy in 72% and indifferent in 28% of the combinations tested. The synergy detected was not specific to any of the antibiotics or the Gram reaction of the bacteria, and no antagonism was detected. We conclude that the aqueous and n-Hexane extract of the husk of *C. nucifera* contains potential broad spectrum antibiotics resistance modulating compounds that could be relevant in the treatment of infections caused by these pathogens. In addition, the husk which is being discarded as agro waste will opens up a vista of opportunities for utilization for therapeutic purposes.

CHAPTER ONE

GENERAL INTRODUCTION.

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CHATPER ONE

GENERAL INTRODUCTION

Plants play a vital role in the existence and survival of man. They supply foods and the much-required oxygen for breathing. Plants also provide fibres for diverse purposes and wood for housing and shelter. It is also known that they provide a tremendous reservoir of various chemical substances with potential therapeutic properties (Lewis and Elvin-Lewis, 1995). The plant kingdom has served as a prolific source of useful drugs, foods, additives, flavoring agents, lubricants, coloring agents and gums from time immemorial (Prescott *et al.*, 2002). Also, the healing power of herbs had been recognized since creation and botanic medicine is one of the oldest practiced professions by mankind (Hugo and Russell, 2003). Medicinal plants have been found useful as antimalarial, antisickling, anti-helmintic, anti-microbial, anti-convultant, anti-hypertensive, and as anti-schistosomal (molluscicidal) agents (Prescott *et al.*, 2002). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Kambizi and Afolayan, 2001). Apart from the use of plants as therapeutic agents, they have also served mankind as a source of economic materials for both social and environmental stabilization (Hugo and Russell, 2003). The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parikh *et al*., 2005).

The curative potentials of these plants are locked-up and embedded in some chemical components that effects physiological response in man (Edeoga *et al*., 2005). Amongst the most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Hill and van

Staden 1992). Many of these medicinal plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 1999, 2001). Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Their role is twofold in the development of new drugs: (1) they may become the base for the development of a medicine, a natural blue print for the development of new drugs, or; (2) a phytomedicine to be used for the treatment of diseases (Iwu *et al*., 1993).

The quest for solutions to the global problems of antibiotic resistance in pathogenic bacteria has often focused on the isolation and characterization of new antimicrobial compounds from a variety of sources including medicinal plants (Sibanda and Okoh, 2008). This is probably because the efficacies of these plant products have been confirmed in different disease situations in different part of the world and that their little or no known side effects have made them succeed where most synthetic and conventional agents have failed. It may also be because scientists have established that crude extracts of some plants and some pure compounds from such plant can potentiate the activity of antibiotics *in-vitro* (Marquez, 2005; Smith *et al.*, 2007).

In Africa, medicinal preparations from plants have been used over a long period for the treatment of ailments. This is because orthodox medicine is not available in some places due to a wide range of reasons among which includes that the first line drugs which are cheap and affordable have become ineffective due amongst other to resistance factor. Now however, these plant preparations are becoming more widely used by people all over the world as they understand the gentle strength in them and the fact that most of them can be used safely without the known side effect of drugs (Steve and Lee, 2004). Now, workers in the field of plant medicine research, regard higher plants as living chemical factories that provide a vast number of unusual chemical substances that display a variety of biological actions

(Oyi and Ames, 1991). Plants are able to produce compounds which though have no apparent function in the primary metabolism of the plant (Robinson and Recio, 1991), have good activity against bacteria pathogens when they are able to find their way into and accumulate in them. These compounds have had an extensive history of use as therapeutic agent (Tyler *et al.*, 1988). A considerable number of works have been done and aimed at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections (both tropical and systemic applications) as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant. During the last ten years the pace of development of new antimicrobial compounds has slowed down while the prevalence of multiple resistances has increased astronomically (Hugo and Russell, 2003). The increase in the number of antibiotic resistant bacteria could no longer be matched by the expansion in the resources of agents available to treat infections.

Literature reports and ethnobotanical records suggest that plants are the sleeping giants of pharmaceutical industry (Hostettmann and Hamburger, 1991). They may provide natural source of antimicrobial drugs that will provide novel or lead compounds that may be employed in controlling some infections globally.

Several studies have been conducted to provide scientific basis for the efficacy of plant in herbal medicines. The development of resistance to most of the available antimicrobial agents and the high costs of treatments consequent upon this resistance has necessitated the search for new, safe, efficient and cost effective ways for the management of infectious conditions. Akinpelu and Onakoya (2006) have suggested that unless concerted efforts are made to acquire new agents, very soon the population of bacteria developing resistance will not match the arsenals to fight. The rising interests in product of natural origin in the developed economies led to the extraction and development of several drugs and chemotherapeutic agents from plants as well as from traditionally used rural herbal remedies (UNESCO,

1998). Extracts of higher plants have served as good source of antibiotics against various bacterial and fungal pathogens (Falodun *et al.*, 2006).

The common view in the society and the medical community is that plant based products are healthier, safer, and more reliable than synthetic products (Benli *et al*., 2008); even though safety and efficacy data are available for only a few number of plant materials. Plants acts generally to stimulate and supplement the bodies' healing forces; they are the natural food of human beings (Ajayi *et al*., 2008). The clinical success of quinine and quinidine isolated from the Cinchona tree bark and recently artemisinin from artemisiaannua in the chemotherapy of malaria have rejuvenated interests in higher plants as potential sources of novel drugs (Igoli *et al.*, 2005). Today, Evolution of pathogenic microorganism towards resistance to antimicrobial agents has led to researches being carried out on medicinal plants. Therefore, more researches should be carried out on these medicinal plants around us. This study is therefore in line with this aspiration.

Remarkably, antimicrobial drugs have proved effective for the control of microbial infections. However, there is an increased attention on extracts and biologically active compounds isolated from plant species used in herbal medicine due to the side effects and the resistance that pathogenic micro-organisms build against conventional antibiotics (Essawi and Srour, 1999). Eloff (1998) reported that the amount of resistant strains of microbial pathogens is increasing since penicillin resistant and multiresistant pneumococci caused a major problem in South African hospitals in 1977. The potential problem of emerging resistance to antimicrobial agents requires careful monitoring. Antimicrobial studies by Palambo and Semple (2001); Kudi *et al*. (1999); Paz *et al*. (1995); and Vlietinck *et al*. (1995) have shown that Gram-negative bacteria exhibits a higher resistance to plant extracts than gram-positive bacteria. This can be as a result of the variation in the cell wall structures of Gram-positive and Gram-negative bacteria (Palambo and Semple, 2001). More specifically, Gram-negative bacteria have an outer

membrane that is composed of high density lipopolysaccharide that serves as a barrier to many environmental substances including antibiotics (Kudi *et al*., 1999).

The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains (Eloff, 1999). As certain antibiotic treatments lead to the development of multiresistant organisms, it is now standard clinical practice to use a combination of two or more antibiotics with different mechanisms of action in an attempt to prevent the development of antibiotic resistance and improve the outcome of therapy (Beringer, 1999).

Usually, infections resulting from strains that are resistant to main groups of antibiotics like the beta-lactams and aminoglycosides are treatable with vancomycin, chloramphenicol or other antibiotics (Hugo and Russell, 2003). But resistance has been developed to these drugs over the years. Infections due to *Staphylococcus aureus* have continued to be a major source of morbidity and mortality in Hospitals and hence a significant cause of concern among physician (Luck *et al.*, 1998). These organisms are now exhibiting multidrug resistance to commonly used antibiotics (Luck *et al*., 1998). Strains of *pneumococci*, once uniformly susceptible to penicillin are currently resistant to it in up to 18% and 40% cases in the United States and South Africa respectively and multiple antibiotic resistances is constantly being reported in virulent type of *Heamophilus influenza* (Tzouvelekis *et al.*, 1998). Although, antibiotic resistant *Klebsiella pneumoniae* is now a common pathogen in urinary tract infections (Dequchi *et al.*, 1998), the increasing frequency of *Neisseria gonorrhoea* isolates with multiple antibiotic resistance is a serious problem worldwide (Takana *et al*., 1997). It has been reported that infections caused by methicillin resistance strains of *Staphylococcus aureus* are often difficult to treat and in an attempt to achieve an optimal outcome, clinicians would use combinations of antibiotic therapies with the aim of achieving antibiotic synergy (Hee *et al*., 2009). There is a continuous and urgent need to develop new

antibiotic and immune modulating compounds with diverse chemical structures and novel mechanisms of action, because there has also been an alarming increase in the incidence of new and re-emerging infectious diseases.

In the developed countries of the world, plants have been widely accepted as the sleeping giants of the pharmaceutical industries that may provide an unlimited source of antibiotics and it is estimated that 27 million South Africans utilize traditional herbal medicines from more than 1020 plant species, more than 80% of those who live in the rural areas now depend on medicinal plants that have been used in folkloric medicine (Stafford *et. al.*, 2004). Many plants have been used for different purposes, such as food, drugs and traditional uses. Typical examples of such plants include our study candidate *Cocos nucifera.*

Cocos nucifera:

Cocos nucifera is an important member of the family Aracaceae (palm family) and it is the only accepted specie of the genus *Cocos*. The English name is Coconut. Coconut palm is grown throughout the tropics for decorations as well as for its culinary and non-culinary uses. Virtually all the parts of coconut palm can be utilized by humans in some manner. It provides almost all the necessities of life-food, drink, oil, medicine, fiber, timber, thatch, mats, fuel, and domestic utensils. For good reason, it has been called the "tree of heaven" and "tree of life" (Chan *et al*., 2006). Today it remains an important economic and subsistence crop in many small Pacific island states. The word Coconut is derived from Portuguese explorers as well as the sailors of vasco da Gama in India while the brown and hairy surface of coconut reminded them of the ghost and witch called Coco. The popular medicinal use of *C. nucifera* aqueous extract in the form of Tea has been reported in a large extent (Esquenazi *et al*., 2002 and

Mendonca- Filho *et al*., 2004). Recent study shows that aqueous extract from *C. nucifera* present antibacterial and antileishmanial properties, against certain gram positive bacteria.

The juice has adequate natural minerals and high quality proteins, which are valuable for growth and repair of the body (Pehowich *et al*., 1992). Medical research has discovered that the coconut oil obtained from the kernel consists of monolaurin and lauric acid, which helps the immune system in a beneficiary manner. The monolauric acids were used by the body to produce high levels of antimicrobial (Mid-American Marketing Corp., 2004). Lauric acid was the basic of monolaurin and was part of the chemical constituent of sodium lauryl sulfate that has been discovered to promote health and used in adjunct treatment of viral diseases. It is also found to be of the active chemicals in controlling Human Immunodeficiency Virus (HIV) disease (Davrit, 2004). Recent study by Alaris *et al*. (2005) has demonstrated that the aqueous and methanol extract possess antibacterial properties against the *Escherichia coli, Shigella flexineri, Shigella sonnei* and *Salmonella* spp. with the latter being more active than the former.

In addition *C. nucifera* extract have antiproliferative effect on animal lymphocytes (Kirszberg *et al*., 2003). It has been demonstrated that *C. nucifera* natural water present antioxidant properties which was correlated with the presence of ascorbic acid (Mantena and Alvies, 2003). Coconut oil has been confirmed to possess antimicrobial, antiviral and antiprotozoal activities (Isaacs and Thormar, 1991; Thormar, 1996; Enig, 2003). Phytochemical studies indicated that lauric acid which is its major fatty acid component was highly responsible for the activities of the oil. Lauric acid has been documented to be converted to monolaurin in the human body and it is the antimicrobial agent found in human milk. Antimicrobial activity of the methanolic extract of the pod shows a strong activity against

Staphylococcus aureus and an inhibition zone was formed against *Bacillus subtilis*. It shows no response against *Escherichia coli* and *Pseudomonas aeruginosa* (Moumita and Adinpunya, 2008); the therapeutic use of *Cocos nucifera* chaffs decoction against various bacterial, viral and protozoan diseases (George and Pamplona-Roger, 1998) has been reported. Nonetheless, there is no information on the anti-listerial and anti-vibrio activities, or their potentials for combination antimicrobial therapy.

JUSTIFICATION OF THIS RESEARCH

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. Bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents and this is a serious cause for concern as the problem of bacterial resistance is growing and the outlook for the use of antibacterial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new and potent drugs of synthetic or natural origin. The ultimate goal is to offer appropriate and efficient antibacterial drugs to the patient. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. However, combination therapy could be an alternative to monotherapy for patients with invasive infections that are difficult to treat, such as those due to multi-resistant species and for those who fail to respond to standard treatment. Antimicrobial compounds used in combination might promote the effectiveness of each agent, with efficacy being achieved using a lower dose of each drug. Pharmacological benefits

would accrue, with one drug clearing infection from one body system while the other clears it from a different site. In addition, synergism in antimicrobials could be utilized in an attempt to prevent or delay the emergence *in vivo* of resistant populations of the pathogenic organisms. Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antimicrobial activity. Esimone *et al*. (2006), Stapleton *et al*. (2004), Bentoni *et al*. (2006) and Braga *et al*. (2005) have demonstrated that plants either contain antimicrobials that can operate in synergy with antibiotics or posses compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective state. Synergism is a positive interaction created when two agents combined and exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. It follows that antibiotic development remains vital if man is to keep ahead of resistance, and it is partly evident that, despite resistance threat, some new agents of plants origin do continue to be developed.

AIMS AND OBJECTIVES

BROAD AIM

The broad aim of this study is to carry out a detailed assessment of the antibacterial potentials of crude extracts of *Cocos nucifera* in relation to its relevance in combination antibacterial chemotherapy and especially against *Listeria* and *Vibrio* pathogens. The specific objectives include:

- To screen aqueous, chloroform and n-hexane extracts of *Cocos nucifera* for antibacterial activities against a panel of extended bacterial pathogens including *Listeria* and *Vibrio* bacteria.
- To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the crude extracts of *Cocos nucifera* and selected conventional antibiotics.
- To determine the rate of kill of the extracts of *Cocos nucifera* against the susceptible bacterial strains.
- To determine the effect of extract - antibiotic combination on their antibacterial potencies.

REFERENCES

Ajayi AO, Balogun SA, Adegbehingbe K (2008). Microorganisms in the crude oil-producing areas of Ondo State Nigeria. *Scientific Research and Essays.* 3 (5): 174–179

Akinpelu DA, Onakoya TM (2006). Antimicrobial activities of medicinal plants used in folklore remedies in south-western. *Afr J Biotechnol.* 5(11): 1078-1081.

Alaries SA, Silva AF, Brandao M, Grandi TSM, Smania EF, Smania Jr A, Zani CL (2005). Biological screening of Brazilian medicinal plants. *Mem Inst. Oswaldo Cruz.* 95: 367-373.

Al-hebshi N, Al-haroni M, Skaug N (2006). *In vitro* antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. *Arch Oral Biol*. 51: 183-188.

Benli A, Ferrara L, Del Pozzo M, Mele G, Sorbo S, Bassi P, Montesano D (2008). Antibacterial and antioxidant activities of ethanol extract from *Paullinia cupana* Mart. *J Ethnopharma.* 102: 32-36.

Beringer PM (1999). New approaches to optimizing antimicrobial therapy in patients with cystic fibrosis. *Cur Opinion Pul Med.* 5: 371-377.

Betoni JEC, Mantovani RP, Barbosa LN, Di-Stasi LC, Fernandes A (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. *Mem Inst Oswaldo Cruz.* 101 no.4.

Braga LC, Leite AAM, Xavier KGS, Takahashi JA, Bemquerer MP, Chartone-Souza E, Nascimento AMA (2005). Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus*. *Canad J Microbio.* 51(7): 541-547.

Chan S, Patron OL, Whang US (2006). Antimicrobial activity and combination effect of drugs to bacterial isolates. *Phytother Research.* 3: 140-144.

Davrit K (2004). Antibiotic susceptibility testing by standardized single disc method. Ame *Jour of Clin Path*. 264: 375–382.

Dequchi KR, Gupta RU, Nathan AU (1998). Studies of antibacterial substances from Melia Azadirachta. *Indian J Med Res*. 28: 211–215.

Edeoga HO, Okwu DE, Mbaebre BO (2005). Phytochemical constituent of some Nigerian Medicinal Plants. *Afr J Biotech.* 4 (7): 685-688.

Eloff JN (1998). It is possible to use herbarium specimens to screen for antibacterial components in some plants. *J Ethnopharm.* 67: 355-360.

Enig M (2003). Know Your Fats—The Complete Primer for Understanding the Nutrition of Fats, Oils and Cholesterol. Bethesda Press, Silver Spring, Maryland.

Esimone CO, Iroha IR, Ibezim, EC, Okeh CO, Okpana EM (2006). *In vitro* evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus. Afr J Biotechnol.* 5(11): 1082-1086.

Esquenazi D, Wigg MD, Miranda MM, Rodriguez HM, Tostes JB, Rozental *et al*. (2002). Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. *Res Microbiol*. 153: 647–652.

Essawi T, Srour M (1999). Screening of some Palestinian medicinal plants for antibacterial activity. *J Ethnopharm*. 70: 343-349.

Falodun A, Usifoh CO, Nworgu ZAM (2006) Smooth muscle relaxant activity of 3 carbomethoxylpyridine from *Pyrenacantha staudtii* leaf on isolated rat uterus. *Afr J Biotech.* 5 (10): 1271-1273.

George D, Pamplona-Roger MD (1998). Encyclopedia of medicinal plants, part Nature and Science. *59 Editorial Safeliz SL Madrid Spain.* 8(9): 267, 425, 435, 710.

Hee V, Victor C, Kaufman D, Budinger GR, Gokhan M (2009). Update in Environmental and Occupational Medicine. *A. Jour of Resp and Critical care medicine.* (42): 240-45.

Hill EN, Van Staden J (1992). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. *J. Ethnopharm*. 87: 35–41.

Hostettmann AK, Hamburger PO (1991). Phytochemical methods – A guide to modern techniques of plant analysis. *Chemico-Biol Int.* 125(1): 1-8.

Hugo WB, Russell AD (2003). Pharmaceutical Microbiology; *6th ed. Blackwell Science Publishers*. 91-129.

Igoli JO, Ogaji OG, Igoli NP, Tor-Anyiin TA (2005). Traditional medicinal practices among the Igede people of Nigeria (part II). *Afri J Tradit Compli Altern Med.* 2(2): 134-152.

Isaacs CE and Thormar H (1991). The role of milk-derived antimicrobial lipids as antiviral and antibacterial agents in Immunology of Milk and the Neonate Plenum Press, New York.

Iwu MM, Duncan AR, Okunji CO (1999). New Antimicrobials of Plant Origin in Perspectives of New Crops and Uses. Ed J Janick ASHS Press.

Kambizi L, Afolayan AJ (2001). An ethnobotanical study of plants used for the treatment of modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana* Level. *J Inf Dis.* 194: 651–660.

Kirszberg C, Esquenazi D, Alviano CS, Rumjanek VM (2003). The effect of catechin-rich extract of *Cocos nucifera* on lymphocytes proliferation. *Phytother Res*. (17): 1054–8.

Kudi AC, Uhoh JU, Eduvie LO, Gefu J (1999). Screening of some Nigerian medicinal plants for antibacterial activity, *Jour of Ethnopharm*. (67): 225-228.

Lewis WH, Elvis-Lewis MP (1995). *Medical Botany* John Wiley and Sons, New York. 808-823.

Luck RE, Moffett TJ, Barnes TG, Gieren WP (1998). Anti- Inflammatory of unusual lupine saponins from medicinal plants. *ApJ*. (115): 605-610.

Mantena P, Alvies J (2003). Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*. *Phytother Res*. (19): 612–617.

Marquez M, Etoh H (2005). Synergistic effects of mupirocin and an isoflavanone isolated from *Erythrina variegata* on growth and recovery of methicillin-resistant *Staphylococcus aureus. Int. J Antimicrob Agents.* (24): 43-48.

