SUSCEPTIBILITIES OF *Salmonella typhi* AND OTHER BACTERIAL PATHOGENS TO ANTIBIOTICS AND HOT AQUEOUS EXTRACT OF *Hibiscus sabdariffa*

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Susceptibilities of *Salmonella typhi* and other Bacterial Pathogens to Antibiotics and hot aqueous extract of *Hibiscus sabdariffa*

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CERTIFICATION

I certify that this research project was carried out by Chukwuemeka Chukwuma K. with the Registration number MB /2008/399 in the Department of Microbiology and Biotechnology, Faculty of Natural Science, Caritas University, Amorji-Nike, Enugu. The department recognises that Chukwuemeka Chukwuma. K. (MB /2008/399) bears full responsibility of this work.

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DEDICATION

This work is dedicated to Almighty God for his mercies and an unending grace upon my life.

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You won't win if you don't begin; for every beginner is a potential winner, God has made me a beginner and a winner. I am grateful.

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ABSTRACT

The susceptibilities of Salmonella typhi and other pathogens to antibiotics and hot aqueous extract of *Hibiscus sabdariffa* were investigated using agar diffusion and agar well diffusion methods respectively. *Salmonella typhi* was sensitive to ampicillin, cetriaxone, ciprofloxacin, gentamycin of ofloxacin and perfloxacin. Nitrofurantoin, ampicillin, clarithomycin and augumentin are resistant. *Escherichia coli, Klebsiella spp*, and *Staphylococcus aureus* were sensitive to 50%, 70% and 60% of the antibiotics respectively. *Pseudomonas aeruginosa* was resistant to all antibiotics. *Hibiscus sabdariffa* extract (0.6g in 6ml of sterile distilled water) was active against *S. typhi* at concentrations of 100mg/ml, 50mg/ml and 25mg/ml (inhibitions zone diameter IZDs = 23mm, 20mm and 16mm respectively). *Staphylococcus aureus* was susceptible to 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml of the extract with IZDs of 29mm, 18mm, 17mm and 14mm respectively. *Klebsiella spp* was susceptible to concentrations of 25mg/ml and 12.5mg/ml of the extract with IZDs of 15mm and 10mm respectively. *Escherichia coli* and *Pseudomonas aeruginosa* were resistant to all the concentrations of *H. sabdariffa* extract. It is therefore imperative to note that the use of medicinal plants is recommended to the Government and Industry.

CHAPTER ONE

INTRODUCTION

Long before mankind discovered the existence of microbes, the idea that plants have some healing potentials, i.e. that they contain what we will currently characterize as antimicrobial principle was well accepted (Doughari, mahmood & Tyoyina, 2011). In whatever manner early man gained his knowledge of the curative powers of plants, one must assume that the was able thereafter to recognize the plant, since the detailed flora available today, were not in existence then (Sofowora, 2008). The use of higher plants and their extracts to treat infectious diseases is an age old practice in traditional African medicine (Onyeagba, ugbogu, Okeke & Iroakasi, 2004). Traditional medicine practice has been known for centuries in many parts of the world (Sofowora, 1984). It is however observed that these practices vary from one country to another (Onyeagba, ugbogu, Okeke & Iroakasi 2004). Nature has been a source of medicinal agents for thousands of years. The use of herbs is the most ancient approach to healing known (Apata, 1979).World Health Organization (WHO) in

1978 defined traditional medicine as the sum total of knowledge or practices whether explicable or inexplicable used in diagnosing and preventing a physical, mental or social disease which may rely exclusively on past experience or observation handed down from generation to generation, verbally or in writings. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of habitual treatment of various maladies (Doughari et al). Numerous plants and herbs are used all over Nigeria as phytomedicine by traditional medicine practioners. Plant extracts are given singly or as concoction for various ailments. The medicine could be either in the form of powders, liquids, liniments and inclusion accoding to Apata. More than 70% of people living in Nigeria depend on these various forms of concoctions and herbal decocotions for the treatment of some diseases (Kimbi-Beyioku, 1996). Many investigators have demonstated the antimicrobial activity of the constituents of some higher plants (Akobundu & Agykara, 1987; Rocio and Rion, 1982; Almagboul et al 1988; Misra et al, 1992; Hablemariam et al*;* 1993) and quite a number

of chemical compounds of plant origin have been shown to possess antimicrobial activity (Corthout, Piefers & Cleays, 1992). One of such plants is *Hibiscus sabdariffa.*

Hibiscus sabdariffa (called Roselle in English or Zobo in the northern part of Nigeria) which belongs to mallow family (*malvaceae*) is native to West Africa. *Hibiscus sadariffa* is cultivated in loamy, well drained soil mainly in tropical climates and requires rainfall averaging about 10 inches (15 cm) each month throughout the growth season. *Hibiscus sabdrriffa* is of several use, it is considered to have anti-hypertensive properties. In some places the plant has been used in folk medicine as diuretic, mild laxative and treatment for cardiac and nerve diseases and cancer to mention but a few.

However, the plant (*Hibiscus sabdariffa*) is rich in anthocyanin. The dried calyces contained flavonoids gossypetin, hibiscentine and sabdaretine. The major flavonoid formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrillin (delphinidin 3- monoglucoside), chrysanthenin (cyanide 3-monoglucoside) and delphinidin are also present (Mohammed,

Fernandez, Pineda & Aguilar, 2007). All these gave it the qualities to be regarded as a chemotherapeutic agent against some microorganisms especially some pathogens of man.

Salmonella typhi is an enteric bacterium responsible for causing typhoid fever which has affected mankind since human population became large enough to contaminate the supply of its water. It is a food borne disease contracted by ingestion of bacteria in contaminated food or water (jerry, 2007). The sources of infection could be through infected food, poor kitchen hygiene, and excretions from either sick people or infected but apparently clinically healthy people and animals, polluted surface water standing water and so on. The signs and symptoms of the disease has 4 phases, first week involves the slow rise in temperature, headache, cough, malaise and abdominal pain. In the second week of the infection, high fever in plateau around $40^{o}C$ ($104^{o}F$) and bradycardiac (Sphygmothermic dissociation) and delirium is frequent. The patient may be calm but sometimes agitated, thus it gave typhoid fever the nickname called "Nervous fever". In the third week, intestinal hemorrhage occurs, Encephalitis,

Neuropsychiatric symptoms, metastatic abscesses and endocarditis is seen. The final week (fourth), the patient enters into the typhoid state. The incidence of *Salmonella* infection may not be perfectly known. This is because the majority of patients are treated as outpatients and therefore hospital based studies will underestimate the true incidence (W.H.O, 2006). However, the incidence of typhoid fever in developing countries is higher compared to other developed countries. The pathogenesis of typhoid fever is a complex process which proceeds through several stages with an asymptomatic incubation period of 7-14days inversely related to the size of the infecting dose during which bacteria invade macrophages spread throughout the reticuloendothelial system. After passing through the pathological stages, necrosis may occur. *Salmonella typhi* infection can be best prevented by sanitation and hygiene. It can be controlled using a wide variety of methods such as the use of vaccines e.g live oral Ty2la vaccine (sold at Vivotif Berna). Diagnosis is by bone marrow or stool culture and with a widal test i.e (demonstration of *Salmonella* antibodies against O and H antigens). Treatment is by the use of

antibiotics such as ampicillin, chloramphenicol, trimethoprim, sulfomethriazole and ciprofloxacin. Since some plants have been shown to have antimicrobial effect against some pathogenic bacteria, especially antibiotic resistant pathogens, this study is aimed at testing the effect of *H. sabdariffa* on *S. typhi* and other pathogens such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumonia*. The aim of the study is to determine the invitro effect of *H. sabdariffa* on a clinical isolate of *S. typhi* and other pathogens. Below are the objectives of this study.

1.1. Objectives

- Collection and identification of *H. sabdariffa* flower
- Antibiotic sensitivity testing of the test organism
- Antimicrobial screening of *H. sabdariffa* extract against the test organisms.
- Investigation of the susceptibility of *S. typhi* and other pathogens to hot water extract of *H. sabdariffa* flower.

CHAPTER TWO

LITERATURE REVIEW

2.1. PLANT EXTRACT

In whatever manner early man gained his knowledge of the curative powers of plants, one must assume that he was able thereafter to recognize the plant, since the detailed flora available today were not in existence then as recoded by Sofowora. It is estimated that there are 250,000 to 500,000 species of plants on earth (Borris, 1996). A relatively small percentage (1-10%) of these is used as foods by both humans and animal species. It is possible that even more are used for medical purposes (Moerman, 1996). Hippocrates (in the late fifth century B.C) mentioned 300-400 medicinal plants (Schultes, 1978). The use of higher plants and their extracts to treat infection is an age old practice in traditional African medicine. Traditional medicine practice has been known for centuries in many parts of the world (Sofowora, 1984). It is however observed that these practices vary from one country to another (Onyeagba et al, 2004). Nature has been a

source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. The plant based, traditional medicine system continues to play an essential role in health care (Owolabi, Omogai & Obasuji, 2007). According to the world health organization, medicinal plants would be the best source of a variety of drugs. Therefore such plants should be investigated to better understand their properties, safety and efficacy (Nascimento, Lacatelli, Freitas & Silva, 2000). The use of herbs is the most ancient approach to healing known. The medicine could be either in form of powders, liquids and liniments according to Apata. Many investigators have demonstrated the antimicrobial activity of the constituents of some higher plants (Akobundu & Agykara, 1987) and quite a number of chemical compound s of plants origin have been shown to possess antimicrobial activity (Corthout, Piefers & Cleays, 1992).

