

**ANTIMICROBIAL ACTIVITY OF *HIBISCUS SABDARIFFA*
AGAINST CLINICAL ISOLATES OF BACTERIA**

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DECLARATION

It is hereby declared that the work in this thesis is original and was carried out by the student and supervised by the supervisors below. Work from other authors where cited have been duly acknowledged. This work has not been concurrently submitted in candidature for any degree.

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ABSTRACT

There have been several reports on the antimicrobial activity of different herbal extracts. Medicinal plants are believed to be the best source for obtaining a variety of drugs. *Hibiscus sabdariffa L.* is a medicinal plant known for its nutritional delicacy and medicinal properties such as the treatment of diseases like hypertension, coughs, biliousness, boils and wounds.

Currently, the main therapy for bacterial infections is the use of synthetic antibiotics. However, the misuse and overuse of antibiotics has become the key factor for the emergence of drug resistant strains of several groups of microorganisms. Hence, the need to investigate the antimicrobial properties of medicinal plants.

The objectives of the study were to investigate the *in vitro* inhibitory activity of *Hibiscus sabdariffa* calyces, leaves and roots preparation against some clinical isolates of bacteria. Secondly, to assess the antimicrobial property of *Hibiscus sabdariffa* against the clinical resistant strains of bacteria. Lastly, to compare the *Hibiscus sabdariffa* aqueous and ethanolic extract effects on clinical isolates of bacteria.

The antibacterial activities of *H. sabdariffa* calyces, leaves and roots extracts (12.5, 25, 50, 100, 200mg/ml) were tested against clinical isolates of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella* sp., *Salmonella typhi*, *Staphylococcus aureus*); with reference strains of *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 49565, *Salmonella typhi* ATCC 19430, *Klebsiella pneumoniae* ATCC 33495, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923; and two each Extended Spectrum Beta-Lactamases and Methicillin-Resistant

Staphylococcus aureus strains using Agar Diffusion and Minimum Inhibitory Concentration Methods.

The calyces of the plant gave the highest inhibitory effect ($P < 0.05$) followed by the leaves. The roots did not exhibit antibacterial activity against the test bacteria ($P > 0.05$).

The calyces extract was potent against all the test organisms except *Proteus mirabilis* which was resistant to all the plant extracts. *Staphylococcus aureus* showed the highest susceptibility to the calyces extract. The aqueous leaves extracts were active against *Pseudomonas aeruginosa* and *Escherichia coli*.

The resistant strains especially MRSA were highly susceptible to the calyces extract with Minimum Inhibitory Concentration Methods values of 6.25mg/ml.

Ethanol extract of the calyces had a slightly higher antibacterial activity while the leaves were effective when extracted by water.

Therefore, the plant's calyces and leaves can be a source for the production of antibiotics which could significantly inhibit the growth of bacteria.

It is recommended that more studies should be done using different solvents for extracting only the important compounds from the *H. sabdariffa* plant. Purification measures would also be necessary. Additionally, there is a need to investigate the phytochemical compounds from the *H. sabdariffa* plant part extracts.

DEDICATION

This work is dedicated to God and my inspiring family especially the loving memory of my father, Mr. Robinson Lovell- Antiaye, who zealously believed in the pursuit of higher education and longed for this time in my life.



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TABLE OF CONTENTS

DECLARATION.....	i
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGEMENT.....	v
TABLE OF CONTENTS	vi
LIST OF FIGURES.....	viii
LIST OF PLATES.....	ix
LIST OF APPENDICES	x
LIST OF ABBREVIATIONS	xii
CHAPTER ONE.....	1
1.0 INTRODUCTION.....	1
1.1 Background.....	1
1.2 Problem Statement.....	2
1.3 Significance of Study	2
1.4 Hypothesis	3
1.5 Aim.....	3
1.6 Specific Objectives	3
CHAPTER TWO.....	5
2.0 LITERATURE REVIEW	5
2.1 Medicinal Plants	5
2.1.1 Uses of Medicinal plants	5
2.1.2 Plants as antimicrobials	6
2.1.2.1 Brief history of plants as antimicrobials.....	7
2.1.2.2 Present use of plants as antimicrobials	8
2.2 Plants with Promising Antimicrobial Activity	9
2.3 <i>Hibiscus sabdariffa</i> Plant	10
2.3.1 Local and Foreign Names.....	11
2.3.2 Uses	12
2.3.2.1 Nutritional use	12

2.3.2.2 Medicinal use.....	13
CHAPTER THREE.....	17
3.0 METHODOLOGY	17
3.1 Study Sites	17
3.2 Study design	17
3.3 Plant and Specimen collection and storage	17
3.4 Laboratory Analysis.....	19
3.4.1 Culture interpretation and Biochemical Identification Test	19
3.4.2 Preparation of extracts	20
3.4.3 Ethanolic Extraction	21
3.4.4 Aqueous Extraction	21
3.4.5 Evaluation of the antimicrobial activity of <i>Hibiscus sabdariffa</i>	22
3.4.5.1 Preparation of extract stock solution	22
3.4.5.2 Agar diffusion method.....	22
3.4.5.3 Micro-dilution method.....	23
3.5 Ethics	25
3.6 Statistical Analysis of Data	25
CHAPTER FOUR	26
4.0 RESULTS.....	26
4.1 Antimicrobial activity of the extracts (Agar diffusion method).....	26
4.2 MIC of the extracts	31
CHAPTER FIVE.....	34
5.0 DISCUSSION.....	34
5.1 LIMITATIONS	39
CHAPTER SIX	40
CONCLUSION AND RECOMMENDATIONS	40
6.0 CONCLUSION	40
6.1 RECOMMENDATIONS	40
REFERENCES	42
APPENDICES.....	49

LIST OF FIGURES

Figure 4.1: Antimicrobial activity of different concentrations of aqueous and ethanol calyces extract of <i>H.sabdariffa</i> against the clinical isolates of bacteria.....	27
Figure 4.2: Antimicrobial activity of different concentrations of aqueous and ethanol calyces extract of <i>H.sabdariffa</i> against the control ATCC strains of bacteria.	28
Figure 4.3: Antimicrobial activity of <i>H. sabdariffa</i> calyces against clinical resistant strains of bacteria	29
Figure 4.4: Antimicrobial activity of <i>H.sabdariffa</i> leaves against clinical isolates of bacteria	29
Figure 4.5: Minimum Inhibitory Concentration of aqueous and ethanol calyces extract of <i>H. sabdariffa</i> on the test bacteria.....	33

LIST OF PLATES

Plate 1:A, B, C showing the antimicrobial susceptibility test of <i>H. sabdariffa</i> leaves, calyces and roots against some test bacteria.....	31
Plate 2: Intact calyces of the <i>Hibiscus sabdariffa</i> plant	53
Plate 3: Roots of <i>Hibiscus sabdariffa</i>	53
Plate 4: Leaves of <i>Hibiscus sabdariffa</i>	54

LIST OF APPENDICES

Appendix 1: Preparation of reagents and chemicals	49
Appendix 2:Preparation of culture media.....	49
Appendix 3: Pictures of <i>H. sabdariffa</i> plant parts.....	53
Appendix 4: Mean zone of inhibition of <i>Hibiscus sabdariffa</i> calyces extract concentration effect on the clinical isolates of bacteria	55
Appendix 5:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> calyces extract concentration effect on the control ATCC strains of bacteria.....	56
Appendix 6: Mean zone of inhibition of <i>Hibiscus sabdariffa</i> calyces extract concentration effect on four resistant strains of bacteria.....	57
Appendix 7:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> leaves extract concentration effect on the clinical isolates of bacteria.	58
Appendix 8:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> leaves extract concentration effect on the control ATCC strains of bacteria.....	59
Appendix 9:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> leaves extract concentration effect on four resistant strains of bacteria.....	60
Appendix 10:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> roots extract concentration effect on the clinical isolates of bacteria	61
Appendix 11:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> roots extract concentration effect on the control ATCC strains of bacteria.....	62
Appendix 12:Mean zone of inhibition of <i>Hibiscus sabdariffa</i> roots extract concentration effect on four resistant strains of bacteria.....	63

Appendix 13: Clinical Test Microorganisms To Reference Antibiotics (Agar Diffusion Test).....	64
Appendix 14: Control ATCC strains to reference antibiotics (Agar Diffusion Test)	65
Appendix 15: Minimum Inhibitory Concentration of some selected test bacteria.....	65
Appendix 16-19: All statistical tables	67
Appendix 20: Ethical Clearance.....	75

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BP	British Pharmacopoeia
CLED	Cystine Lactose Electrolyte Deficient
CLSI	Clinical and Laboratory Standards Institute
CPMR	Centre for Plant Medicine Research
DCA	Deoxycholate Citrate Agar
DMSO	Dimethyl sulfoxide
ESBL	Extended Spectrum Beta-Lactamases
GHS	Ghana Health Service
INT	Iodonitrotetrazolium Chloride
KBTH	Korle- Bu Teaching Hospital
LF	Lactose Fermenters
MDR	Multidrug Resistance
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>

NLF Non-Lactose Fermenters

UNESCO United Nations Educational, Scientific and Cultural Organization

WHO World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

According to WHO 2008, “Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences, indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses”. Herbal medicine is the most common form of traditional medicine that uses plants or other plant materials as active ingredients (WHO, 2008).

WHO report 2008 estimates that about 80% of the population in Asia and Africa depend on traditional medicine for primary health care. In Ghana, the doctor to population ratio in 2009 was 1:11,929. Greater Accra region had the best doctor to population ratio of 1:5,103 with the Northern region having the worst of 1:50,751 (GHS, 2010). This suggests that some people especially those in areas with low doctor to population ratio mostly rely on medicinal plants which are more accessible and less costly to them.

Hibiscus sabdariffa is a medicinal plant that belongs to the Malvaceae family, commonly found in the tropics and subtropics. It is known as roselle, sorrel, karkade and Florida cranberry (Morton, 1987). Locally, the plant is called *Rarna*, *Sule* (Hausa), *Sakpa* (Ga) *Evema* (Ewe) (Dokosi, 1998). The *H. sabdariffa* plant is consumed as food and also useful in herbal medicine (Cisse *et al.*, 2009). The young shoots and leaves are used as vegetables and potherbs. The calyces used to make beverages and jams. The calyces are also used as colouring matter (Dokosi, 1998). *Hibiscus sabdariffa* is medicinally used as a

laxative, an anticarcinogenic, an antihypertensive, and a cholesterol lowering medicine. It exhibits great antioxidant activity, lowers hepatotoxicity and reduces fever. In some parts of Africa it is used as a remedy for abscesses, bilious conditions, cough, sores, wounds, dysuria, and scurvy (Morton, 1987). According to Fullerton *et al.* (2011), *Hibiscus sabdariffa* also holds a great promise as an antimicrobial agent. There is therefore the need for more research to be done to assess the effectiveness of this medicinal plant.

1.2 Problem Statement

Currently, the main therapy for bacterial infections is the use of synthetic antibiotics. However, the misuse and overuse of antibiotics has become the key factor for the emergence of drug resistant strains of several groups of microorganisms. Drug resistance is now a global public health threat that involves all major microbial pathogens and antimicrobial drugs (Stuart and Bonnie, 2004). Therefore, researchers are now turning their attention to herbal products, investigating for new hints to develop better drugs against resistant strains (Braga *et al.*, 2005).

Plant-based therapeutics are known to be easily biodegradable, having no or minimal adverse side effects, and being easily accessible at low prices as such there is a high demand for it in both developing and developed countries (Fullerton *et al.*, 2011). There is therefore the need to find new herbal antimicrobial agents in this era of rapid global spread of resistant isolates to commonly used antibiotics.