Mendonca-Filho RR, Rodrigues IA, Alviano DS, Santos ALS**, Soares RMA, Alviano *et al.* (2004).** Leishmanicidal activity of polyphenolic-rich extract from husk fiber of *Cocos nucifera* Linn. (Palmae). *Res Microbiol*. 155:136–43.

Mid-American Marketing Corp (2004). Coconut oil online profile. 1531 US Route 35E Eaton, Ohio.

Moumita C, Adinpunya M (2008). The antioxidant and antimicrobial properties of methanolic extract from *C. nucifera* mesocarp.*Jour of Agric & Food chem*. (47): 1620-1624.

OKWU DE (1999). Flavouring properties of spices on Cassava Fufu. *Afr J Rts Tuber Crp.* 3(2): 19-21.

OKWU DE (2001). Evaluation of the chemical composition of indigenous Spices and flavouring Agents. *Global J Appl.* 7(3): 455-459.

Oyi AA, Ames BN (1991). Endogenous mutagens and the causes of aging and cancer. *Mutat. Res.* 250: 3–16.

Palambo EA, Semple SJ (2001). Antibacterial activity of traditional Australian medicinal plants. *J Ethnopharm*. 77: 151-157.

Parikh UM, Barnas DC, Faruki H, Mellors JW (2005). Antagonism between the HIV-1 37 Reverse-Transcriptase Mutation K65R and Thymidine-Analogue Mutations at the Genomic Level. *J Inf Dis.* 194: 651–660.

Paz EA, Cerdeiras MP, Fernandez J, Ferreira F, Moyna P, Soubes M, Vazquez A, Vero S, Zunino L (1995). Screening of Uruguayan medicinal plants for antimicrobial activity. *J. Ethnoph.* (45): 67-70.

Pehowich TG, Paulsen IT, Gillespie MT, Tennent JM, Midgley M, Jones IG, Purewal AS, Skurray RA (1992). Substrate specificity and energetics of antiseptic. *Res Microbiol.* 152: 106–110.

Prescott LM, Harley JP, Klein DA (2002). Microbiology 6th ed. McGraw Hill Publishers.

Robinson JL, Recio MC (1991). Medicinal plants and antimicrobial activity. *J Ethnopharm*. 100: 80-84.

Sibanda T, Okoh AI (2008). *In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics. *Afr J Biotech.* **7**(11): 1672-1678.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. *Phytochem.* 68(2): 210-217.

Stafford GI, Jager AK, Staden J (2004). Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants. *J Ethnopharma.* 52: 1240-1246.

Stapleton PD, Shah S, Anderson JC, Hara Y, Hamilton-Miller JMT, Taylor PW (2004). Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int J Antimic Agents*. 23(5): 462-467.

Steve AJ, Lee KM (2004). *In vitro* activity of β-lactams in combination with other antimicrobial agents against resistant strains of *Pseudomonas aeruginosa. Int J Antimicrob Agents.* 21: 8-12.

Takana OP, Vlinient AM, kuren AF (1997). Medicinal plants of importance: A review of the traditional uses, biological activity and Phytochemistry. *J Ethnopharm.* 109: 613-617.

Thormar PK (1996). Handbook on Coconut Palm. Oxford & IBH Publishing Co. New Delhi.

Tyler R, Bustmante B, Bauer J, Fernandez I, Alban J (1988). Antimicrobial activity of selected Peruvian medicinal plants. *J Ethnopharm*. 88: 199-204.

Tzouvelekis LS, Tzelepl E, Prinarakis E, Gazouli M, Katrahoura A, Glakkoup P, Paniara O, Legakis NJ (1998). Sporadic Emergence of *Klebsiella pneumonia* strains Resistant to 161 Cafepime and Cefpirome in Greek Hospitals. *J Clin Microbiol.* 36: 266-268.

UNESCO (1998). FIT/504-RAF-48 Terminal Report: Promotion of Ethnobotany and the Sustainable Use of Plant Resources in Africa, Paris.

Vlietinck AJ, Hoof V, Totte L, Lasure J, Vanden A, Berghe D, Rwangabo PC, Mvukiyumwani J (1995). Screening of a hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J Ethnopharm.* (46): 31-47.

CHAPTER TWO

LITERATURE REVIEW

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LITERATURE REVIEW

INTRODUCTION

Ethnobotany, the study of traditional plant use, is a field of growing interest to research scientists and pharmaceutical companies looking to develop new and more effective drugs. No one knows exactly how many different medicinal plants are used in the world today, but we do know that medicinal plants are enormously important in both traditional and Western medicine (Parikh *et al*., 2005). Analyzing the Phytochemicals in medicinal plants provides scientists with insight into how effective plants are medicinally, and understanding how and why their effectiveness can lead to the development of new medicines (Parikh *et al*., 2005). People have used medicinal plants throughout human history, and long before good records were kept about plant use. There is evidence that humans have been using medicinal plants not just for centuries, but for thousands of years (Rio and Recio 2005). Otzi the "Ice-man," a 5,000-year-old man found preserved in the retreating glaciers of Europe was carrying a pouch of mushrooms that have phytochemicals known to fight intestinal parasites, and analysis showed Otzi did indeed have intestinal parasites when he died from injuries (Rio and Recio 2005). Various articles and journals from ancient India and China also contain descriptions of countless plant-derived medicines. Kamboj (2000) estimated that about 85% of Indians use higher plants as effective anti-microbial for the treatment of various diseases. A large number of anti-microbial agents derived from traditional medicinal plants are available for treating various diseases caused by micro-organisms (Jain, 1994). Craig, (1998) suggested that the therapeutically useful novel agents should inhibit the germs and exhibit greater

selective toxicity towards the infecting germ than the host cells. The mode of action for plant-derived agent should target biochemical features of the invading pathogens that are not possessed by the normal host cell (Prescott *et al*., 2002). Some of the factors important for anti-microbial treatment include methods such as sensitivity of the infecting micro-organism to a particular agent (Pellecure *et al*., 2006). Phytochemical analysis of medicinal plants has shown that numerous compounds in plants traditionally used for medicinal purposes have chemical properties effective at treating illness (Lupetti *et al*., 2002). In the book, "Modern Phytomedicine: Turning Medicinal Plants into Drugs," the authors state that Phytochemicals extracted from medicinal plants include alkaloids, tannins, flavonoids, sterols and numerous other chemicals. Tannins, flavonoids and alkaloids are chemicals that are known to have anti-bacterial properties. Hence, abundant medicinal plants have been used in many forms over the years to treat, manage or control man's ailments (Prescott *et al.*, 2002); therefore any effort to further explore the medicinal or natural products from man's botanical flora towards improving health care delivery deserves attention.

MECHANISM OF ACTION OF ANTIMICROBIAL COMPOUNDS.

Antimicrobial compounds are chemical substance that kills or inhibits the growth of microorganisms. Such an antimicrobial compounds may either be a synthetic chemical or a natural product (Pelczar *et al*., 2001). Antimicrobial agents are either bacteriostatic or bactericidal. The bacteriostatic effects do irreversibly inhibit microorganism and rely on the host

defense mechanism for the final eradication of the infecting organism whereas the bactericidal effect shows irreversible lethal action on their target organism (Pelczar *et al*., 2001).

Some antimicrobial drugs have a narrow range of different microbial type they affect, these are called narrow spectrum. Penicillin for example is known to affect Gram-positive bacteria but very few Gram-negative bacteria. Antimicrobial drugs that affect broad range of Gram-positive and Gram-negative bacteria are therefore referred to as broad spectrum antimicrobial drugs (Tortora *et al*., 2001). It has been observed that Gram positive bacteria are usually more sensitive to antimicrobial drugs than Gram-negative bacteria (Pelczar *et al*., 2001). Antimicrobial compounds exert their inhibitory activity on microorganisms in different ways by interfering with various activities of the microbial cells and invariably causing their death (Pelczar *et al*., 2001). Antimicrobial agents are often categorized according to their principal mechanism of action. Mechanisms include interference with cell wall synthesis (e.g. beta-lactams and glycopeptides agents), inhibition of protein synthesis (macrolides and tetracycline), interference with nucleic acid synthesis (fluoroquinolones and rifampin), inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole), and disruption of bacterial membrane structure (polymyxins and daptomycin) Tenover *et al*. (2006).

Inhibition of cell wall synthesis

Bacteria cell walls are unique in that they contain peptidoglycan, a three dimensional structure composed of alternating subunits of N-acetyl glucosamine and N-acetylmuramic acid (NAM). The strands called glycan chains are interconnected through Peptide Bridge between the amino acid chains of NAM. The intact structure provides the cell the rigidity to maintain integrity, because peptidoglycan is specific to bacteria. Compounds that interfere solely with cell wall synthesis do not affect eukaryotic cells, examples of such compounds includes Beta-lactams drugs such as penicillin, cephalosporin, vancomycin and bacitracin (Prescott *et al*., 2002).

The β-lactams drugs irreversibly inhibit enzymes involved in final steps of the cell wall synthesis. The enzymes by β-lactams drugs mediate the formation of the peptide bridges between adjacent strands of peptidoglycan; they are called penicillin binding proteins (PBPs) (Prescott *et al*., 2002). The β-lactams ring of the penicillin and other similar drugs bear structural similarities to the normal substrate of the PBPs and thus competitively inhibit their enzymatic activity. This causes a disruption in the cell wall biosynthesis, leading to a series of events that ultimately causes the cell to lyses (Meyer and Dilika, 2007).

Damage to cell membrane

The cell or cytoplasmic membrane of bacteria which encloses the cytoplasm plays a significant role in the cell by controlling the movement of materials between the internal and external environment of the cell. It also ensures that metabolites and nutrients are concentrated within the cell and serve as a site for respiratory and some biosynthesis activities (Pelczar *et al*., 2001). There are some antibiotics which interfere with one or more of these functions, thus leading to serious disruption of the viability of the organism and these results in the leakage of purine, pyrimidine, nucleotide and proteins from the cell (Jawetz *et al*., 2004).

Some agents that act primarily on the cell membrane are amphotericin B, miconazole, ketonazole, nystatin and polymyxins. The polyenes (amphotericin B and nystatin) selectively inhibit organism whose cell membrane contain sterol and so interfere with the osmotic barriers of the cells. These agents therefore, are active only against fungi, yeast and some other eukaryotic cells and not prokaryotic cells since their membrane lacks sterol (Onawunmi *et al*., 2007). Polymyxins acts as cationic detergents and

bind specifically to the surface of the cell membrane, thus altering the structure and osmotic properties of the cell (Pelczar *et al*., 2001).

Inhibition of nucleic acid synthesis

One of the main antibiotic group that inhibit DNA synthesis are called fluoroquinolone and rifampin which inhibit one or more topoisomerase which are enzymes needed for bacteria nucleic acid synthesis. In Gram positive bacteria, the main target of fluoroquinolone is the DNA gyrase, an enzyme responsible for the super coiling of bacterial DNA during DNA replication whereas in Gram negative bacteria the primary target is topoisomerase II which is an enzyme responsible for relaxation of super coil circular DNA. Rifampins are more widely used in chemotherapy because they are more selective toxic (Tortora *et al*., 2001).

Inhibition of protein synthesis

Several types of antibacterial drugs inhibit prokaryotic protein synthesis while all cells synthesize proteins. The structure of the 70s ribosome which is composed of a 30s and a 50s subunit is different enough from the eukaryotic 80s ribosome to make it a suitable target for selective toxicity of some of these drugs. The major classes of antibiotic that inhibit protein synthesis are aminoglycosides, tetracycline and macrolides. The aminoglycosides irreversibly bind to the 30s ribosomal subunits, causing it to distort and malfunction. This blocks the initiation of translation and causes misreading of mRNA by ribosome that has already passed the initiation step. Examples of aminoglycosides include streptomycin, gentamycin, tobramycin, and amikacin (Prescott *et al*., 2002). Tetracycline do not affect

the mRNA rather, they inhibit protein synthesis by interfering with the binding of aminoacyl + RNA and f-met tRNA to the acceptor site on the ribosome (Pelczar *et al*., 2001).

MECHANISM OF RESISTANCE OF MICROORGANISM TO ANTIMICROBIAL COMPOUNDS.

The treatment of bacterial infections is increasingly complicated by the ability of bacteria to develop resistance to antimicrobial agents. Antimicrobial agents are known to inhibit or prevent multiplication of microorganism. Notwithstanding, the bacteriostatic or bactericidal effect of antimicrobial agents can be resisted by the microorganism. The discovery and subsequently introduction of antimicrobial agents opened a new era in the life of mankind since life threatening infectious disease can now be treated (Sonntag *et al*., 1991). Bacteria may be intrinsically resistant to more than one class of antimicrobial agents, or may acquire resistance by de novo mutation or via the acquisition of resistance genes from other organisms. Acquired resistance genes may enable a bacterium to produce enzymes that destroy the antibacterial drug, to express efflux systems that prevent the drug from reaching its intracellular target, to modify the drug's target site, or to produce an alternative metabolic pathway that bypasses the action of the drug (Shanmugan *et al*., 2008). Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation, transformation, or transduction, with transposes often facilitating the incorporation of the multiple resistance genes into the host's genome or plasmids (Tenover *et al*., 2006). Resistance has been defined as the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would destroy or inhibit other members of the strain (Chong and Pagano, 1997). Bacteria may be

described as resistant when they are not susceptible to a concentration of antibacterial agent used in practice (Shibata *et al*., 2005). Traditionally, resistance refers to instances where the basis of increased tolerance is a genetic change, and where the biochemical basis is known (Cloete, 2003). Antimicrobial substances target a range of cellular loci, from the cytoplasmic membrane to respiratory functions, enzymes and the genetic material. The mechanisms of resistance that have been reported include; (Cloete, 2003).

- Limited diffusion of antimicrobial agents through the biofilm matrix (Cloete, 2003).
- Interaction of the antimicrobial agents with the biofilm matrix (cells and polymer) (Cloete, 2003).
- Enzyme mediated resistance (Cloete, 2003).
- Level of metabolic activity within the biofilm (Cloete, 2003).
- Genetic adaptation (Cloete, 2003).
- Efflux pumps (Cloete, 2003) and
- Outer membrane structure (Cloete, 2003).

Some Medicinal plants and their Antimicrobial activities.

Microbial infection of man is not a new problem but the situation has worsened dramatically within the last two decades with infectious diseases now attacking man on multiple fronts (Prescott *et al*., 2002). They represent the world's leading cause of premature death and our well being depends on the production of new clinically useful antibiotics to curtail and / or eradicate pathogens in our communities (Hugo and Russell, 2003). For over a decade now, the pace of development of new antimicrobial has

slowed down while the prevalence of resistance has grown at an astronomical rate. The increase in number of antibiotics recalcitrant bacteria is no longer matched by a parallel expansion in the arsenal of agents used to treat infections (Prescott *et al*., 2002). It is making a growing number of infections virtually untreatable both in the hospitals and the general community with nosocomial infections becoming more rampant and deadly (Hugo and Russell, 2003).

In the 16th century, botanical gardens were created to grow medicinal plants for medical schools (Akerele, 1993). Herbal medicine practice flourished until the 17th century when more "scientific pharmacological remedies were favoured (Trevelyan *et al*., 2003). Around the world, the history of herbal use begins when health care was provided by women in the home. Initially they used home-made botanical remedies and later purchased similar products as "patent medicines." In the early 19th century, scientific methods became more advanced and preferred, and the practice of botanical healing was dismissed as quackery. In the 1960s, with concerns over the iatrogenic effects of conventional medicine and desire for more self-reliance, interest in "natural health" and the use of herbal products increased (Trevelyan *et al*., 2003). Worldwide, herbal use again became popular and in 1974 the World Health Organization (WHO, Geneva, Switzerland) encouraged developing countries to use traditional plant medicines to "fulfill a need unmet by modern systems." (Trevelyan *et al*., 2003). Despite the availability of different approaches for the discovery of drugs, plants still remain the main reservoirs of natural medicines. It is estimated that about 30% of the drugs in the modern pharmacopeias were derived from plants and many others, which are synthetic analogues, were built on prototype compounds isolated from plants (Kim, 2005) and over the years there have been increasing interests in the use of herbal therapeutics worldwide.

Hugo and Russell (2003) asserted that 80 percent of the populations in the developing countries now use medicinal plants. As a result of the importance of herbs in the lives of people, the World Health

organization devoted 27 centers, out of 915 collaborating centers worldwide, to traditional medicine (WHO, 2001).

The plant kingdom has served as a prolific source of useful drugs, foods, additives, flavoring agents, lubricants, colouring agents and gums from time immemorial (Parikh *et. al*, 2005). The healing power of herbs had been recognized since creation and botanic medicine is one of the oldest practiced professions by mankind (Kambizi and Afolayan, 2001). Medicinal plants have been found useful as antimalarial, antisickling, anti-helmintic, anti-microbial, anti-convultant, anti-hypertensive, and as anti-schistosomal (molluscicidal) agents (Prescott *et. al.*, 2002). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen - substituted derivatives (Kambizi and Afolayan, 2001). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinolones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoids capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Pecere *et. al*., 2000). As a result of the importance of herbs in the lives of people, the World Health organization devoted 27 centers, out of 915 collaborating centers worldwide, to traditional medicine (WHO, 2001). Bacteria play a vital role in the environment, and are economically important in the food, pharmaceutical industries as well as agents of disease. The spread of multidrug resistance strains of bacteria and the limited number of drugs available makes it necessary to discover new classes of antibacterial agents and compounds and inhibit these resistance mechanisms (Abad *et al*., 2007). This has led to a search or therapeutic alternatives, particularly among medicinal plants and compounds isolated from them used for their empirically antibacterial properties. In these natural source, a series of molecules with antibacterial activity against different strains of bacteria have been found, which are of

great importance to humans and plants (Abad *et al*., 2007). Demand for plant-derived medicines has created a trade in indigenous plants in South Africa currently estimated to be worth hundreds of millions of rands annually (Mander 1998). Medicinal plants have become the focus of intense study recently in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore (Cocks, 1997).

Plants antimicrobials have been found to be **s**ynergistic enhancers in that though they may not possess any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (Kamatou *et al.,* 2006). The synergistic effect from the association of antibiotic and plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al.*, 2000). The use of synergistic combinations in antimicrobial chemotherapy is often used commercially in the treatment of various infections (e.g. Augmentin). Traditional healers often use combinations of plants to treat or cure diseases (Kamatou *et al.,* 2006). One notable example from the ethnobotanical literature is the concomitant administration of various *Salvia* species with *Leonotis leonurus* to treat various infections (Masika and Afolayan, 2003). Kamatou *et al.* (2006), in their study confirmed the existence of synergism between *Salvia chamelaeagnea* and *Leonotis leonurus*, when these two plants were tested individually against *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*. Kamatou *et al.* (2006) reported synergism when the tincture of *L. leonurus* and various *Salvia* species were used against influenza. Boik (2001), conducted a large number of combination studies using various natural substances and the results strongly suggest that when used in combination, natural substances can produce synergistic effects. It is thought that phenolic compounds such as flavonoids may increase the biological activity of other compounds by synergistic or other mechanisms Williamson, (2001).

Experimental evidence of synergistic actions between plants was also shown in a clinical study on the formulation of Chinese herbs used to treat eczema (Williamson, 2001). To halt the trend of increased emerging and resistance infectious diseases, it will require a prolonged approach that includes the development of new drugs. An important aspect of this research focus on how we can assess the therapeutic potentials of plants from the traditional African system of medicine as well as to how best these plants can be used in the treatment of diseases.

Synergism between bioactive plant product and Antimicrobial compound with specific mechanism of Action.