2.1.1. Brief History of Medicinal Plants

The bible offers description of approximately 30 healing plants. Indeed frankincense and myrrh probably enjoyed status of great worth due to their medicinal properties. Reported to have antiseptic properties, they were even employed as mouth washes (Majorie, 1999). The fall of ancient civilization forestalled western advances in the understanding of medicinal plants, with much of the documentation of plant pharmaceuticals being destroyed or lost (Stockwell, 1988). During the dark ages, the Arab world continued to excavate their own works and to build upon them. Of course, Asian cultures were also busy compiling their own pharmacopoeia. However, medicinal plants are a source of great economic value in the Indian subcontinent. India is the largest producer of medicinal herbs and is called botanical garden of the world (Uma & Sudersaman, 2011). A large number of diverse types of plants grow in different parts of the country. India is rich in all three levels of biodiversity namely, species diversity, genetic diversity and habitat diversity. In India, thousands of species are known to have medicinal value and the

use of different parts of several medicinal plants to cause specific ailments has been in vogue since ancient times. Ayurveda, Unani, Siddha and folk (also called tribal) medicines are major systems of Indian medicines (Uma & Sudersaman, 2011). North America's history of plant medicinal use follows two sides i.e. their use by indigenous cultures (Native Americans), dating from prehistory (Weiner, 1980) and an alternate movement among Americans of European origin, beginning in the 19th century. Native Americans use of plant medicinal has been reviewed expensively in a series of articles by Moerman. He reported that 1,625 species of plants have been used by various Native American groups as food and 2564 have found use as drugs (Klink, 1997). Speculation as to how and why a selected number of plant species came into use for either food or drugs is fascinating but outside the scope of review as reviewed by Marjorie in 1999.

According to Okigbo and Mmeka (2006), traditional medicine is a major African socio-cultural heritage has been in existence for hundreds of years. In African countries, hunters have been reported as

original custodians of some effective traditional herbal recipes. Such knowledge could have been acquired when for example a hunter shot an Elephant, if the Elephant ran away, chewed leaves from a specific plant as a specific antidote for wound, or for relieving pains. Similar observations were made by Apata in villages where for example domestic animals chewed leaves of specific plant when the animal was ill and later recovered or when another animal accidentally chewed a leave and died. It was once to believe to be primitive and wrongly challenged by foreign religion dating back during the colonial rule in Africa and subsequently by the conventional or orthodox medical practitioners as recorded by Okigbo and Mmeka. W.H.O, defined traditional medicine as the sum total of knowledge or practices explicable or inexplicable used in diagnosing, preventing or eliminating a physical, mental or social disease, which may rely exclusively on past experience or observations handed down from generation to generation verbally or in writing.

In Nigeria, the new health agenda focuses on the institutionalization of traditional medicine in parallel with orthodox medicine into

national health care scheme in order to move health agenda forward since effective health cannot be achieved in Nigeria by orthodox medicine alone unless it has been complimented with traditional medicine. (Elujoba, Odeleye & ogunyemi 2005). Numerous plants and herbs are used all over Nigeria as phytomedicine by traditional medicine practitioners. Plants extracts are given singly or as concoction for various ailments. More than 70% of the people living in Nigeria depend on these various forms of concoction and herbal deconcoction for the treatment of some diseases (Kimbi & Fagbenro-Beyioku, 1996). According to legend, the first man to practice the act of healing was Orumnila in the Yoruba speaking part of Nigeria. Orunmila was endowed with the knowledge of the gods. Orunmila had a younger brother Osanyin who gained knowledge of medicinal herbs through assisting his elder brother to compound drugs. The two brothers were separated during inter tribal wars, but Orunmila continued to heal with herbs. One day, a slave in the household commented on some medicinal herbs to Orumila's wife Gbinrinbiti, whom reported the fact to her husband. During an interview, it was

revealed to Orunmila that this slave was his own brother Osanyin. These two legendary figures were believed to be the first to practice medicine in Nigeria by Sofowora. Long before mankind discovered the existence of microbes, the idea that plants have some healing potentials, indeed that they contain what we will currently characterize as antimicrobial properties was well accepted (Doughari et al, 2011). Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies as seen by Doughari, et al. For example, the use of bearberry (*Arctostaphyles uvaursi*) and cranberry juice (*Vaccinium microcarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy while species such as lemon balm (*Mellisa Officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleica alternifonia*) are described as blood spectrum antimicrobial agents (Rio & Rocio, 2005). Chaulmorgra oil from species of hydno carpus gaeth is one of the earliest recorded herbal medicines used. This was known to be effective in the treatment of leprosy. Such as use was recorded in the pharmacopoeia

of Emperor Chen Nung of china between 2730 and 3000BC.It's not known however, who was the discoverer of this medicinal uses of the oil nor was the time of discovery reviewed. Similarly, the seed of the "opinum" poppy (*Papaver somniferum*) were excavated from some ancient Egyptians far back as 1500BC (Sofowara). The records available in such papyri as the ebers papyrus also confirmed that medicinal plants were used at that time in Egypt. Opium seeds and Castro oils seeds were used for the same medicinal purposes as they are today but the discoverer and the exact date of discovery is not known. According to Sofowora who said that some 3000 years B.C, man was already aware of the medicinal properties and some toxic effects of some plants grown around him. Below are some African medicinal plants with their medicinal value.

Table 1 Some African medicinal plants

Plants	Disease cured	Action	Usage
Xylopia aethiopica	intestinal spasms, cough, post partum tonic, for lactation, stomach remedy, bronchitis, biliousness, dysentery, headache, female hygiene	soothing, antispasmodic, remove biliousness, emollient, sedative	poultice of the plant
Garcinia kola	bronchitis, throat infections, relieve colic, head or chest cold, cough, liver disorder	antibiotic, antispasmodic, soothing, sedative, ease cough, expectorant, choleretic	eating the seed of the plant
Crytolepis sanguinoleta	fever, malaria, urinary and upper respiratory tract infection, rheumatism, venereal diseases	antiplasmodial, antiviral, antispasmodic, expectorant, anti-inflammatory	hot poultice of dried root

2.1.2 Phytochemical components with antimicrobial activity

i. Alkaloids

The name "alkaloids" was introduced in 1819 by the German chemist Carl F.W.Meissner. Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen containing compounds that are found in over 20% of plant species. Alkaloids contain a ring with nitrogen. Many alkaloids have dramatic effects on the central nervous system. Caffeine is an alkaloid that provides a mild lift but the alkaloids in Datura cause severe intoxication and even death *(http//en.wikipedia.org./wiki/herbalism)*. They are widely used in medicine for the development of drugs (Okwu, 2005).

ii. Terpenes

Terpenes are a large and varied class of organic compounds, produced primarily by wide varieties of plants, particularly conifers. Vitamin A is an example of a terpene.When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids (Uma and Sudarsunam, 2011). Each terpene consists of two paired isoprenes.

The names monoterpenes, sesquiterpenes, diterpenes and triterpenes are based on the number of isoprene units (*http//en.wikipedia.org./wiki/herbalism).*

iii. Phenolics

In organic chemistry, phenols,sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol also called as carbolic acid C_6H_5OH. They have higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen.

iv. Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants, in response to microbial infection (Dixon & Dey, 1983), it should not be surprising that they have been found in vitro to be

effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinines by Majorie.

2.1.3. Characteristics Of Phytomedicine

Phytomedicine has some characteristics that make them unique and different from synthetic drugs (Calixto, 2000).

- The active principle is frequently unknown.
- The availability and quality control are frequently problematic.
- Standardization, stability and quality control are feasible but not easy.
- They have a wide range of therapeutic use and are suitable for chronic treatments.
- Well-controlled double blind clinical and toxicological studies to prove their efficacy and safety are rare when compared with synthetic drugs.
- They are cheaper than synthetic drugs.

2.1.4. Why the Demand For Phytomedicine?

For years, public interest has increased for natural therapies (mainly phytomedicine) all over the world including Africa (Blumenthal, 1999 & Grunwald, 1995). According to Calixto and Grunwald, there are several factors that lead to the preference and growth of phytotherapeutic market worldwide and they include

- Preference of consumers for natural therapies
- Great interest in alternative medicine
- The belief that phytomedicine is devoid of side effect since millions of people all over the world have been using phytomedicine for thousands of years
- The belief that phytomedicine is used for the treatment of certain diseases where conventional medicine fails
- Improvement in the quality, proof of efficacy and safety of phytomedicine and
- High cost of synthetic drugs.

2.1.5. Challenges in the Use and Development of Phytomedicine

There are many factors hindering the development of phytomedicine in Africa and these problems have to be fully addressed so as to move the African Health Agenda forward. Such problems include:

- Development of drug from its natural source is not an easy task and is more difficult than synthetic drug development; formulation of phytomedicine particularly in crude-drug form requires a specialized expert area that requires training and experience (Elujoba et al, 2005).
- Lack of standardization and quality control of the herbal drugs used in clinical trials (Calixto & Makhubu, 2006) and occult practices.
- The risk of side effect due to toxicity, over-dosage, interaction with conventional drugs as recorded by Calixto. Several manufacturing problems such as misidentification of plants as recorded by Calixto, lack of standardization, failure of good manufacturing practice, contamination as a result of field microbial contamination, poor packaging, chemical used, the environmental condition (temperature, light exposure) (Elujoba et al, 2005 &

Calixto, 2000), substitution and adulteration of plants, incorrect preparation and dosage.

- Imprecise diagnosis and dosage for phytomedicine (Calixto, 2000).
- There is lack of collaborative research among TMP's, Orthodox medical practitioners and scientists (Elujoba et al, 2005 & Makhubu, 2006).

As a result, there is a danger of losing valuable ethno-medical knowledge that the TMPs have concerning the plant and other aspects of the medicinal system that are intrinsically part of their lives as recorded by Makhubu.

- Inadequate randomizations in most studies. Patients are not properly selected and the numbers of patients used in most trials are insufficient for the attachment of statistical significance (Calixto, 2000).
- Problem of serious attention, energy, resource mobilization commitment and the required political will (Elujoba et al, 2006).
- Communication problem is an obstacle between the TMPs and the scientists (Makhubu, 2006).

• There is wide variation in the duration of treatment using herbal medicine

Calixto.

• Domestication: It is difficult to convince members of a community to trust phytomedicine after a long use of Orthodox medicine, as assessed by Makhubu.

• There is absence or inadequate record of what is available and many species are becoming extinct because they are not cultivated and protected from indiscriminate harvesting (Makhubu & McGee, 1998). Also, the traditional healers are of advancing age and dying (Elujoba, 2003).

• Unfavorable legislation such as witchcraft Act of 1901as recorded by Makhubu.

