Original Research Article

Determination of antimicrobial susceptibility pattern of bacteria isolated from herbal drugs hawked in Onitsha Anambra State Nigeria

ABSTRACT

This study aimed at the determination of antimicrobial susceptibility pattern of bacteria isolated from herbal drugs hawked in Onitsha Anambra State. The microbial quality and antibacterial properties of six Nigerian herbal remedies with such claimed efficacy of curing all manners of microbial diseases were assessed. The herbal remedies were discovered to be contaminated with the following microorganisms: *Bacillus subtilis*, *Staphylococcus* sp, *Salmonella* spp and *E. coli*. Qualitative phytochemical screening of the herbal remedies revealed the presence of saponin, tannins, alkaloids, anthraquinone and cardiac glycosides which suggest possible antimicrobial effect. The maximum activity of the herbal remedies in the test isolates was observed on *Staphylococcus aureus,* which showed clear zones of inhibition with diameters ranging from 7.00 to 13mm for the six herbal drugs respectively while it had low activity on *E coli*, with clear zones of inhibition of 6.00mm, to 14.00mm. The herbal extracts have shown appreciable antimicrobial activities comparable to the currently prescribed modern drugs tested. Accordingly, further studies on clinical efficacy trial, safety, toxicity and affordability analyses have to be instigated promptly, so as to proceed to the final step to synthesize precursor molecules for new effective antimicrobials.

INTRODUCTION

Background of the study

Herbal medicine, a form of complementary and alternative medicine, is becoming increasingly popular in both developing and developed countries (Czech *et al.,* 2011). A World Health Organization (WHO) survey indicates that about 70-80% of the world population particularly in the developing countries rely on non-conventional medicines mainly of herbal sources in their primary healthcare. WHO has described traditional medicine as one of the surest means to achieve total health care coverage of the world's population. In pursuance of its goal of providing accessible and culturally acceptable health care for the global population, WHO has encouraged the rational use of traditional plant based medicines by member states and has developed technical guidelines for the assessment of herbal medicine (Twari, *et al.,* 2019).

A medicinal plant is any plant which in one or more of its organs contains an active ingredient which is used for therapeutic purposes or contains foundation compounds that can be used for synthesis of useful drugs (Afolabi and Afolabi, 2013). Doughari (2012) explained that, medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for chemo-pharmaceutical semi synthesis. Medicinal plants naturally synthesize and accumulate some secondary metabolites, like alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenics, tannins, resins, lactones, volatile oils as well as others (Motaleb *et al*., 2011).

The part of plants in used in the formulations of herbal medicine include; leaves, roots, rhizomes, stems, barks, flowers, fruits, grains, or seeds and these contains chemical components which are used for control and treatment of a diseases. According to Motaleb *et al*., (2011) researchers have found that people in different parts of the world tend to use the same or similar plants for treating the same illness. WHO estimated that 80% of the people globally rely on herbal medicines, partially for their primary health care.

The pharmacological efficacy of all herbal medicines is attributed to its phytochemical constituents. Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals with sufficient antimicrobial efficacy can be used for the cure of microbial infections.

An antimicrobial is a substance that kills or inhibits the growth of microbes such as bacteria, fungi, protozoa or viruses. Antibiotics are those substances which are produced by microorganism that kills or prevents the growth of another micro-organism. Antibiotics are generally used against bacteria, antiviral are used specifically for treating viral infections. Antifungal are used to treat fungal infections, some of these side effects can be life threatening if the drug is not used properly.

Several microorganisms derived antibiotics are currently in use to treat a variety of human disease, therefore the action must be taken to control the use of antibiotics, develop new drugs either synthetic or natural, for a long period of time, plant have a valuable source of natural products for maintaining human health. India has a rich tradition in use of medicinal plants to develop drugs. According to world health organization (WHO), any plant which contain substances that can be used for therapeutic purpose or which are precursor of chemo-pharmaceuticals semi synthetic new drugs is referred as medicinal plant (Iwu *et al,* 1999].