The use of medicinal plant as remedies for many infectious diseases is on the increase, hence the need for more searches for substances with antimicrobial activity. Infectious diseases still represent an important cause of morbidity and mortality among humans, especially in developing countries. Even though pharmaceutical industries have produced a number of new antimicrobial drugs in the last years, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents (Nascimento *et al*. 2000). Antibiotics have been effective in treating infectious diseases, but resistance to these drugs has led to the emergence of new and the reemergence of old infectious diseases, Combination of antibiotics and bioactive plant substance is a novel concept and have often been used for the treatment of drug resistant infections. Several plant extracts have exhibited synergistic activity against microorganisms so also combination of drugs, such as [beta]-lactams together with [beta]-lactamase inhibitors (Shanmugan *et al*., 2008). Combinations of antimicrobials that demonstrate an *in vitro* synergism against infecting

strains are more likely to result in successful therapeutic result. Thus, evidence of *in vitro* synergism could be useful in selecting most favorable combinations of antimicrobials for the practical therapy of serious bacterial infections (Aiyegoro *et al*., 2009).

Studies by Stermitz *et al*. (2000) proved that, in addition to the production of intrinsic antimicrobial compounds, plants also produce multi-drug resistant (MDR) inhibitors which enhance the activity of the antimicrobial compounds. Tegos *et al.* (2002) also showed that the activity of presumed plant antimicrobials against Gram-positive and Gram-negative organisms was significantly enhanced by synthetic MDR inhibitors of associated efflux proteins. The findings provided a basis that plants can be prospective sources of natural MDR inhibitors that can modulate the performance of antibiotics against resistant strains.

Antimicrobial screening of crude plant extracts for synergistic interaction with antibiotics is expected to provide ways for the isolation of MDR inhibitors. The ability of crude extracts of plants to potentiate the activity of antibiotics has been observed by some researchers and it is anticipated to form the basis for the bioassay directed fractionation of potential resistance modulators from plants (Darwish *et al*., 2002; Sibanda and Okoh 2008; Betoni *et al*., 2006; Lewis and Ausubel, 2006; Ball *et al*., 2006, Aiyegoro *et al*., 2009).

Darwish *et al*. (2002) in their studies on some plants demonstrated that the efficacy of the antibiotics, gentamycin and chloramphenicol against *S. aureus* were reportedly improved by the use of plant materials. Ahmad and Aqil, (2006), in their search also reported that crude extracts of Indian medicinal plants demonstrated synergistic interaction with tetracycline and ciprofloxacin against extended spectrum β-lactamase (ESβL)-producing multidrug-resistant enteric bacteria. Betoni *et al*. (2006) also

observed synergistic interactions between extracts of Brazilian medicinal plants in combination with eight antibiotics on *S. aureus.*

The use of *Catha edulis* extracts at subinhibitory levels, has been reported to reduce the minimum inhibitory concentration (MIC) values of tetracycline, and penicillin G against resistant oral pathogens, *Streptococcus oralis, Streptococcus sanguis* and *Fusobacterium nucleatum* (Al- hebshi *et al*., 2006). Aiyegoro *et al*. (2008, 2009) in their studies has also demonstrated some levels of synergism between the extracts of *H. pedunculatum and H. longifolium* and eight frontline antibiotics.

A number of compounds with an *in vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols (epicatechin gallate and catechin gallate) have been reported to reverse beta-lactam resistance in Methicillin Resistant *S. aureus* (MRSA) (Stapleton *et al*., 2004). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus* (Marquez *et al*., 2005; Smith *et al*., 2007; Shibata *et al*., 2005 and Oluwatuyi *et al*., 2004).

The synergies detected in the studies mentioned in this subsection were not specific to any group of organisms or class of antibiotics. This suggests that plant crude extracts are blend of 30 compounds that can enhance the activity of different antibiotics (Shanmugan *et al*., 2008). Plants have been known to contain myriads of antimicrobial compounds (Iwu *et al*., 1999) such as polyphenols and flavonoids. The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies such as Cushnie and Lamb (2005), Sato *et al*. (2004).

Some of these compounds including polyphenols have been shown to exercise their antibacterial actions/activities through membrane perturbations. This disruption of the cell membrane coupled with the action of beta-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al*., 2006). It has also been revealed that some plant-derived compounds can improve the *in vitro* activities of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao *et al*., 2001).

While the above explanations may account for the synergy between the extracts and beta-lactam antibiotics that act on the cell wall, it might not apply in the case of the observed synergy with other classes of antibiotics with different targets such as tetracycline, erythromycin, ciprofloxacin and chloramphenicol. Bacterial efflux pumps are responsible for a considerable level of resistance to antibiotics in pathogenic bacteria (Kumar and Schweitzer, 2005). Some plant derived compounds have been observed to argument the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria (Tegos *et al*., 2002). 5'-methoxyhydnocarpin is an example of an inhibitor of the NorA efflux pump of *S. aureus* isolated from *Berberis fremontii* (Stermitz *et al*., 2000b). Such compounds are likely to be broad spectrum efflux inhibitors considering that the synergistic effect of the extract was observed on both Gram-positive and Gram-negative organisms as well as in combination with cell wall inhibiting and protein synthesis inhibiting antibiotics. Importantly, some broad spectrum efflux pump inhibitors have been isolated from some plants (Stermitz *et al*., 2000b). Jacqueline *et al*. (2001) reported a new efflux inhibitor pyridoquinoline derivative as potential inhibitors of the fluoroquinolone efflux pump in resistant *Enterobacter aerogenes* strains.

REFERENCES

Abad I, Streit JM, Fritsch TR (2007). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J Ethnopharm.* 74: 113–23.

Ahmad I, Aqil F (2006). *In vitro* efficacy of bioactive extracts of 15 Medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. *Microbio. Res*. 1-12.

Aiyegoro OA, Afolayan AJ, Okoh AI (2008). Studies on the *In vitro* time-kill assessment of crude aqueous and acetone extracts of *Helichrysum pedunculatum* leaves. *Afr J Biotech.* 7(20): 3721-3725.

Aiyegoro OA, Afolayan AJ, Okoh AI (2009). Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. *Biol Res.* 42: 327-338.

Akerele O (1993). Nature's medicinal bounty: don't throw it away. *World Health Forum*. 14: 390-395.

Al-hebshi N, Al-haroni M, Skaug N (2006). *In vitro* antimicrobial and resistance-modifying activities of aqueous crude *khat* extracts against oral microorganisms. *Arch Oral Biol.* 51: 183-188.

Ball S, Zhang L, Huang PL, Chang YT, Huang PL (2006). Anti-HIV activities of olive leaf extract (OLE) and modulati**on** of host cell gene expressi**on** by HIV-1 infection and OLE treatment. *Biochem Biophys Res Commun*. 30: 1029–37.

Betoni JEC, Mantovani RP, Barbosa LN, Di Stasi LC, Fernandes A Jnr (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. *Mem Inst Oswaldo Cruz.* 101(4): 387-390.

Boik J (2001). Natural Compounds in Cancer Therapy. Princeton, MN: *Oregon Medical Press*.

Chong KT, Pagano PJ (1997). *In vitro* combination of PNV-140690, a Human Immunodeficiency Virus type 1 protease inhibitor with Ritonavir against Ritonavir-sensitive and Resistant Clinical 35 Isolates. *Antimicrob Agents Chemo.* 41(2): 2367-2377.

Cloete TE (2003). Resistance mechanism of bacteria to antimicrobial compounds**.** *Inter Biodeter & Biodegra*. 51: 277 – 282.

Cock TA, (1997). A study with seed oil of "*N.sativa*". *J Fac Phar Gazi.* 2: 1-7.

Craig WA (1998). Pharmacokinetics / Pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clin Infect Dis.* 26: 1–12.

Cushnie TPT, Lamb AJ (2005). Antimicrobial activity of flavonoids. *Int J Antimic Agents.* 26(5): 343-356.

Darwish RM, Aburjai T, Al-Khalil S, Mahafzah A (2002). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. *J Ethnopharm.* 79: 359-364.

Esimone CO, Iroha IR, Ibezim, EC, Okeh CO, Okpana EM (2006). *In vitro* evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus. Afr J Biotechnol*. 5(11): 1082-1086.

Hugo WB, Russell AD (2003). Pharmaceutical Microbiology; 6th ed. Blackwell Science Publishers. 91-129.

Iwu MM, Angela R, Duncan A, Chris O, Okunji (1999). New Antimicrobials of Plant Origin in Perspectives of New Crops and Uses, Ed. J. Janick, ASHS Press,

Jacqueline AP, Salgueiro LR, Goncalves MJ, Proenca da Cunha A, Vila R, Canigueral S (2001). Essential oil composit**ion** and **antimicrobial** activity of *Santiria trimera* bark. *Planta Medica.* 69: 77–9.

Jawetz AR, Zamnaizadehnajari S, Azar PA, Tahara S (2004). Antibacterial diterpenoids from *Astragalus bachystachys. J Antimicro Chem*. 50: 945–952.

Kamatou GPP, van Zyl RL, van Vuuren SF, Viljoen AM *et al.* (2006). Chemical Composition, Leaf Trichome Types and Biological Activities of the Essential Oils of Four Related Salvia Species Indigenous to Southern Africa. *J Ess Oil Res.* 18: 72-79.

Kambizi L, Afolayan AJ (2001). An ethnobotanical study of plants used for the treatment of sexually transmitted diseases (njovhera) in Guruve district, *Zimbabwe J Ethnopharm.* 71: 5 - 9.

Kamboj VP (2000). Herbal medicine. *Curr Sci*. 78: 35–9

Kim JD (2005). Nutriti**o**nal toxicology of tannins and related polyphenols in forage legumes. *J Animal Sci*. 73: 1516–28.

Kumar A, Schweitzer HP (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Adv Drug Deliv Rev.* 57: 1486-1513.

Lewis K, Ausubel FM (2006). Prospects for plant-derived antibacteria. *Nat Biotech Inst. Oswaldo Cruz.* 24(12): 1504- 1507.

Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S (2002). Molecular basis of resistance to azoles antifungal. *Trends Mol Med.* 8**:** 76–81.

Mander CM (1998). Resistance to antimicrobial drugs: A worldwide calamity. *Ann Intern Med.* 118: 557-561.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, Sant Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. *Phytochem.* 66: 1804-1811.

Masika PJ, Afolayan AJ (2003). An Ethnobotanical Study of Plants Used for the Treatment of Livestock Diseases in the Eastern Cape. South *Africa Pharm Biol.* 41(1): 16-21.

Meyer JJM, Dilika F (2007). Antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites. *J Ethnopharm.* 53: 51-54.

Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz J Microbiol.* 31: 247-256.

Oluwatuyi M, Kaatz GW, Gibbons S (2004). Antibacterial and resistance modifying activity of *Resmarinus officinalis*. *Phytochem.* 65: 3249–3254.

Onawunmi GO, Yisak WA, Ogunlana EO (2007). Effect of lemon grass powder and oil on mold deterioration and aflatoxin contamination of melon seeds (*Colocynth citrullus L*). *Afr J biotech.* 3(1): 52-59.

Parikh UM, Barnas DC, Faruki H, Mellors JW (2005). Antagonism between the HIV-1 37 Reverse-Transcriptase Mutation K65R and Thymidine-Analogue Mutations at the Genomic Level. *J Inf Dis.* 194: 651–660.

Pecere S, Whitefield PJ, Sanderson L, Bartlett A (2000). The plant molluscide *Millettia thonningii* (Leguminosae) as a topical antischistosomal agent. *J Ethnophar*. 47: 49–54.

Pelczar MJ, Chan EC, Kruz NR (2001). Microbiology. 5th edition. Teta McGraw-Hill Publishing Company Ltd. New Delhi.

Pellecure S, Allegrini J, Buochberg S (2006). *Huiles essenticlles* bactericides et fungicides. *Revue del Institute Pasteur de Lyon.* 9: 135–159.

Prescott LM, Harley JP, Klein DA (2002). Microbiology 6th ed. McGraw Hill Publishers. 808-823.

Ríos JL, Recio MC (2005). Medicinal plants and antimicrobial activity. *J Ethnopharm*. 100: 80-84.

Sato Y, Shibata H, Arai T, Yamamoto A, Okimura Y, Arakaki N, Higuti T (2004). Variation in synergistic activity by flavones and its related compounds on the increased susceptibility of various strains of methicillin-resistant *Staphylococcus aureus* to β-lactam antibiotics. I*nt J Antimicrob Agents.* 24: 226-233.

Shanmugan H, Anil K, Mukesh D (2008). Synergism between natural products and antibiotics against infectious diseases. *J phytomed.* (15): 639-652.

Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, Higuti T (2005). Alkyl gallates, intensifiers of ß-lactam susceptibility in Methicillin-Resistant *Staphylococcus aureus. Antimic Agents Chemo*. 49 (2): 549-555.

Sibanda T, Okoh AI (2008). *In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics. *Afr J Biotech.* **7** (11): 1672-1678.

Smith RA, Calviello CM, Der Marderosian A, Palmer ME (2007). Evaluation of antibacterial activity of *Belizea* plants: an improved method. *Pharm Biol*. 38: 25–29.

Sonntag F, Monache F, Smania E, Gil ML, Benchetrit LC, Cruz FS (1991). Antibacterial activity of substances produced by the fungus *Pyrocarpus sanguineus. Murr Ethnopharm*. 45: 177-181.

Stapleton PD, Shah S, Anderson JC Hara Y, Hamilton-Miller JMT, and Taylor PW (2004). Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int J Antimic Agents.* 23(5): 462-467.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, and Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Appl Bio Sciences*. 97(4): 1433-1437.

Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials, *Antimicrob. Agents Chemother.* 46(10): 3133-3141.

Tenover FC, Weigel LM, Appelbaum PC, McDougal LK, Chatram J, McAllister S, Clark N, Killgore G, O'Hara CM, Jevitt L, Patel JB, Bozdogan B (2006). Vancomycin-resistant Staphylococcus aureus isolate from a patient in Pennsylvania, *Antimicrob agents Chemother*. 48: 275-80.

Tortora GJ, Funke BR, Case CL (2001). Microbiology: An Introduction, Benjamin Cummings, San Francisco.

Trevelyan H, Sato M, Miyazaki T, Fujiwara S, Tanigaki S, Ohyama M, et al. (2003). Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. *J Ethnopharm.* 50: 27–34.

Williamson EM (2001). Synergy and other interactions in phytomedicine. *Phytomed.* 8(5): 401-409.

World Health Organization (2001). Essential Drugs and Medicines Policy. WHO Geneva

Zhao W, Hu Z, Okubo S, Har Y, Shimamura T (2001). Mechanism of synergy between epigallocatechin gallate and b-Lactams against methicillin-resistant *Staphylococcus aureus. Antimicrob Agents Chemother*. 45 (6): 1737–1742.

CHAPTER THREE

In-vitro antilisterial properties of crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera*

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CHAPTER THREE

In-vitro antilisterial properties of crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera*

Abstract

The *in vitro* antilisterial activities and time kill regimes of crude aqueous and n-Hexane extracts of the husk fiber of *Cocos nucifera* were assessed. The aqueous extracts were active against 29 of the test *Listeria* isolates while the n - Hexane extract was active against 30. The minimum inhibitory concentrations (MICs) for all the susceptible bacteria ranged between 0.6 and 2.5 mg/ml for the aqueous fraction and between 0.6 and 5.0 mg/ml for the n - Hexane extract. The average log reduction in viable cell count in the time kill assay ranged between 0.32 Log_{10} and 3.2 Log_{10} CFU/ml after 4 hr of interaction, and between 2.6 Log_{10} and 4.8 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC (aqueous extract); and between 2.8 Log_{10} and 4.8 Log_{10} CFU/ml after 4 hr of interaction, and 3.5 Log_{10} to 6.2 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC for the n–Hexane extract. The extract was bactericidal against one of the test bacteria at 1 × MIC and against three of the test bacteria at 2 × MIC from 8 hour interaction period for the aqueous extract while for the n-Hexane fraction; the extract was bactericidal against all the five test bacteria at both MICs after the 8 hour interaction period. We suggest that the crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera* could be bacteriostatic or bactericidal depending on time of exposure and concentration.

Key words: *Cocos nucifera*, n - Hexane extract, aqueous extract, MIC, time-kill.

3.1 INTRODUCTION

Listeriosis is a serious disease of humans. The overt form of the disease has mortality greater than 25 percent (Kenneth, 2008) and a serious infection caused by eating food contaminated with *Listeria* species. Listeriosis has been recognized as an important public health problem in the United States (Kenneth, 2008). The disease affects primarily pregnant women, newborns, and adults with weakened immune systems (Kenneth, 2008). The causative agents in most instances appear to be members of the indigenous microbiota and, thus, the infections might be thought of as endogenous (Socransky and Haffajee, 2002). The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual of bacterial infections (Kenneth, 2008). *Listeria* species have many opportunities to enter food production and processing environments. Consequently, outbreaks and sporadic cases of Listeriosis have been traced to different foodstuffs, such as dairy products (Allerberger and Wagner, 2010; Denny *et al*., 2008).

The use of medicinal plants in the treatment of infections caused by *Listeria* pathogen remains an important approach to the development of new antimicrobial drug. Esquenazi *et al*. (2002) has reported the antibacterial, antifungal and antiviral properties of the extract of *Cocos nucifera* plant and an extensive range of several medicinal uses of this plant has been reported (Duke, 1992). In recent years, resistance to multiple drug in both human and plant pathogenic microorganisms have developed due to indiscriminate use of conventional antibiotics commonly applied in the treatment of infectious diseases (Loper *et al*., 1999). This situation has encouraged further exploration for new antimicrobials from

various sources, including medicinal plants (Cordell, 2000) that could be used in the treatment of infections by drug resistant pathogens.

Cocos nucifera (English name: coconut) is an important food crop and medicinal plant in tropical and subtropical countries. It belongs to the family Palmae. The coconut palm is found throughout the tropics, where it is interwoven into the lives of the local people. It is particularly important in the low islands of the Pacific where, in the absence of land-based natural resources, it provides almost all the necessities of life - food, drink, oil, medicine, fiber, timber, thatch, mats, fuel, and domestic utensils (Edward and Craig, 2006). For good reason, it has been called the "tree of heaven" and "tree of life" to this day remains an important economic and subsistence crop in many small Pacific island states (Edward and Craig, 2006).

The husk fiber of *Cocos nucifera* has been reported to be rich in catechin and epicatechin together with condensed tannins, which confers to its aqueous extract a potent antioxidant characteristics (Alviano *et al*., 2004). It has also been reported to have antibacterial, antiviral, antidysenteric, antifungal, antileishmanial, antilymphoproliferative and antineoplasic activities (Esquenazi *et al*., 2002; Mendonca-Filho *et al*., 2004; Kirszberg *et al*., 2003; Koschek *et al*., 2007). In Brazil, the husk fiber decoction is used in traditional medicine for treatment of diarrhea and arthritis (Esquenazi *et al*., 2002) and in India, heating the coconut shells gives oil that is used against ringworm infections (Chakraborty, 2008). In this paper, we explored the potentials of the crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera* for the treatment of listerial infections.

3.2 Materials and methods

3.2.1 Plant material

The plant specimens were collected from the vicinity of the Research Farm of the Obafemi Awolowo University, Ile Ife, Nigeria and identified by the curator of the Herbarium at the Department of Botany, Obafemi Awolowo University, and a voucher specimen kept there.

3.2.2 Preparation of Extracts

The husk of the coconut was sun–dried, milled and sieved manually to obtain the fine powdered particles. About 50 g dried powdered husk of the plant was added to 200 ml of 95% n-Hexane using Soxhlet extraction method at room temperature and for 48 hr. The mixture was then filtered using Whatman 1filter paper. The filtrates of each extraction were pooled together and concentrated to dryness *in vacuo* using a rotary evaporator to remove the n - Hexane. The concentrated extract was then allowed to dry at room temperature to a constant weight. For the aqueous extract, about 50 g of the powdered extract was dissolved in 500 ml of sterile distill water for 24 hr with shaking. The resultant extracts were centrifuged at 3000 rpm for 5 min at 4 ^{o}C. The supernatant was filtered through a Whatman No. 1 filter paper and the filtrate was lyophilized.