2.1.6. Possible Solutions

The quality and stability of phytomedicine is achieved by the use of fresh plants, regulated physical factors like temperature, light, water availability, cultivation of plants in place of wild-harvested plants, because they show smaller variation in their constituents. The

standardization of phytomedicine can also be achieved by the use of chromatography, infra-red and ultraviolet (UV) spectrometry (Calixto, 2000).

The African pharmacognosists, pharmacologists, pharmacists, physicians have to learn, acquire, document and use traditional medicine to help curtail the extinction of plants and human resources (Elujoba). Workshops with TMPs have to be conducted to break the communication problem between the TMPs and Scientists, and human resources can be obtained through individual contacts as recorded by Makhubu. Collaborative work could be achieved through staff exchange and training and funding for capital building; the government should help in funding researches on phytomedicine; the private sector as well as non-governmental agencies should also help finance researches; organization of seminars to raise awareness to the general public on the benefits of medicinal plants and also remove the perception that scientists are out to harness their knowledge for money making; abandoning outdated legislation (such as witchcraft Act, 1901) and passing new legislation to protect

2.2. A REVIEW OF *Hibiscus sabdariffa*

*Hibiscus sabdariffa***,** also called rosella, Jamaican sorrel, or java jute, (*roselle*), plant of the *hibiscus*, or mallow, family (*Malvaceae*), and its fibre is one of the vast fibre groups. *H. sabdariffa* is probably native to West Africa and includes *H. sabdariffa* variety *altissima*, grown for fibre, and *H. sabdariffa* variety *sabdariffa*, cultivated for the edible external portion of its flower (calyx). The plant, known in the West Indies early in the 16th century, was growing in Asia by the 17th century. Extensive cultivation in the Dutch East Indies (now Indonesia) began in the 1920s under a government-subsidized program established to obtain fiber for sugar-sack manufacture (The scientific classification is below)

2.2.1 *Scientific* classification

Kingdom: Plantae

(Unranked): Angiosperms

(Unranked): Eudicots

(Unranked): Rosids

Order: Malvales

Family: *Malvaceae*

Genus: *Hibiscus*

Species: *sabdariffa*

Binomial name: *Hibiscus sabdariffa*

2.2.2. Description

Although a perennial, *B* is usually grown as an annual and propagated from seed. It grows best in loamy, well-drained soil, mainly in tropical climates, and requires rainfall averaging about 10 inches (25 cm) each month throughout the growing season. Stalks and leaves range from dark green to reddish color; flowers are creamy white or pale yellow. For fiber crops, seeds are sown close together, producing plants 10 to 16 feet (3 to 5 meters) high, with little branching. The stalks, cut when buds appear, are subjected to a retting process, then stripped of bark or beaten, freeing the fiber. In some areas retting time is reduced by treating only the bark and its adhering fiber. Plants for fruit crops,

more widely spaced, are shorter and many-branched, and their calyxes are picked when plump and fleshy. The fibre strands, 3 to 5 feet (1 to 1.5 metres) long, are composed of individual fibre cells. Roselle fibre is lustrous, with colour ranging from creamy to silvery white, and is moderately strong. It is used, often combined with jute, for bagging fabrics and twines. India, Java, and the Philippines are major producers (*Encyclopedia Britannica, 2012).*

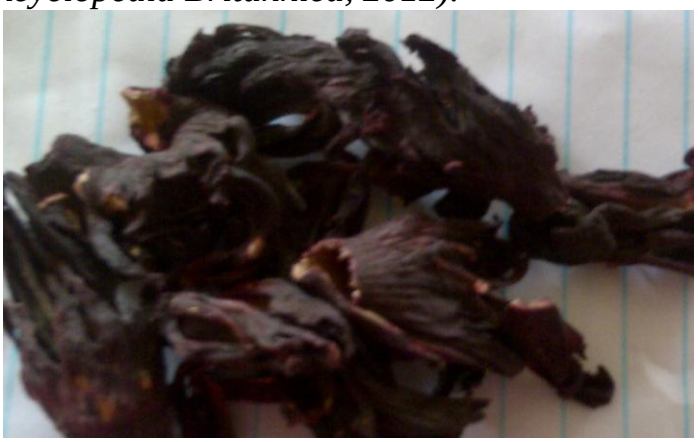

Fig. 1 *Hibiscus sabdariffa* calyces

2.2.3. Origin and Distribution

Roselle is native from India to Malaysia, where it is commonly cultivated, and must have been carried at an early date to Africa. It has been widely distributed in the Tropics and Subtropics of both

hemispheres, and in many areas of the West Indies and Central America has become naturalized.

The Flemish botanist, M. de L'Obel, published his observations of the plant in 1576, and the edibility of the leaves was recorded in Java in 1687. Seeds are said to have been brought to the New World by African slaves. Roselle was grown in Brazil in the 17th Century and in Jamaica in 1707. The plant was being cultivated for food use in Guatemala before 1840. J.N. Rose, in 1899, saw large baskets of dried calyces in the markets of Guadalajara, Mexico.In 1892, there were 2 factories producing *H. sabdariffa* jam in Queensland, Australia, and exporting considerable quantities to Europe. This was a short-lived enterprise. In 1909, there were no more than 4 acres (1.6 ha) of edible roselle in Queensland. A Mr. Neustad of San Francisco imported seeds from Australia about 1895 and shared them with the California State Agricultural Experiment Station for test plantings and subsequent seed distribution. It was probably about the same time that Australian seeds reached Hawaii. In 1904, the Hawaiian Agricultural

Experiment Station received seeds from Puerto Rico. In 1913 there was much interest in interplanting *H. sabdariffa* with Ceara rubber (*Manihot glaziovii* Muell. Arg.) On the island of Maui and there were some plantations established also on the island of Hawaii, altogether totaling over 200 acres (81 ha). The anticipated jelly industry failed to materialize and promotional efforts were abandoned by 1929 (Morton, 1987). P.J. Wester believed that *H. sabdariffa* was brought to Florida from Jamaica about 1887. Plants were grown by Dr. H.J. Webber at the United States Department of Agriculture's Subtropical Laboratory at Eustis, Florida, in the early 1890's, but all the roselle was killed there by a severe freeze in 1895.Today, *H. sabdariffa* is attracting the attention of food and beverage manufacturers and pharmaceutical concerns who feel it may have exploitable possibilities as a natural food product and as a colorant to replace some synthetic dyes.

2.2.4. Various Names of *H. sabdariffa*

The *H. sabdariffa* is known as the rosella or rosella fruit in Australia. Its close relative, *Hibiscus cannabinus* is also known as meśta/meshta

on the Indian subcontinent, Tengamora among assamese and "mwitha" among Bodo tribals in Assam, Gongura in Telugu, Pundi in Kannada, Ambadi in Marathi, LalChatni or Kutrum in Mithila Mathipuli in Kerala, chin baung in Burma, KraJiabDaeng in Thailand, som phor dee in Lao PDR, bissap in Senegal, Guinea Bissau, Mali, Burkina Faso, Ghana, Benin and Niger, the Congo and France, dah or dah bleni in other parts of Mali, wonjo in the Gambia, zobo in western Nigeria (the Yorubas in Nigeria call the white variety Isapa (pronounced Ishapa), Zoborodo in Northern Nigeria, Chaye-Torosh in Iran, karkade. In Egypt, Saudi Arabia, and Sudan, omutete in Namibia, sorrel in the Caribbean and in Latin America, Flor de Jamaica in Mexico, Saril in Panama, grosella in Paraguay, rosela in Indonesia, asam belandain. Malaysia. In Chinese it is (Luo Shen Hua). In Zambia the plant is called lumanda in ciBemba, katolo in kiKaonde, or wusi in chiLunda. In certain West Indian islands, Grenada and Trinidad for example, it is called Sorrel.

2.2.5. Climate

Roselle is very sensitive to frost. It succeeds best in tropical and subtropical regions from sea-level up to 3,000 ft (900 m) with a rainfall of about 72 in (182 cm) during its growing season. Where rainfall is inadequate, irrigation has given good results. It can be grown as a summer crop in temperate regions. The fruits will not ripen, but the herbage is usable (Morton, 1987).

2.2.6. Cultivation and Harvesting

While deep, fairly fertile sandy loam is preferrable, *H. sabdariffa* grew and produced well over many years in the oölitic limestone of Dade Country. Wester observed that the high pinelands were far more suitable than low-lying muck soils. The plants tended to reseed themselves and on some properties they spread so extensively they became a nuisance and were eradicated. *H. sabdariffa* is usually propagated by seed but grows readily from cuttings. The latter method results in shorter plants preferred in India for inter-planting with tree crops but the yield of calyces is relatively low. Seedlings may be raised in nursery beds and transplanted when 3 to 4 in (7.5-10 cm)

high, but seeds are usually set directly in the field, 4 to 6 to a hill, the hills 3 to 6 ft (0.9-1.8 m) apart in rows 5 to 10 ft (1.5-3 m) apart. When 2 or 3 leaves have developed, the seedlings are thinned out by 50%. If grown mainly for herbage, the seed can be sown as early as March, and no early thinning is done. *H. sabdariffa is* a short-day plant and photoperiodic. Unlike kenaf, *H. sabdariffa* crops cannot be grown successively throughout the year (Morton, 1987).

For herbage purposes, the plants may be cut off 6 weeks after transplanting, leaving only 3 to 4 in (7.5-10 cm) of stem in the field. A second cutting is made 4 weeks later and a third after another 4 weeks. Then the shorn plants are thinned out–2 of every 3 rows removed–and the remaining plants left to grow and develop fruit as a second product. The fruits are harvested when full-grown but still tender and, at this stage, are easily snapped off by hand. They are easier to break off in the morning than at the end of the day. If harvesting is over due and the stems have toughened, clippers must be used. The fruits of *H. sabdariffa* ripen progressively from the lowest to the highest. Harvesting of seeds takes place when the lower and middle tiers of the

last of the fruits are allowed to mature, at which time the plants are cut down, stacked for a few days, then threshed between canvas sheets as recorded by Morton.