1.3 Significance of Study

The emergence of resistant strains of microbes to commonly used synthetic drugs has become a global concern. Some reports have indicated that herbal products may help

alleviate this problem. In Africa, most people use herbal products to cure ailments. However, there are few published reports of effectiveness of these products. It is certain that some of these herbal products will provide the solution to drug resistance. There is therefore the need to investigate the antimicrobial properties of medicinal plants.

In view of the above stated reasons, the study was intended to investigate the *in vitro* inhibitory activity of *Hibiscus sabdariffa* preparation against some clinical isolates of bacteria. Secondly, it assessed the *Hibiscus sabdariffa* aqueous and ethanolic extract and its effects on some clinical isolates of bacteria. Lastly, the study may provide useful information on the antimicrobial property of *Hibiscus sabdariffa* against the resistant strains of clinically isolated bacteria.

1.4 Hypothesis

The study hypothesizes that *Hibiscus sabdariffa* plant has antimicrobial properties that are effective against some clinical isolates of bacteria.

1.5 Aim

This research work aims to determine the antimicrobial activity of *Hibiscus sabdariffa* leaves, calyces and roots against clinical isolates of selected bacteria.

1.6 Specific Objectives

The specific objectives of this work are:

1. To evaluate the antimicrobial activity of *Hibiscus sabdariffa* leaves, calyces and roots extracts against some clinical isolates of bacteria from KBTH.
2. To assess antimicrobial effect of the extracts of *Hibiscus sabdariffa* leaves, calyces and roots against typed resistant clinical isolates.

3. To compare the antimicrobial activity of aqueous and ethanolic extracts of *Hibiscus sabdariffa* leaves, calyces and roots against clinical isolates of bacteria from KBTH.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Medicinal Plants

“A medicinal plant is any plant in which one or more of its organ, contains a substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs” (Sofowora, 1982). Plants are acknowledged to be the richest bio-resource of drugs for traditional and modern medicines, as well as pharmaceutical precursors and chemical entities for synthetic drugs (Sukhdev *et al.*, 2008).

2.1.1 Uses of Medicinal plants

According to the World Health Organization (WHO) almost 80% of the human population depends on medicinal plants. This indicates the importance of plants for therapeutic purposes. Medicinal plants, since ancient times as described in Vedas and Bible texts, have been used by different cultures as a source of medicine. UNESCO (1996) observed that in most developing countries the use of traditional medicine and medicinal plants is a basis for health maintenance. In the industrialized world, the use of medicinal plants has been traced to the extraction and development of several drugs and chemotherapeutics from these plants (UNESCO, 1998).

Traditional information on medicinal plants has provided many important drugs such as the antimalarial drugs Quinine and Artemisinin derived from *Cinchona* sp. and *Artemisia annua* plants respectively (Joy *et al.*, 2001).

Traditional medicine can historically be traced to every region. The practice of traditional medicine is deeply entrenched in a specific social-cultural context, which varies between communities (Rukangira, 2001). In Ghana, both rural and urban regions consider medicinal plants as important. Studies by Irvine (1961) and Abbiw (1990) have emphasized the importance of medicinal plants in Ghana. The use of herbal drugs is generally associated with quality and availability of health care resources. In rural Ghana, the low doctor to population ratio compels inhabitants to rely mostly on medicinal plants which are more accessible and less costly to them (Abbiw, 1996). Therefore, herbal medicines are generally the first line of treatment in rural areas, where as in the urban areas, herbal medicines are used for persistent problems for which patients cannot find a cure from modern medicine.

2.1.2 Plants as antimicrobials

Over the last ten years, lots of researches have been aimed at the discovery of antimicrobial agents from plants. One of the key reasons for these studies is the emergence of multidrug resistance (MDR) bacteria to drugs which are currently available, which can be life threatening. Another reason is the discovery that, besides plant extracts possessing antimicrobial agents against a wide range of microorganisms; they also have other medicinal benefits (González- Lamothe *et al.*, 2009).

Plants naturally produce a substance known as phytoanticipins which is constantly being synthesized by plants that act as chemical barriers to microorganisms. Phytoalexin is also a plant material which is produced in response to many stimuli, and can impede microbial attack (VanEtten *et al.*, 1994). Studies are underway to discover if these substances could contribute to the production of new antimicrobials (González- Lamothe *et al.*, 2009).

Additionally, antibacterial plant products such as phenolic acids, polyphenols, phenanthrenes, flavonoids, and terpenoids have been studied by a number of researchers but currently, no product has been accepted for systemic use against bacterial infections, partly due to the non-specific mode of action of these purified compounds. Also, the presence of a mixture of different compounds with variable degrees of bioactivity and cytotoxicity has made it challenging to isolate specific active components from plant extracts (Rios and Recio, 2005).

Gram negative bacteria are the most common pathogens of plants. However, purified plant products show low activity against such organisms. Gram positive bacteria, on the other hand, are often sensitive to these biologically active plant products and this suggests that the susceptibility of Gram negatives and Gram positives organisms to purified plant products is due to their cell wall and membrane arrangement. This also suggests that antibacterial activity against Gram negative bacteria can be executed by the use of a mixture of plant natural compounds. Advances in the understanding plant products and their mode of action by experimental methods will provide evidence of the bioactivities of plant extracts that could enhance the probability of some therapeutic applications (González- Lamothe *et al.*, 2009).

2.1.2.1 Brief history of plants as antimicrobials

The concept that plants contain therapeutic agents is prehistoric. Worldwide, humans have used infusions of indigenous plants for different purposes (Cowan, 1999). Since the creation of the world, plants have been used as food by human beings and some animals. It is assessed that there are 250,000 to 500,000 species of plants (Borris, 1996) of which only about 1 to 10% is used as food, and more than 80,000 are medicinal (Joy *et al.*, 2001). In

the late fifth century B.C, Hippocrates stated 300 to 400 medicinal plants (Moerman, 1996). The Bible also mentions about 30 medicinal plants such as frankincense and myrrh. The fall of ancient civilizations led to the loss of the pharmaceutical plant documents which averted the improvements of medicinal plants (Stockwell, 1988). However, the Arabs and Asians are discovering their antiquated works on medicinal plants to build upon them. In North America, the use of medicinal plants can be traced to the Native Americans who used plants extensively for medicinal purposes (Weiner, 1980). The answer to the harmful medical practices, in the 19th century, such as the use of mercury baths to treat syphilis and hallucinogens as tuberculosis “cure” as observed by Oliver Wendell Holmes, led to the consideration of plants for therapeutic purposes (Holmes, 1861).

The usage of plants in medicine is gaining popularity. For example, in cancer treatment, plants with antileukaemic alkaloids, vinblastine and vincristine such as *Catharanthus roseus* *syn. Vinca roseus* are used (Nelson, 1982). As an antimicrobial agent, plants are being targeted for the production of novel drug compounds for the treatment of infectious diseases. Some examples of drugs of plant origin known for their antimicrobial properties are isoquinoline alkaloid emetine, an amoebicidal drug, obtained from the underground part of *Cephaelis ipecacuanha*. Quinine from the bark of *Cinchona* tree is used as an antimalarial drug (Iwu *et al.*, 1999).

2.1.2.2 Present use of plants as antimicrobials

Today, crude forms of plant materials used in traditional healing practices are being modified and used as commercially available drugs. It is estimated that 50% Western drugs are derived from plant materials (Robbers, 1996). The advantages of using plant derived

medicines are that they are safer than synthetic drugs, affordable and available, and they offer great therapeutic value.

Therapeutic Benefit

The use of natural products as antimicrobials arises from microbial sources. Penicillin discovery led to later discoveries of streptomycin and aureomycin. Most of the clinical antibiotics are produced by soil microorganisms or fungi. Plants such as Lichens have bacteriostatic and antifugicidal properties and garlic (*Allium sativum*) produces allinine, which is an antibacterial agent (Trease and Evans, 1972).

Economic Benefit

Over the last decade, there has been a surge of interest in natural products globally. This is due to the belief that natural products are effective than conventional medicines. Herbal products worldwide have increased jobs employment and revenue to countries. In the United States, botanical products have reached \$3.1 billion of the \$10.4 billion dollar dietary supplement industry (NBJ, 1998).

International trade of *Hibiscus sabdariffa* calyces (roselle) has also increased over the last ten years, with 15,000 tonnes per year entering the world market. In 1998, United States and Germany, the large importers, paid 1200–1700 US\$ per tonne for Egyptian and Sudanese roselle. This further goes to highlight the economic benefit of plants to countries that cultivate them (McClintock and El Tahir, 2004).

2.2 Plants with Promising Antimicrobial Activity

A number of plants are believed to possess biological activity against a wide variety of microorganisms. Many of these plants are believed to be found in Africa such as *Garcinia kola* (*Guttiferae*), *Acanthospermum hispidum*, *Nauticlea latifolia*, *Bridelia atroviridis* and

Zanthoxylum gilletis, *Phyllanthus niruri*, *Cassia alata*, *Ageratum conyzoides*, and *Sida acuta*. These medicinal plants have been studied and are known to have anti-infective activity against Gram positive and Gram negative organisms (Hoffman *et al.*, 2004; Agyare *et al.*, 2006).

Many medicinal plants of Africa are still under investigation for their chemical component and bioactivity against microorganisms. One plant that holds an enormous promise of an antimicrobial agent is the *Hibiscus sabdariffa* plant (Fullerton *et al.*, 2011).

2.3 *Hibiscus sabdariffa* Plant

The *Hibiscus sabdariffa* plant belongs to the Malvaceae family. There are two main types of the plant: *H. sabdariffa* var. *sabdariffa* and *H. sabdariffa* var. *altissima*. The latter is a tall unbranched plant about 300-480cm high grown for fibre. *Hibiscus sabdariffa* var. *sabdariffa* is a bushy shrub with red or pale green stems and red or pale yellow inflated edible calyces (Dokosi, 1998). *Hibiscus sabdariffa* plant is believed to have originated from Africa specifically Sudan. It was then introduced to India and America in the 17th century, and Sri Lanka, Thailand, Malaysia and Java at the start of the 20th century. The plant is distributed throughout the tropics and subtropics especially in tropical Africa, Asia and some parts of India. In tropical Africa, it is especially common in the West and Central Africa. However, truly wild plants of *Hibiscus sabdariffa* have been collected in Ghana, Niger, Nigeria and Angola (McClintock and El Tahir, 2004).

It is an annual plant usually grown mainly in April or in late August and requires about four months to mature. The plant is cultivated from the seed or some cut plant parts (Stephens, 2012).

Hibiscus sabdariffa is an erect, slightly branched herb. The plant may have a smooth or hispid stem with glabrous, long-stalked, palmate leaves. The palmately lobed leaves are divided into 3-5 lobes with serrate margins. The pedunculate flowers are large and coloured red to yellow. The ½ to 1½ inches in diameter red calyces are fleshy when matured. The root is a deep penetrating tap root (Dokosi, 1998).



Figure 2.3. *Hibiscus sabdariffa* plant (Retrieved, from http://toptropicals.com/cgi-bin/garden_catalog/cat.cgi?uid=Hibiscus_sabdariffa, November 19, 2013)

2.3.1 Local and Foreign Names

The local names of *Hibiscus sabdariffa* plant indicate that the plant is very popular among the people of Northern, Western and certain parts of the southern Ghana. The plant is called *Digbemre* or *Injamgbam* (Dagbani); *Rarna* or *Sule* (Hausa); *Bito* (Moshi); *Riaripari*

(Guan -Krachi); *Nangana* (Frafra); *Tingyanbam* (Konkomba); *Sakpa* (Ga); *Evema* (Ewe) (Dokosi, 1998).