3.2.3 Test bacterial strains

The bacterial isolates used in this study included 37 *Listeria* isolates which were isolated from waste-water effluents in the Eastern Cape Province, South Africa as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The isolates comprise of *Listeria ivanovii* (33), *Listeria grayi* (3), and *Listeria monocytogenes* (1).

3.2.4 Antibacterial susceptibility test

The susceptibility screening of the test bacteria to both crude extracts and standard antibiotics were done in accordance with the method of Irobi *et al.* (1994). The inoculum size of each test strain was standardized at 5×10^5 CFU/ml using McFarland Nephelometer standard. Sterile Mueller-Hinton agar plates were seeded with test bacterial strains and allowed to stand at 37 ^{o}C for 3 hr. Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells filled with the solution of the extracts and antibiotics taking care not to allow spillage of the solution onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract and antibiotics into the media and thereafter incubated at 37 ^{o}C for 24 h, after which they were observed for zones of inhibition. The effects of the extracts on the test bacterial isolates were compared with those of tetracycline and ampicillin standard antibiotics at a concentration of 1mg/ml and 10 μg/ml respectively.

3.2.5 Determination of minimum inhibitory concentration (MIC)

The MIC of the crude aqueous and n - Hexane extract was carried out using the method of Akinpelu and Kolawole (2004). Two-fold dilutions of the extracts were prepared and 2 ml aliquot of different concentrations of the solution were added to 18 ml of pre-sterilized molten Mueller - Hinton agar at 40 ^{o}C to give final concentration regimes of 5.0 to 0.156 mg/ml. The media were then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry under a laminar flow before streaking with 18 hr old bacterial cultures. The plates were later incubated at 37 ^{o}C for up to 72 hr after which they were examined for the presence or absence of growth. The MIC was taken as the least concentration of extracts that prevented the visible growth of the test bacteria.

3.2.6 Time-Kill assay

Determination of the kill rate of the crude extracts was done following the procedure as described by Okoli and Iroegbu (2005). Inocula were prepared following the described guidelines of EUCAST (2003). The resultant suspension were diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of Mueller Hinton broth incorporated with extracts at MIC and 2 × MIC to a final cell density of approximately 5×10^5 CFU/ml. The flasks were incubated at 37 ^{o}C on an orbital shaker at 120 rpm. A 500 µl sample were removed from the cultures at the appropriate time interval of 0, 4 and 8 hr respectively, and transferred to 4.5 ml of nutrient broth recovery medium containing 3% “Tween 80” to neutralize the effects of the antimicrobial compounds carry-overs from the test suspensions. The suspensions were diluted serially and 100 µl of the diluted samples were plated on Mueller Hinton agar

plates and incubated at 37 ^{o}C for 24 hr. Controls included extract-free Mueller Hinton broth seeded with the test inoculum.

3.3 Results

The results of the antilisterial activities of the crude aqueous and n–Hexane extract of the husk of *Cocos nucifera* against the test organisms are as shown in Table 3.1. Twenty-nine of test organisms were susceptible to the aqueous extract while thirty were susceptible to the n-Hexane extract both at the screening concentration of 25 mg/ml. The diameters of the zones of inhibition ranged between 12 and 17 mm for the aqueous extract; and between 12 and 24 mm for the n - Hexane extract; while those of the two control antibiotics (ampicillin and tetracycline) ranged between 20 and 50 mm; and 22 and 46 mm respectively (Table 3.1).

The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.6 and 5.0 mg/ml. Specifically, MICs of the aqueous extract ranged between 0.6 and 2.5 mg/ml while that of the n-Hexane was between 0.6 and 5.0 mg/ml (Table 3. 1).

The results of time-kill studies are presented in Table 3.2. Data are presented in terms of the $\log_{10}$ CFU/ml reduction in viable count. For the aqueous extract, average log reduction in viable cell count ranged between 0.32 Log_{10} and 4.8 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC. For the n-Hexane extract, the log reduction ranged between 2.4 Log_{10} and 6.2 Log_{10} CFU/ml after 8 hr

interaction in 1 × MIC and 2 × MIC. The greatest reductions in cell counts achieved with the aqueous extract was on *Listeria ivanovii* LEL_9 with the average log reduction of 4.8 Log_{10} CFU/ml, while the greatest reduction achieved by the n-Hexane extract was on *Listeria ivanovii* LEL_{30} with the average log reduction of 6.2 Log_{10} CFU/ml. Log reduction in viable cell counts of *Listeria ivanovii* LEL_9 was 4.8 Log_{10} CFU/ml after 8 hr of interaction at 1 × MIC, the log reduction was constant until after 8 hr even at 2 × MIC, thus suggesting that the total population of the bacteria had been wiped out by the forth hour at both 1 × MIC and 2 × MIC.

3.4 Discussion

Emergence of food borne bacterial illnesses caused by strains of *Listeria* pathogens continues to be a significant threat to public health, and thus the need for a reliable preservative and control measures that limits the proliferation of this psychotropic pathogen in refrigerated food and drinks. The development of suitable control strategies for this pathogen would benefit from the availability of functional, effective antilisterial control measures. Many natural compounds found in dietary and medicinal plants, such as extracts of herbs and fruits, and essential oils of various spices, possess antimicrobial activities against *Listeria* pathogens (Kim *et al*., 1995 and Hao *et al*., 1998). Hence our findings on the antilisterial efficacy of *Cocos nucifera* further corroborate the potentials of plant remedies in antilisterial infections.

Cocos nucifera is a well known antimicrobial solution, but a review of literature on the extract found antimicrobial activity against a range of bacteria, but very few assays has been performed on the antilisterial efficacy of this plant. The results from this study show the antilisterial activity of the n-

Hexane extract to be stronger than that of the aqueous extract, virtually against all the test bacteria. This is in accordance with the findings of Al-Reza *et al*. (2009), who revealed a strong antilisterial effect of the n-Hexane extract of *Zizyphus jujuba* against all strains of *L. monocytogenes* tested with zones of inhibition ranging between 11 – 18 mm.

Our aqueous extract had lower antilisterial activity in comparison to the n-Hexane extract in support of other reports (Esquenazi *et al*., 2002; Zakaria *et al*., 2006). Results from this study also suggest n-Hexane to be a better solvent for the extraction of bioactive compounds of *Cocos nucifera* than water. Al-Reza *et al*. (2009) in their superoxide radicals scavenging activity assay observed the extract of n-Hexane to be relatively efficient in extracting bioactive compounds in terms of potency and diversity of compounds extracted.

The antilisterial activities observed in this study could be related to the documented chemical composition of the plant. Alviano *et al*. (2004) has reported on the husk fiber of *Cocos nucifera* to be rich in catechin and epicatechin together with condensed tannins which confers on it potent antioxidant characteristics. Also Paschuka *et al*. (1998) and Peng *et al*. (2001) in their studies indicated that catechin one of the compounds present in the extract of *Cocos nucifera* plant is capable of inhibiting tumor cell lines.

The time-kill characteristics of the two extracts were assessed against some selected listerial isolates. The effectiveness of an antibacterial agent is measured by its ability to inhibit and kill bacteria (Nostro *et al.,* 2001). At higher concentration (2 × MIC) and longer duration of interaction (8 hr), more bacteria

were killed, thus corroborating the observation of Rhodes (2004) in his comparison study on the antilisterial properties of red grape juice and red wine.

The n-Hexane extract showed good bactericidal activity at 2 × MIC against the 4 test *Listeria ivanovii* isolates after 4 hr of exposure, and after 8hr, all the bacteria were eliminated. For the aqueous extract, bactericidal activity was observed against 3 of the tested Listeria strains at a concentration of 2 × MIC after 8 hr exposure period. Alviano *et al*. (2004) had reported a similar finding on the bactericidal efficiency of the aqueous extract of *Cocos nucifera* husk plant which revealed a 64.1% reduction in the bacterial count. The results of the time kill studies suggest that the effect of the extract of *C. nucifera* could either be static or cidal depending on concentration and duration of exposure, also that the plant represent a promising source of chemotherapeutic agents against *Listeria* pathogens.

In vitro time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance (Nostro *et al*., 2001). The *in vitro* data corroborates the reported efficacies of the several different crude extracts of *C. nucifera* on a wide range of microorganisms. For example, Wager *et al*. (2008) reported on the efficacy of this plants in the prevention and treatment of oral and periodontal diseases caused by some planktonic organism, thus supporting the folkloric uses of this plant in the treatment of different topical ailments (Nostro *et al*., 2001). The use of plant extracts with medicinal properties represents a concrete alternative for the treatment of different pathological conditions.

3.5 Conclusion

To the best of our knowledge, this is the first report on the antilisterial activities of the crude n – Hexane extract of the husk of *Cocos nucifera*. The levels of antilisterial activities observed suggest the plant to be a potential source of bioactive compounds that could be relevant in antilisterial drugs formulation which is a subject of on-going research in our group. Besides, Moumita and Adinpunya (2007) observed that the husks of the coconut palm are discarded as waste and it is considered as one of the major agro wastes of the tropical countries. Therefore, our study will definitely open up a scope for future utilization of these agro wastes for therapeutic purposes.

Table 3.1. Anti-listerial activities of crude aqueous and n-Hexane husk extracts of *C. nucifera.*

Isolate Identity	Inhibition zone (mm) / MIC		Antibiotics Inhibition zone (mm)	
	Aqueous Extract	n-Hexane Extract	AMP	TET
***L. ivanovii* LEL_1**	15 / 2.5	18 / 0.625	21	31
***L. ivanovii* LEL_2**	15 / 0.625	16 / 1.25	26	30
***L. ivanovii* LEL_3**	15 / 0.625	19 / 0.625	25	34
***L. ivanovii* LEL_4**	20 / 2.5	24 / 2.5	22	40
***L. ivanovii* LEL_5**	- / ND	12 / 2.5	25	34
***L. ivanovii* LEL_6**	12 / 0.625	- / ND	30	32
***L. ivanovii* LEL_7**	14 / 1.25	18 / 1.25	21	29
***L. ivanovii* LEL_8**	12 / 1.25	- / ND	27	29
***L. ivanovii* LEL_9**	16 / 2.5	21 / 0.625	24	38
***L. ivanovii* LEL_{10}**	- / ND	14 / 0.625	22	30
***L. ivanovii* LEL_{14}**	15 / 1.25	21 / 2.5	34	40
***L. ivanovii* LEL_{15}**	15 / 1.25	12 / 1.25	30	36
***L. ivanovii* LEL_{17}**	15 / 2.5	18 / 0.625	25	32
***L. ivanovii* LEL_{18}**	- / ND	12 / 2.5	20	22
***L. ivanovii* LEL_{30}**	- / ND	15 / 1.25	21	29
***L. ivanovii* LAL_1**	13 / 0.625	- / ND	20	28
***L. ivanovii* LAL_2**	- / ND	- / ND	22	35

***L. grayi* LAL$_3$**	12 / 2.5	14 / 0.625	28	33
***L. ivanovii* LAL$_4$**	14 / 0.625	20 / 2.5	26	41
***L. ivanovii* LAL$_5$**	18 / 2.5	20 / 5.0	28	44
***L. ivanovii* LAL$_6$**	16 / 2.5	15 / 2.5	21	30
***L. ivanovii* LAL$_7$**	- / ND	- / ND	20	31
***L. monocytogenes* LAL$_8$**	13 / 1.25	16 / 1.25	20	34
***L. ivanovii* LAL$_9$**	13 / 0.625	15 / 0.625	42	42
***L. ivanovii* LAL$_{10}$**	17 / 0.625	16 / 1.25	32	46
***L. ivanovii* LAL$_{11}$**	12 / 1.25	12 / 1.25	24	40
***L. grayi* LAL$_{12}$**	13 / 1.25	12 / 2.5	24	26
***L. ivanovii* LAL$_{14}$**	16 / 2.5	14 / 2.5	22	34
***L. grayi* LAL$_{15}$**	12 / 0.625	14 / 2.5	24	26
***L. ivanovii* LDB$_3$**	17 / 0.625	18 / 5.0	50	46
***L. ivanovii* LDB$_6$**	14 / 1.25	14 / 2.5	21	23
***L. ivanovii* LDB$_7$**	12 / 0.625	- / ND	25	22
***L. ivanovii* LDB$_8$**	12 / 0.625	14 / 1.25	26	38
***L. ivanovii* LDB$_9$**	12 / 2.5	- / ND	28	32
***L. ivanovii* LDB$_{10}$**	- / ND	14 / 2.5	38	34
***L. ivanovii* LDB$_{11}$**	15 / 2.5	15 / 5.0	40	42
***L. ivanovii* LDB$_{12}$**	- / ND	13 / 2.5	29	34

Key: - represent no bacterial activity; MIC represents minimum inhibitory concentration, ND represents not determined, AMP- ampicillin, TET- tetracycline.

Table 3.2. Nature of inhibition of crude aqueous and n-Hexane extracts of *C. nucifera* husk against *Listeria* pathogens.

	Aqueous Extract					n-Hexane Extract				
Susceptible isolate	MIC (mg/ml)	Log_{10}Kill (MIC)		Log_{10}Kill (2*MIC)		MIC (mg/ml)	Log_{10}Kill (MIC)		Log_{10}Kill (2*MIC)	
		4hr	8hr	4hr	8hr		4hr	8hr	4hr	8hr
L. ivanovii **LEL_3**	0.625	0.32	2.6	2.8	3.0*	0.625	3.2*	4.8*	4.0*	5.2*
L. ivanovii **LEL_9**	2.5	1.4	2.8	3.2*	4.8*	0.625	2.6	2.8	2.4	4.8*
L. ivanovii **LEL_{15}**	NA	NA	NA	NA	NA	2.5	2.8	3.0*	3.8*	5.6*
L. ivanovii **LEL_{17}**	2.5	0.48	2.8	2.2	3.4*	0.625	4.0*	4.0*	4.8*	5.0*
L. ivanovii **LEL_{30}**	NA	NA	NA	NA	NA	1.25	3.2*	3.5*	4.6*	6.2*

Key: MIC represents minimum inhibitory concentration; * represents bactericidal effect;

NA represents no activity.

REFERENCES

Akinpelu DA, Kolawole DO (2004). Phytochemistry and antimicrobial activity of leaf extract of *Piliostigma thonningii* (Schum). Sci. Focus 7: 64-70.

Allerberger F, Wagner M (2010). Listeriosis: a resurgent foodborne infection. *Clin Microbiol Infect.* 16(1): 16-23.

Al-Reza SM, Vivek KB, Kang SC (2009). Antioxidant and antilisterial effects of seed essential oil and organic extracts from *Zizyphus jujuba*. *J food and chem Tox.* (47): 2374-2380.

Alviano DS, Rodrigues KF, Leita˜o SG, Rodrigues ML, Matheus ME, Fernandes PD, et al (2004). Antinociceptive and free radical scavering activities of *Cocos nucifera L*. (Palmae) husk fiber aqueous extract. *J Ethnopharm*. (92): 269–73.

Chakraborty T (2008). Intracellular gene expression profile of Listeria monocytogenes. *J ASM Org.* 74: 1323-1338.

Cordell GA (2000). Biodiversity and drug discovery a symbiotic relationship. *Phytochem*. 55: 463–480.

Denny J, McLaughlin J. Human (2008). Listeria monocytogenes infections in Europe - an opportunity for improved European surveillance. *Euro Surveill.* 13: 8082-8087.

Duke JA (1992). Handbook of Phytochemical Constituents of Gras Herbs and Other Economic Plants, CRC Press Service, Antibiotics that resist resistance. *Sci pharm.* 270: 724–727.

Edward C, Craig RE (2006). *Cocos nucifera* species Profile for Pacific Island Agroforestry. *Permanent Agric Res Hawaii*.

Esquenazi D, Wigg MD, Miranda MM, Rodrigues HM, Tostes JB, Rozental S *et al.* (2002). Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. *Res Microbiol*. 153:647–52.

EUCAST (European Committee for Antimicrobial Susceptibility Testing (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin Microb Infect.* 9(8): 1-7.

Hao YY, Brackett RE, Doyle LE (1998). Inhibition of *Listeria monocytogenes* and *Aeromonas hydrophila* by plant extracts in refrigerated cooked beef. *J Food Prot*. 61: 307–312.

Irobi ON, Young M, Anderson WA (1994). Antimicrobial activity of Annato (*Bixa orella*) extract. *Int J Pharmacog*. 34: 87-90.

Kenneth (2008). Effect of food borne pathogens on stainless surfaces and cross-contamination to food. *J Food Microbiol*. 85: 230-238.

Kim JM, Marshall MR, Wei C (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. *J Agric Food Chem.* 43: 2839–2845.

Kirszberg C, Esquenazi D, Alviano CS, Rumjanek VM (2003). The effect of catechin-rich extract of Cocos nucifera on lymphocytes proliferation. *Phytother Res.* 17: 1054–1058.

Koschek PR, Alviano DS, Alviano CS, Gattass CR (2007). The husk fiber of Cocos nucifera L. (Palmae) is a source of antineoplasic activity. *Braz J Med Biol Res.* 40: 1339–43.

Loper JE, Henkel MD, Roberts RG, Grove GG, Willett MJ, Smith TJ (1999). Evaluation of streptomycin, oxatetracycline, and copper resistance of *Erwinia amylovora* isolated from pear orchards in Washington State. *Plant Dis*. (75): 287–290.

Mendonca-Filho RR, Rodrigues IA, Alviano DS, Santos ALS, Soares RMA, Alviano CS et al. (2004). Leishmanicidal activity of polyphenolic-rich extract from husk fiber of *Cocos nucifera* Linn. (Palmae). *Res Microbiol*. 155: 136–43.

Moumita C, Adinpunya M (2007). The antioxidant and antimicrobial properties of methanolic extract from *C. nucifera* mesocarp. *J Agric & Food chem*. 47: 1620-1624.

Nostro A, Germarno MP, D'Angelo V, Marino A, and Canatelli MA (2001). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Lett in Appl Microbiol*. 30: 379-384.

Okoli S, Iroegbu CU (2005). *In vitro* antibacterial activity of *Synclisa scabrida* whole root extract. *Afr. J. Biotechnol*. 4(9): 946-952.

Paschuka AR, Butler R, Young CYF (1998). Induction of apoptosis in prostate cancer cell lines by green tea component, (-)-epigallocatechin- 3-gallate, *Cancer Lett*. 130: 1–7.

Peng Z, Hayasaka Y, Iland PG, Sefton M, Hoj P, Waters EJ (2001). Quantitative analysis of polymeric procyanidins (tannins) from grape (*Vitis vinifera*) seeds by reverse phase high-performance liquid chromatography. *J Agr Food Chem.* 49: 26–31.

Rhodes PL (2004). Antimicrobial factor from grape. Thesis submitted to the Dept of Micro. Uni of Auckland, New Zealand.

Socransky SS, Haffajee AD (2002). Dental biofilm difficult therapeutic targets Periodontology. *J Pharmaco and Toxico.* (28): 12–55.

Wager AR, Waisbren BA, Martins RR, Batayias GE (2008). A method for determining sensitivities of antiviral drugs *in vitro* for possible use as clinical consultation, *Am J Clin Path*. 56: 667–692.

Zakaria ZA, Reezal I, Mat Jais AM, Somchit MN, Sulaiman MR, Marmin AHI, Sidek H, Husin SH, Rahim MHA, Abdul Rahman L (2006). The anti-inflammatory, anti-pyretic and wound healing activities of *Cocos nucifera* fresh juice and kernel extract in experimental animals. *J. Pharmaco and Toxico.* 1(6): 516-526.