2.2.7. Pests and Diseases

Roselle's major enemy is the root-knot nematode, *Heterodera rudicicola*. Mealybugs may be very troublesome. In Australia, 3 beetles, *Nisotra breweri*, *Lagris cyanea*, and *Rhyparida discopunctulata*, attack the leaves. The "white" roselle has been found heavily infested with the cocoa beetle, *Steirastoma breve* in Trinidad, with a lighter infestation of the red roselle in an intermixed planting. Occasional minor pests are scales, *Coccus hesperidum* and *Hemichionaspis aspidistrae*, on stems and branches; yellow aphid, *Aphis gossypii*, on leaves and flower buds; and the cotton stainer, *Dysdercus suturellus*, on ripening calyces.

In Florida, mildew (Oidium) may require control. Late in the season, leaves on some Philippine plants have appeared soft and shriveled; and Phoma sabdariffae has also done minimal damage (Morton, 1987).

2.2.8. General Uses of *H. sabdariffa*

The plant is considered to have antihypertensive properties. In some places, the plant is primarily cultivated for the production of vast fibre from the stem of the plant. The fibre may be used as a substitute for jute in making burlap (*hort.purdue.edu, 2008*). *Hibiscus*, specifically Roselle, has been used in folk medicine as a diuretic, mild laxative, and treatment for cardiac and nerve diseases and cancer (*drugs.com*, 2008). The red calyces of the plant are increasingly exported to America and Europe, where they are used as food colorings. Germany is the main importer. It can also be found in markets (as flowers or syrup) in some places such as France, where there are Senegalese immigrant communities. The green leaves are used like a spicy version of spinach. They give flavour to the Senegalese fish and rice dish thiéboudieune. Proper records are not kept, but the Senegalese government estimates national production and consumption at 700 t (770 short tons) per year (*http.www.en.m.wikkipedia; 2012)* In East Africa, the calyx infusion, called "Sudan tea", is taken to relieve

coughs. Roselle juice, with salt, pepper, asafetida and molasses, is taken as a remedy for biliousness.

The heated leaves are applied to cracks in the feet and on boils and ulcers to speed maturation. A lotion made from leaves is used on sores and wounds. The seeds are said to be diuretic and tonic in action and the brownish-yellow seed oil is claimed to heal sores on camels. In India, a decoction of the seeds is given to relieve dysuria, strangury and mild cases of dyspepsia. Brazilians attribute stomachic, emollient and resolute properties to the bitter roots. *H. sabdariffa* fruits are best prepared for use by washing, then making an incision around the tough base of the calyx below the bracts to free and remove it with the seed capsule attached. The calyces are then ready for immediate use. They may be merely chopped and added to fruit salads. In Africa, they are frequently cooked as a side-dish eaten with pulverized peanuts. For stewing as sauce or filling for tarts or pies, they may be left intact, if tender, and cooked with sugar. The product will be almost indistinguishable from cranberry sauce in taste and appearance. For making a finer-textured sauce or juice, sirup, jam, marmalade, relish,

chutney or jelly, the calyces may be first chopped in a wooden bowl or passed through a meat grinder. Or the calyces, after cooking, may be pressed through a sieve. Some cooks steam the *H. sabdariffa* with a little water until soft before adding the sugar, then boil for 15 minutes. *H. sabdariffa* sauce or sirup may be added to puddings, cake frosting, gelatins and salad dressings, also poured over gingerbread, pancakes, waffles or ice cream. It is not necessary to add pectin to make a firm jelly. In fact, the calyces possess 3.19% pectin and, in Pakistan, *H. sabdariffa* has been recommended as a source of pectin for the fruit-preserving industry. The young leaves and tender stems of *H. sabdariffa* are eaten raw in salads or cooked as greens alone or in combination with other vegetables or with meat or fish. They are also added to curries as seasoning. The leaves of green *H. sabdariffa* are marketed in large quantities in Dakar, West Africa. The juice of the boiled and strained leaves and stems is utilized for the same purposes as the juice extracted from the calyces. The herbage is apparently mostly utilized in the fresh state though Wester proposed that it be evaporated and compressed for export from the Philippines.

The seeds are somewhat bitter but have been ground to a meal for human food in Africa and have also been roasted as a substitute for coffee. The residue remaining after extraction of oil by parching, soaking in water containing ashes for 3 or 4 days, and then pounding the seeds, or by crushing and boiling them, is eaten in soup or blended with bean meal in patties. It is high in protein (Morton, 1987). Many parts of the plant are also claimed to have various medicinal values. They have been used for such purposes ranging from Mexico through Africa and India to Thailand. Roselle is associated with traditional medicine and is reported to be used as treatment for several diseases such as hypertension and urinary tract infections (*http://www.utirose.com*). There is currently insufficient evidence to demonstrate any beneficial effect of *H. sabdariffa* on raised blood pressure or on blood lipid lowering. Experimental results are contradictory.

Hibiscus sabdariffa has shown in vitro antimicrobial activity against *E. coli*. A recent review stated that specific extracts of *H. sabdariffa*

exhibit activities against atherosclerosis, liver disease, cancer, diabetes and other metabolic syndromes. The constituents of *H. sabdariffa* are written in the table below.

Table 2: Constituents of *H. Sabdariffa*

Constituents'	percentage (%)
➢ Moisture	7.6
➢ Crude extract	24
➢ Fat	22.3
➢ Fiber	15.3
➢ Ash	0.3
➢ Carbon	0.6
➢ Phosphorous	0.4
➢ myristic	2.1
➢ Palmitic	35.2
➢ Palmitholeic	2.0
➢ Stearic	3.4
➢ Oleic	34.0
➢ Linoleic	14.4
➢ Fatty acids	4-5
➢ Cholesterol	5.1
➢ Malvalic	1.3
➢ Sistosterol	61.3

2.2.9. Phytomedicine

The plants are rich in anthocyanins, as well as protocatechuic acid. The dried calyces contain the flavonoids gossypetin, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), Chrysanthenin (cyanidin 3-monoglucoside), and delphinidin are also present. Roselle seeds are a good source of lipid-soluble antioxidants, particularly gamma-tocopherol.

2.2.9. Various Research on *H. sabdariffa*

Scientists all over the world have sought to know the capabilities of this plant (*B*). Below are some of the findings of those scientists.

- ✓ Protective effects of dried flower extracts of *Hibiscus sabdariffa* oxidative stress in rat primary hepatocytes.

Dried flower extracts of *Hibiscus sabdariffa*, was found to possess anti-oxidant activity. The results indicated that the dried flower extracts (specifically the chloroform – soluble fraction and ethyl

acetate – soluble fraction) protect rat hepatocytes from free radical toxicity and genotoxicity.

- ✓ Investigation of the anti-spasmodic potential of *Hibiscus sabdariffa* calyces

Addition of an aqueous extract of *Hibiscus sabdariffa* calyces (2.5ml / bath approximately 125mg of starting crude material) inhibited the tone of various isolated muscle preparations (rabbit aortic strip rhythmically contracting rat uterus, guinea pig tracheal chain and rat diaphragm). Other muscles were stimulated (quiescent rat uterus and frog rectus abdominis), intravenous injection of the extract to anaesthetized cats lowered the blood pressure in a dose-response manner.

The inhibitory effects were resistant to a number of standard receptor blockers but the hypotensive influence was practically blocked by atropine.

- ✓ Hypocholesterolemic action of *Hibiscus sabdariffa*

A remarkable lowering effect in the level of different lipid fractions was noticed in spite of the continued cholesterol and cholic acid loading during the treatment.

2.3. DESCRIPTION OF *Salmonella typhi*

Typhoid or enteric fever caused by *Salmonella* Species is an ancient disease which has afflicted mankind since human population has been large enough to contaminate water of its supply. *Salmonella* injection is one of the most food-borne infections worldwide. Typhoid fever is referred to as the major health problem in developing countries where safe water supply and adequate sewage disposal are often lacking (Hornick, 1985). Wain, in 2002 viewed typhoid fever as an infection associated with water contaminated by feaces of infected humans or person to person contact.

A further explanation of typhoid fever is a disease that is characterized by progressing inversive infection but eventually leads to septicaemia (Dunston et al. 2007). A Salmonella infection is

thought to be a multi systemic disease with protean manifestation and initial lesions in the bowel (Fin et al. 2000). Typhoid fever is also defined by the delirium, obtundation, stupor, coma, or shock which may lead to death.

2.3.1. Scientific Classification of *Salmonella typhi*

Superkingdom: Bacteria

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Graminaproteobacteria

Order: Enterobacteriales

Family: *Enterobacteriacea*

Genus: *Salmonella*

Species: *typhi*

2.3.2. History of Typhoid Fever

Typhoid fever was not well understood in the ancient, world probably because its symptoms are not primarily diarrheal but rather systemic and non-specific (Wiley et al 2008). Typhoid fever, also known as

enteric fever, bilious fever or yellow jack is an illness caused by the bacterium called *Salmonella* enterica servar *typhi*.

Around 430- 426 B.C, a devastating plaque which some believed to have been typhoid fever killed one third of the population of the Athens. Ancient historian Thucy also contracted the disease but survived to write about the plaque. The cause of the plaque has long been disputed with modern academic and medical scientists considering typhus the most likely cause. It was only in the mid-19th century that physicians began to distinguish it from *typhus* and malaria. However a 2006 study detected DNA sequence similar to that of the bacterium responsible for typhoid fever. The disease is commonly transmitted through poor hygiene habit and poor public sanitation condition (Ashbolt, 2004).

In 1891, the rate of typhoid fever was 174 per 100,000 persons. The most notorious type of typhoid fever was Mary Mallon also known as Typhoid Mary a young Irish cook that lived in New York City was responsible for 53 cases of typhoid fever over a period of 15 years. In early 1990's, public health authorities told her to give up working as

cook or have her gall bladder removed. She did but returned later with a false name. She was later detained and quarantined after another typhoid fever outbreak. She died 26 years been quarantined (W.H.O, 2006).

2.3.3. Causative Agent

Typhoid fever is caused by *Salmonella typhi*, previously a pathogen specific only to human, as well as by certain non-typhoid *salmonella* (NTS) particularly paratyphoid strain A, B and C. (Yoons, 2004). The milder form of the disease, paratyphoid fever is caused by *S. paratyphi* A, B and C is also known as Horsellafeldil other servicers such as *S. typhimurium* can lead to a form of human gastroenteritis sometimes referred to as Salmonellosis.