For decades, the screening of medicinal plant materials for their therapeutic values has continued to represent potential sources of new effective medicines. Immense benefits have been derived by man from using medicinal herbs in disease management because they are relatively safer, more affordable and sometimes offer better therapeutic value than synthetic drugs. The increasing discovery of more medicinal plants has necessitated increased scientific scrutiny of their bioactivity in order to provide data that will help physician and patients make wise decision before using them.

Statement of problem

Despite the availability of modern drug formulations, traditional medicine is still the predominant means in the third world for the preservation of health of the rural majority who constitute over 70% of the total population. However, adequate knowledge of the methods of preparation, the possible toxicological effects of some chemical species present in the ingredients and the side effects of the various mixtures are usually unknown to many who patronize herbal medicines.

Also, herbs contain hundreds of plant chemicals in varied concentrations) which if not properly utilized can pose a threat to the health of the consumer and could lead to the damage of vital organs such as liver and kidney that are involved in the metabolism and evacuation of toxic chemicals in the body. Unfortunately the belief in the efficacy of herbal remedies by an average Nigerian outweighs individual knowledge of the pharmacological effects of these medicines and this could prove fatal in many cases. Therefore, the need to study the efficacy of some herbal preparations sold in our environs is essential.

Aim of the study

The overall aim of this current study is the determination of antimicrobial susceptibility pattern of bacteria isolated from herbal drugs hawked in Onitsha Anambra State Nigeria.

Objectives of the study

The study will achieve the following specific objectives

1. To evaluate the phytochemical compositions of some herbal preparations sold in Onitsha metropolis and environments in terms of its flavonoid, saponin, tannin, alkaloid, glycoside and phenolic contents.
2. To determine the efficacy of each herbal formulation sold in Onitsha metropolis through antimicrobial analysis against the causative microbes of the diseases they claimed it cures.

3. To compare the efficacy and antimicrobial strength of the herbal formulations and commercial antibiotic drugs sold in Onitsha metropolis.

LITERATURE REVIEW

2.1 Concept of Traditional Medicine

Traditional medicines (TMs) are a critical component of healthcare in sub-Saharan Africa and greater integration between traditional and biomedical health systems may be needed. In Nigeria, for instance, alongside increasing access to biomedicine, nearly 70% of people still frequently access healthcare through traditional healers or vendors (Aluko, 2015). Because of their importance, understanding how and why people in Nigeria use TMs is necessary. Others have highlighted the complex dynamics of TM use in sub-Saharan Africa. Cultural belief models, high cost and limited access of biomedicine, disease understanding, safety concerns of biomedicine, and perceptions of TMs as more effective have all been studied as determinants for the use of TM (Adepoju, 2005). However, TM use among general populations is less well-characterized especially as many of the studies across the region have focused on use in low-income areas, rural settings, healthcare-based samples, or among specific issues such as malaria, human immunodeficiency virus (HIV) infection, mental health disorders, bone setting, or midwifery (Asenso-Okyere, 1996).

Herbal medicines

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants, or other plant materials, or combinations.

- **Herbs:** crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.
- **Herbal materials:** in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages or other materials.
- **Herbal preparations:** the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes.
- **Finished herbal products:** herbal preparations made from one or more herbs. If more than one herb is used, the term mixture herbal product can also be used. Finished herbal products and mixture

herbal products may contain excipients in addition to the active ingredients. However, finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

2.2.1 Traditional use of herbal medicines

Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities.

Therapeutic activity

Therapeutic activity refers to the successful prevention, diagnosis and treatment of physical and mental illnesses; improvement of symptoms of illnesses; as well as beneficial alteration or regulation of the physical and mental status of the body.

Active ingredient

Active ingredients refer to ingredients of herbal medicines with therapeutic activity. In herbal medicines where the active ingredients have been identified, the preparation of these medicines should be standardized to contain a defined amount of the active ingredients, if adequate analytical methods are available.