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In-vitro antibacterial properties of crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera*

Abstract

The increasing antibiotic resistance cases among pathogenic bacteria such as *Vibrio* species poses a major problem to food and aquaculture industries. Most of the antibiotics were no longer effective in controlling pathogenic bacteria affecting these industries. Therefore, this study was carried out to assess the antibacterial potentials of crude aqueous and n-Hexane extracts of the husk of *Cocos nucifera* against some selected *Vibrio* species and other bacterial pathogens including those normally implicated in food and wound infections. The crude extracts were screened against forty-five strains of *Vibrio* pathogens and twenty-five other bacteria isolates made up of ten Gram positive and fifteen Gram negative bacteria. The aqueous extract was active against 17 of the tested bacterial and 37 of the *Vibrio* isolates; while the n-Hexane extract showed antimicrobial activity against 21 of the test bacteria and 38 of the test *Vibrio* species. The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.6 – 5.0 mg/ml for both extracts. Specifically, MICs for the aqueous and n-Hexane extracts ranged between 0.6 – 5.0 mg/ml and 0.3 – 5.0 mg/ml respectively, while the time kill study result for the aqueous extract ranged between 0.12 Log_{10} and 4.2 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC. For the n-Hexane extract, the log reduction ranged between 0.56 Log_{10} and 6.4 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC. This study revealed the huge potentials of extract of *C. nucifera* as alternative therapies against microbial infections.

Key words: *C. nucifera, Vibrio* specie, antibacterial, n - Hexane extract, aqueous extract

4.1 INTRODUCTION.

Medicinal plants contain large varieties of chemical substances with important therapeutic properties that can be utilised in the treatment of human diseases. Consequently, there is the increasing justified assumption that says traditional medicine is cheaper and more effective than modern medicine. The studies of medicinal plants used in folklore remedies have therefore attracted immense attention in the scientific world in an attempt to finding possible solutions to the problems of multiple resistances to the existing synthetic and conventional antimicrobials. The discovery of antibiotics had eradicated the infections that once ravaged the humankind, but their indiscriminate use has led to the development of multidrug-resistant pathogens (Shanmugan *et al*., 2008).

Vibrios are gram-negative, curved, rod-shaped bacteria that are natural inhabitants of the marine environment (McLaughlin *et al*., 1995). The centre for disease control (CDC) estimates that 8028 *Vibrio* infections and 57 deaths occur annually in the United States (Mead *et al*., 1999). Transmission of *Vibrio* infections is primarily through the consumption of raw or undercooked shellfish or exposure of wounds to warm seawater (Morris and Black, 1985; Levine and Griffin, 1993). The most common clinical presentation of *Vibrio* infection is self-limited gastroenteritis, though wound infections and primary septicemia may also occur (Levine and Griffin, 1993). Patients with liver disease are at particularly high risk for significant morbidity and mortality associated with these infections (Hlady and Klontz, 1993).

Many cases of *Vibrio* associated gastroenteritis are under-recognized due to application of inadequate diagnostic procedures (Morano *et al*., 2000). The enterotoxin produced by these *Vibrio* strains causes copious, painless, watery diarrhea leading to vomiting, severe dehydration, and even death if treatment is not prompt (World Health Organization, 2001). Early detection and initiation of treatment of these

infections are very important, particularly for cholera and invasive *Vibrio* infections which has high mortality potentials, (Vollberg and Heirara, 1997). The Centers for Disease Control and Prevention (CDC) in 2005 estimated that the average annual incidence of all *Vibrio* infections increased by 41% between 1996 and 2005. Treatments such as antibiotics injections, aggressive wound therapy and supportive care had been adopted over the years but persistent resistance and immunocompromising conditions recorded from patient with this infection calls for immediate attention and a need to search for more potent and new antimicrobial compounds of natural origin to combat the activities of these pathogens, which forms the basis for this research.

Cocos nucifera belongs to the family Aracaceae. The English name is coconut; the plant is mainly used as Staple food crop, wood, handicrafts, among many others; thought by many to be the "world's most useful plant and medicinal plant in tropical and subtropical countries (Edward and Craig, 2006). *C. nucifera is* found throughout the tropics, where it is interwoven into the lives of the local people (Edward and Craig, 2006). Esquenazi *et al*. (2002) in their studies reported that in the traditional medicine in northeastern Brazil, coconut husks have been used for the treatment of diarrhea and arthritis. Today, coconut oil, obtained from the fruit of coconut palm has been relegated mainly to non food uses in the developed countries but retains its importance in producing countries for traditional uses (Edward and Craig, 2006). Coconut oil has been confirmed to possess antimicrobial, antiviral and antiprotozoal activities (Isaacs and Thormar, 1991; Thormar, 1996; Enig, 2003). In this paper, we report on the antibacterial properties of the aqueous and n-Hexane extracts of the husk of *C. nucifera* against some *Vibrio* pathogens and other bacteria as part of our exploration for new and novel bioactive compounds.

4.2. Materials and methods

4.2.1 Plant material

The plant specimens were collected from the vicinity of the Research Farm of the Obafemi Awolowo University, Ile Ife, Nigeria and identified by the curator of the Herbarium at the Department of Botany, Obafemi Awolowo University, and a voucher specimen kept there.

4.2.2 Preparation of Extract

The husk of the coconut was sun–dried, milled and sieved manually to obtain the fine powdered particles. About 50 g dried powdered husk of the plant was added to 200 ml of 95% n-Hexane using Soxhlet extraction method at room temperature and for 48 hr. The mixture was then filtered using Whatman 1filter paper. The filtrates of each extraction were pooled together and concentrated to dryness *in vacuo* using a rotary evaporator to remove the n - Hexane. The concentrated extract was then allowed to dry at room temperature to a constant weight. For the aqueous extract, about 50 g of the powdered extract was dissolved in 500 ml of sterile distill water for 24 h with shaking. The resultant extracts were centrifuged at 3000 rpm for 5 min at 4 ^{o}C. The supernatant was filtered through a Whatman No. 1 filter paper and the filtrate was lyophilized.

4.2.3 Test bacterial strains

The bacterial isolates used in this study included forty-five *Vibrio* strains and twenty-five bacteria pathogens as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The *Vibrio* species were isolated from waste-water effluent in the Eastern Cape Province, South Africa. The bacterial isolates include reference strains (9) obtained from the South African Bureau of Standard (SABS), environmental strains (12) and clinical isolates (4).

4.2.4 Antibacterial susceptibility test

The susceptibility screening of the test bacteria to both crude extracts and standard antibiotics were done in accordance with the method of Irobi *et al.* (1994) and Akinpelu *et al*. (2008). The inoculum size of each test strain was standardized at 5×10^5 CFU/ml using McFarland Nephelometer standard. Sterile Mueller-Hinton agar plates were seeded with test bacterial strains and allowed to stand at 37 ^{o}C for 3 hr. Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells filled with the solution of the extracts and antibiotics taking care not to allow spillage of the solution onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract and antibiotics into the media and thereafter incubated at 37 ^{o}C for 24 hr, after which they were observed for zones of inhibition. The effects of the extracts on the test bacterial isolates were compared with those of tetracycline and ampicillin standard antibiotics which serve as a negative and positive control at a concentration of 1mg/ml and 10 μg/ml respectively.

4.2.5 Determination of minimum inhibitory concentration (MIC)

The MIC of the crude aqueous and n - Hexane extract was carried out using the method of Akinpelu and Kolawole (2004). Two-fold dilutions of the extracts were prepared and 2 ml aliquot of different concentrations of the solution were added to 18 ml of pre-sterilized molten Mueller - Hinton agar at 40 ^{o}C to give final concentration regimes of 5.0 to 0.156 mg/ml. The medium was then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry under a laminar flow before streaking with 18 h old bacterial cultures. The plates were later incubated at 37 ^{o}C for up to 72 hr after which they were examined for the presence or absence of growth. The MIC was taken as the least concentration of extracts that will prevent the visible growth of the test bacteria.

4.2.6 Time-Kill assay

Determination of the kill rate of the crude extracts was done following the procedure as described by Okoli and Iroegbu (2005). Inocula were prepared following the described guidelines of EUCAST (2003). The resultant suspension were diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of Mueller Hinton broth incorporated with extracts at MIC and 2 × MIC to a final cell density of approximately 5×10^5 CFU/ml. The flasks were incubated at 37^{o}C on an orbital shaker at 120 rpm. A 500 µl sample were removed from cultures at 0, 4 and 8 hr, diluted serially and 100 µl of the diluted samples were plated on Mueller Hinton agar plates and incubated at 37^{o}C for 24 hr. Controls included extract-free Mueller Hinton broth seeded with the test inoculum.

4.3 Results

The antibacterial activity of *Cocos nucifera* husk extract was investigated. Seventeen of the test bacteria were susceptible to the aqueous extract with a zone of inhibition value ranging between 11 and 20 mm, while twenty-one bacteria were susceptible to the n-Hexane extract with inhibition zone ranging between 12 and 18 mm at the test concentration of 25 mg/ml (Table 4.1). Thirty-seven of the *Vibrio* isolates were susceptible with inhibition zone diameters ranging between 10 and 18 mm for the aqueous extracts; while thirty-eight were susceptible to the n-Hexane extract with inhibition zone diameters ranging between 12 and 21 mm (Table 4.1).

The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.6–5.0 mg/ml for both extracts. Specifically, MICs for the aqueous and n-Hexane extracts ranged between 0.6–5.0 mg/ml and 0.3–5.0 mg/ml respectively (Table 4.1). The n - Hexane extracts showed stronger antimicrobial activity when compared with the aqueous extracts.

The results of time-kill studies are presented in Table 4.2. Datas are presented in terms of the Log_{10} CFU/ml reduction in viable cell count and interpretations are based on the conventional bactericidal activity standard, which is, a 3 Log_{10} CFU/ml or greater reduction in the viable colony count (Pankey and Sabbath, 2004). For the aqueous extract, average log reduction in viable cell count in time kill assay ranged between 0.12 Log_{10} and 4.2 Log_{10} CFU/ml after 8 hr interaction at 1 × MIC and 2 × MIC. For the n-Hexane extract, the log reduction ranged between 0.56 Log_{10} and 6.4 Log_{10} CFU/ml after 8 hr interaction in 1 × MIC and 2 × MIC. The greatest reductions in cell density achieved with the aqueous extract were on *Vibrio vulnificus* EL039 with the average value of 4.2 Log_{10} CFU/ml, *S. aureus*

OKOH2B (clinical strain) with the average reduction in viable cell count of 3.46 $\log_{10}$ CFU/ml, while the greatest reduction in viable cell volume achieved by the n-Hexane extract were on the environmental strain *Bacillus substilis* with the average value of 6.40 $\log_{10}$ CFU/ml and reference strain *Escherichia coli* ATCC 8739 with the average reduction in viable cell count of 5.6 $\log_{10}$ CFU/ml.

The crude aqueous extract was bactericidal against *B. substilis, V. vulnificus* EL039 *and V. fluvialis* EL041 at 1 × MIC and 2 × MIC after an 8 hr interaction period and bacteriostatic during the first 4 hr of interaction at both MIC levels, while the n-Hexane extract was bactericidal against nine of the test bacteria *E.coli, A. calcaoceticus anitratus* CSIR, clinical strain *Staphylococcus aureus, environmental strain B. substilis* and the *Vibrio strains V. metschnkovii* EL008, *V. specie* EL009, *V. vulnificus* EL039 and *V. fluvialis* at both MIC levels after 8 hr of interaction but was bacteriostatic against *S. faecalis* ATCC 29212 after 8 hr of interaction.

4.4 Discussion

The use of plant extracts with medicinal potentials represents a valid alternative for the treatment of different ailments and diseases. The antivibriol and antibacterial properties of the husk of *C. nucifera* were investigated against a number of *Vibrio* pathogens and other bacteria pursuant to contributing to our body of knowledge on the potentials o the plant in the management of *Vibrio* and other bacterial infections in support of previous report (Alviano *et al*., 2004).

The aqueous and n-Hexane extracts of the husk of our study plant exhibited potent antivibriol and antibacterial activity against about 90 % of the bacteria strains tested. The result from this study confirms that both the aqueous and n-Hexane fraction of the husk possess antimicrobial properties against Vibrio species and other bacteria thus supporting the traditional use of this plant in the treatment of wound, respiratory and gastro intestinal tract infections.

The diameters of the zones of inhibition exhibited by the extracts against the text bacteria are similar to those reported elsewhere such as Ravikumar *et al.* (2010) and Chandrasekaran *et al.* (2009) who reported on the chloroform extracts of *Exoecaria agallocha* leaves; as well as methanol and aqueous extracts of mangrove respectively. The limited activity of the aqueous extract in comparison to the n-Hexane extract corroborate the findings of Wei *et al.* (2006) and Alanis *et al.* (2005) where they reported lower activity in the aqueous extracts compared to other solvent extracts.

The MICs values observed in this study varied depending on the strain and ranged from 0.6 to 5.0 mg/ml for the *Vibrio* bacteria and from 0.3 to 5.0 mg/ml for the other bacterial isolates. The observation that some of the *Vibrio* and bacteria strains were susceptible to the plant extract at a concentration as low as 0.3125 mg/ml strongly suggest that of *C. nucifera* plant can be effective in the treatment of infections caused by these pathogens.

Similar result was reported by Sharma *et al.* (2009), in their studies on the vibriocidal activities of 16 Indian medicinal plants, wherein 70 % of the *Vibrio* pathogens tested were susceptible to the plant extract at a concentration ranging between 2.5 and 20 mg/ml. The bactericidal activities of the aqueous extracts of this plant at 2 × MIC after 8 hr exposure against *S. aureus* OKOH2B (a clinical isolate from

wound sepsis); *V. vulnificus* and *V. fluvialis* is worth noting and further supports its use in folklore remedy. At 1 × MIC, the n-Hexane extract showed bactericidal activity against 3 of the 6 bacteria species tested as well as the entire *Vibrio* isolates. At 2 × MIC the entire population of the *Vibrio* and the other bacteria species (except *S. faecalis*) tested had been wiped out after 8 hr exposure. It would appear that the observed bactericidal or bacteriostatic activity of this plant is both time and concentration dependent.

Considering the crude nature and low toxicities of the solvent extracts used in this study, our results allow us to conclude that the crude extract from *C. nucifera* exhibited significant antibacterial activity and properties that support folkloric use in the treatment of some food borne diseases as well as its potentials in wound healing activities. Plants that have tannins as their components are astringent in nature and are used for treating intestinal disorder such as dysentery and diarrhea (Dharmananda, 2003) thus exhibiting antimicrobial activity. Esquenazi *et al*. (2002) reported that *C. nucifera* aqueous extract is rich in catechin and epicatechin together with condensed tannin.

The presence of tannin in *C. nucifera* supports the traditional medicine use of this plant in the treatment of different ailments. Mortar *et al*. (1985) revealed the importance of tannins for the treatment of inflamed or ulcerated tissues. Li *et al*. (2003) also reviewed the biological activities of tannins and observed that tannins have remarkable activities in cancer prevention and anticancer, thus suggesting that this plant *C. nucifera* has potentials as useful source of important bioactive molecules for the treatment and prevention of cancer.

To further buttress the phytochemical importance of *C. nucifera*, Zakaria *et al.* (2006) administered the coconut juice extract as part of a dietary supplement at low concentrations and also the coconut cream and oil. The application of *C. nucifera* extract as food supplement is both an economical and an eco-friendly alternative in antimicrobial chemotherapy. Although coconut fruit is meant for human consumptions, this present study suggests the need for characterizing the antibacterial active principle(s) of *Cocos nucifera*. Understanding the chemical nature of the active principle(s), it will provide an opportunity to synthesize new and effective antibacterial (including antivibriol) drug.

4.5 Conclusion

This study has demonstrated the antibacterial activities of *C. nucifera* especially against *Vibrio* bacteria and suggests that the plant has immense potentials as an alternative to synthetic antibiotics in the management of *Vibrio* and other bacterial infections. Further studies are needed to elucidate the active components and their modes of action as well as their potentials in combination chemotherapy with synthetic drugs which is the subject of ongoing research in our group.

Table 4.1 Antivibriol activities of crude Aqueous and n-Hexane extracts of *C. nucifera* husk on *Vibrio* pathogens.

Isolate Identity	Inhibition zone (mm) / MIC		Antibiotics Inhibition zone (mm)	
	Aqueous Extract	n-Hexane Extract	AMP	TET
***Vibrio vulnificus* EL047**	17 / 0.625	18 / 0.625	25	18
***Vibrio* specie EL014**	15 / 0.625	- / ND	26	16
***Vibrio* specie EL031**	15 / 2.5	16 / 0.625	24	20
***Vibrio metschnkovii* EL003**	- / ND	20 / 2.5	20	20
***Vibrio* specie EL006**	16 / 1.25	15 / 2.5	16	20
***Vibrio fluvialis* EL049**	14 / 0.625	- / ND	20	19
***Vibrio* specie EL027**	13 / 1.25	- / ND	40	22
***Vibrio* specie EL052**	16 / 1.25	17 / 1.25	15	32
***Vibrio fluvialis* EL007**	16 / 2.5	18 / 0.625	15	29
***Vibrio vulnificus* EL051**	13 / 0.625	- / ND	27	17
***Vibrio fluvialis* EL036**	14 / 1.25	12 / 2.5	14	30
***Vibrio fluvialis* EL015**	- / ND	15 / 1.25	16	18
***Vibrio vulnificus* EL017**	15 / 2.5	16 / 0.625	17	18
***Vibrio* specie EL013**	16 / 2.5	18 / 2.5	28	17
***Vibrio metschnkovii* EL028**	18 / 1.25	14 / 1.25	22	16
***Vibrio vulnificus* EL039**	12 / 0.625	- / ND	30	21
***Vibrio metschnkovii* EL008**	14 / 0.625	12 / 1.25	19	28

***Vibrio fluvialis* EL035**	13 / 1.25	12 / 0.625	12	26
***Vibrio vulnificus* EL002**	- / ND	12 / 1.25	15	30
***Vibrio vulnificus* EL005**	16 / 0.625	18 / 0.625	13	15
***Vibrio* specie EL021**	16 / 2.5	20 / 2.5	20	29
***Vibrio vulnificus* EL018**	- / ND	21 / 2.5	21	20
***Vibrio vulnificus* EL043**	10 / 0.625	- / ND	12	18
***Vibrio vulnificus* EL045**	18 / 1.25	12 / 1.25	15	28
***Vibrio parahaemolyticus* AL045**	14 / 1.25	15 / 1.25	16	18
***Vibrio vulnificus* EL040**	12 / 2.5	16 / 0.625	20	16
***Vibrio vulnificus* EL012**	14 / 1.25	12 / 2.5	19	17
***Vibrio fluvialis* EL034**	- / ND	14 / 0.625	12	18
***Vibrio vulnificus* EL044**	15 / 2.5	18 / 2.5	16	16
***Vibrio vulnificus* EL053**	- / ND	16 / 2.5	12	40
***Vibrio fluvialis* EL042**	18 / 1.25	18 / 1.25	26	40
***Vibrio fluvialis* EL041**	15 / 0.625	18 / 0.625	28	40
***Vibrio vulnificus* EL048**	15 / 0.625	21 / 0.625	21	34
***Vibrio vulnificus* EL050**	16 / 2.5	18 / 0.625	22	35
***Vibrio vulnificus* EL010**	13 / 0.625	15 / 1.25	21	35
***Vibrio* specie EL009**	13 / 5.0	17 / 2.5	20	21
***Vibrio* specie AL046**	13 / 2.5	13 / 2.5	22	20
***Vibrio* specie EL054**	15 / 1.25	13 / 1.25	22	40
***Vibrio vulnificus* EL036**	13 / 1.25	16 / 1.25	24	38

***Vibrio vulnificus* EL039**	14 / 2.5	18 / 0.625	24	26
***Vibrio vulnificus* EL033**	- / ND	14 / 0.625	13	18
***V. fluvialis* AL019**	14 / 1.25	12 / 2.5	12	29
***V. fluvialis* EL032**	18 / 1.25	18 / 0.625	30	32

Key: - represents no antibacterial activity; MIC represents minimum inhibitory concentration; ND represents not determined, AMP – ampicillin, TET – tetracycline.