Salmonellosis is genus in the family *Enterobacteriaceae*. They are Gram-negative flagellate, non-sporulating, facultative aerobic bacilli that ferment glucose, reduce nitrate to nitrite and synthesize peritrichous flagella when motile with diameters around 0.7 to 1.7um lengths from 2-5um.

They are chemoorganotrophs obtaining their energy from oxidation and reduction reactors organic sources, and are facultative anaerobes. Most species produce sulfide (Clark & Barret, 1998) which can readily be detected by growing item on media containing ferrous sulfonate such asTSI. Most isolates exists in two phases: a motile phase 1 and a non-motile phase Cultures are non-motile phase by Cragie tube. *Salmonella*, closely related to be *Escherichia* genus are found worldwide in cold and warm-blooded animals (including humans), and in the environment they cause illness like typhoid fever, paratyphoid fever and other food-borne illness (Ryan &Ray,2004). *Salmonella* is named after a pathro Daniel Elmor Salmon.

2.3.4. Mode of Transmission

Typhoid fever is contracted by the infection of the bacteria in contaminated food or water (Jerry). The sources of infection include:

- Infected food, often originating at the production site but then introduced into the stream of commerce.

- Poor kitchen hygiene, especially problematic in institutional kitchen and restaurants because this can lead to significant outbreaks.
- Excretions from either sick or infected but apparently clinically healthy people and animals (especially endangered are caregirers and animals)
- Polluted surface water and standing water (such as in shower houses or unused water dispensers)
- Unhygienically bared fowl (the melt water, contains many bacteria)

An association with reptiles (pet tortoise, snakes and frogs, but primarily aquatic turtles (Simon, 2011)

Since *Salmonella* can survive several weeks in dry environment and several months in water, they are frequently found in polluted water, contamination in excrement of carrier animals being particularly important. Aquatic vertebrates, notably birds and reptiles are important vectors of salmonella. Poultry, cattle and sheep frequently being agents of contamination, *Salmonella* can be found in food,

especially in milk, meats and sometimes in games which have crave (Humphrey, 1994).

2.3.5. Signs and Symptoms

Typhoid fever is characterized by a sustained fever as high as 40^{o}C (104^{o}F), profuse sweating, gastroenteritis and non bloody diarrhea. Classically symptoms of typhoid fever are divided into four stages, each lasting approximately one week. In the first week, there is a slow rise in temperature with severe headache, cough and malaise abdominal pain is seen also. A bloody nose (epitaxial) is seen in a quarter of cases. There is leucopemia and relative lymphocytosis, a positive reaction and blood cultures are positive for *Salmonella typhi*. The classic Widal test is in the next week .In the second week of the infection, the patient lies prostrate with high fever in plateau around 40^{o}C (104^{o}F) and bradycardia (Sphygmothermic dissociation) classically with a dicrotic pulse wave. Delirium is frequently calm but sometime agitated; this delirium gives to typhoid the nickname "nervous fever". Rose spots appear on the lower chest and abdomens

in around a bird of patients. There are rhonchi in lung bases. The abdomen is distended and painful in the right lower quadrant where borborvgmin can be heard. Diarrhea can occur in this stage. Six to eight stools in a day, green with a characteristic smell, comparable to pea soup. However, constipation is also frequent. The spleen and liver are enlarged (Hepatosplenomegaly) and tender, and there is elevation of liver bransaminase. The widal reaction is strongly positive with anti O and anti H-antibodies.

In the third week of typhoid fever, a number of constipation can occur

a. Internal hemorrhage due to bleeds in congested prefers patch, this can be very serious but it's usually not fatal.
b. Internal perrforation in the distal ileum. This is very serious and is frequently fatal.
c. Encephalitis.
d. Neuropsychiatric symptoms.
e. Metastatic abscesses, cholecystitis and endocarditis.

The fever is still high and oscillates, very little over 24 hours, dehydrahm ensures and use patient is activated to typhoid state. By

the end of third week, the fever has started reducing this (Defervescence). This carries on into the fourth and final week (Giannella, 1996).

2.3.6. Epidemiology of Typhoid Fever

The evidence of *Salmonella typhi* may not be perfectly known, this is because the majority of patients are treated as out patients and therefore, hospital based studies will under estimate the true evidence as reported by W.H.O. However, in the united state, an estimated 1.4 million cases and more than 500 human deaths occur annually. The cases are more in developing world and (Santos et al, 2003) of an estimated evidence of 33 million cases of thyphoidal salmonellosis occur in developing countries of Africa and south-east Asia. Worldwide, there are an estimated of 20 million and 700,000 deaths annually due to typhoid illness constituting a major health problem in developing countries (Kumar, Surendram, & Thampuram, 2009). In contrast to that seen in developed countries, the incidence of typhoid fever in developing countries Nigeria for example is at higher rate.

2.3.7. Pathogenesis of Typhoid Fever

The patho physiology of typhoid fever is a complex process which proceeds through several stages. The disease with an asymptomatic incubation period 7-14days inversely related to the size of infecting dose, during which bacteria invade macrophages spread throughout the reticuloendothelial system. The first of symptomatic disease is characterized by progressive elevation of the temperature followed by bacteremia. The second week begins with the development of rose spots, abdominal pain and splenomegaly. The third week is marked by a more intense inflammatory part which can result in perforation and hemorrhage (Weiner, 1980). The pathogenesis starts when the bacteria must have survived the acidic nature of the stomach, the invading organism pass through the intestinal epithelia cell and come into contact with phagocytic cells. However, the macrophages do not kill the bacteria (Patel, Rossanesse & Galan, 2005). Bacteria inject effectors' protein into the cells of the innate immune system (macrophages and Natural killer cells) through a type 3 protein

secretions system which stimulate both pro and anti inflammatory responses. After incubation period of 7-14days, the bacteria proliferate and spread through the blood stream to the liver, spleen, bone marrow and gall bladder. The inflammatory response of this process gives rise to necrosis which is a prominent feature of typhoid fever.

2.3.8. Prevention and Control

Sanitation and hygiene are critical measures that can be taken to prevent typhoid fever. Typhoid can only spread in environments where human feces or urine are able to come in contact with the food or drinking water. Careful food preparation and washing of Hands are therefore crucial to prevent typhoid fever (WHO 2008). Generally, filtration and chlorination of water are effective methods that could be applied in preventing the transmission of typhoid fever since even the food we eat is being prepared with water. Again, uses of several vaccines available are effective in decreasing the risk of typhoid fever disease as reported by W.H.O. They exists an older killed whole cell vaccine that is still used in

countries where the newer preparation are not available, but this vaccine is no longer recommended for use because it has a higher rate of side effects (mainly pain and inflammation at the site of the injection (WHO 2008). There are 2 vaccines currently recommended by world health organization for the prevention of typhoid; these include

a) Live oral Ty2la vaccine (sold as Vivotif Berna) and

b) The ingestible vi capsular polysaccharides vaccine (sold as typhim VI). Both are 50%-80% protective and are recommended for traveler's to area where typhoid is endemic.

2.3.9. Diagnosis

Diagnosis is made by blood, bone marrow or stool cultures and also with the Widal test (demonstration of *Salmonella* antibodies against O-somatic and H-flagella) remains an important public health problem in many parts of the world.

Although several serological assays for detecting *Salmonella typhi* antigens or antibodies have been used for their simplicity and rapidity, no non culture tests for typhoid fever has been repeatedly been shown

to be highly sensitive and specific (Edelman and Levine, 1986). The classical and the most commonly used serological method; the widal test is particularly unreliable with the single titers in endemic areas (Levine et al, 1978 & Wicks et al, 1974). Confirmation of typhoid fever requires the identification of *S. typhi* in clinical specimens. *S. typhi* can be isolated from more than 90% of patient with typhoid fever if blood, stool, rose spots and bone marrow aspirates are cultured. However, numerous other serological tests have been developed such as Enzyme-linked immune sorbent assay (ELISA) and dipstick assay.

2.3.4.1. Risk Factors

The age range considered to be at greatest risk was 11-30 years. However, this has been questioned in a study from WHO which found that typhoid fever affects both adults and children of all ages and it also affects both and female by W.H.O.

2.3.4.2. Treatment

Typhoid fever in most cases are not fetal, however, antibiotics such as ampicillin, chloramphenicol, trimethoprim, sulfomethazole and

ciprofloxacin have been used to treat typhoid fever in developed countries prompt of the disease with antibiotics reduces the case fatality rate to approximately 1% W.H.O.

2.3.4.3. Other pathogens

Other pathogens include *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*.

E. coli, named after Theodor Escherich is a gram negative rod shaped bacterium that is commonly found in the liver intestine of warm blooded organisms. Most strains of *E. coli* are harmless but some such as serotype 0157:H7 can cause serious food poisoning in human beings. The harmless strains are part of normal flora of the gut and can benefit their host by producing Vit.K_2 and by preventing the establishment of pathogenic bacteria with the intestine (Feng, & Weagant, 2002).

Scientific classification of *E. coli*

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteria

Family: *Enterobacteriaceae*

Genus: *Escherichia*

Specie: *coli* (Migula, 1895).

E. coli is not always conformed to the intestine and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test the environmental samples for feacal contamination. The bacteria can also be grown easily and its genetics are comparatively simple and easily manipulated or duplicated through a process of mutagenesis, making it one of the best studied prokaryotic model organism and an important specie in biotechnology and microbiology (Bentley & Meganathan, 1982).

E. coli is a gram negative, facultative anaerobic and non sporulating cells, typically rod shaped and about 2 micrometer(μm) long and 0.5μm in diameter, with a cell volume of 0.6-0.7 $(\mu m)^3$. It can live on wide varieties of substrate. *E. coli* use mixed acid fermentation in anaerobic condition, producing lactate, succinate, ethanol acetate and carbondioxide, since many pathways in mixed acid fermentation

produce hydrogen gas, these pathways require the levels of hydrogen gas, these pathways require the levels of hydrogen to be low, as in the case when *E. coli* lives together with hydrogen consuming organisms such as methanogenic sulphate reducing bacteria. *E. coli* has an optimum growth of 37^{o}C (98.6^{o}F).