Hibiscus sabdariffa has diverse foreign names which suggest the presence of the plant in several parts of the world. In most French-speaking West African countries, *Hibiscus sabdariffa* is commonly known as *Bissap*. In Nigeria: *Zobo*; Sierra Leone: *Sour – sour*; Sudan: *Furundu*, *Sudan Tea*; Japan, Malaysia, West Indies the plant is known as *Roselle*; Arabia, Egypt, Switzerland: *Karkarde*; Guinea, Jamaica: *Red Sorrel*; India: *Mesta*; Iran: *Sour Tea*; Thailand: *Thai Red*; Florida: *Florida cranberry* (Morton, 1987).

2.3.2 Uses

2.3.2.1 Nutritional use

The plant *H. sabdariffa* var. *sabdariffa* is mainly used as a vegetable and for preparation of beverage. The calyces of *Hibiscus sabdariffa* are mainly used for the preparation of beverages, especially as tea and coffee substitute for people who are sensitive to stimulants (Irvine, 1961). It is also used in preserves, jelly, juice, or as a sauce (Stephens, 2012) and also in making roselle wine, gelatin, pudding and cakes. The calyces can be used for colouring food and drinks (Duke and Atchley, 1984). In Ghana, a drink known as *Sooboro* (Hausa) is made by boiling the calyces in water, sweetened with sugar and may flavored with ginger and other local spices. The leaves and calyces are also used as a cooked vegetable or used to make a soup. In Senegal, sauces made with the leaves and calyces, and eaten with tuber and cereals. Additionally, in the United States of America, the leaves are used for salads, and the red fleshy calyx used in fruit salads. Also, during the dry season,

especially in Côte d'Ivoire, the dried calyces are ground to powder and used in sauces. The plant is used as feed for livestock after the harvest of calyces.

The leaves of *Hibiscus sabdariffa* are also used in soups in Northern Ghana (Dokosi, 1998). In many West African countries, the green leaves of the plant are eaten as a potherb. The *H. sabdariffa* leaves are added to a soup prepared with groundnut and “dawadawa” (ground, fermented seeds of *Parkia clappertoma*).

The dried leaves are kept in pots, and used in soup preparation during the dry season. When cooking, the water is changed many times to reduce the sliminess of the potherb. This water may be used in preparing millet or guinea-corn porridge which keeps a few more days in good condition than that prepared with ordinary water. This proposes preservation properties of the leaves.

The *Hibiscus sabdariffa* seeds are eaten roasted as a snack or ground into meal (McClintock and El Tahir, 2004). The seeds are used as groundnut substitute in times of scarcity. The fermented seeds are used as a substitute for “dawadawa” (Dokosi, 1998). The Nigerians ferment roselle seeds to make ‘sorrel meat’ cake. The oil of roselle seed is extracted and used for cooking. However, the seed oil is claimed to contain some toxic substances and may be better used in the soap and cosmetics industries. In Chad, Tanzania and China, the oil is used for soap and cosmetics. This oil is also used as a substitute for castor oil. The residue, after removal of the oil, is used as food, either in soup or mixed with beans and other ingredients into cakes (McClintock and El Tahir, 2004).

2.3.2.2 Medicinal use

Hibiscus sabdariffa has been found to possess several health benefits. *H. sabdariffa* is medically used as an antiseptic, antibilious, antiscorbutic, aphrodisiac, astringent, diuretic,

emollient, purgative, sedative and tonic. It is also a folk remedy for abscesses, cough, and dysuria (Perry, 1980).

The calyces of plant are used to lower high blood pressure. The calyces of *Hibiscus sabdariffa* have been found to contain gossypetin, anthocyanin, glycoside, hibiscin and citric acid, which confer the diuretic, choleric and antihypertensive properties of the plant (McKay *et al.*, 2010). In Nigeria, the red calyces are soaked in water and the liquid, drunk as a tonic.

The leaves and seeds are used in the treatment of scurvy, which suggests the presence of vitamin C. It may also have antioxidant properties (Duke & Atchley, 1984). The mucilaginous leaves are used in pharmaceutical and cosmetics industries. Angolans used it as emollient and a soothing cough remedy. In Senegal, the leaves are used in the treatment of conjunctivitis. Leaves are applied as a poultice to heal sores and ulcers especially in Central Africa (McClintock and El Tahir, 2004).

The seeds of *Hibiscus sabdariffa* also act as an aphrodisiac (Irvine, 1961). After oil extraction of *Hibiscus sabdariffa* seeds, the residue can be moistened and applied to wounds. Taiwanese regard the seed as diuretic, laxative and tonic. In India, a decoction of the seeds was given to relieve dysuria and many cases of dyspepsia and debility.

The roots of *Hibiscus sabdariffa* are also used in treating syphilitic sores or chancre. The roots also act as aperients and tonic (Mehta, 1994).

The constituents of the plant include citric, tartaric acids, hibiscin, and malic acids, delphinidin, anthocyanin hibiscitrin, gossypitrin, sabdaritrin and cyanidin. The antimicrobial activity of the plant can be linked to the flavonoids (Badreldin *et al.*, 2005). Flavonoids are polyphenolic compounds which have been known to possess antimicrobial

activity (Cowan, 1999). The antimicrobial mechanisms of flavonoids can be classified as the inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism (Cushnie and Lamb, 2005).

The *H. sabdariffa* plant has also exhibited antifungal, antiparasitic and antibacterial actions. Studies indicate that an ethanol extract of the dried leaves of the plant has been shown to have an *in vitro* inhibitory effect against some fungi that include *Aspergillus fumigatus*, *Rhizopus nigricans* and *Trichophyton* (Guerin and Reveillere, 1984). An aqueous extract of dried sepals of *H. sabdariffa* was active against *Schistosoma mansoni* (Elsheikh *et al.*, 1990).

In Ghana, little work has been done on its medicinal use. Frimpong (2008) studied the suitability of *Hibiscus sabdariffa* calyx extract as colouring agent for paediatric syrups. She found out that the colour conformed to the BP standard. The study also revealed that the plant has antimicrobial properties with methanolic extracts showing a better antimicrobial activity than the aqueous extract.

According to Sharaf *et al.*, 1966, the colouring matter of the calyces is said to be lethal to *Mycobacterium tuberculosis*. Other works done by Sharaf *et al.*, 1966, similarly showed that the aqueous extracts of *H. sabdariffa* prevented the growth of *Pasteurella*, *Pseudomonas*, *Proteus* and *Streptococcus* bacteria.

Oboh and Elusiyan (2004) also studied the nutrient composition and antimicrobial activity of sorrel drinks against *P. aeruginosa*, *Lactobacillus* sp., *Bacillus* sp., and *Corynebacterium* and found out that the aqueous extracts of *H. sabdariffa* inhibited the growth of *P. aeruginosa*, *Lactobacillus* sp., *Bacillus* sp., and *Corynebacterium* sp.

Fullerton *et al.* (2011) determined the antimicrobial activity of sorrel (*Hibiscus sabdariffa*) on *Escherichia coli* O157:H7 isolated from food, veterinary, and clinical samples. Ethanolic extract of *Hibiscus sabdariffa* calyces was studied and showed to have antimicrobial agents effective in inhibiting *E. coli* O157:H7. The *Hibiscus sabdariffa* plant therefore holds a great promise as an antimicrobial agent.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Sites

The study was carried out at the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, Eastern Region; and the Microbiology Department, Central Laboratory, Korle-Bu Teaching Hospital (KBTH), Greater Accra Region.

The CPMR focuses on research into herbal medicines. The centre has an extraction laboratory as well as a microbiology laboratory in which the preparation of the plant extract and the microbial analysis of these extracts were done. The Korle-Bu Teaching Hospital, the third largest hospital in Africa and the leading national referral centre in Ghana, aided in the clinical isolation of the selected bacteria in their well-equipped bacteriology laboratory (Retrieved, from <http://kbth.gov.gh/index.php?id=126>, March 28, 2014).

3.2 Study design

This study was a quantitative experimental research.

3.3 Plant and Specimen collection and storage

The fresh *Hibiscus sabdariffa* leaves and roots were acquired from a farm in Kpotame, Sogakope in the Volta Region of Ghana while the *Hibiscus sabdariffa* calyces were obtained from the Madina local market in the Greater Accra Region. All the plant parts were authenticated by CPMR botanist. Samples of the leaves, calyces and roots of the plant (Voucher number: CPMR 0214) are being kept in the CPMR herbarium.

Of the twenty eight different types of bacteria used for this study, six are American Type Culture Collection (ATCC) namely: *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 49565, *Salmonella typhi* ATCC 19430, *Klebsiella pneumoniae* ATCC 33495, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923; eighteen are clinical isolates, three each of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which were obtained from the Microbiology Department, Central Laboratory, Korle-Bu Teaching Hospital (KBTH) from December 2013 to February 2014. Lastly, two strains each of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and ESBL (Extended Spectrum Beta-Lactamases) isolates were received from Beverly Egyir who worked on ‘Antimicrobial Resistance, Epidemiology and Population Structure of *Staphylococcus aureus* in Ghana’ and Noah Obeng-Nkrumah, a PhD student, who is working on the ‘Molecular Epidemiology of Beta-Lactamase producing *E.coli* and *Klebsiella pneumoniae*’; both with Clinical Microbiology Department, School of Biomedical and Allied Health Sciences, University of Ghana.

Gram staining and biochemical tests of indole, urease, citrate, oxidase, and Triple Sugar Iron were performed on samples to confirm the identification of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella* sp. and *Pseudomonas aeruginosa*. For the confirmation of *Staphylococcus aureus*; Gram staining, catalase and coagulase tests were done. Confirmed isolates of *Escherichia coli*, *Klebsiella* sp. and *Staphylococcus aureus* were purified on Cystine Lactose Electrolyte Deficient (CLED) agar; and that of *Salmonella typhi*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* on MacConkey agar. The pure cultures were then stocked by inoculating in Eppendorf tubes containing 2mls of

Mueller Hinton agar (Oxoid, United Kingdom) slants. The inoculated Eppendorf tubes were incubated at 35-37°C for 18-24 hours, and then kept frozen at a temperature of -20°C.

3.4 Laboratory Analysis

3.4.1 Culture interpretation and Biochemical Identification Test

The collected isolates were thawed, and cultured on the agar plates. Differential media of CLED, DCA, and MacConkey agars aided in distinguishing Gram negative bacteria that are lactose fermenters (LF) and non-lactose fermenters (NLF). *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella* sp., with the exception of *Pseudomonas aeruginosa*, were identified using biochemical and microbiological procedures, as defined by Cowan and Steel (1993), as Enterobacteriaceae.

Pseudomonas aeruginosa was identified by oxidase test. The bacteria oxidize the reagent, tetramethyl-*p*-phenylenediamine dihydrochloride, to a deep purple colour within 10 seconds. The test was performed by soaking a piece of filter paper with a few drops of oxidase reagent, a colony of the bacteria was picked with a wooden loop and smeared on the filter paper; the inoculated area of paper was observed for colour change to deep purple colour (Gordon and McLeod, 1928).

Isolated colonies from pure GNR bacteria were picked using sterile bacteriological straight wire and inoculated into tubes of peptone water (Liofilchem, Roseto Degli Abruzzi, Italy), urea agar slants (Liofilchem, Roseto Degli Abruzzi, Italy) citrate slants (Liofilchem, Roseto Degli Abruzzi, Italy), Triple Sugar Iron agar slants (Liofilchem, Roseto Degli Abruzzi, Italy) and motility test media. Inoculated biochemical media were incubated aerobically at 35-37°C for 18 to 24 hours. Biochemical reactions were examined after

incubation. Drops of Kovac's reagent (Oxoid, Hampshire, UK) were added to peptone water cultures for reactions suggestive of indole production. For each isolate, reactions of biochemical tests were compared to that of reference strains for species identification.