The earliest evidence of human's use of plant for healing dates back to the Neanderthal period (Forest, 2009). Herbal medicinal is now being used by an increasing number of patients who typically do not report to their clinicians concomitant use. There are multiple reasons for patients turning to herbal therapies. Often cited is a "sense of control, a mental comfort from taking action," which helps explain why many people taking herbs have diseases that are chronic or incurable viz. diabetes, cancer, arthritis or AIDS. In such situations, they often believe that conventional medicine has failed them. When patients use home remedies for acute, often self-limiting conditions, such as cold, sore throat, or bee sting, it is often because professional care is not immediately available, too inconvenient, costly or time-consuming (Kumar, 2004).

Natural plant products are perceived to be healthier than manufactured medicine. Additional, report of adverse effect of conventional medications are found in the lay press at a much higher rate than reports of herbal toxicities, in part because mechanisms to track adverse effect exist for conventional medicines whereas such data for self treatment is harder to ascertain. Even physicians often dismiss herb as harmless placebos (Rocha, 2010).

Difference between Herbal medicine and Conventional Drugs

Although superficially similar, herbal medicine and conventional pharmacotherapy have three important differences:

Use of Whole Plants- Herbalists generally use unpurified plant extracts containing several different constituents. It is claimed that these can work together synergistically so that the effect of the whole herb is greater than the summed effects of its components. It is also claimed that toxicity is reduced when whole herbs are used instead of isolated active ingredients (“buffering”). Although two samples of a particular herbal drug may contain constituent compounds in different proportions, practitioners claim that this does not generally cause clinical problems. There is some experimental evidence for synergy and buffering in certain whole plant preparations, but how far this is applicable to all herbal products is not known (Vickers and Zollman, 2005).

Herb Combining- Often several different herbs are used together. Practitioners say that the principles of synergy and buffering apply to combinations of plants and claim that combining herbs improves efficacy and reduces adverse effect. This contrasts with conventional practice, where polypharmacy is generally avoided whenever possible (Vickers and Zollman, 2005).

Diagnosis- Herbal practitioners use different diagnostic principles from conventional practitioners. For example, when treating arthritis, they might observe, “under functioning of a patient’s symptoms of elimination” and decide that the arthritis results from “an accumulation of metabolic waste products”. A diuretic, cholerectic or laxative combination of herbs might then be prescribed alongside herbs with anti-inflammatory properties (Vickers and Zollman, 2005).

2.3 Phytochemical constituents of herbal medicine

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventing properties. They are chemical compound that occurs naturally in plants and responsible for color and organoleptic property. They are non-essential nutrient, meaning that they are not required by human body for sustaining life (Iwu *et al*., 1999).

The Flavonoids

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. To date about 3000 varieties of flavonoids are known (Kuhnau, 2016). Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity (Cesarone *et al*., 1992). Other biological effects exhibited by flavonoids include anti-inflammatory, anti-hepatotoxic and anti-ulcer actions (Robak *et al*., 1988). They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are potent antioxidants and have free radical scavenging abilities. Many have anti-allergic, antiviral actions and some of them provide protection against cardiovascular mortality (Hertog *et al*., 1993). They have been shown to inhibit the growth of various cancer cell lines in vitro and reduce tumour development in experimental animals.

Alkaloid

Alkaloids occur in many different species in numerous genera and families of vascular plants as well as in certain species of fungi. It has been estimated that some fifteen percent or more of all vascular plants contain alkaloids. A number of amines produced by animals possess physical and chemical properties rather similar to those of alkaloids. By traditions and conventions, these animals amines are generally not considered as alkaloids. The occurrence of alkaloids in different plant organs and tissues and their relationship to aspects of the physiology of the plants are interesting part of alkaloids (Babalola, 2009).

Phenolics

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL). They are very important to plants and have multiple functions. The most important role may be in plant defence against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections (Puupponen- Pimiä *et al.*, 2008). They are classified into (i) phenolic acids and (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolies. Caffeic acid is regarded as the most common of phenolic compounds distributed in the plant flora followed by chlorogenic acid known to cause allergic dermatitis among humans (Kar, 2007). Phenolics essentially represent a host of natural antioxidants, used as nutraceuticals, and found in apples, green-tea, and red-wine for their enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents. Other examples include flavones, rutin, naringin , hesperidin and chlorogenic (Nwokocha and Peter, 2011).