Staphylococcus aureus called the golden cluster seed or the seed gold is a facultative anaerobic, gram positive *coccus*, and also most common cause of *staphylococcus* infections. It is found frequently in the nose and the skin. About 20% of the human populations are long time carriers of *S. aureus*. It can also be seen in the intestine when it is ingested to stomach with a poisoned or spoilt food containing a toxin, in the process called food poisoning. The carotenoid pigment staphyloxanthin is responsible for the characteristic golden colour of *S. aureus* colonies. The pigment acts as a virulent factor with an antioxidative action that helps the microbe to invade host by reactive oxygen species use by the host immune system (Liu etal., 2005).

S. aureus was identified by Alexander Ogston in pus from surgical abscesses in the Aberdeen Scotland. Each year, about 500, 000

patients in Nigerian hospitals contract a staphylococcal infection (Ogston, 1984).

Scientific Classifications

Domain: Bacteria

Kingdom: Bacteria

Phylum: Firmicles

Class: Bacilli

Order: Bacillale

Family: *Staphylococcaceae*

Genus: *Staphylococcus*

Specie: *aureus*

Binomial name: *Staphylococcus aureus* (Rosenbach, 1884)

CHAPTER THREE

MATERIALS

3.1.1 Equipments

Bunsen burner, Incubator (DNP-9022A), Autoclave (model. yx280A), Automatic weighing balance (Scout pro 6001 S.P.U), 4mm Cork borer, Refrigerator (FR-330), water bath, Beaker, Conical flask, Bijour Bottles, Amber bottles, cotton wool, Glass rods, Slides, Syringes, Applicator Sticks, Swab Sticks, Spread Sticks, Calibrated cylinder, whatman no 1 filter paper Foil, (Aluminum), Latex gloves, and Test tubes.

3.1.2 Soft wares

Distilled water, oxidase reagent, Barium Chloride (1.17%), Sulfuric and (1%), Normal saline, crystal violent, Safranin, hydrogen peroxide, acetone Alcohol, lugol's iodine, kovac's reagent.

3.1.3 Media

Nutrient agar, peptone water, (Methods of preparation are in the appendix 1).

3.2. Collection and confirmatory tests of test Organisms

Pure culture of *Salmonella typhi* was collected from the Department of pharmaceutics, faculty of Pharmacy, University of Nigeria Nsukka. *Escherichia coli, Staphylococcus aureus, Klebsiella spp and Pseudomonas aeruginosa* were obtained from the Microbiological laboratory unit of University of Nigeria Teaching Hospital. Ituku-ozalla, Enugu State. The microorganisms were subcultured and pure isolates of the resulting growth were confirmed using gram staining and some biochemical methods.

3.2.1 Gram staining: - This is a method used to differentiated bacteria cells into gram positive and gram negative cells. This differentiation is based on the color reaction, exhibited by bacteria.

Procedure:-

- A flamed wireloop was used to pick a colony from a plate and a thin film of the organism as made on a clean grease free glass slide.
- The film was heat fixed by waving over a flame of a Bunsen burner.

- The smear was flooded with primary stain, crystal violet for 1 minute and then rinsed with water.
- The smear was flooded with lugol's iodine and allowed for about 1minute and then rinsed with water.
- Acetone which acts as a decolorizing agent was briefly added for 10 seconds and was quickly rinsed with water to avoid over decolourization.
- A counter stain safranin was then applied to impact a contracting colour.

Result

- Gram positive cells appear purple.
- Gram negative cells appear red.

3.2.2 Biochemical Tests

- Indole Test
- Oxidase Test
- Catalase Test
- Coagulase Test
- Sugar fermentation Test

- **Indole Test:** - This test was used to used to identify the presence of *E coli*, which has the ability to split indole from amino acid tryptophan present in the peptone water.

Method

- Tubes of peptone water were inoculated with young culture of the Isolates.
- The tubes were incubated at 37^0C for 24hours
- After incubation, about 4 drops of kovac's reagent were added into 1ml of each of the culture tubes.

Result

Red surface layer (like ring) – positive indole test

No red surface layer – negative indole test.

- **Oxidase test**:- This test was used to determine the microorganisms capable of producing the enzyme oxidase. It is also a hallmark test for the *Neisseria spp.* it can also be used to determine the presence of *Pseudomonas spp*. In order to carryout this test, oxidase reagent (blue in colour) is used to impregnate a filter paper. The test organisms are smeared on the

impregnated filter paper. If there is a colour change after about 30seconds to 1minute, from blue to purple, it signifies a positive result but if there is no colour change, then the test organism is negative to enzyme oxidase.

- **Catalase Test:** - This test was used to demonstrate which of the test organisms could produce the enzyme catalase which releases oxygen from hydrogen peroxide. The test was used to differenciate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative). Catalase acts as a catalyst in the breakdown of hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O) the organism used were brought in contact with hydrogen peroxide bubbles of O_2 was released.

Method

- A loopful of the pure culture colony was transferred into a plane, clean, grease free glass slide.
- The sample was then mixed with a drop of 3%v/v hydrogen peroxide with the aid of a wireloop.

Note: care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells.

- The reaction was observed immediately for bubbling.

Result: - Presence of gas bubbles; positive catalase test (*Staphylococcus spp*).

Absence of gas bubbles, negative catalase test

- **Coagulase Test:** This was used to determine the organism which is capable of producing the enzyme coagulase. The coagulase enzyme causes plasma to clot by converting fibrinogen to fibrin. Bound coagulase converts fibrinogen directing to fibrin without requiring a coagulase reacting factor. It can be detected by clumping of bacterial cells in the slide test (Cheesbrough, 2004).

Method

a. A drop of distilled water was placed on two separate clean grease free glass slides.
b. A colony of the test organism was emulsified in each of the drops to make 2 thick suspensions.

c. A loopful of plasma was added to one the suspension, and mixed gently.

d. The slides were observed for clumping of the suspension which was used to indicate the presence of coagulase.

Result

Clumping within 10 seconds – *Staphylococcus aureus*

No clumping within 10seconds – *Staphylococcus spp*

- **Sugar fermentation Test:** - This test was done to detect the acidity resulting from fermentation e.g. sugar incorporated into the medium it also detects gas and acid production. Most bacteria especially gram negative bacteria utilize different sugar as source of carbon and energy with the production of either acid or gas.

Method

- Peptone water was prepared according to manufacture's guide.
- Drops of methyl red indicator were added into the sterile peptone water.
- The test organisms were used for the fermentation of sugar.

- For each beaker, pipette 5ml of the peptone broth containing sugar into different screwed capped test tube.
- Durham tube was inserted into different test tubes and all the test tubes containing peptone broth was sterilized at 121^0c for 15minute and then it was allowed to cool.
- Different organisms were inoculated into different sterile 1ml distilled water and mixed thoroughly.
- Using micro pipette, 1ml of the sterile distilled water containing organisms was dispensed into the 5ml of sterilized peptone broth and then it was incubated for 5-7days at 35^0c watching daily for colour changes.
- Acid production was indicated by the appearance of yellow colour in both media and gas production was indicated by the presence of bubble solace in the inverted Durham's tube (positive test)
- If colour remains red and there was no bubble, it signifies that the test is negative.

- Presence of bubble in Durham's tube without colour changes signifies gas production.

3.3 Collection and identification of plant material: Dried calyces of *Hibiscus sabdariffa* (zobo) flowers were bought from Ogbete market in Enugu metropolis enclosed in a black polytene bag and transferred to the Microbiology and Biotechnology laboratory of Caritas University for analysis. It was identified by Emeritus Prof. J.C. Okafor of the department of botany in the University of Nigeria Nsukka.

3.4 Extraction of plant material

Using maceration method, the following procedure was employed.

- 25g of *H. sabdariffa* was weighed on a scout proelectronic weighing balance and transferred into a conical flask.
- 125ml of hot distilled water (100^{o}C) measured using a glass caliberated cylinder was gradually poured into the conical

flask containing *H. sabdariffa* calyces and stored in a cupboard for 24hours.

- The mixture was filtered out with a Whatman No 1 filter paper.
- The filtrate was then poured into 6 glass Petri dishes.
- The Petri dishes containing the filtrate was allowed to dry in a laboratory dry oven at 45^{o}C to 70 ^{o}C, to concentrate the extract.
- The extract was scrapped using a clean spatular into a sterilized Amber bottle and then stored in the refrigerator.

3.5 Preparation of the Mcfarland's Standard:

The preparation involves the mixture of both Tetraoxosulphate(vi) acid (sulphuric H_2S0_4) (1%) and Barium Chloride (1.17%). 9.95ml of H_2S0_4 was added to 0.05ml of Bacl to form precipitate suspension. This served as the 0.5 Mcfarland's standard which served as turbidity standard for the test organisms.

3.6. Preparation of the cell suspension (inocular)

The Test organisms were sub cultured on nutrient agar plate and incubated at 37^{o}C for 15-24 hours. The growth from each plate was transferred into a test tube containing 5ml of 0.9% sterile saline and the volume was adjusted to attain a turbidity which matches that of 0.5 Mcfarland's standard. This means that the cell suspension contains appropriately 1.5 x 10^{8} colony forming unit per ml (cfu/ml).

3.7 Serial dilutions of the extract

An electronic weighing balance was used to weigh 0.6g of *H. sabdariffa* extract. This was transferred into a sterilized bijour bottle containing 6ml of distilled water. It was stirred using a glass rod to help it dissolve. This gives a concentration of 100mg/ml. Serial dilution was made by withdrawing 3ml of the concentration from this bijou bottle into another bijou bottle containing 3ml of sterile distilled water and mixed to obtain 50mg/ml solution. This procedure was used to prepare concentrations of 25mg/ml, 12.5mg/ml and 6.25mg/ml.