For *Staphylococcus aureus*, Gram staining showed Gram positive cocci in clusters. The slide catalase test was done using a drop of hydrogen peroxide (H₂O₂) on a microscope slide. *Staphylococcus* produces the enzyme catalase, which breaks down the H₂O₂ to oxygen and water; as such bubbles of oxygen were released. The tube coagulase test was performed, with negative and positive controls, to help identify *Staphylococcus aureus* which produces the enzyme coagulase. One millilitre of diluted plasma (1 part of plasma to 9 parts of peptone water) was pipetted in three test tubes labelled positive control, negative control and test. The positive tube was inoculated with standard *Staphylococcus aureus* ATCC 25923 strain. The negative test tube was not inoculated, and the last test tube with the test organism under investigation. The tubes were incubated aerobically at 35- 37°C for 18-24 hours. The test and positive control showed clotting of tube contents or fibrin clot which confirms *Staphylococcus aureus*.

3.4.2 Preparation of extracts

Extraction Techniques used were Maceration and Decoction.

Macroscopic evaluation in *Hibiscus sabdariffa*

One kilogram each of *Hibiscus sabdariffa* calyces, leaves and roots were examined macroscopically for any foreign matter such as moulds, insects and undesirable plant parts.

Preliminary Preparation

One kilogram each of *Hibiscus sabdariffa* calyces, leaves and roots were washed and dried in a shed at the CPMR, Mampong- Akuapem for about two weeks. The completely dried plant parts were then pulverized.

3.4.3 Ethanolic Extraction

Sukhdev (2008) method for the extraction of plant compound was followed. One hundred and fifty grams of dried *Hibiscus sabdariffa* calyces, leaves and roots were weighed out using an analytical weighing scale. One and half litres of 70% ethanol was added to it and left to macerate for 5 days at room temperature, with occasional stirring. After 5 days, the 70% ethanol was decanted and filtered through a Whatman No. 1 filter paper. The marc was pressed, filtered and added to the first filtrate. The extracts were then concentrated under reduced pressure using the Eyela rotary evaporator N-1200 (Tokyo Rikakikai Company Limited, Japan); after which they were freeze-dried and altered into powdered form at the College of Agriculture, University of Ghana, and Legon.

3.4.4 Aqueous Extraction

Two hundred and fifty grams each of the dried *Hibiscus sabdariffa* leaves, roots and calyces were brought to boil in 2.5 litres of distilled water on a hot plate for about thirty minutes and then strained. The aqueous extracts were filtered using Whatman No. 1 filter paper and subsequently cooled for 45 minutes. After cooling, the extracts was freeze-dried and re-constituted into powder at the College of Agriculture, University of Ghana, Legon.

3.4.5 Evaluation of the antimicrobial activity of *Hibiscus sabdariffa*

3.4.5.1 Preparation of extract stock solution

A concentration of 200mg/ml aqueous extracts of the *Hibiscus sabdariffa* leaves, roots and calyces was prepared by dissolving 2g of the powdered extracts in 10ml of sterile distilled water. Similarly, 2g of ethanolic extract was dissolved in 10ml of 5% Dimethyl sulfoxide (DMSO). This concentration was chosen based on preliminary susceptibility test on these bacteria using these extracts.

Alternatively, other concentrations of the extract were made by use of the following formula:

$$C1/ C2= V1/V2,$$

Where V1 = volume of stock reagent

C1 = concentration of the stock reagent

V2 = final volume needed

C2= final concentration needed (Efiok, 1993).

Sterilization of the prepared extract solutions was done by using 0.22µm membrane filtration (Millex GP filter unit, Millipore, Ireland) and stored in aliquots at 2-8°C.

3.4.5.2 Agar diffusion method

The disc diffusion method (Clinical and Laboratory Standards Institute, CLSI) was used to screen for antibacterial activity of *Hibiscus sabdariffa* calyces, leaves and roots. About

three to four isolated colonies of similar morphology were picked from 18-24 hour agar plate of pure cultures of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*, *Klebsiella* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* using a sterile loop; and then inoculated individually into 4mls peptone broth (Sigma, P0556, Sigma-Aldrich Inc., USA). The density of the inoculum was adjusted to 0.5 McFarland turbidity standard, resulting in a suspension of 1×10^7 colony forming units.

Mueller Hinton agar plates (Oxoid, CM0337, Oxoid Ltd, United Kingdom) were seeded with the test organisms and the plates left to dry for some few minutes. After drying, wells were made in the agar using sterile cork borer measuring 5 mm in diameter. Eighty millilitres of the prepared serial concentrations of 12.5, 25, 50, 100 and 200mg/ml of aqueous and ethanolic extract of leaves, roots and calyces were dispensed into the labelled wells. The plates were then kept in the refrigerator for one hour for the extract to diffuse into the medium. Ciprofloxacin (5 μ g) and amikacin (30 μ g) antibiotics (Himedia, India) were used as positive controls to compare the zones of inhibition with that of the extracts. Sterile distilled water and 5% DMSO were used as negative controls. The plates were incubated aerobically overnight at 37C. The following day, the zones of inhibition were measured in millimetres. Analysis was done in duplicate.

3.4.5.3 Micro-dilution method

The Minimum Inhibitory Concentration was determined for active plant extracts against some selected susceptibility bacteria. Ciprofloxacin and amikacin (MAST Diagnostics, Bootle ,United Kingdom) were used to perform the micro-dilution test. Twenty five microlitres stock solution of 800 μ g/ml ciprofloxacin and 3.2mg/ml of amikacin were prepared by following manufacturer's instruction. The stock solutions were stored at 2-8°C

for a maximum of 7 days. Mueller Hinton broth (Liofilchem, Roseto Degli Abruzzi Italy) was used as the diluent for the Minimum Inhibitory Concentration (MIC) test because it acted as a medium for the growth of the bacteria. Each test isolate was emulsified in the Mueller-Hinton broth (MHB) and incubated aerobically for 8-12 hours at 35-37°C. The inoculum was then adjusted to 0.5 McFarland standard. The suspension was further diluted to provide an inoculum of 1×10^5 cfu/ml.

The MIC dilution method as described by Eloff (1998) was followed. For each active plant extract, 96-well sterile microtitre plates (Sumilon Multi Well Plate, MS-30964, S.B. Medical. Co. Ltd, Japan) were labelled with appropriate plant extract dilutions in rows. A two fold serial dilution of the extract with an initial concentration of 100mg/ml was used. One hundred microlitres of the diluted extract was dispensed into the wells in rows. One hundred microlitres of the inocula were then added to each of the dilution to make a final volume of 200µl in each well. Un-inoculated wells of extract and broth were used to determine sterility. Reference antibiotic dilutions of initial concentration of 20µg/ml ciprofloxacin (MAST Diagnostics, Bootle, United Kingdom) and 80µg/ml amikacin (Mast Diagnostics, Bootle, United Kingdom) were tested against test bacteria in each row.

Preparations were covered and incubated aerobically at 35-37°C for 18 to 24 hours. After incubation, 40 µl of 0.2 mg/ml INT (Iodonitrotetrazolium Chloride, Fluka, Austria) was added to each of the wells after incubation. Microtitre plates were examined after 30 to 120 minutes incubation. Bacterial growth was indicated by a red colour of INT reduced to the formazan. The lowest concentration at which a decrease in the red colour was apparent compared to the next dilution was taken as the MIC value. MICs were recorded as the least dilution which inhibited visible growth of the bacteria.

3.5 Ethics

Clinical isolates were assigned arbitrary numbers. This study received ethical clearance from the Ethical and Protocol Review Committee of the University of Ghana Medical School (UGMS).

3.6 Statistical Analysis of Data

Results were analyzed with Genstat Ninth Edition Statistical Software and SPSS version 16.0 to address the objectives of the study. The mean zones of inhibition for each isolate were analyzed and the mean difference between the extract types was compared using Tukey's Studentized range test and ANOVA at a significance level of $P < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Antimicrobial activity of the extracts (Agar diffusion method)

The outcome of this study conducted to evaluate the antimicrobial activity of *Hibiscus sabdariffa* (leaves, calyces and roots) extracts using six clinical and control ATCC strains of bacteria (*Escherichia coli*, *Salmonella typhi*, *Klebsiella* sp., *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*); and also four resistant strains (Two ESBL and two MRSA organisms) are shown in Figure 4.1 to 4.4 and Appendix 4 to Appendix 12.

Of six concentrations (0, 12.5, 25, 50, 100 and 200mg/ml), extracts of 50,100 and 200mg/ml had significant inhibitory results in each of the plant part's extract. The differences among growth inhibition at low concentrations (0, 12.5 and 25mg/ml) extracts against all the test microorganisms were not significant because the plant extracts at that concentration had no microbial inhibitory effect. This investigation also revealed that antibacterial activity increased significantly ($P < 0.05$) with increase in extract concentration (Figure 4.1 to 4.3 and Appendix 4 to Appendix 6).

The antimicrobial activity of the calyces of *Hibiscus sabdariffa* showed higher growth inhibition than the leaves and roots. The roots of the plant tested against all the test bacteria at various concentrations had no microbial growth inhibition. However, only aqueous extract of the leaves was more potent in terms of growth inhibition against *Pseudomonas aeruginosa* and *E. coli* (Figure 4.4 and Appendix 7, Appendix 8).

Additionally, this research revealed that only *Proteus mirabilis* was resistant to all the *H.*

sabdariffa plant extracts at all the test concentrations as compared to the other test bacteria. At 200mg/ml ethanol extract of calyx, *S. aureus* recorded the highest mean zone inhibition of clinical isolates (18.33 ± 0.3), as well as that for resistant strain MRSA 5038 (24 ± 0.3) and ATCC strain (23.00 ± 0.6) (Figure 4.1 to 4.3 and Appendix 4 to 6).

Among the Gram negative bacteria, the aqueous calyx showed peak growth inhibition (16.67 ± 0.3) against clinical isolate *Pseudomonas aeruginosa* (Fig 4.1, Appendix 4); the ethanol calyx extract showed high growth inhibition against clinical isolate *E. coli* (16.67 ± 0.3) (Fig 4.1, Appendix 4).