Table 4.2 Antibacterial activities of crude Aqueous and n - Hexane extracts of *C. nucifera* husk.

Isolate Identity	Inhibition zone (mm) / MIC		Antibiotics zone (mm)	
	Aqueous extract	n-Hexane extract	AMP	TET
***Escherichia coli* ATCC 8739**	11 / 0.625	12 / 5.0	28	27
***Pseudomonas aeruginosa* ATCC 19582**	- / ND	13 / 1.25	22	25
***Streptococcus faecalis ATCC* 29212**	15 / 0.625	14 / 0.312	21	27
***Pseudomonas aeruginosa* ATCC 7700**	16 / 2.5	18 / 2.5	15	20
***Klebsiella pneumoniae* ATCC 10031**	22 / 1.25	15 / 2.5	21	30
***Klebsiella pneumoniae* ATCC 4352**	13 / 1.25	- / ND	24	33
***Proteus vulgaris* CSIR 0030**	20 / 1.25	16 / 1.25	24	35
***Bacillus subtilis* KZN**	- / ND	12 / 2.5	28	22
***Pseudomonas aeruginosa* KZN**	16 / 2.5	18 / 0.625	28	36
***Enterococcus faecalis* KZN**	14 / 0.625	13 / 0.625	22	32
***Escherichia coli* KZN**	13 / 1.25	12 / 2.5	26	38
***Staphylococcus aureus* KZN**	- / ND	- / ND	27	36

***Staphylococcus aureus* OKOH1**	**14 / 2.5**	**14 / 0.625**	**23**	**34**
***Staphylococcus aureus* OKOH2A**	13 / 2.5	16 / 2.5	19	28
***Staphylococcus aureus* OKOH2B**	- / ND	12 / 2.5	28	32
***Staphylococcus aureus* OKOH3**	- / ND	- / ND	25	35
Micrococcus kristinae	- / ND	- / ND	22	32
***Serratia marsecens* ATCC 9986**	15 / 0.625	16 / 1.25	25	27
***A. calcaoceuticus anitratus* CSIR**	- / ND	14 / 2.5	14	32
***Klebsiella pneumoniae* KZN**	15 / 0.625	12 / 0.625	14	21
***Shigella flexineri* KZN**	- / ND	15 /1.25	16	30
***Salmonella* specie KZN**	15 / 1.25	14 / 2.5	17	25
***Staphylococcus epididirmis* KZN**	18 / 0.625	12 / 0.625	17	18
Micrococcus luteus	14 / 1.25	12 / 0.625	24	33

Key: - represents no antibacterial activity; MIC represents minimum inhibitory concentration; ND represents not determined.

Table 4.3. **Nature of inhibition of crude Aqueous and n-Hexane extracts of *C. nucifera* husk against some bacterial isolates and *Vibrio* pathogens.**

	Aqueous extract					n-Hexane extract				
Susceptible isolate	MIC (mg/ml)	Log_{10}Kill (MIC)		Log_{10}Kill (2*MIC)		MIC (mg/ml)	Log_{10}Kill (MIC)		Log_{10}Kill (2*MIC)	
		4hr	8hr	4hr	8hr		4hr	8hr	4hr	8hr
Vibrio metschnkovii EL008	0.625	2.0	2.2	2.6	2.4	1.25	3.0*	3.4*	2.8	3.2*
Vibrio specie EL009	5.0	1.2	2.4	2.0	2.2	2.5	3.4*	3.8*	4.0*	4.2*
Vibrio vulnificus EL039	0.625	2.4	2.8	2.6	4.2*	0.625	3.2*	4.0*	4.2*	4.2*
Vibrio fluvialis EL041	0.625	1.8	2.4	2.4	3.4*	0.625	4.0*	4.6*	4.2*	5.0*
Escherichia coli ATCC8739	0.625	0.64	0.72	0.70	0.92	5.0	3.5*	5.6*	5.6*	5.6*
Streptococcus faecalis ATCC 29212	0.625	0.12	0.48	1.24	1.48	0.312	1.02	1.28	1.22	2.46
Acinetobacter calcaoceticus anitratus CSIR.	NA	NA	NA	NA	NA	2.5	2.1	2.2	4.2*	4.2*

Bacillus substilis$^{\infty}$	**NA**	**NA**	**NA**	**NA**	**NA**	**2.5**	**0.56**	**4.22***	**2.84**	**6.40***
Shigella flexineri $^{\infty}$	NA	NA	NA	NA	NA	1.25	4.2*	4.3*	4.3*	4.3*
Staphylococcus aureus	0.625	1.40	1.62	2.21	3.46*	1.25	1.20	2.40	2.04	3.40*

Key: MIC represents minimum inhibitory concentration; * represents bactericidal effect;

NA represents no activity; α represent clinical strains; ∞ represent environmental strains.

REFERENCES

Akinpelu DA, Aiyegoro OA, Okoh AI (2008). *In vitro* antibacterial and phytochemical properties of crude extract of stem bark of *Afzelia africana* (Smith). *Afr J Biotech.* 7(20): 3662-3667.

Akinpelu DA, Kolawole DO (2004). Phytochemical and antimicrobial activity of leaf extract of *Piliostigma thonningii* (Schum.). *Sci Focus J*. 7: 64-70.

Alanis AD, Calzada F, Cervantes JA, Torres J, Ceballos GM (2005). Antimicrobial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *J Ethnopharm.* 100: 153-157.

Alviano DS, Rodrigues KF, Leita˜o SG, Rodrigues ML, Matheus ME, Fernandez PD *et al.* (2004). Antinociceptive and free radical scavering activities of *Cocos nucifera* L. (Palmae) husk fiber aqueous extract. *J Ethnopharm*. 92: 269–73.

Centers for Disease Control and Prevention (2005). Preliminary Food Net data on the incidence of infection with pathogens transmitted commonly through food—10 states, United States. *MMWR Morb Mortal Wkly Rep*. 55: 392–5.

Chandrasekaran M, Kannathasan K, Venkatesalu V, Prabhakar K (2009). Antibacterial activity of some salt marsh halophytes and mangrove plants against methicillin resistant *Staphylococcus aureus. World J Microbiol Biotech*. 25: 155-160.

Dharmananda S (2003). Golinuts and the uses of tannins in Chinese Medicine. In proceedings of Institute for Traditional Medicine Portland, Oregon.

Edward C, Craig RE (2006). *Cocos nucifera* species Profile for Pacific Island Agroforestry. *Permanent Agric Res, Hawaii*.

Esquenazi D, Wigg MD, Miranda MM, Rodrigues HM, Tostes JB, Rozental S *et al*. (2002). Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. *Res Microbiol*. (153): 647–52.

EUCAST (European Committee for Antimicrobial Susceptibility Testing), 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin Microb Infect.* 9(8): 1-7.

Hlady WG, Klontz KC (1993). The epidemiology of *Vibrio* infections in Florida. *J Infect Dis.* 173: 1176-1183.

Irobi ON, Young M, Anderson WA (1994). Antimicrobial activity of Annato (*Bixa orella*) extract. *Int J Pharmacog*. 34: 87-90.

Isaacs CE and Thormar H (1991). The role of milk-derived antimicrobial lipids as antiviral and antibacterial agents in Immunology of Milk and the Neonate Plenum Press, New York.

Levine WC, Griffin PM (1993). *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. *J Infect Dis.* (167): 479-483.

Li H, Wang Z, Liu Y (2003). Review in the studies on tannins activity of cancer prevention and anticancer. *Zhong-Yao-Cai*. 26(6): 444-448.

McLaughlin JC, Murray PR, Baron EJ, Pfaller *et al.* (1995). *Manual of Clinical Microbiology.* 6th ed. Washington, DC: *ASM Press*; 465-476

Mead PS, Slutsker L, Dietz *et al.* (1999). Food-related illness and disease in the United States. *Emerg Infect Dis.* 5: 1-20.

Morano NN, Daniels NA, Easton AN, et al. (2000). Stool culturing practices for *Vibrio* species at clinical laboratories in Gulf Coast states. *J Clin Microbiol.* 38: 2267-2270.

Morris J, Black R (1985). Cholera and other *vibrioses* in the United States. *N Engl J Med.* 312: 343-350.

Mortar MLR, Thomas G, BarbosaFillo JM (1985). Effects of *Anacardium occidentale* stem bark extract on *in vivo* inflammatory models. *J Ethnopharm*. 95(2-3): 139-142.

Okoli S, Iroegbu CU (2005). *In vitro* antibacterial activity of *Synclisa scabrida* whole root extract. *Afr J Biotechnol.* 4(9): 946-952.

Pankey GA, Sabbath LD (2004). Clinical relevance of bacteriostatic versus bactericidal Mechanisms of action in the treatment of gram-positive bacterial infections. *Clin Infect Dis.* (38): 864-870.

Ravikumar S, Muthuraj M, Sivaperumal P, Gnanadesigan M (2010). Antibacterial activity of mangroves leaves Exoecaria agallocha against selected fish pathogens. *Asian J Med Sci* 2(5): 211-213.

Shanmugan H, Anil K, Mukesh D (2008). Synergism between natural products and antibiotics against infectious diseases. *Jour of phytomed.* 15: 639-652.

Sharma AC, Patel VK, Chaturvedi AN (2009). Vibriocidal activity of certain medicinal plants used in India folklore medicine. *Indian J Pharmacol.* 41(3): 129-133.

Thormar PK (1996). Handbook on Coconut Palm. Oxford & IBH Publishing Co. New Delhi.

Vollberg CM, Herrara JL (1997). *Vibrio vulnificus* infection: an important cause of septicemia in patients with cirrhosis. *South Med J.* 90: 1040-1042.

Wei LS, Najiah M, Wendy W (2006*).* *In vitro* antimicrobial activity of *Colocasia esculenta* extract against *Vibrio* spp. *J. Agric* 42: 5-7

World Health Organization (2001). Essential Drugs and Medicines Policy; WHO Geneva.

Zakaria ZA, Reezal I, Mat Jais AM, Somchit MN, Sulaiman MR, Marmin AHI, Sidek H, Husin SH, Rahim MHA, Abdul Rahman L (2006). The anti-inflammatory, anti-pyretic and wound healing activities of *Cocos nucifera* fresh juice and kernel extract in experimental animals. *J Pharmaco and Toxi.* 1(6): 516-526.

CHAPTER FIVE

In vitro assessment of the interactions between the extracts of *Cocos nucifera* husk and some antibiotics

CHAPTER FIVE

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In vitro assessment of the interactions between the extracts of *Cocos nucifera* husk and some antibiotics.

Abstract

This study has been done to assess and evaluate the interactions between the crude extract of *Cocos nucifera* and six front line antibiotics such as ampicillin sodium salt (Calbiochem), penicillin G sodium (Duchefa), amoxicillin (Duchefa), chloramphenicol (Duchefa), ciprofloxacin (Fluka) and tetracycline hydrochloride (Duchefa), against some pathogenic organisms. The assessment was done by use of the time-kill and checkerboard method. Results from the time kill study showed that the highest bactericidal activity was observered on *Vibrio fluvialis* EL041, with a -5.6±0.12 $\log_{10}$ CFU/ml reduction in cell density when the extract and chloramphenicol were combined at 2 x MIC. Synergisms using the time kill method constituted about 72%, while indifference constituted about 28%. The checkerboard method using the FIC indices showed some level of interaction of the extracts in combination with the antibiotics with a synergistic interaction value of 67% and indifference value of 33%.The observed synergy was not specific to a particular class of antibiotics. This investigation suggests the crude extract of *Cocos nucifera* to be a potential broad spectrum antimicrobial compound.

Key words: synergism, indifference, antibiotics, time-kill, checkerboard.

5.1 INTRODUCTION.

Infectious diseases still represent an important cause of morbidity and mortality among humans, especially in developing countries. Even though pharmaceutical companies have produced a number of new antibacterial drugs in the last years, resistance to these drugs by bacteria has increased and has now become a global concern (Betoni *et al*., 2006). In general, bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents (Nascimento *et al*., 2000). Drug-resistant pathogens are on the rise. In the recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented (Jones *et al*., 2004).

These multidrug-resistant bacteria have also created immense clinical problems in cancer and immune compromised patients. Among the various diarrheagenic serotypes of *E.coli*, enterohaemorrhagic *E. coli* O157:H7 is implicated in a large number of food borne outbreaks in many parts of the world (Mead *et al*., 1999). *Listeria* species has also been isolated from various environment and it is reported to cause about 25% of all the death resulting from food borne outbreaks in the United states annually (CDC, 1995).Therefore, the importance of identifying new effective antimicrobial agents cannot be overemphasized. In rational drug therapy, the concurrent administration of two or more drugs is often essential and sometimes mandatory in order to achieve the desired therapeutic goal or to treat co-existing diseases.

However, the drug interaction may have different effects on the host as well as the infecting microorganism. The potential benefits of using combined antimicrobial therapy can be treatment of mixed infections, therapy of severe infections in which a specific causative organism is known, enhancement of antibacterial activity, reducing the time for long-term antimicrobial therapy and prevention of the emergence of resistant microorganisms (Hugo *et*

al., 2003; Levinson and Jawetz, 2002). Drug synergism between known antimicrobial agents and bioactive plant extracts is a novel concept and has been recently reported by several authors. (Nascimento *et al*., 2000; Aburjai *et al*., 2001; Shiizu *et al*., 2001; Aqil *et al*., 2005; Junior *et al*., 2005; Betoni *et al*., 2006; Esimone *et al*., 2006; Ibezim *et al*., 2006; Ali *et al*., 2007; Chang *et al*., 2007; Horiuchi *et al*., 2007, Aiyegoro *et al*., 2009).

Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antimicrobial activity (Pankey and Ashcraft, 2005). Infections resulting from strains that are resistant to main groups of antibiotics like the β-lactams and aminoglycosides are treatable with vancomycin, chloramphenicol or other antibiotics (Hugo and Russell, 2003). Yet resistance to these drugs is fast growing. Over the years, resistance to fast acting fluoroquinolone drugs were known in certain bacteria by mutation but recent reports by (Wang *et al*., 2003; Cheung *et al*., 2005) has confirmed the plasmid-mediated quinolone resistance qnrA gene in *Escherichia coli* and other members of enterobacteriaceae conferring low level resistance to ciprofloxacin and other fluoroquinolones.

Prospective antibacterial actions of plant extracts have been documented which include inhibition of MDR-efflux pump (Stermitz *et al*., 2000) and β-lactamase activity (Yam *et al*., 1998), antibiotic resistance properties and R-plasmid elimination (Beg and Ahmad, 2001). Similarly, some plant extracts and phytocompounds exhibited synergistic interaction with antibiotics against Gram-positive bacteria (Zhao *et al*., 2001; Aqil *et al*., 2005;). The discovery of new compounds that prevents or blocks resistance mechanisms can improve or eradicate the activities of these multidrug resistance pathogens (Sibanda and Okoh, 2008).

This study is taken to assess the combination potentials of *Cocos nucifera* husk extract with some antibiotics. The research is aimed at enhancing the potentials of the antimicrobial properties of the plant with a view to discover new antimicrobial drugs effective against some pathogenic organisms.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

The plant specimens were collected from the vicinity of the Research Farm of the Obafemi Awolowo University, Ile Ife, Nigeria and identified by the curator of the Herbarium at the Department of Botany, Obafemi Awolowo University, and a voucher specimen kept there.

5.2.2 Preparation of Extract

The husk of the coconut was sun–dried, milled and sieved manually to obtain the fine powdered particles. About 50 g dried powdered husk of the plant was added to 200 ml of 95% n-Hexane using Soxhlet extraction method at room temperature and for 48 hr. The mixture was then filtered using Whatman no1filter paper. The filtrates of each extraction were pooled together and concentrated to dryness *in vacuo* using a rotary evaporator to remove the n - Hexane. The concentrated extract was then allowed to dry at room temperature to a constant weight. For the aqueous extract, about 50 g of the powdered extract was dissolved in 500 ml of sterile distill water for 24 h with shaking. The resultant extracts

were centrifuged at 3000 rpm for 5 min at 4 ^{o}C. The supernatant was filtered through a Whatman No. 1 filter paper and the filtrate was lyophilized.

5.2.3 Test bacterial strains

The bacterial isolates used in this study included reference, environmental and clinical strains (6) obtained from the South African Bureau of Standard (SABS), *Vibrio* (6) and *Listeria* pathogens (6). The Inocula of the test organisms were prepared using the colony suspension method (EUCAST, 2000). Colonies picked from 24 hr old cultures grown on nutrient agar were used to make suspension of the test organisms in saline solution to give an optical density of approximately 0.1 at 600nm. The suspension was then diluted 1:100 by transfer of 0.1 ml of the bacterial suspension to 9.9 ml of sterile nutrient broth to give approximately 5×10^5 CFU/ml bacteria.

5.2.4 Antibiotics used in this study

The following antibiotics were used in this study: ampicillin sodium salt (Calbiochem), penicillin G sodium (Duchefa), amoxicillin (Duchefa), chloramphenicol (Duchefa), ciprofloxacin (Fluka) and tetracycline hydrochloride (Duchefa).

5.2.5 Antibacterial susceptibility test.

The susceptibility screening of the test bacteria to both crude extracts and standard antibiotics were done in accordance with the method of Irobi *et al.* (1994) and Akinpelu *et al*. (2008). The inoculum size of each test strain was standardized at 5×10^5 CFU/ml using McFarland Nephelometer standard. Sterile Mueller-Hinton agar plates were seeded with test bacterial

strains and allowed to stand at 37 ^{o}C for 3 hr. Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells filled with the solution of the extracts and antibiotics taking care not to allow spillage of the solution onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 1 hr to allow proper diffusion of the extract and antibiotics into the media and thereafter incubated at 37 ^{o}C for 24 hr, after which they were observed for zones of inhibition. The effects of the extracts on the test bacterial isolates were compared with those of tetracycline and ampicillin standard antibiotics at a concentration of 1mg/ml and 10 μg/ml respectively.

5.2.6 Determination of the minimum inhibitory concentrations (MIC)

The MIC of the crude aqueous and n - Hexane extract was carried out using the method of Akinpelu and Kolawole (2004). Two-fold dilutions of the extracts and antibiotics were prepared and 2 ml aliquot of different concentrations of the solution were added to 18 ml of pre-sterilized molten Mueller - Hinton agar at 40 ^{o}C to give final concentration regimes of 5.0 to 0.156 mg/ml and 0.01 to 0.5 mg/ml. The media were then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry under a laminar flow before streaking with 18 hr old bacterial cultures. The plates were later incubated at 37 ^{o}C for up to 72 hr after which they were examined for the presence or absence of growth. The MIC was taken as the least concentration of extracts that prevented the visible growth of the test bacteria.