3mls of solution

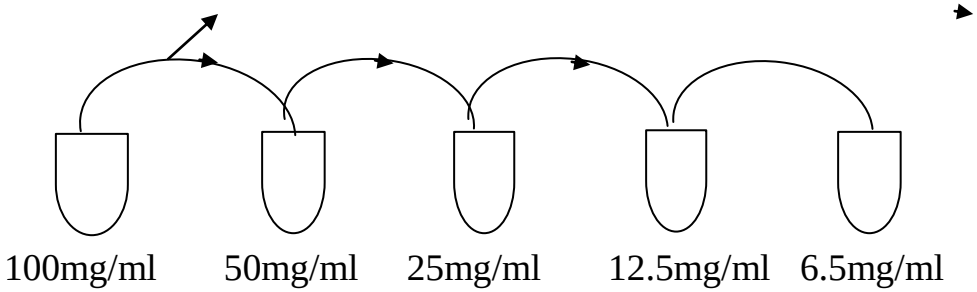

Fig: 2 Serial dilutions of the plant extract

3.8 Susceptibility testing of the test organisms with antibiotics using disc diffusion method

The Susceptibility of the test organisms to antibiotics was tested. The tests were carried out in nutrient agar plate and antibiotics (optan wig) were aseptically placed on the media after innoculating the agar plate with the organism in an anticlockwise manner. The gram negative organisms were screened against ciprofloxacin 10µg, ofloxacin 10µg, perfloxacin 30µg, nitrofurantoin 100µg, cetriaxone 30µg, gentamycin 10µg, augumentin 30µg, Ampicillin 30µg, chloramphenicol 10µg, clarithomycin 30µg.

The gram positive organism was screened against erythromycin 10µg, cetriaxone 3oµg, ampicillin and cloxacillin 35µg, levofloxacin 5µg,

norfloxacin 10µg, ciprofloxacin 5µg, gentamycin 10µg, ofloxacin 5µg and clindamycin 10µg.

The Kirby-bauer disc diffusion method was employed. The cell suspension formally prepared in section 3.2.6 was used to swab the entire surface of the nutrient agar plate as follows a sterile swab stick was dipped into the bacterial suspension and was dispensed in the nutrient agar surface. The swab stick was used to swab the nutrient agar surface all over while rotating it in an anticlockwise manner. Multidiscs containing the antibiotics named above were aseptically placed on the nutrient agar plate inoculated with the test organism. The discs were allowed for 1hour to diffuse through the plate and were then incubated at 37^{o}C for 24 hours. After incubation, Inhibition Zone Diameters (IZDs) around each antibiotic were measured. Inhibition Zone Diameters were measured in mm and were recorded by calculating the mean of IZDs in duplicate plates. The results were interpreted according to CLSI standards.

3.9 Susceptibility testing of the Test organisms with *Hibiscus sabdariffa* extracts using agar well diffusion method.

Nutrient agar plates were inoculated with the suspension of test organisms using the swab method as previously described in section3.8. The Susceptibilities of the test organisms to *H. sabdariffa* extract were tested using agar well diffusion method. Wells were made aspecially near a Bunsen burner using a sterile cork borer (8mm) and then 0.1ml of the solution of different concentrations of *H. sabdariffa* extract was dispensed in the labeled well i.e (100mg/ml, 50mg/ml,25mg/ml,12.5mg/ml and 6.25mg/ml). The plates were allowed to stand on the table 1hour for proper diffusion and were incubated for 24hours at 37^{o}C. After incubation, the plates were examined and inhibition zone diameters (IZDs) measured using a ruler caliberated in mm to determine the degree of susceptibility of the test organism. The IZDs (mm) was recorded by calculating the mean of inhibition zone diameter for each set of duplicate plates.

CHAPTER FOUR

4.1. RESULTS

Table 3: Results of identification test.

Organism	Gram	INDO	CAT	COA	OXI	CIT	GLU
S. typhi	-	-	+	-	-	-	AG
E. coli	-	+	+	-	-	-	-
P. aeruginosa	-	+	+	-	+	-	-
S. aureus	+	-	+	+	-	+	AG
Klebsiella. spp	-	-	+	-	-	+	-

Key: Positive Result = + and Negative = -, AG = Acid Gas, A^+ = Acid positive , G. = Gas , A = Acid OXI- oxidase test, CAT- catalase test, INDO- indole test, COA- coagulase test, CIT- citrate test, GLU- glucose test and Gram- Gram reaction test.

Table 4: Inhibitions zone diameters (IZDs) of antibiotic agents on the test Organisms (mm).

Test organism	C	AM	N	CT	CIP	GN	OF	AU	PF	CM	%R	%S
S. typhi	0	12	0	15	26	16	20	0	≥28	14	50	50
E. coli	0	0	20	28	20	14	0	0	20	20	50	50
Klebsiella spp	0	13	11	22	22	16	24	16	≥28	18	30	70
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	100	0

	E	CT	AP	CE	LV	NB	CIP	GN	OF	CD	%R	%S
S. aureus	18	12	0	10	30	14	28	18	≥28	28	40	70

Keys for gram negatives	Keys: for gram positive organism
C - chloramphenicol	E - Erythromycin
Am - ampicillin	Ct - cetiaxone
N - nitrofurantoin	Ap - ampicllin
Ct - cetriaxone	Ce - cifixmine
Cip - ciprofloxacin	Lv - levofloxacin
Gn - gentamycin	Nb - norfloxacin
Of - ofloxacin	Cip - ciproflocacin
Au - augmentin	Gn - gentamycin
Pr - perfloxacin	Of - ofloxacin

Fig 3: Agar plate showing the effect of antibiotics on *S. typhi*.

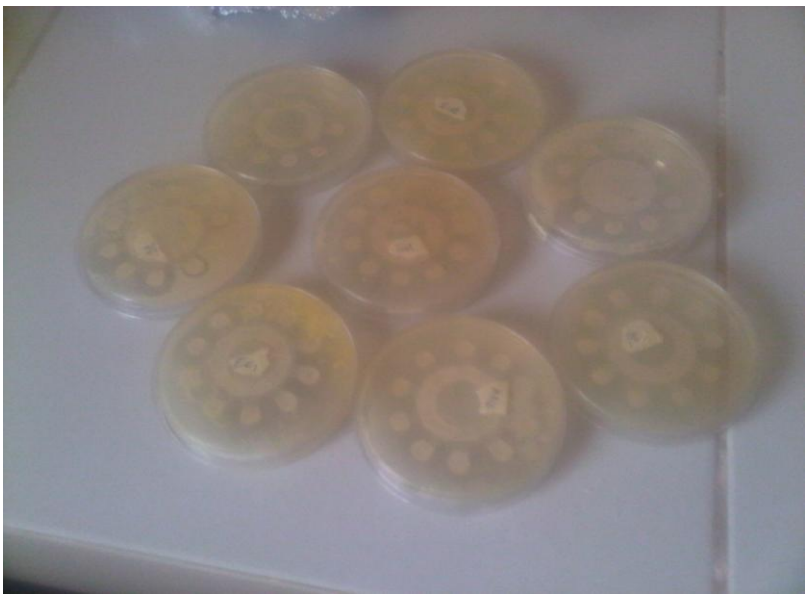

Fig 4: Agar plates showing the effect of antibiotics on other test organisms

Table 5: Inhibitions zone diameters (IZDs) of concentrations of *H. sabdariffa* on the test organisms measured in mg⁄ml.

Test organism	100	50	25	12. 5	6.25
S. typhi	23	20	16	0	0
E. coli	0	0	0	0	0
K. Spp	0	0	15	10	0
P. aeruginosa	0	0	0	0	0
S. aureus	29	18	17	14	0