Figure 4.1: Antimicrobial activity of different concentrations of aqueous and ethanol calyces extract of *H.sabdariffa* against the clinical isolates of bacteria

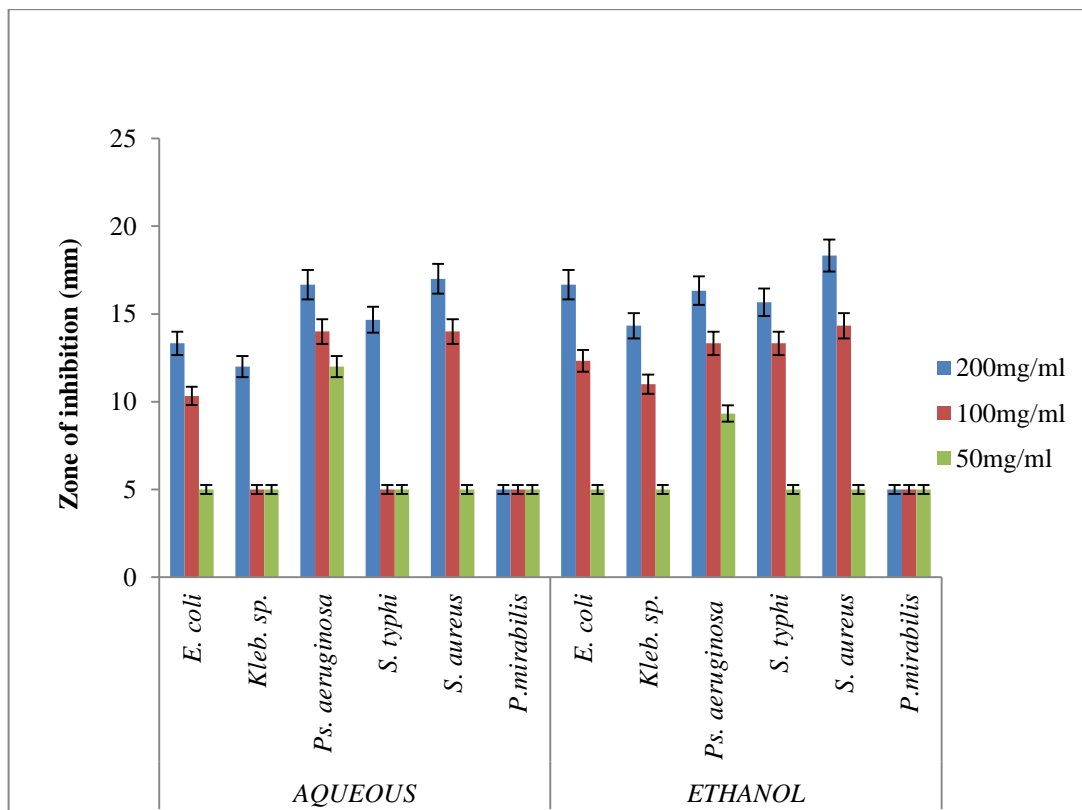


Figure 4.2: Antimicrobial activity of different concentrations of aqueous and ethanol calyces extract of *H.sabdariffa* against the control ATCC strains of bacteria

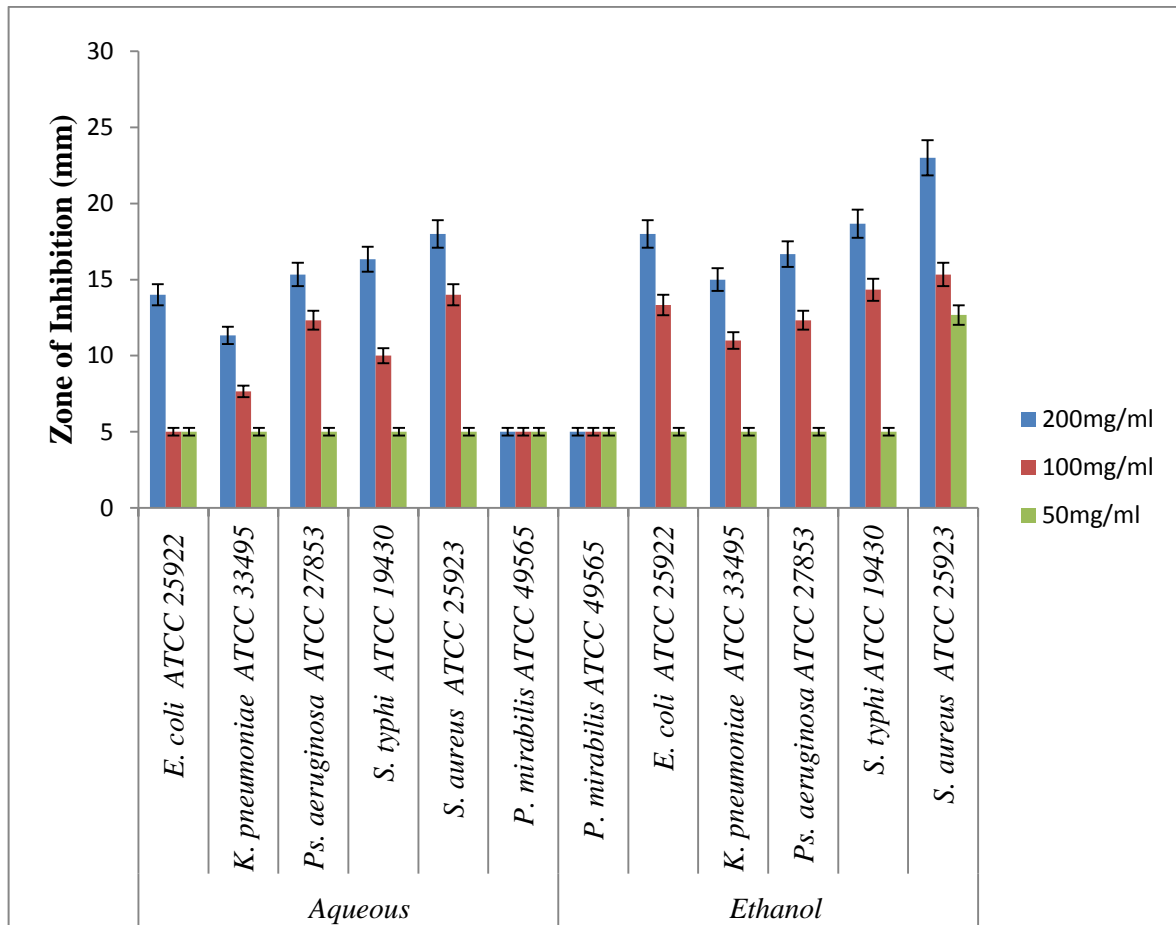


Figure 4.3: Antimicrobial activity of *H.sabdariffa* calyces against clinical resistant strains of bacteria

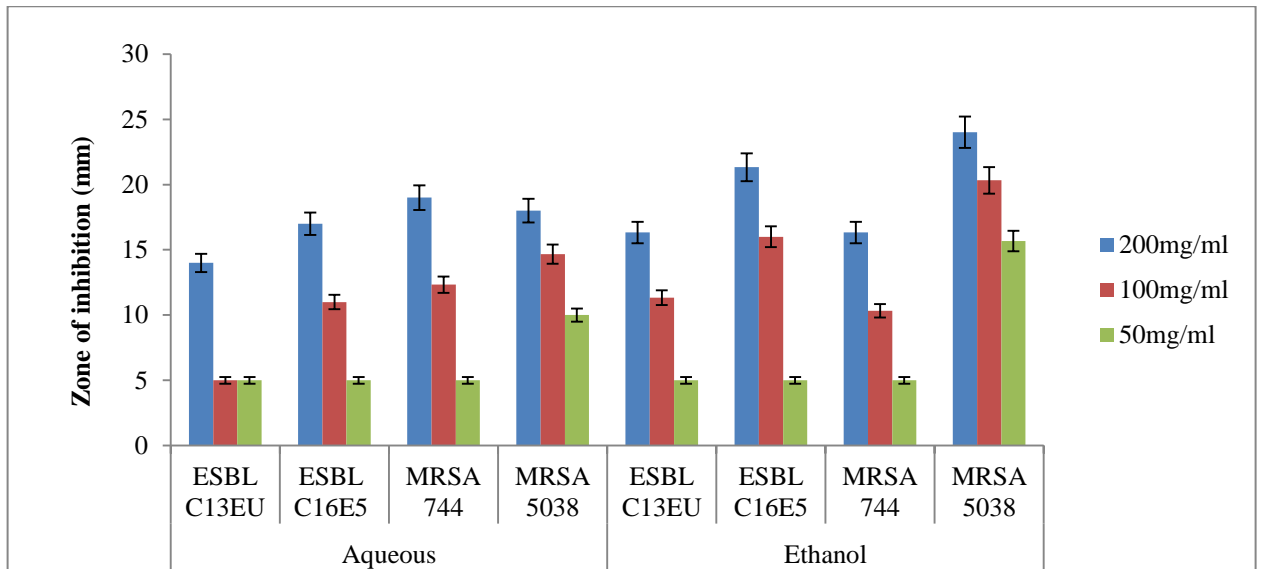
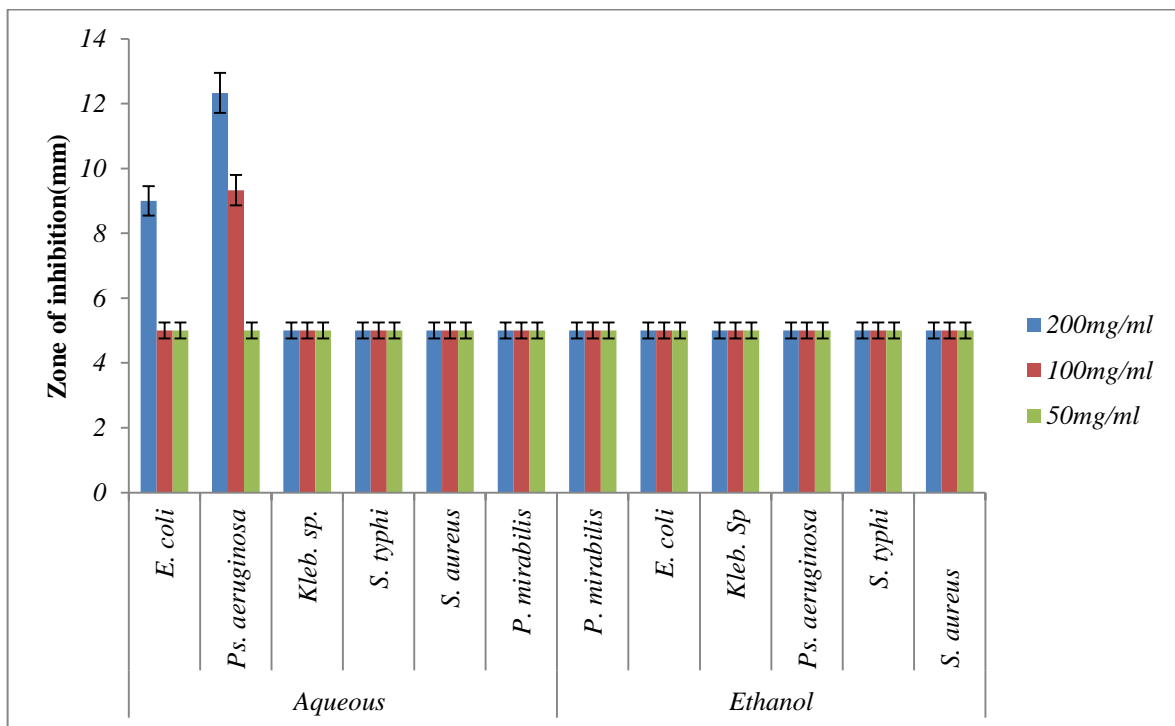


Figure 4.4: Antimicrobial activity of *H.sabdariffa* leaves against clinical isolates of bacteria



In Figure 4.3, the water extract of calyces against ESBL C13EU recorded the lowest mean zone of inhibition at all the concentrations. However, plant extracts documented high inhibitory effect against MRSA 5038.

In comparing the antimicrobial activity of aqueous and ethanol extracts of *Hibiscus sabdariffa* calyces, the results of the Agar Diffusion Assay against five control ATCC strains and clinical isolates (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*); as well as four resistant strains (ESBL C13EU, ESBL CI6E5, MRSA 744, MRSA 5038) are seen in Figure 4.1 to 4.3 and Appendix 4 to 6.

In regards to the extraction type of calyx against all the microorganisms at various concentrations, there was no significant ($P > 0.05$) difference between the aqueous and ethanol extract.