5.2.7 Antibiotic-extract combination experiment

5.2.7.1 The time-kill method

The effect of combinations of the crude extracts and antibiotics was assessed and evaluated using time-kill assay method (Pankey and Ashcraft, 2005). Controls consisting of nutrient broth incorporated with the extract and the respective antibiotic without the test organism at the test concentrations were included in each experiment. The test and control flasks were inoculated with each test standardized organism to a final inoculum density of approximately 5×10^5 CFU/ml. Immediately after inoculation, aliquots (100 µl) of the negative control flasks were taken, serially diluted in sterile physiological saline and plated on nutrient agar in order to determine the zero hour counts. The test flasks were incubated at 37 ^{o}C with shaking at 120 rpm. After 24 hr of incubation, samples were taken from control and each test flasks. The samples from the test flask were transferred to a recovery medium containing 3% "Tween-80" to neutralize the effects of the crude extracts and antibiotics carry-overs from the test suspensions. Both samples from the recovery medium and the control flasks were then serially diluted in sterile physiological saline and plated on nutrient agar in duplicates. The plates were incubated at 37 ^{o}C for 24 hr; numbers of colonies were enumerated and expressed as Log_{10}.

5.2.7.2 The checkerboard method

The assay was done as described by (Mandal *et al*., 2004). Plates were inoculated with standardized cultures by streaking in duplicates and incubated for 24 hr at 37 °C after which the MIC values were estimated.

The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic and extract combination permitting no visible growth of the test organisms on the plates (Mandal *et al*., 2004).

The FIC value for each agent was calculated using the formula:

FIC (antibiotic) = MIC of antibiotic in combination / MIC of antibiotic alone

FIC (extract) = MIC of extract in combination / MIC of extract alone

The interactions between the antibiotics and the extracts were assessed in terms of the FIC indices calculated using the formula:

FIC Index = ΣFIC = FIC (antibiotic) + FIC (plant extract)

5.2.8 DATA ANALYSIS

The means of the two methods (checkerboard and time-kill) were compared using independent t test of significance ($P<0.05$).

5.3 RESULTS

The zones of inhibition diameter (IZD) of the aqueous extract of *Cocos nucifera* husk ranged from 11 mm to 20 mm for the *Listeria* isolates, 10 mm to 22 mm for *Vibrio* isolates, 10 mm to 22 mm for the reference, environmental and clinical strains. While the inhibition zone diameter (IZD) of the n-Hexane extract ranged from 10 mm to 24 mm against *the Listeria* isolates, 09 mm to 21 mm for the *Vibrio* isolate and between 10 mm to 18 mm for the

reference, environmental and clinical strains. The antibiotics, tetracycline and ampicillin yielded zones of inhibition of 22-46 mm and 20-50 mm for the *Listeria* isolates, 10-40 mm and 10-40 mm for the *Vibrio* isolates and 18-38 mm and 14-28 mm for the reference, environmental and clinical strains respectively.

The results of these experiments revealed that crude extracts of the husk of *Cocos nucifera* exhibited antibacterial activities against almost all the test bacterial isolates comprising of both Gram-negative and Gram-positive bacteria made up of reference, clinical, environmental strains, as well as *Vibrio* and *Listeria* pathogens at a screening concentration of 5 mg/ml (Tables 5.1).

The MICs of the plant extracts and the antibiotics varied between 1 µg/ml and 5.0 mg/ml (Table 5.2). Specifically, the MICs of the aqueous extracts ranged from 0.6 to 2.5 mg/ml for the Listeria isolates; 0.6 to 5.0 mg/ml for Vibrio isolates; and 0.5 to 5.0 mg/ml for reference, environmental and clinical isolates. For the standard antibiotics, the ranges were 0.01 to 0.50 mg/ml for penicillin G, 0.01 – 0.25 mg/ml for amoxicillin; 0.01 to 0.016 mg/ml for ciprofloxacin; 0.01 to 0.50 mg/ml for chloramphenicol; 0.01 to 0.25 mg/ml for ampicillin; 0.01 to 0.50 mg/ml for tetracycline. The minimum bactericidal concentration (MBC) were also determined for all the susceptible organism and it ranged from 2.5 mg/ml and above (Table 5.2)

The time-kill data on the effects and interactions of the extracts and the antibiotics singly and in combinations are shown in Table 5.3. The interactions were considered synergistic if there was a decrease of $\geq$ 2 log10 CFU/ml in colony counts after 24 hr by the combination compared to the most active single agent (Pankey and Ashcraft, 2005). Additivity or indifference was described as a < 2 log10 CFU/ml change in the average viable counts after

24 hr for the combination, in comparison with the most active single drug. Antagonism was defined as a ≥ 2 log10 CFU/ml increase in colony counts after 24 hr by the combination compared with that by the most active single agent alone (Lee *et al*., 2006; Aiyegoro *et al*., 2009). The extracts showed ability to improve the bactericidal effect of the antibiotics on both Gram-positive and Gram-negative organisms. The highest bactericidal activity with a 5.6 Log_{10} reduction in cell density was produced by the combination of plant extract and Chloramphenicol against *Vibrio fluvialis*.

Synergy rate of 70% (Extract + Ampicillin; Extract + Amoxicillin), 90% (Extract + Penicillin G; Extract + Chloramphenicol), 80% (Extract + Ciprofloxacin) and 40% (Extract + Tetracycline) were observed on all the test isolates. Overall, synergistic response constituted about 72%, while indifference constituted about 28% respectively of all types of combinations of extract and antibiotics against all test organisms using the time kill method.

Table 5.4 shows the interactions of the extract-antibiotic combinations using the checkerboard method, combinations were classified as synergistic, if the FIC indices were < 1, additive if the

FIC indices were = 1, indifferent if the FIC indices were between 1 and 2 and antagonistic if the FIC indices were >2 (Kamatou *et al.*, 2006; Aiyegoro *et al*., 2009). Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC value was expressed as the average of the individual FIC values (Pankey and Ashcraft, 2005). About 67% of all the interactions were synergistic, while indifference interactions constituted about 33%. A comparison of the data for the time kill and checkerboard methods (Table 5.5) revealed that the degree of agreements between the two methods ranges from 50% to absolute agreement (100%).

5. 4 DISCUSSION

Combinations of plant extracts with antibiotics are considered to be a fundamental therapy in the treatment of infections and diseases. This experiment was carried out to assess and establish the combination potentials between antimicrobial drugs such as (fluoroquinolone and β-lactam) and plant extract against some pathogenic bacteria. The antimicrobial activity of plant extract with antibiotics against some bacteria pathogens were confirmed in this study and it was observed that synergism was possible with all the antimicrobial drugs tested, No antagonism was observed and the degree of agreement between the plant extract and all the antibiotics using the checkerboard method ranged between 50-100 percent. The chequerboard study is done in order to confirm the time-kill method while the time kill studies reveal the ability of the extract to improve the bactericidal effects of the antibiotics both on Gram negative and Gram positive bacteria.

Our time-kill studies are based on comparing the killing rate of combination to that of the individual agent. Synergy was observered at 1/4 MIC level of combination of (Extract + penicillin G; chloramphenicol and ciprofloxacin) against the entire tested organism. Suggesting that the magnitude of the inhibition at 24 hr was consistent with the criteria of synergism. The inhibitory effect of the combination of (Extract + ciprofloxacin; ampicillin, penicillin G and chloramphenicol) at 2 times the MIC persisted against all tested isolate except for *Vibrio vulnificus* and *Escherichia coli.*

The *in vitro* efficacy of Extract + Penicillin G; Extract + Chloramphenicol; Extract + Ciprofloxacin was superior to that of Extract + Tetracycline in producing synergy against all tested bacteria, a similar observation was reported by Otsuki and Nishino (1996), and this has

been attributed to the interaction of quinolones with the outer membrane as chelating agents raising the permeability of the outer membrane to β-lactam antibiotics. The mechanism by which such combinations achieve synergy is believed to be the facilitation of entry of β-lactam antibiotics into cells after partial disruption of the cell wall through the action of quinolones (Otsuki and Nishino, 1996). It is likely that the activities of substances found in plant extracts on ribosome structure and bacterial enzymes inhibition could bring about the observed synergism profile between plant extracts and inhibitors of protein and cell wall synthesis; however, the understanding of synergism mechanism is fundamental to development of new pharmacological agents to treat infectious diseases. Our observation suggests the clinical usefulness of this antimicrobial combination therapy be further established by developing an animal model to investigate this phenomenon. It would be an additional knowledge if clinical studies are carried out to test the relevance of our findings. As an alternative method the checkerboard method was done to observe changes in the MIC values (Mandal *et al*., 2004). Using the FIC indices, significantly, synergy was detected in all manner of combination of extract with all the antibiotics (except penicillin) against *Escherichia coli* and *Vibrio vulnificus* (Gram negative organisms) this is similar to the findings of Esimone *et al*. (2006). The enhanced antimicrobial effect of the combination could be attributed to the perturbation of the cell membrane coupled with the action of β-lactams on the transpeptidation of the cell membrane structure.

As seen in table 5.3, the antimicrobial mechanism of the drugs used in this study varies. the cell wall synthesis inhibitors (Penicillin) were those that presented the strongest synergistic effect of 90% followed by the protein synthesis inhibitor (chloramphenicol) and then the nucleic acid inhibitor (ciprofloxacin) using both the time-kill and checker board assay method. The synergistic potentials were promising for the combinations between the extract

of *C. nucifera* and tetracycline in both assay methods. The presence of condensed tannin contained in the extract of *Cocos nucifera* husk was comprised of flavonoids subunits catechin, epicatechin, epigallocatechin, and epicatechin-3-*O*-gallate (Alviano *et al*., 2004) which has antimicrobial and resistance modifying potentials. The mechanism by which this naturally occurring Tannins work has been studied and they are reported to act by iron deprivation, hydrogen binding or specific interactions with vital proteins such as enzymes in microbial cells. (Akinpelu *et al.*, 2008) this study is similar to such.

The use of medicinal plants to treat infectious diseases has been reported by several researchers. Antimicrobial combination therapy may be used frequently for diagnosis purpose such as to extend spectrum coverage, prevent the emergence of resistant mutants and gain synergy between antimicrobials (Kamatou *et al*., 2006). Combination therapy is often recommended for empirical treatment of bacterial infections in intensive care units, where monotherapy is not likely to cover all potential pathogens, and the emergence of resistance is a potential threat.

Zhao *et al*. (2001); Aiyegoro *et al*. (2009) has revealed in their studies that some plant derived compounds can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site in the cell wall. Kumar and Schweitzer, (2005) also attributed effect of antibiotic resistance in pathogenic organism to bacteria efflux pump system. For example Stermitz *et al*. (2000) reported the compound 5'-methoxyhydnocarpin, isolated from *Berberis fremontii* against *S. aureus* to be an inhibitor of efflux pump NorA, so it may be that the husk of *C.nucifera* may contain broad spectrum efflux pump inhibitor compounds which could enhance its combination interaction with antibiotics against both Gram positive and Gram negative bacteria.

It has been suggested that, plants also produce multi-drug resistance (MDR) inhibitors in addition to the production of intrinsic antimicrobial compounds, which enhance the activity of the antimicrobial compounds (Stermitz *et al*., 2000). This report was demonstrated by Tegos *et al*. (2002), who showed that the activity of some plant antimicrobials against gram positive and gram negative organisms was significantly enhanced by synthetic MDR inhibitors of MDR efflux proteins. These findings by Tegos *et al*. (2002) suggested to us that plants can be potential sources of natural MDR inhibitors that can potentially improve the performance of antibiotics against resistant strains.

The results of the present study seem to be promising and may enhance its use as a natural product, showing the potential of this plant in the treatment of infectious diseases caused by organisms implicated in food and wound infections, this corroborate the findings of Monica (2000) who suggested that coconut oil contains antimicrobial agents which could make it suitable for medicinal purposes like the treatment of wound infection and urinary tract infection. The antimicrobial activities of plant extracts on *Vibrio* and *Listeria* strains were confirmed and synergism was possible with all the antimicrobial drugs tested. All antibiotics presented synergism with the extracts of *C. nucifera* against all tested bacteria, although with vary antimicrobial activity profile.

Nevertheless, our research experiment has suggested the potential usefulness of extract of *Cocos nucifera* plant and some front line antibiotics as a combination therapy for the treatment of *V. vulnificus, V. fluvialis, L. ivanovii* and some reference strain bacterial infections. The detection of synergy in this experiment demonstrates the ability of this plant as a potential source of antibiotic resistance modifying compounds. Hence the need to further

analyze and identify the possible compounds in the plant that could be responsible for the synergism observed as well as an *in vivo* studies of the mechanism of action of the compounds in combination therapy.

5.5 Conclusion

In summary, the combination interactions of extract of *Cocos nucifera* husk with six front line antibiotics were studied as far as synergy were concerned in a number of some pathogenic organism implicated in food and wound infections. The result from this study indicates that the husk of *C. nucifera* possess some antimicrobial properties with a greater potency when used concurrently with antibiotics against the test pathogens indicating the extract to be a promising plant to new choice of antimicrobial compound for the treatment of infectious disease. Although *in vivo* studies need to be confirmed by *in vitro* findings, the data obtained support the use of antimicrobial combinations including glycopeptides, fluoroquinolones and β-lactams with plant extract in initial empirical therapy of severe infections potentially sustained by strains of these pathogenic organisms such as *Vibrio* and *Listeria* pathogens.

Table 5.1. Antibacterial activity profile of crude extracts of the husk fiber of *Cocos nucifera.*

Bacterial Isolate.	Aqueous Extract (5mg/ml)	n-Hexane Extract (5mg/ml)	Tetracycline 1mg/ml	Ampicillin 10µg/ml
***Listeria ivanovii* LEL1**	15 ± 0.0	18 ± 0.0	31 ± 0.4	21 ± 0.2
***Listeria ivanovii* LEL2**	15 ± 0.1	16 ± 0.0	30 ± 0.2	26 ± 0.1
***Listeria grayi* LAL3**	16 ± 0.0	19 ± 0.1	34 ± 0.5	25 ± 0.2
***Listeria ivanovii* LEL17**	18 ± 0.2	18 ± 0.0	32 ± 0.1	25 ± 0.4
***Listeria ivanovii* LAL10**	17 ± 0.1	16 ± 0.0	46 ± 0.2	32 ± 0.2
***Listeria monocytogenes* LAL8**	16 ± 0.2	14 ±0.1	34 ± 0.4	22 ± 0.1
***Vibrio vulnificus* EL047**	17 ± 0.2	18 ± 0.1	18 ± 0.2	25 ± 0.0
***Vibrio metschnkovii* EL008**	14 ± 0.0	12 ± 0.0	28 ± 0.2	19 ± 0.2
***Vibrio* specie EL009**	13 ± 0.2	17 ± 0.1	21 ± 0.4	20 ± 0.2
***Vibrio fluvialis* EL041**	14 ± 0.1	11 ± 0.0	22 ± 0.2	21 ± 0.1
***Vibrio vulnificus* EL039**	14 ± 0.0	11 ± 0.2	26 ± 0.1	24 ± 0.2
***Vibrio fluvialis* AL019**	16 ± 0.2	18 ± 0.0	30 ± 0.1	20 ± 0.4
***E.coli* ATCC 8739 Ω**	11 ± 0.2	12 ± 0.0	27 ± 0.0	28 ± 0.1
***S. faecalis* ATCC 29212 Ω**	15 ± 0.1	14 ± 0.2	27 ± 0.2	21 ± 0.1
Acinetobacter calcaoceuticus anitratus CSIR Ω	15 ± 0.2	14 ± 0.2	32 ± 0.4	14 ± 0.1
Bacillus substilis ∞	11 ± 0.1	12 ± 0.0	22 ± 0.2	28 ± 0.1
Shigella flexineri ∞	14 ± 0.2	15 ± 0.0	30 ± 0.1	16 ± 0.2
Staphylococcus aureus α	13 ± 0.2	16 ± 0.1	28 ± 0.0	19 ± 0.2

α- clinical strains; ∞- environmental strains; and Ω- reference strains.

Table 5.2 Determination of the MIC & MBC of the susceptible organisms on *C.nucifera husk* fiber extract.

Test organism	Gram reaction	Aqueous extract	n-Hexane extract
***Listeria ivanovii* LEL1**	+	2.5 / >5.0	0.625 / 2.5
***Listeria ivanovii* LEL2**	+	0.625 / 2.5	1.25 / 5.0
***Listeria grayi* LEL3**	+	0.625 / 5.0	0.625 / 2.5
***Listeria ivanovii* LEL17**	+	2.5 / >5.0	0.625 / 5.0
***Listeria ivanovii* LAL10**	+	0.625 / 1.25	5.0 / >5.0
***Listeria monocytogenes* LAL8**	+	2.5 / >5.0	2.5 / 5.0
***Vibrio vulnificus* EL047**	-	0.625 / 2.5	0.625 / 2.5
***Vibrio metschnkovii* EL008**	-	0.625 / 5.0	1.25 / 2.5
***Vibrio* specie EL009**	-	5.0 / >5.0	2.5 / 5.0
***Vibrio fluvialis* EL041**	-	0.625 / 2.5	0.625 / 2.5
***Vibrio vulnificus* EL039**	-	0.625 / 2.5	0.625 / 5.0
***Vibrio fluvialis* AL019**	-	ND	1.25 / 2.5
***E.coli* ATCC 8739 Ω**	-	0.625 / 2.5	5.0 / >5.0
***S. faecalis* ATCC 29212 Ω**	+	0.625 / 5.0	0.312 / 1.25
Acinetobacter calcaoceuticus anitratus CSIR Ω	-	ND	2.5 / 5.0
Bacillus substilis∞	+	ND	2.5 / 5.0
Shigella flexineri∞	-	ND	1.25 / 2.5
Staphylococcus aureusα	+	0.625 / 5.0	1.25 / 5.0

ND = Not determined; Ω = reference strain; α = clinical strain and ∞= environmental strain.

Table 5.3. The minimum inhibitory concentrations (MICs) of selected antibiotics against bacterial isolates.

Bacterial isolates	**AMP**	**PEN G**	**AMOX**	**CHLOR**	**CIPRO**	**TET**
***E.coli* ATCC 8739**	0.0312	0.031	0.016	0.125	0.016	0.016
***S. faecalis* ATCC 29212**	0.0312	0.0625	0.062	0.016	0.016	0.016
Listeria ivanovii	0.016	0.016	0.016	0.016	0.016	0.016
Vibrio vulnificus	0.25	0.016	0.25	0.031	0.016	0.016
Vibrio fluvialis	0.25	0.50	0.25	0.50	0.016	0.50

AMP=ampicillin; PEN G= penicillin G; AMOX= amoxicillin; CHLO= chloramphenicol; CIPRO= ciprofloxacin; TET= tetracycline.

Table 5.4. *In vitro* antibacterial activity of extracts-antibiotic combinations by Time-Kill method.