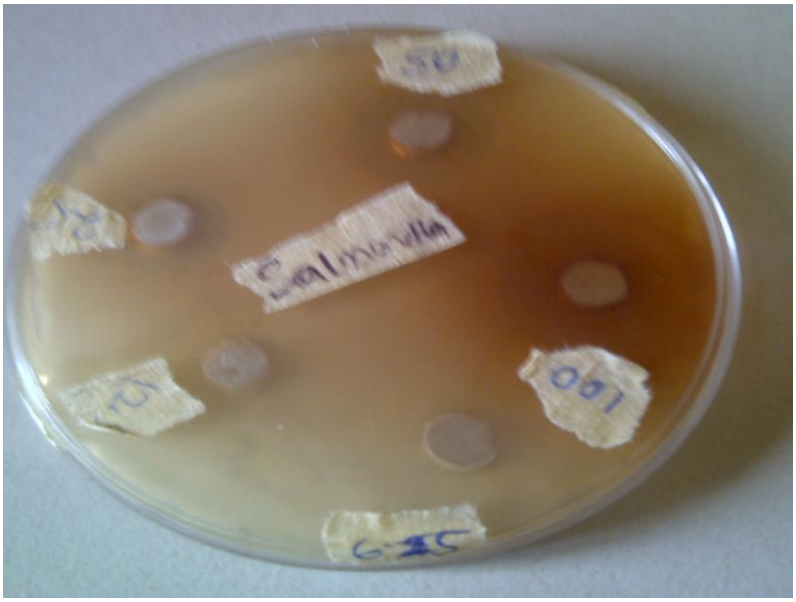

Fig 5: Agar plate showing effect of *H. sabdariffa* extract on *S. typhi*

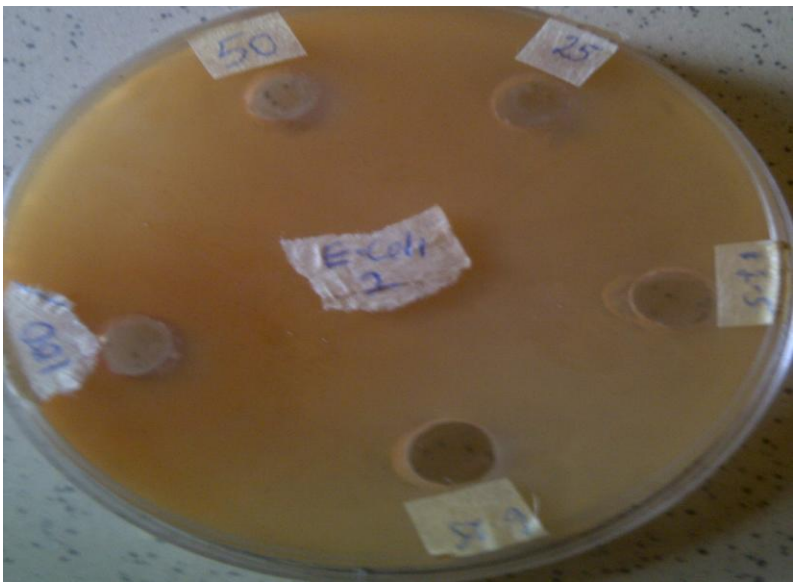

Fig 6: Agar plate showing the effect of *H. sabdariffa* on *E. coli*

Fig 7: Agar plate showing the effect of *H. sabdariffa* on *S. aureus*

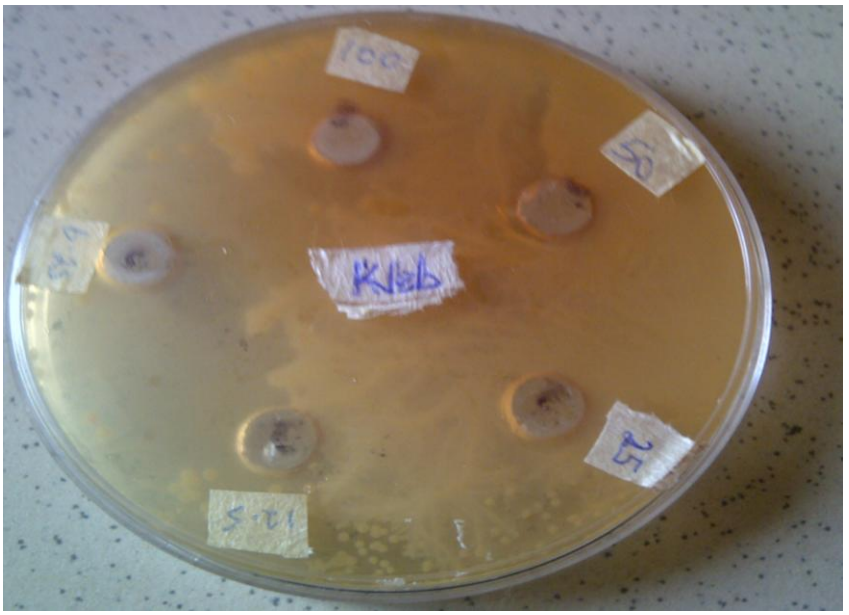

Fig 8: Agar plate Effect of *H. sabdariffa* on *klebsiella sp*

CHAPTER FIVE

5.1 DICUSSIONS OF RESULTS

The results obtained from this study showed that aqueous extract of the calyces of *Hibiscus sabdariffa* have effective antimicrobial activities. The organisms tested were *Salmonella typhi, Escherichia coli, Klebsiella pneumonia, and Pseudomonas aeruginosa and Staphylococcus aureus.*

Antibiotics are chemically synthesized substances which have the capacity to inhibit or if possible kill microorganisms. Antibiotics can also be said to be restricted to bacteria and fungi. Their mechanisms of action could be cell wall inhibition (penicillin), protein synthesis, nucleic acid synthesis nuclear membrane synthesis etc. However, certain microorganisms have been seen to be resistant to some of these antibiotics, which imply that further investigation and development of substances capable of inhibiting if not killing these microorganisms should be developed if human beings must attain the level of health as defined by the world health organization as a state of physical, mental and social well

being. The antibiotic sensitivity test carried on the test organism shows that there are some organisms which are sensitive and some which are resistant to antibiotics. *Salmonella typhi* was sensitive to cetriaxone, ciprofloxacin, gentamycin, ofloxacin and perfloxacin. It showed resistance to nitrofurantoin, ampicillin, chloramphenicol and augumentin. *Escherichia coli* on the other hand was sensitive to nitrofurantoin, cetriaxone, ciprofloxacin, perfloxacin and clarithomycin but showed resistance to chloramphenicol ampicillin ofloxacillin, gentamicin and augumentin. *Klebsieilla spp* was sensitive to six antibiotics but resistant to four. *Pseudomonas aeruginosa* was resistant to all the antibiotics while *Staphylococus aureus* was sensitive to six tested antibiotic and resistant to four antibiotics. It is therefore imperative to note that orthodox medicine cannot fill the space of microbial resistance alone. As a result of this, there is need for medicinal plants to aid antibiotics in the treatment of various diseases caused by these microorganisms.

H. sabdariffa, a plant that is native to West Africa and used as drink has also been shown to possess some antimicrobial

capabilities. It contains some constituents such as 7.6% of moisture, 24% of crude protein 22.3% of fat, 15.3% fiber, 0.3% of Ash, 0.6% of carbon, 0.4% of phosphorous, 5.1% cholesterol, 3-2% of erqosterol, 0.004-0.005% of mixture of citric and malic acid. In as much as it is used as native drinks, it still possess some antimicrobial properties which are conferred on it by it's constituents such as anthocyanins and flavonoids. The results of the screening of these test organisms with *H. Sabdariffa* extract showed that the plant's extract was active against some of these organisms.

For *Salmonella typhi,* the concentrations of 100mg/ml 50mg/ml and 25mg/ml showed inhibition zone diameters (IZDs) of 23mm, 20mm and 16mm respectively. The minimum inhibitory concentration of the extract was measured using a calibrated ruler. Note that MIC is the lowest concentration of the chemotherapeutic agent capable of inhibiting the growth of a microorganism. The MIC for *S. typhi* is 25mg/ml. In *Escherichia coli* all concentrations showed resistance. The inhibition zone diameter (1ZDs) for

Klebsiella spp was seen in 12.5mg/ml and 6.25mg/ml as 15mm and 10mm respectively. The Mic is 12.5mg/ml showing 10mm IZD. *Pseudomonas aeruginosa* was all resistant to concentrations of *H. sabdariffa* extract. *Staphylococcus aureus* was most sensitive with diameters of 29mm, 18mm, 17mm and 14mm with decreasing concentration of t*he H. sabdariffa* extract are 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml respectively.

From the discussion above, it was shown that *Staphylococcus aureus* was sensitive to the *H. sabdariffa* aqueous water extract followed by *Salmonella typhi* and *Klebsiella spp* which was in concord to the work done by Marbel, 1996. *Pseudomonas aeruginosa* and *Escherichia coli* showed resistance to all the concentrations of *H. sabdariffa* extract. The result showed that there is a variation in the degree of antibacterial activities of the extracts. It is of great interest to note that some of the antibiotic-resistance organisms were susceptible to various concentrations of *H. sabdariffa* extract. The results of the study carried out suggest that there is a vast number of species of medicinal plants in our

environment which can be exploited in the development of new antibiotics.

5.2. CONCLUSION

Based on the findings of this great research work, aqueous extract of *H. sabdariffa* possess antimicrobial activity. The extract exhibited the highest activity on *S. aureus*, followed by *S. typhi* and *K. spp*. *E. coli* and *P. aeruginosa* were all resistant. From the results of these findings, *H. sabdariffa* can inhibit as well as promote the growth of certain microorganisms. This is because it is very rich in varieties of constituents which promote the growth of certain organisms such as moisture, crude protein, fat, fiber, carbon, phosphorous to mention but a few. *H. sabdariffa* should be produced in industrial scale since it has nutritional as well as therapeutic properties. The industrialists should endeavor to maintain standard operational procedures (SOP) and also pasteurize the finished product in order to reduce the Microbial load. I conclude by saying that *H. sabdariffa* has immense benefits

which can be exploited to enhance the health as well as economy of the Nation.

3.3. RECOMMENDATION

From the results of this study, I recommend that government should fund research on *H. sabdariffa* with a view of using it in the development of novel antibiotics for the treatment of some infectious diseases. Its potentials as well as accepted beverage drink has already been highlighted for the attention of entrepreneurs interested in industrial scale production of Zobo drink.

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APPENDIX I

MEDIA PREPARATION

Nutrient Agar (NA)

The medium was used for the enumeration of bacterial cells and also to maintain pure cultures.

Preparation

The preparation involves measuring twenty-eight grams (28g) of the powder on a weighing balance and suspending it into 1 litre of distilled water (or equivalent w/v for lower volumes of distilled water). This was boiled over a Bunsen burner to dissolve completely and subsequently sterilized by autoclaving at 121^{o}c for 15minutes. After cooling to about 47^{o}C, the sterile molten medium was distributed i.e. about 20ml each into sterile Petri dish.

Composition:

The medium composed of the following

Beef extract 3.0g/l

Peptone 5.0g/l

Sodium Chloride 8.0g/m

PH 7.3 $\pm$ 0.2

Batch number 110/24/147

Expiring date 2014/05

The medium was used to enrich and develop the inocular that were used to innoculate the agar plates. It was also used to maintain cultures for some biochemical tests.

APPENDIX II

Table 5: Reagents used for the study

Regent used	**Uses**	**Manufacturer**
Crystal violet	Used as primary Stain for gram stain	Juhel pharmaceutical Nig Ltd
Acetone	Used in the Decolourization and as a solvent for extraction	Gauce pharmaceutical Nig Ltd
Hydrogen peroxide	Used for catalase test	Zubite pharmaceutical Nig Ltd
Safranin	Used as counter stain for gram stain	Lab limited tapley house, 52 wash cane, bury, lane cashier BL9 6as, United Kingdom
Peptone water	Used for biochemical test	Mark and Zehl Medics Nig Ltd
Kovac's regret	Used for biochemical test	Mark and Zehl Medics Nig Ltd

APPENDIX III

Zone Diameter interpretative chart for antibiotic testing.

Antibiotic and code	Disc content	R	I	S
Ampicillin (PN)	10 µg	≤ 13	14-16	≥ 17
Augumentin (AU)	30 µg	≤ 13	14-17	≥ 18
Ciprofloxacin (CIP)	5 µg	≤ 15	16-20	≥ 21
Clindamycin (CO)	2 µg	≤ 16	17-20	≥ 21
Erythromycin	15 µg	≤ 13	14-22	≥ 23
Gentamycin (GN)	10 µg	≤ 12	13-14	≥ 15
Nalidixic acid (NA)	30 µg	≤ 13	14-18	≥ 19
Ofloxacin (OFX)	5 µg	≤ 14	15-17	≥ 18
Perfloxacin (PER)	10 µg	≤ 12	13-16	≥ 17
cephaplaxin	30 µg	≤ 14	15-17	≥ 18

Keys

R – Resistance

I - Intermediate

S – Sensitive.