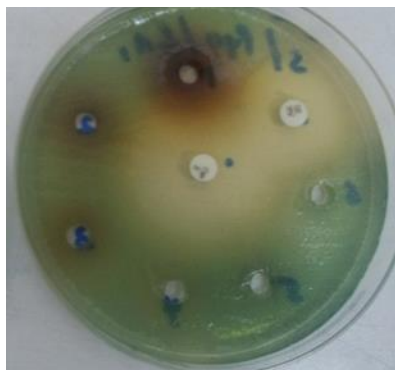
However, interaction between *Staphylococcus aureus* and ethanol extraction had higher growth inhibition than the other bacteria (Figure 4.1 to 4.3).

Generally, the inhibitory effect of the antimicrobial activity of the aqueous and ethanol extracts of the *Hibiscus sabdariffa* seems to have an effect on both Gram positive and Gram negative bacteria. This suggests the broad spectrum nature of the plant against bacteria.

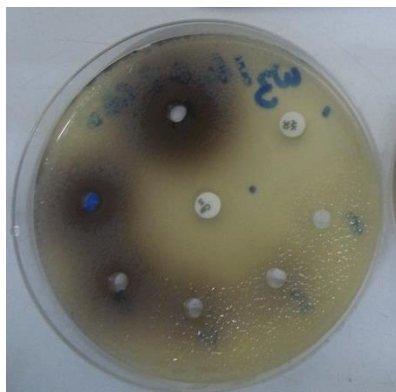
Antimicrobial activity of different concentrations of aqueous and ethanol leaf extract of *H.sabdariffa* against the clinical isolates of bacteria showed that the aqueous extract produced the highest zone of inhibition of 12mm against *Ps. Aeruginosa*, and *E.coli* 9mm at 200mg/ml (Figure 4.4). There was a difference between the aqueous and ethanol extract

type. The inhibitory activity of the leaves at 200mg/ml against test organisms was less than the reference antibiotics; Ciprofloxacin and Amikacin (Appendix13 and 14). Five percent DMSO and sterile distilled used as control showed no activity against the test organisms.

Plate 1: A, B, C showing the antimicrobial susceptibility test of *H. sabdariffa* leaves, calyces and roots against some test bacteria



A. *H. sabdariffa* leaves showing inhibition against *Pseudomonas aeruginosa* ATCC 27853 at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.



B. *H. sabdariffa* calyces showing inhibitory effects against clinical isolate *E. coli* at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.



C. *H. sabdariffa* roots showing inhibitory effects against *Proteus mirabilis* ATCC49565 at 200, 100, 50, 25 and 12.5 mg/ml with negative and positive control.

4.2 MIC of the extracts

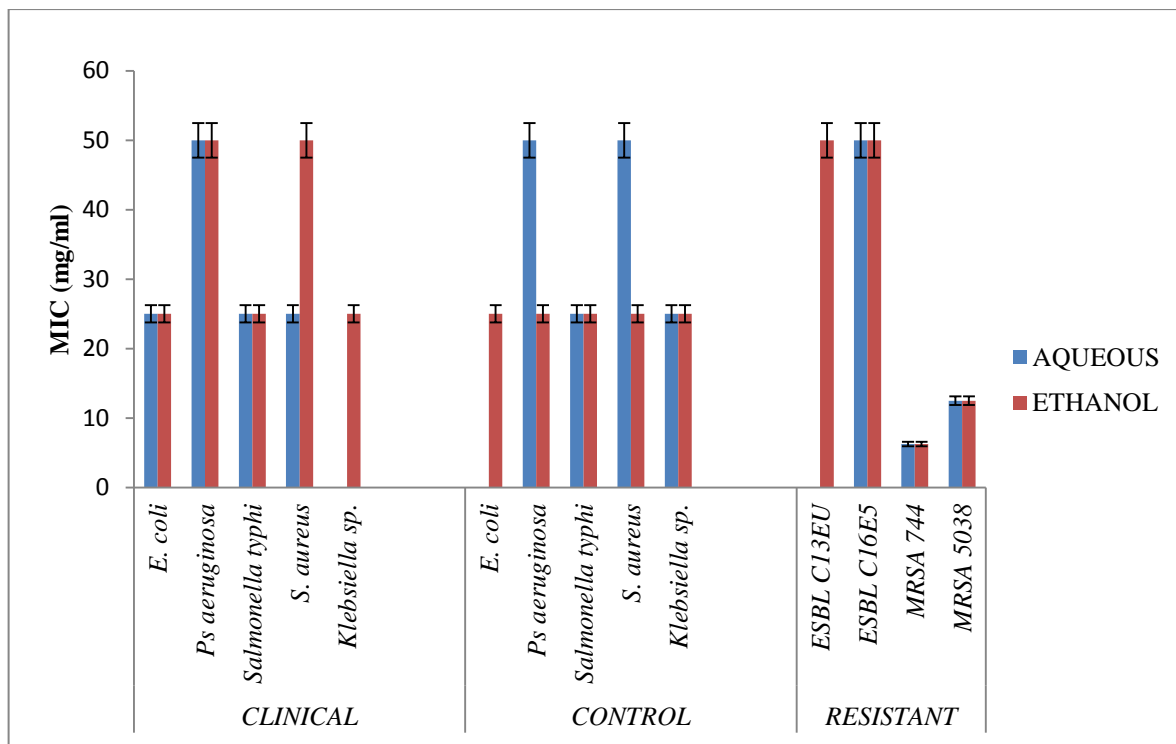
The results of the MIC values of the *Hibiscus sabdariffa* calyces extract determined against selected susceptible bacteria are shown in Fig 4.5. Generally, the MIC of the ethanol and aqueous extract for the various organisms ranged between 6.25 and 50mg/ml; higher concentrations of 50mg/ml *Hibiscus sabdariffa* calyces extracts were required to inhibit *Pseudomonas aeruginosa*, *Staphylococcus aureus*, ESBL C16ES, ESBL CI3EU in comparison to the other isolates. The extraction type had no influence on the MIC test against the selected bacteria.

In Fig 4.5, the MIC values of the *H. sabdariffa* calyces aqueous and ethanol extract against some selected test bacteria are shown. The MIC of the ethanol extract for the different control ATCC organisms was 25mg/ml and that for the aqueous extract ranged between 25 and 50 mg/ml.

Additionally, the MIC values of the *H. sabdariffa* calyces both aqueous and ethanol extract against clinical isolates of bacteria ranged between 25 and 50 mg/ml.

Unpredictably, the MRSA 744 and MRSA 5038 showed a more robust activity with MIC values of 6.25 and 12.5mg/ml respectively.

Figure 4.5: Minimum Inhibitory Concentration of aqueous and ethanol calyces extract of *H. sabdariffa* on the test bacteria.



CHAPTER FIVE

5.0 DISCUSSION

The therapeutic use of plants especially as antimicrobials has been reported by many scientists (Cowan, 1999; Sharaf *et al.*, 1966, González-Lamothe *et al.*, 2009). Reports of antimicrobial activity of *H. sabdariffa* show various levels of microbial growth inhibition against Gram positive and Gram negative bacteria. This indicates the broad spectrum nature of the plant extract. Studies show that the plant is effective against *E.coli* 0157:H7 (Fullerton *et al.*, 2011) as well as pathogenic bacteria of *Pseudomonas aeruginosa* and *Escherichia coli* (Khalaphallah and Wagdi, 2014), and *Klebsiella pneumoniae* (Alshami and Alharbi, 2014).

In the present study, antimicrobial activity of *Hibiscus sabdariffa* calyces and leaves extracted by water and ethanol extracts was effective against some clinical isolates of bacteria. Among the three plant parts extract of *H. sabdariffa*, calyces extract were found to be more active than the leaves and roots extracts. This is probably why much work has been done on the calyces as compared to the other parts.

The calyces extract were notably effective against *Escherichia coli*, *Salmonella* sp., *Klebsiella* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These findings were similar to studies done by Sharaf *et al.* (1966), Oboh and Elusiyan (2004) who found *H. sabdariffa* plant to be effective against *Pseudomonas aeruginosa*; Frimpong (2008) also detected the plant extract to be effective against *S. aureus*, *E. coli* and *Pseudomonas aeruginosa*. Recently, Khalaphallah and Wagdi (2014) have showed the extract of the plant is effective against *Pseudomonas aeruginosa*, *E. coli* and *Bacillus subtilis*.

On the contrary, all the *H. sabdariffa* extracts were not effective against *Proteus mirabilis*. Sharaf *et al.* (1966), however, discovered that the *H. sabdariffa* plant prevented the growth of *Proteus*. In this study, the *P. mirabilis* resistance to the plant extract could be due to the strains of *Proteus* included in these researches, the concentrations of the extracts and methodology used at that time which was different to the current CLSI method used. The swarming phenomenon of *Proteus mirabilis* also is presumed to be a factor of its supposed resistance since CLSI (2012) stated that “Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp., ignore the thin veil of swarming growth in an otherwise obvious zone growth inhibition”. Yet in this case the swarming was not a thin envelope; and probably the presence of certain constituents in the plant extract could have encouraged it to swarm the more. The variation in the degrees of antimicrobial activities of the extracts on the isolates is presumed to be due to differences in responses by the isolates to different active compounds present in the plant.

There is little literature about the antimicrobial property *H. sabdariffa* plant though some scientists have attributed it to the presence of the secondary metabolites (alkaloids, flavonoids, phenolics, and biterpenoids) in the extract (VanEtten *et al.*, 1994; Badreldin *et al.*, 2005; Rios and Recio, 2005; Olaleye, 2007). Flavonoids are phenolic compounds; C6–C3 unit linked to an aromatic ring which is known to be synthesized by plants in response to microbial infection, and so it is not astonishing that they are antimicrobial substances against a wide array of microorganisms. Cowan (1999), reported that the number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity.

The mechanism of action of the extract may be by inhibition of electron transport, protein translocation, phosphorylation steps, and other enzyme-dependent reactions, followed by an increase in plasma membrane permeability and finally ion leakage from the bacterial cells (Walsh *et al.*, 2003) and may be related to the permeability of the bacteria cell surface to the extracts (Cowan, 1999).

H. sabdariffa calyces also contain proanthocyanidins. Proanthocyanidins is recognized to either combine to bacterial cells P-fimbriae or transform the structural entity of P- fimbriae and inhibit formation of the adhesion of P-fimbriated *E. coli* to the uroepithelium, also inhibiting the formation of biofilm *in vitro* (Gupta *et al.*, 2012). The fimbriae of *Klebsiella* sp., *Salmonella* sp., *Pseudomonas aeruginosa* also adhere to the epithelial cells and bind to specific protein receptors to initiate infection; by inference, proanthocyanidins in *H. sabdariffa* may also prevent the biofilm formation by these bacteria.

The same effect is seen in *H. sabdariffa* calyces through a recent study done by Alshami and Alharbi (2014) who discovered that *H. sabdariffa* calyces extract may inhibit biofilm formation capacity against uropathogenic isolates. Biofilm forming bacteria also are known to be associated with long term persistence and resistance to antibiotics. The effectiveness of *H. sabdariffa* against these bacteria gives a basis for the potency against resistant strains such as ESBL in this present study.

The high potency of *H. sabdariffa* against these bacteria gives scientific basis for its uses in folk medicine in the treatment of abscesses, bilious conditions, cough, dysuria and scurvy (Morton, 1987; Perry, 1980; Ross, 2003).

Both the agar diffusion test and MIC using the micro-dilution method were used in the study to generate quantitative results that provided a clearer interpretation. The study revealed that MIC gave the minimum concentration of the extract that can inhibit microbial growth. The susceptibility of the microorganisms against the extract was better determined by MIC, with values ranging from 6.25mg/ml to 50mg/ml. According to Allen *et al.* (1991), low levels of antimicrobial activity of plant extracts are not detectable in agar disc diffusion method. MIC is a reliable and appropriate method that has been identified as more accurate than agar disc diffusion (Eloff, 1998). This could explain the variation in the MIC and agar diffusion test in which low concentration of the extracts (25mg/ml) gave an inhibitory effect in *Salmonella sp.* and *Staphylococcus aureus* as compared to the Agar diffusion test which gave an inhibitory effect at 100mg/ml.