Bacterial isolate	EXT + AMP		EXT + PEN G		EXT + AMOX		EXT + CHLO		EXT + CIPRO		EXT + TET	
	¼ MIC	2 MIC	¼ MIC	2 MIC	¼ MIC	2 MIC	¼ MIC	2 MIC	¼ MIC	2 MIC	¼ MIC	2 MIC
***Escherichia coli* ATCC 8739**	-1.3± 0.11 (I)	-2.4± 0.14 (S)	-2.6± 0.12 (S)	-1.4± 0.10 (I)	-2.4± 0.24 (S)	-1.8± 0.22 (I)	-3.2± 0.40 (S)	-1.4± 0.22 (I)	-4.4± 0.14 (S)	-4.6± 0.42 (S)	-1.6± 0.14 (I)	-2.4± 0.12 (S)
***Streptococcus faecalis* ATCC 29212**	-2.4± 0.22 (S)	-2.6± 0.20 (S)	-2.2± 0.10 (S)	-2.0± 0.11 (S)	-1.2± 0.14 (I)	-3.8± 0.12 (S)	-2.0± 0.11 (S)	-3.4± 0.10 (S)	-3.8± 0.18 (S)	-4.2± 0.22 (S)	-1.8± 0.11 (I)	0.56± 0.12 (I)
***Listeria ivanovii* LEL_3**	-3.2± 0.14 (S)	-4.4± 0.32 (S)	-4.0± 0.20 (S)	-3.2± 0.10 (S)	-3.6± 0.23 (S)	-2.8± 0.12 (S)	-2.4± 0.11 (S)	-2.0± 0.10 (S)	-4.6± 0.20 (S)	-2.8± 0.11 (S)	-2.0± 0.21 (S)	-2.8± 0.20 (S)
***Vibrio vulnificus* EL039**	-2.8± 0.16 (S)	-1.6± 0.11 (I)	-4.2± 0.23 (S)	-3.4± 0.17 (S)	-2.2± 0.21 (S)	-1.2± 0.11 (I)	-3.8± 0.40 (S)	-4.0± 0.31 (S)	-2.1± 0.21 (S)	-1.4± 0.23 (I)	0.56± 0.12 (I)	-1.2± 0.22 (I)
***Vibrio fluvialis* EL041**	-1.9± 0.10 (I)	-2.3± 0.11 (S)	-3.6± 0.22 (S)	-2.4± 0.10 (S)	-1.8± 0.40 (I)	-3.4± 0.11 (S)	-3.2± 0.20 (S)	-5.6± 0.22 (S)	-3.0± 0.10 (S)	-1.8± 0.21 (I)	-1.4± 0.11 (I)	-2.3± 0.20 (S)

I= indifferent; S= synergy; MIC=minimum inhibitory concentrations; AMP= ampicillin; PEN G= penicillin G; AMOX= amoxicillin; CHLO= chloramphenicol; CIPRO= ciprofloxacin, TET=tetracycline

Table 5.5. ***In vitro*** **antibacterial activity of extracts-antibiotic combinations by Chequerboard method.**

Bacterial isolate	**EXT + AMP**	**EXT + PEN G**	**EXT + AMOX**	**EXT + CHLO**	**EXT + CIPRO**	**EXT + TET**
E. coli **ATCC 8739**	0.3 (S)	1.9 (I)	0.6 (S)	0.4 (S)	0.4 (S)	0.8 (S)
S. faecalis **ATCC 29212**	1.6 (I)	0.8 (S)	0.8 (S)	1.4 (I)	0.8 (S)	0.3 (S)
L. ivanovii **LEL_3**	1.8 (I)	0.9 (S)	1.4 (I)	0.4 (S)	0.4 (S)	1.2 (I)
V. vulnificus **EL039**	0.6 (S)	1.2 (I)	0.7 (S)	0.6 (S)	0.6 (S)	0.6 (S)
V. fluvialis **EL041**	0.4 (S)	0.8 (S)	1.4 (I)	1.2 (I)	1.1 (I)	0.4 (S)

I= indifferent; S= synergy; AMP= ampicillin; PEN G= penicillin G; AMOX= amoxicillin; CHLO= chloramphenicol; CIPRO= ciprofloxacin, TET=tetracycline

Table 5.6. Comparison of results by time kill and checkerboard methods.

	NUMBER OF TEST STRAINS FROM A TOTAL OF TEN											
	EXT + AMP		**EXT + PEN G**		**EXT + AMOX**		**EXT + CHLO**		**EXT + CIPRO**		**EXT + TET**	
OBSERVATIONS												
	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB
Synergy	7	6	9	6	7	6	9	6	8	8	4	8
Indifference	3	4	1	4	3	4	1	4	2	2	6	2
Means	5	5	5	5	5	5	5	5	5	5	5	5
(Tables 5.3 & 5.4)												

TK= time kill; CB= checker board; EXT= extract; AMP= ampicillin; PEN G= penicillin G; AMOX= amoxicillin; CHLO= chloramphenicol; and TET= tetracycline.

REFERENCES

Aburjal T, Darwish RM, Al-Khalil S, Mahgzah A, Al-Abbdi A (2001). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Pseudomonas aeruginosa*. *J Ethnopharm. 76*: 39-44

Aiyegoro OA, Afolayan AJ, Okoh AI (2009). Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. *Biol Res.* 42: 327-338.

Akinpelu DA, Aiyegoro OA, Okoh AI (2008). *In vitro* antibacterial and phytochemical properties of crude extract of stem bark of *Afzelia africana* (Smith). *Afr J Biotech.* 7(20): 3662-3667.

Akinpelu DA, Kolawole DO (2004). Phytochemical and antimicrobial activity of leaf extract of *Piliostigma thonningii* (Schum). *Sci. Focus J*. 7: 64-70.

Ali NH, Kazmi SU, Faizi S (2007). Activity of synergistic combination of Amoxy-*cassia* against Salmonella. *Pak J Pharm Sci*. 20: 140-145.

Alviano DS, Rodrigues KF, Leitão SG, Rodrigues ML, Matheus ME, Fernandes PD, Antoniolli AR, Alviano CS (2004). Antinociceptive and free radical scavenging activities of *Cocos nucifera* L. (Palmae) husk fiber aqueous extract, *J Ethnopharmacol.* 92: 269–273.

Aqil F, Khan MSA, Owais M, Ahmad I (2005). Effect of certain bioactive plant extracts on clinical isolates of ß-lactamase producing methicillin resistant *Staphylococcus aureus. J Basic Microbiol. 45*: 106-114.

Beg AZ, Ahmad I (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J Ethnopharm. 74*: 113-123.

Betoni JEC, Mantovani RP, Barbosa LN, Di Stasi LC, Fernandes A Jnr (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. *Mem Inst. Oswaldo Cruz.* 101(4): 387-390.

Centers for Disease Control and Prevention (2005). Preliminary Food Net data on the incidence of infection with pathogens transmitted commonly through food—10 states, United States. *MMWR Morb Mortal Wkly Rep*. 55: 392–5.

Chang PC, Li HY, Tang HJ, Liu JW, Wang JJ, Chuang YC (2007). *In vitro* synergy of baicalein and gentamicin against Vancomycin resistant *Enterococcus. J Microbiol Immunol Infect*. 40: 56-61.

Cheung CR, Chou JS, Shaw FJ (2005). Multiple forms and functions of *Candida rugosa* lipase. *Biotech and Applied Biochem.* (19): 93-97.

Esimone CO, Iroha IR, Ibezim, EC, Okeh CO, Okpana EM (2006). *In vitro* evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus. Afr J Biotechnol.* 5(11): 1082-1086.

EUCAST (2000) of the European Society of Clinical Microbiology and Infectious Diseases.

Horiuchi K, Shiota S, Kuroda T, Hatano T, Yoshida T, Tsuchiya T (2007). Potentiation of antimicrobial activity of aminoglycosides by carnosol from *Salvia officinalis*. *Biol Pharm Bull* 30: 287-290.

Hugo CJ, Segers P, Vancanneyt M, Kersters K (2003). *Chryseobacterium joostei* species isolated from dairy environment. *Int J Syst Evol Microbiol* (53): 771-777.

Hugo WB, Russell AD (2003). Pharmaceutical Microbiology; 6th ed. *Blackwell Science Publishers, Oxford,* United Kingdom. 91-129.

Ibezim EC, Esimone CO, Nnamani PO, Onyishi IV, Brown SA, Obodo CE (2006). *In vitro* study of the interaction between some fluoroquinolones and extracts of *kola nitida* seed (2006). *Afr J Biotech.* (19)5: 1781-1784.

Irobi ON, Moo-Young M, Anderson WA, Daramola SO (1994). Antimicrobial activity of the bark of *Bridelia ferruginea* (Euphorbiaceae). *Int J Pharmacog.* 34: 87-90.

Jones AL, Hulett MD, Altin JG, Hogg P, Parish CR (2004). Plasminogen is tethered with high affinity to the cell surface by the plasma protein, histidine-rich glycoprotein. *J Biol chem.* (37): 38267-76.

Junior AF, Balestrin EC, Betoni JE, Orsi Rde O, da Cunha Mde L, Montelli AC (2005). Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem. Inst. Oswaldo. Cruz*. 100: 563-566.

Kamatou GPP, van Zyl RL, van Vuuren SF, Viljoen AM et al. (2006). Chemical Composition, Leaf Trichome Types and Biological Activities of the Essential Oils of Four Related Salvia Species Indigenous to Southern Africa. *J Ess Oil Res.* 18: 72-79.

Kumar A, Schweizer HP (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake. Adv. Drug Deliv. Rev. 57: 1486-1513

Lee JY, Oh WS, Ko KS, Heo ST, Moon CS, Ki HK, Kiem S, Peck KR, Song JH (1998). Synergy of arbekacin-based combinations against vancomycin hetero-intermediate *Staphylococcus aureus*. *J Korean Med Sci.* 21: 188-192.

Levinson W, Jawetz E (2002). Medical microbiology and immunology: Examination and board review. International ed (7th), Lange Medical Books/McGraw-Hill, New York, pp 73-83.

Mandal S, Mandal MD, Pal NK (2004). Evaluation of combination effect of ciprofloxacin and cefazolin against *Salmonella enterica* serovar *typhi* isolates by *in vitro* methods. *Cal Mel J.* 2(2): 22-26.

Mead PS, Slutsker L, Dietz *et al.* (1999). Food-related illness and disease in the United States. *Emerg Infect Dis.* (5):1-20.

Nascimento GGF, Locatelli J, Freitas PC, Silva GL (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz. J. Microbiol* 31: 247-256.

Otsuki M, T Nishino (**1996).** The synergic effects of quinolones and oral cephem antibiotics on *Serratia marcescens. J Antimicrob Chemother*. 38: 771-776.

Pankey G, Ashcraft D, Patel N (2005). *In vitro* Synergy of Daptomycin plus Rifampin against *Enterococcus faecium* Resistant to both Linezolid and Vancomycin. *Antimicrob Agents Chemother.* 49: 5166-5168.

Shiizu M, Shiota S, Mizushima T, Ito H, Hatano T, Yoshida T, Tsuchiya T (2001). Marked potentiating of activity of beta-lactams against methicilin-resistant *Staphylococcus aureus* by corilagin. *Antimicrob Agents Chemother*. 45: 3198-3201.

Sibanda T, Okoh AI (2008). *In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics. *Afr J Biotech.* 7 (11): 1672-1678.

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K (2000). Synergy in a medicinal plant: Antimicrobial action of Berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Appl Bio Sciences*. 97(4): 1433-1437.

Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials, Antimicrob. Agents Chemother 46(10): 3133-3141.

Wang BC, Yang XS, Yang ZC, Wang Q, Ran L (2003). The synergistic activity of antibiotics combined with eight traditional Chinese medicines against two different strains of *Staphylococcus aureus. Colloids and surfaces B Biointerf.* 41(2-3): 79-81.

Yam TS, Hamilton-Miller JM, Shah S (1998). The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. *J Antimicrob Chemother*. 42: 211-216.

Zhao W, Hu Z, Okubo S, Har Y, Shimamura T (2001). Mechanism of synergy between epigallocatechin gallate and b-Lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 45 (6): 1737–1742.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION.

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CHAPTER SIX

DISCUSSION AND CONCLUSION

6.1 Discussion

Diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms of resistant to antibiotic therapies (Levy and Marshall, 2004). Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns (Iwu *et al.,* 1999). The global emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Hancock, 2005).

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being (Iwu *et al.,* 1999). Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent (Betoni *et al.,* 2006; Shibata *et al.,* 2005). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel, 2006; Cowan, 1999).

A number of plant derived compounds have been reported to exhibit *in vitro* synergy with antibiotics against resistant strains of pathogenic bacteria. The synergy has been reported in a

few cases, to involve dual targeting or mutual interference between the plant compounds and antibiotics (Zhao *et al.,* 2001; Yam *et al.,* 1998). To a greater extent, however, the synergy has been attributed to inhibition of antibiotic MDR efflux proteins (Smith *et al.,* 2007; Marquez *et al.,* 2005; Oluwatuyi *et al.,* 2004; Aiyegoro *et al*., 2009). It is the prospects of obtaining potent broad spectrum MDR efflux pump inhibitors from plants that has been considered attractive (Lomovskaya and Bostain, 2006; Lewis and Ausubel, 2006). Obtaining drug preparations based on combinations between efflux pump inhibitors and antibiotics can help to recover the clinical efficacy of old antibiotics that have been rendered ineffective due to resistance. The consistent use and unending demand for traditional therapies has brought about the need to ascertain the safety and efficacy of medicinal plants.

In this study, we investigated the husk extract of *Cocos nucifera*, a plant of the family Aracaceae which is widely distributed in West and Central Africa where it is valued for its medicinal properties against some food borne pathogenic infection as well as their combination potential with synthetic drugs. In the antimicrobial activity assays, the aqueous and n–Hexane extracts of the husk were evaluated for their activity against *Listeria* and *Vibrio* pathogens as well as some other bacteria isolates. In the antilisterial activity assay, the aqueous extract showed activity against 29 of the tested strains wit MIC values ranging from 0.6 – 2.5 mg/ml while the n–Hexane extract was active against 30 of the *Listeria* pathogen tested with and MIC value ranging from 0.6 – 5.0 mg/ml. The listericidal activities of the extract suggest that the crude aqueous and n-Hexane extracts of *Cocos nucifera* could be bacteriostatic or bactericidal depending on time of exposure and concentration.

In the antivibriol activity assay, the aqueous and n–Hexane extract of the husk were investigated for their activity against forty–five *Vibrio* strains isolated from waste water effluent within the Eastern Cape Province, South Africa. 37 of the tested *Vibrio* pathogens were susceptible to the aqueous extract at an MIC range of 0.6 – 5.0 mg/ml and 38 susceptible to the n–Hexane extract at an MIC value of 0.3 – 5.0 mg/ml. Also from the total of 25 bacteria strains tested, 17 were susceptible to the aqueous extract and 21 susceptible to the n–Hexane extract all at an MIC value ranging between 0.6 – 5.0 mg/ml respectively.

In the study of the effect of combinations between the extracts of the plant and antibiotics, combinations of the n-Hexane extract and the antibiotics penicillin G, amoxicillin, tetracycline, chloramphenicol, ampicillin and ciprofloxacin were investigated by means of the fractional inhibitory concentration (FIC) indices as well as by the use of time-kill assays. The time kill assay was used to assess the effect of combinations of the husk extracts of the plant and antibiotics. The FIC method was based on a comparison of the killing rate of the combination to that of the individual agents. Overall, synergy rate of above 50% was detected for combinations involving all the antibiotics with the plant extract. Since synergy was not specific to any class of antibiotics in this experiment, this suggests that crude extracts of this plant could contain a mixture of compounds that can enhance the activity of different antibiotics. Alternatively, checkerboard method was also used to detect synergy; this is based on the increased susceptibility of the test organism to the presence of both antimicrobial agents which is reflected by changes in the MIC values (Odds, 2003). In comparison with the time kill assay, the checkerboard method, also detected synergy against some of the tested bacteria revealing some degree of agreements between the two methods ranges from 50% to absolute agreement (100%) for the plant extract.

The time-kill assay and the agar dilution checkerboard method are the preferred methods used in combinations involving crude plant extract and antibiotics as they provides detailed information on the bactericidal activity of the antibiotic combination (Darwish *et al*., 2002). The findings of Tegos *et al*. (2002) have provided a platform for us to base the potential actions of plant derived antimicrobial compounds and other compounds with no intrinsic antimicrobial value. Marquez *et al*. (2005) and Smith *et al*. (2007) in their investigations has established that crude extracts of some medicinal plants and some pure compounds from such plants can potentiate the activity of antibiotics *in vitro.* The *in vitro* antimicrobial profile of our study plant has been reported in this work as well as by some other authors (Esquenazi *et al*., 2002; Alviano *et al*., 2004). Nevertheless, further investigation is needed in order to identify and purify the active components in the extracts.

It is therefore recommended that further investigation should address a bioassay guided fractionation of the extract of the plant in a bid to isolate the compounds responsible for the antimicrobial as well as the potentiating of the antibiotic activity.

6.2 Conclusion.

The results from this research revealed some significant findings on the therapeutic potentials of the husk of *Cocos nucifera* and also tend to confirm the saying by Lewis and Elvin – Lewis, (1995) that says plants play a vital role in the existence and survival of man. The search for more natural product especially of plant origin is a continuous efforts taking place in different research laboratories all over the universe, with the expectation of reducing and

totally combating the existing and emerging drug resistant pathogens. The following conclusion from the work therefore indicates:

- A vital *in vitro* time kill antilisterial activities exhibited by the plant against a wide range of food borne pathogenic organism supporting its use in folklore remedy against infections caused by this organisms.
- The anti*vibrio* activities of the crude extract of the plant of *Cocos nucifera* supporting its use for the treatment of human tropical infections.
- The *in vitro* extract – antibiotics interaction demonstrate the potentials of this plant as a candidate for bioprospecting in food and pharmaceutical industry.
- The antimicrobial potentials of this plant has open up a scope for future utilization of these agro wastes for therapeutic purposes.

REFERENCES

Aiyegoro OA, Afolayan AJ, Okoh AI (2009). Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. *Biol. Res.* 42: 327-338.

Alviano DS, Rodrigues KF, Leita˜o SG, Rodrigues ML, Matheus ME, Fernandes PD *et al* (2004). Antinociceptive and free radical scavering activities of *Cocos nucifera* L. (Palmae) husk fiber aqueous extract. *J Ethnopharm:* (92) 269–73.

Betoni JEC, Mantovani RP, Barbosa LN, Di-Stasi LC, Fernandes A (2006). Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. *Mem Inst Oswaldo Cruz.* 101 No. 4

Cowan MM (1999). Plant Products as Antimicrobial Agents. *Clin Microbiol Rev*. 12(4): 564-582.

Darwish RM, Aburjai T, Al-Khalil S, and Mahafzah A (2002). Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. *J Ethnopharm*. 79: 359-364.

Esquenazi D, Wigg MD, Miranda MM, Rodriguez HM, Tostes JB, Rozental S *et al*. (2002). Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (Palmae) husk fiber extract. *Res Microbiol*. 153:647–52.

Hancock EW (2005). Mechanisms of action of newer antibiotics for Gram-positive pathogens. *Lancet Infect. Dis*. 5(4): 209-218.

Iwu MW, Duncan AR, Okunji CO (1999). New antimicrobials of plant origin. Janick J (ed.), Perspectives on new crops and new uses. 457-462.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med*. 10: S122-S129.

Lewis K, Ausubel FM (2006). Prospects for plant-derived antibacterials. *Nat Biotech*. 24(12): 1504-1507.

Lewis WH, Elvis-Lewis MP (1995). Medical Botany John Wiley and Sons, New York. 808-823

Lomovskaya O, Bostian KA (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic - A vision for applied use. *Biochem Pharmacol*. 7(1): 910-918.

Marquez B, Neuville L, Moreau NJ, Genet JP, Santos AF, Andrade MCC, Sant Ana AEG (2005). Multidrug resistance reversal agent from *Jatropha elliptica*. *Phytochem*. 66: 1804-1811.

Odds FC (2003). Synergy, antagonism, and what the chequerboard puts between them. *J Antimicro Chemo.* 52(1): 1-10.

Oluwatuyi M, Kaatz GW, Gibbons S (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. *Phytochem.* 65(24): 3249-3254.

Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, Higuti T (2005). Alkyl Gallates, Intensifiers of β-Lactam Susceptibility in Methicillin-Resistant *Staphylococcus aureus* Antimicrob. *Agents Chemother.* 49(2): 549-555.

Smith ECJ, Williamson EM, Wareham N, Kaatz GW, Gibbons S (2007). Antibacterials and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. *Phytochem.* 68(2): 210-217.

Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials. *Antimicrob Agents Chemother.* 46(10): 3133-3141.

Yam TS, Hamilton-Miller JM, Shah S (1998). The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2 synthesis and beta-lactamase production in *Staphylococcus aureus*. *J Antimicrob Chemother.* 42(2): 211-216.

Zhao WH, Hu ZQ, Okubo S, Hara Y, Shimamura T (2001). Mechanism of synergy between Epigallocatechin gallate and β-Lactams against methicillin resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 45(6): 1737-1742.