Glycosides

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemiacetal entity of the carbohydrate must essentially take part in the condensation. Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Kar, 2007). Alcohol, glycerol or phenol represents aglycones. Glycosides are neutral in reaction and can be readily hydrolyzed into its components with ferments or mineral acids.

Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action.

Saponins

Saponins are steroid or triterpenoid glycosides that are present in many feedstuffs. They have a bitter taste, can form foams in aqueous solutions and haemolyse red blood cells. They are known to depress growth performance in both poultry and swine. Their ant nutritional properties seem related to their ability to form complexes with sterols, in particular those in membranes of animal cells. This appears to result in increased permeability of the intestinal mucosa. Poultry, compared to other monogastrics, are more sensitive to saponins. Significant saponin levels are present in alfalfa meal with minor levels in other legumes such as soya beans, rapeseed and various varieties of peas. In general saponins are of minor concern in monogastric animals because they are present at only law levels in common feedstuffs (Batran, *et al.,* 2006).

Terpenes

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins. Terpenoids includes hydrocarbons of plant origin of general formula $(C_5H_8)n$ and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monterpenes include terpinen-4-ol, thujone, camphor, eugenol and menthol (Griebel *et al.*, 1995).

The triterpenes (C30) include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. Common triterpenes: amyrins, ursolic acid and oleanic acid sesquiterpene *(C15*) like monoterpenes, are major components of many essential oils (Harborne, 2013).

Steroids

Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs. The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids (anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness (Haristoy *et al.*, 2015).

Antimicrobials

In an attempt to combat the various forms of disease that have continued to plague humans from time immemorial to this day, different types of antimicrobials have been developed to fight the pathogens responsible for these diseases. Antimicrobials, which are substances that kill or inhibit the growth of microorganisms, could be in the form of antibiotics, which are products of microorganisms or synthesized derivatives antimicrobial peptides produced by complex organisms as well as some microbes and medicinal plants, which appear to be the focus of mainstream medicine today (Alarcon *et al.,* 1994).

Types and Sources of Antimicrobials

Different types of antimicrobials exist: antibiotics, anti-viral, anti-fungal, anti-protozoan etc. Antibiotics are used in the treatment of bacterial infections and can be obtained from either natural or synthetic sources. Examples of those with a natural origin are phenyl propanoids (chloramphenicol), polyketides (tetracycline), aminoglycosides (streptomycin, gentamycin), macrolides (erythromycin), glycopeptides (vancomycin) and second-generation β-lactams (cephalosporins). Those from synthetic sources are sulphonamides, quinolones and oxazolidinones. Most antibiotics exert their action either by inhibition of the bacterial cell wall or protein synthesis (Nascimento *et al.,* 2000).

Exceptions are the quinolones that inhibit DNA synthesis, and the sulphonamides that inhibit the synthesis of metabolites used for the synthesis of deoxyribonucleic acid (DNA) (Singh and Barrett 2006). Most anti-viral, anti-fungal, anti-protozoa and anti-cancer drugs however are obtained from synthetic sources.

Because of the re-occurring resistance of pathogenic microorganisms to antibiotics, as well as the side effects presented by these antibiotics, investigation of other sources of antimicrobials, such as medicinal plants, for their antimicrobial properties is gaining ground. Plants produce secondary metabolites (phytochemicals), which have demonstrated their potential as antibacterials when used alone and as synergists or potentiators of other antibacterial agents. Phytochemicals frequently act through different mechanisms than conventional antibiotics and could therefore be of use in the treatment of resistant bacteria (Abreu *et al.*, 2012).

Mechanism of Actions of Natural Antimicrobials

Phenolic compounds are the main antimicrobial agents in plants. Even though the exact antimicrobial mechanism of phenolic compounds is not clear, phenolic compounds are commonly known for their antimicrobial effects. The ability of phenolic compounds to alter microbial cell permeability, thereby permitting the loss of macromolecules from the cell interior, could help explain some of the antimicrobial activity (Kar, 2007). Another explanation might be that phenolic compounds interfere with membrane function and interact with membrane proteins, causing deformation in structure and functionality. A combination of phenolic compounds can provide synergistic antimicrobial effects and can contribute to a better antimicrobial reaction as compared to the reaction of an individual compound (Firn, 2010). In addition, the effect of phenolic compounds can be concentration dependent; at low concentration, phenols affect enzyme activity while at high concentrations they cause protein denaturation. It has been reported that the antimicrobial activity of isothiocynates derived from onion and garlic is related to the inactivation of extracellular enzymes through oxidative cleavage of disulfide bonds and that the formation of the reactive thiocyanate radical was proposed to mediate the antimicrobial effect (Sarker and Nahar, 2007).