The slightly higher potency of the ethanolic extract over the aqueous extract in the Agar Diffusion assay agrees with the reports of Frimpong (2008) and Khalaphallah and Wagdi (2014) where ethanol extracts also showed a higher growth inhibition as compared with water extract. This may be attributed to the fact that ethanol was able to extract constituents from *Hibiscus sabdariffa* calyces with more potent anti-microbial activity than water. This detected difference may be due to insolubility of the active compounds in water. The MIC values for both the ethanol and water extract was generally the same suggesting that both extracts were generally the same with few non- polar compounds from the ethanol one.

According to Sharaf *et al.* (1966) the coloring matter of the calyces is said to be lethal and this colouring matter may have been fully extracted in both water and ethanol.

The study also shows that the *S. aureus* even MRSA is very susceptible to the calyces. Most plant products are more active against Gram positive bacteria than Gram negative bacteria (González-Lamothe *et al.*, 2009). Gram negative bacteria are the most common pathogens of plants. However, Gram positive bacteria, on the other hand, are often sensitive to biologically active plant products and this proposes that the susceptibility of Gram negatives and Gram positives organisms to plant products is due to their cell wall and membrane arrangement (Rocío *et al.*, 2009) and their permeability to the extracts. Nair and Chanda (2006), also found similar effects, and reported that ATCC strains of Gram positive bacteria were more sensitive than Gram-negative ones toward the plant extracts.

The aqueous leaves extract was active against only *Pseudomonas aeruginosa* and *Escherichia coli*. The results show the possible phytochemical compounds present in the dark green to red leaves. The little colouring matter could also be a factor. In folk medicine, the leaves of *Hibiscus sabdariffa* are also used in soups in Northern Ghana. The water extract of the leaves used in soups keeps a few more days in good condition than that prepared with ordinary water. This proposes antimicrobial/ preservative properties of the leaves (Dokosi, 1998).

Studies indicate that leaves of the plant have an *in vitro* inhibitory effect against some microorganisms (Guerin and Reveillere, 1984). In the present study, aqueous leaves extract activity against only *Pseudomonas aeruginosa* and *Escherichia coli* might be that these bacteria strains were susceptible to the leaves constituents. Ewansiha (2014) confirms the antimicrobial property of the aqueous extract of *H. sabdariffa* leaves against *Klebsiella pneumoniae*, *Salmonella typhi* and *Shigella dysenteriae* but resistant to the hexane extract.

The *Hibiscus sabdariffa* roots extract, in this study, did not prevent the growth of all the test microorganisms. This is similar to investigations that show that Petroleum ether extracts of leaves, stem and root of the plant that did not show any activity at all in *E. coli*, *Pseudomonas* sp., *S. aureus*, *P. vulgaris*, *Salmonella* spp. (Arvind and Alka, 2011). These disparities are perhaps due to phytochemical constituents which are extracted differently based on the polarity of solvent used (Ewansiha, 2014). Additionally, Alo *et al.* (2012) has also reported that the inactivity of plant extracts may be due to age of plant, extracting solvent, method of extraction and time of harvesting of plant materials.

The results of the present study support the use of *H. sabdariffa* and the importance of the use of plant extracts worldwide for therapeutic purposes since the prolonged use of antibiotics has led to drug resistant clinical isolates. Additionally, very few antimicrobials are produced yearly. The discovery of antimicrobial plant extracts would be an effective means of controlling clinical infections.

5.1 LIMITATIONS

The calyces used in the study were not from the same plant as the roots and leaves since the ones harvested at the time of research was not matured and so the calyces were bought from another source.

Despite the above reason, the results of this study offer basic information needed to exploit the possibility of using *H. sabdariffa* as an antimicrobial agent against clinical isolates.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.0 CONCLUSION

Microbiological tests revealed that *Hibiscus sabdariffa* plant extract has antibacterial properties thus verifying folklore medicine in the treatment of abscesses, bilious conditions, cough, dysuria and scurvy (Morton, 1987; Perry, 1980 Ross, 2003). The extraction type of aqueous and ethanol generally had the same potency with ethanol extracts having a slightly better antimicrobial activity than aqueous extract in the agar diffusion test.

It can be concluded from this research that, of all the plant parts tested, *H. sabdariffa* calyces showed the best antimicrobial activity against all of the test organisms except *Proteus mirabilis*. The leaf extracts followed with activity seen in *E. coli* and *Pseudomonas aeruginosa* only. The roots, however, showed no antimicrobial activity against all the test bacteria. Therefore, the plant's calyces and leaves may be used for the production of antimicrobial agents which will significantly inhibit the growth of bacteria.

6.1 RECOMMENDATIONS

Further studies using solvents other than water and ethanol for extracting the important compounds from the *H. sabdariffa* plant as well as purification measures would be necessary.

Also, investigations of the plant extracts against more pathogenic bacteria as well as fungi.

Investigations done on the active constituents of the plant and side effects in addition to toxicology have to be studied.

Additionally, there is a need to study the phytochemical compounds from various part extracts of the plant.

The stem and seeds of the *Hibiscus sabdariffa* plant has to be experimented on also.

Finally, studies should be done *in vivo* to assess the clinical efficacy of the extract.

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APPENDICES

APPENDIX 1

Preparation of reagents and chemicals

A: Preparation of 5% DMSO Stock solution

A 5ml of DMSO (Sigma, D5879, Germany) was measured into a 100ml volumetric flask and diluted with sterile distilled water up to the 100ml mark. The solution was stored at 2-8°C.

B: Preparation of INT stock solution

INT (Iodonitrotetrazolium chloride, Fluka Biocheika, 58030, Sigma- Aldrich, Austria) stock was prepared by dissolving 2mg of INT crystals in 10ml of sterile distilled water. The stock solution was kept in a refrigerator (2-8°C) in a brown sterile bottle.

APPENDIX 2

Preparation of culture media

A: Bacteriological Peptone (Sigma- Aldrich, P0556, Germany)

i. Formula (g/litre)

Peptone 10.0g

Sodium chloride 5.0

pH 7.2± 0.2

ii. Preparation

This medium was prepared according to manufacturer's standard. The medium was heated until completely dissolved and then distributed into capped test tubes before autoclaving at 121 °C for 15 minutes.

B: Mueller- Hinton Agar (Oxoid, CM0337, Oxoid Ltd, England)

i. Composition (g/litre)

Beef, dehydrated infusion 300

Casein hydrolysate 17.5

Starch 1.5

Agar 17.0

pH 7.4±0.2

ii. Preparation

This medium was prepared by the producer's instruction. It was boiled to dissolve completely after which it was sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the medium was allowed to cool to 50°C and poured aseptically. Approximately 25ml volumes were dispensed into an 90mm sterile Petri Dishes and allowed to set. The agar plates were stored at 2-8°C. Sterility check was done on each batch of plates by incubating a plate at 37 °C aerobically for 18-24 hours. The media quality was checked by inoculating randomly selected media with control bacteria.

C: Mueller Hinton broth

i. Composition(g/litre)

Beef infusion solids 2.0

Acid hydrolyzed casein 17.5

Starch 1.5

Calcium ions 0.05

Magnesium ions 0.02

pH 7.3 ± 0.1

ii. Preparation

Broths were prepared according to the manufacturer's instruction (Liofilchem, Italy). The mixture was warmed until completely dissolve and then dispensed into appropriate bottles before autoclaving at 121°C for 15 minutes.

D: MacConkey Agar

i. Composition (g/ litre)

Peptone 20.0

Lactose 10.0

Bile salts 5.0

Neutral red 0.075

Agar 12.0

pH 7.4± 0.2

ii. Preparation

Media was prepared according to the manufacturer's instruction (Liofilchem, Italy). After sterilization, the media was cooled to about 55°C, and then approximately 25ml volumes were dispensed into 90mm sterile Petri dishes and allowed to set.

E: Cystine Lactose Electrolyte Deficient (CLEED) Agar

i. Composition (g/litre)

Peptone 4.0

Lab- Lemco 3.0

Tryptone 4.0

Lactose 10.0

L-cystine 0.128

Bromothymol blue 0.02

Agar 15.0

pH 7.3±0.2

ii. Preparation

Manufacturer's standard (Liofilchem, Italy) was followed. The medium was boiled to dissolve completely. Sterilization was done at 121°C for 15 minutes. After the medium had cooled, it was mixed and dispensed aseptically into sterile petri dishes and allowed to set. Sterility and media quality checks were done on them.

F: Blood and Chocolate Agar

i. Composition (g/litre)

`Lab-Lemco' powder 10.0

Peptone Neutralised 10.0

Sodium chloride 5.0

Agar 15.0

pH 7.3 + 0.2

ii. Preparation

Blood agar (Liofilchem, Italy) was prepared according to manufacturer's instruction. The dehydrated blood agar base was heated to dissolve entirely. After sterilization at 121°C for 15 minutes, the medium was cooled to 50°C and 7% sterile sheep blood was added to the agar based, mixed well and poured aseptically into Petri dishes.

Chocolate agar: A double strength blood agar base was prepared. Sterile 2% w/v haemoglobin powder added to the double strength agar base, mixed and poured aseptically into 90mm Petri dishes.

APPENDIX 3:

Pictures of *H. sabdariffa* plant parts

Plate 2: Intact calyces of the *Hibiscus sabdariffa* plant



Plate 3: Roots of *Hibiscus sabdariffa*



Plate 4: Leaves of *Hibiscus sabdariffa*



APPENDIX 4: Mean zone of inhibition of *Hibiscus sabdariffa* calyces extract concentration effect on the clinical isolates of bacteria

Clinical		Zone of inhibition (mm)					
Isolates	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i>	Aqueous	13.33±0.3a	10.33±0.3a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Aqueous	12.00±0.0b	5.00±0.0b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginos</i>	Aqueous	16.67±0.3c	14.00±0.0c	12.00±0.6b	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Aqueous	14.67±0.3d	5.00±0.0 b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Aqueous	17.00±0.0e	14.00±0.6c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i>	Ethanol	16.67±0.3c	12.33±0.3d	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Ethanol	14.33±0.6d	11.00±0.6e	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Ethanol	16.33±0.3c	13.33±0.6d	9.33±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Ethanol	15.67±0.3c	13.33±0.3d	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Ethanol	18.33±0.3f	14.33±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

abcdef Different letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of calyces extract

APPENDIX 5: Mean zone of inhibition of *Hibiscus sabdariffa* calyces extract concentration effect on the control ATCC strains of bacteria

Control Strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i> ATCC 25922	Aqueous	14.00±0.6a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K. pneumoniae</i> ATCC 33495	Aqueous	11.33±0.3b	7.66±0.3 b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i> ATCC 27853	Aqueous	15.33±0.3c	12.33±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i> ATCC 19430	Aqueous	16.33±0.3c	10.00±0.0d	5.0±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i> ATCC 25923	Aqueous	18.00±0.6d	14.00±0.6e	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i> ATCC 49565	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i> ATCC 49565	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i> ATCC 25922	Ethanol	18.00±0.6d	13.33±0.6c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K. pneumoniae</i> ATCC 33495	Ethanol	15.00±0.6c	11.00±0.0d	5.0±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i> ATCC 27853	Ethanol	16.67±0.3c	12.33±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i> ATCC 19430	Ethanol	18.67±0.3d	14.33±0.3e	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i> ATCC 25923	Ethanol	23.00±0.6e	15.33±0.3e	12.67±0.3b	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

abcde Different letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of calyces extract.

APPENDIX 6: Mean zone of inhibition of *Hibiscus sabdariffa* calyces extract concentration effect on four resistant strains of bacteria.

Resistant

Strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
ESBL C13EU	Aqueous	14.00±0.6a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Aqueous	17.00±0.6b	11.00±0.6b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Aqueous	19.33±0.3c	12.33±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Aqueous	18.00±0.6b	14.67±0.3d	10.00±0.0b	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C13EU	Ethanol	16.33±0.3e	11.33±0.3b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Ethanol	21.33±0.3f	16.00±0.0e	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Ethanol	16.33±0.3e	10.33±0.3b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Ethanol	24.00±0.3g	20.33±0.3f	15.67±0.3c	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

abcdefg Different letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of leaves extract.

APPENDIX 7: Mean zone of inhibition of *Hibiscus sabdariffa* leaves extract concentration effect on the clinical isolates of bacteria.

Clinical Isolates	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i>	Aqueous	9.00±0.6b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Aqueous	12.33±0.9c	9.33±0.3b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

‘abc’ Different letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of leaves extract

APPENDIX 8: Mean zone of inhibition of *Hibiscus sabdariffa* leaves extract concentration effect on the control ATCC strains of bacteria

Control strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Aqueous	10.67±0.3b	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K.pneumoniae</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K.pneumoniae</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

‘ab’ Different letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of leaves extract

APPENDIX 9: Mean zone of inhibition of *Hibiscus sabdariffa* leaves extract concentration effect on four resistant strains of bacteria.

Resistant Strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
ESBL C13EU	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C13EU	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

‘a’ Different letters denote there is no differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of roots extract

APPENDIX 10: Mean zone of inhibition of *Hibiscus sabdariffa* roots extract concentration effect on the clinical isolates of bacteria

Clinical Isolates	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Kleb.sp.</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

'a' Different letters denote there is no differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of roots extract

APPENDIX 11: Mean zone of inhibition of *Hibiscus sabdariffa* roots extract concentration effect on the control ATCC strains of bacteria

Control strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
<i>E. coli</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K.pneumoniae</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>P. mirabilis</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>E. coli</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>K.pneumoniae</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>Ps. aeruginosa</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. typhi</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
<i>S. aureus</i>	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

‘a’ Different letters denote there is no differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of roots extract

APPENDIX 12: Mean zone of inhibition of *Hibiscus sabdariffa* roots extract concentration effect on four resistant strains of bacteria.

Resistant Strains	Extraction type	Zone of inhibition (mm)					
		200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	0mg/ml
ESBL C13EU	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Aqueous	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C13EU	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
ESBL C16E5	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 744	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a
MRSA 5038	Ethanol	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a	5.00±0.0a

(Data are mean ± SEM values)

‘a’ letters denote differences in zones of inhibition for 200mg/ml, 100mg/ml and 50mg/ml of roots extract.

APPENDIX 13: Clinical Test Microorganisms To Reference Antibiotics (Agar Diffusion Test)

Test organisms	Mean Zone of Inhibition (mm)	
	Standard Antibiotics	
	Ciprofloxacin(5µg)	Amikacin (30µg)
<i>Escherichia coli</i>	9	19
<i>Pseudomonas aeruginosa</i>	25	25
<i>Proteus mirabilis</i>	31	21
<i>Klebsiella sp.</i>	9	22
<i>Salmonella typhi</i>	32	29
<i>Staphylococcus aureus</i>	30	25

Bacteria tested in MHA medium

APPENDIX 14: Control ATCC strains to reference antibiotics (Agar Diffusion Test)

Test organisms	Zone of Inhibition (mm)	
	Standard Antibiotics	
	Ciprofloxacin(5µg)	Amikacin (30µg)
<i>Escherichia coli</i> ATCC 25922	28	25
<i>Proteus mirabilis</i> ATCC 49565	33	22
<i>Salmonella typhi</i> ATCC 19430	28	22
<i>Klebsiella pneumoniae</i> ATCC 33495	25	22
<i>Pseudomonas aeruginosa</i> ATCC 27853	28	26
<i>Staphylococcus aureus</i> ATCC 25923	28	24

Bacteria tested in MHA medium

APPENDIX 15: Minimum Inhibitory Concentration of some selected test bacteria

Test organisms	Ethanol (mg/ml)	Aqueous	Ciprofloxacin (μ g/ml)	Amikacin
<i>Escherichia coli</i>	25	25	>40	>80
<i>Escherichia coli</i> (ATCC 25922)	25	-	20	>80
<i>Salmonella typhi</i>	25	25	>40	>80
<i>Salmonella typhi</i> (ATCC 19430)	25	-	>40	>80
<i>Klebsiella</i> sp.	25	-	>40	>80
<i>Klebsiella pneumoniae</i> (ATCC 33495)	25	25	40	80
<i>Pseudomonas aeruginosa</i>	50	50	40	>80
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	25	50	40	>80
<i>Staphylococcus aureus</i> (ATCC 25923)	25	50	>40	80
<i>Staphylococcus aureus</i>	50	25	>40	80
ESBL C13EU	50	-	40	>80
ESBL CI6E5	50	50	>40	>80
MRSA 744	6.25	6.25	>40	40
MRSA 5038	12.5	12.5	>40	40

Ciprofloxacin- Initial concentration of 20 μ g/ml; Amikacin - Initial concentration 80 μ g/ml

APPENDIX 16-19: All statistical tables**APPENDIX 16**

Independent sample test comparing the zone of inhibition between aqueous and ethanolic extract of *Hibiscus sabdariffa* calyces against clinical isolates of bacteria

	t-test for Equality of Means					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Mean zone of Equal variances assumed inhibition (mm)	-.581	34	.565	-1.16556	-5.24031	2.90920

ANOVA comparing the zone of inhibition of the concentration of *Hibiscus sabdariffa* calyces against clinical isolates of bacteria

Mean zone of inhibition (mm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	366.704	2	183.352	6.909	.003
Within Groups	875.712	33	26.537		
Total	1242.416	35			

Post-Hoc**Multiple comparisons**

Mean zone of inhibition (mm)

LSD

(I) Concentration (mg/ml)	(J) Concentration (mg/ml)	Mean Difference (I- J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
200 mg/ml	100 mg/ml	3.52917	2.10304	.103	-.7495	7.8078
	50 mg/ml	7.80583*	2.10304	.001	3.5272	12.0845
100 mg/ml	200 mg/ml	-3.52917	2.10304	.103	-7.8078	.7495
	50 mg/ml	4.27667	2.10304	.050	-.0020	8.5553
50 mg/ml	200 mg/ml	-7.80583*	2.10304	.001	-12.0845	-3.5272
	100 mg/ml	-4.27667	2.10304	.050	-8.5553	.0020

*. The mean difference is significant at the 0.05 level.

APPENDIX 17

Independent sample test comparing the zone of inhibition between aqueous and ethanolic extract of *Hibiscus sabdariffa* calyces against resistant isolates of bacteria

		t-test for Equality of Means					
		t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Mean zone of inhibition (mm)	Equal variances assumed	-1.025	22	.317	-2.51667	-7.60932	2.57599

ANOVA comparing the zone of inhibition of the concentration of *Hibiscus sabdariffa* calyces against resistant isolates of bacteria

Mean zone of inhibition (mm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	511.891	2	255.945	16.688	.000
Within Groups	322.082	21	15.337		
Total	833.973	23			

Post-Hoc**Multiple comparisons**

Mean zone of inhibition (mm)

LSD

(I) Concentration (mg/ml)	(J) Concentration (mg/ml)	Mean Difference (I- J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
50	100	-5.65000*	1.95814	.009	-9.7222	-1.5778
	200	-11.31250*	1.95814	.000	-15.3847	-7.2403
100	50	5.65000*	1.95814	.009	1.5778	9.7222
	200	-5.66250*	1.95814	.009	-9.7347	-1.5903
200	50	11.31250*	1.95814	.000	7.2403	15.3847
	100	5.66250*	1.95814	.009	1.5903	9.7347

*. The mean difference is significant at the 0.05 level.

APPENDIX 18

Independent sample test comparing the zone of inhibition between aqueous and ethanolic extract of *Hibiscus sabdariffa* calyces against control ATCC strains of bacteria

		t-test for Equality of Means					
		t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Mean zone of inhibition (mm)	Equal variances assumed	-.891	34	.379	-1.89278	-6.21078	2.42523

ANOVA comparing the zone of inhibition of the concentration of *Hibiscus sabdariffa* calyces against control ATCC strains of bacteria

Mean zone of inhibition (mm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	763.852	5	152.770	7.053	.000
Within Groups	649.844	30	21.661		
Total	1413.695	35			

Post-Hoc**Multiple comparisons**

Mean zone of inhibition (mm)

LSD

(I) Concentration (mg/ml)	(J) Concentration (mg/ml)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
200 mg/ml	100 mg/ml	4.11917	2.24744	.076	-.4533	8.6916
	50 mg/ml	8.30250*	2.24744	.001	3.7300	12.8750
100 mg/ml	200 mg/ml	-4.11917	2.24744	.076	-8.6916	.4533
	50 mg/ml	4.18333	2.24744	.072	-.3891	8.7558
50 mg/ml	200 mg/ml	-8.30250*	2.24744	.001	-12.8750	-3.7300
	100 mg/ml	-4.18333	2.24744	.072	-8.7558	.3891

*. The mean difference is significant at the 0.05 level.

APPENDIX 19

Independent sample test comparing the zone of inhibition of aqueous extract of *Hibiscus sabdariffa* leaves against test bacteria

		t-test for Equality of Means					
		t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Mean zone of inhibition (mm)	Equal variances not assumed	-1.475	10	.182	-2.21667	-5.73887	1.130554

ANOVA comparing the zone of inhibition of the concentration of *Hibiscus sabdariffa* leaves against two test bacteria.

Mean zone of inhibition (mm)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.065	2	19.532	4.051	.056
Within Groups	43.398	9	4.822		
Total	82.462	11			

Post-Hoc**Multiple comparisons**

Mean zone of inhibition (mm)

LSD


(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
200 mg/ml	100 mg/ml	3.17500	1.55273	.071	-.3375	6.6875
	50 mg/ml	4.25000*	1.55273	.023	.7375	7.7625
100 mg/ml	200 mg/ml	-3.17500	1.55273	.071	-6.6875	.3375
	50 mg/ml	1.07500	1.55273	.506	-2.4375	4.5875
50 mg/ml	200 mg/ml	-4.25000*	1.55273	.023	-7.7625	-.7375
	100 mg/ml	-1.07500	1.55273	.506	-4.5875	2.4375

*. The mean difference is significant at the 0.05 level.

APPENDIX 20: ETHICAL CLEARANCE

UNIVERSITY OF GHANA MEDICAL SCHOOL
COLLEGE OF HEALTH SCIENCES
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My Ref. No: **MS-AA/C.2/Vol.18^A**



P O Box 4236
Accra
Ghana

2nd December, 2013

Your Ref. No.

Ms. Priscilla A.N.A. Lovell-Antiaye
M.Phil Candidate
Dept. of Microbiology
UGMS

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.2 – P 4.5/2013-2014

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 29th November, 2013 unanimously approved your research proposal.

TITLE OF PROTOCOL: "Antimicrobial Activities of Hibiscus Sabdariffa against Clinical Isolates of Bacteria"

PRINCIPAL INVESTIGATOR: Ms. Priscilla A.N.A. Lovell-Antiaye

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.


Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till October, 2014.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: 
PROFESSOR JENNIFER WELBECK
(CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: As. Dean
Head of Department
Research Office