For peptides, the mechanism of action of antimicrobial peptides seems to involve multiple targets. The plasma membrane is the most cited target by peptides whereas recent studies have suggested intracellular targets to be more likely for some peptides. Most antimicrobial peptides have nonspecific mechanisms and they may display some selectivity between different microorganisms. Antimicrobial peptides can assume amphipathic structures, which are able to interact directly with the microbial cell membrane. This action rapidly disrupts the membrane in several locations and result in the leaching out of vital cell components. Studies on the mechanism of action of pleurocidin revealed that this peptide exhibits a strong membrane translocation and pore-formation ability reacting with both neutral and acidic anionic phospholipid membranes (Firn, 2010).

Factors Influencing the Antimicrobial Activity of Natural Products

The antimicrobial activity of natural compounds could be influenced by number of factors including botanical source, time of harvesting, stage of development, and method of extraction in addition to the composition, structure, and functional groups of the natural compounds (Samy *et al.*, 2006).

Mechanisms of antimicrobial

Antibiotics affect micro organisms in several ways with variation from one antibiotic to the other. They can be grouped as those that:-

i) Inhibit cell wall synthesis (ampicillin, cephalosporin, β-lactam, Vancomycin, Bacitracin)

ii) Inhibit nucleic acid function (Nitroimidazole, Nitrofurans, Quinolones, Rifampicin) or intermediate metabolism (Sulphonamides, trimethoprin)

iii) Damage cell membrane hence interfere with its function (Polymyxin, polygene)

iv) Inhibit protein synthesis (Aminoglycosides, Fenicols, Lincosamides, Macrolides, Streptogramins, Pleuromutilins, Tetracyclines)

v) Inhibit respiration that is antagonism of metabolic pathways

Antibacterial resistance

Resistance is the ability of a micro-organism to withstand the effects of antibiotics. Antibiotic resistance may evolve via natural selection acting upon random mutation. It can also be engineered by applying an evolutionary stress factor on a population of bacteria. Once a gene is generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals of same species) by plasmid exchange. If a bacterium carries several resistance genes, it is described as multi resistant or informally, a superbug. The antibiotic action against the pathogen can be seen as an environmental pressure to the pathogenic bacteria such that those which have a mutation allowing them to survive will live to reproduce (Firn, 2010).

They will then pass these traits to their offspring resulting in a fully resistant colony. Resistance to antibiotics may be either intrinsic or acquired. Intrinsic resistance is when the organism lacks the target site for the agent or has other features that always render it resistant to the antibiotic. Acquired resistance is applicable to those organisms that were previously susceptible to the antibiotic in question. This later form of resistance causes great concern because of its potential for reducing the range of previously useful antibiotics available. Resistance may also be either phenotypic or genotypic.

Mechanisms for inter-bacterial transfer of resistance

Three mechanisms have been identified for inter- and intra-transfer of genetic material, including resistance genes.

These mechanisms are:

(i) Transduction- This is common in Gram positive bacteria. Transmission of genetic material from one bacterium to another is by bacteriophages.

(ii) Transformation- This involves direct transfer of free DNA originating for example from lysed bacteria.

(iii) Conjugation- This is common in Gram- negative bacteria. The latter is the most important mechanism of inter- and intra-bacterial transfer of resistance. A plasmid or other genetic material is transferred from the donor bacterium to the recipient via cytoplasmic bridge (pilus). Conjugation may occur between bacteria of the same species, within species of the same genera or between species of different families (Stuart, 1998).

Factors that influence antibiotic resistance of bacteria

Pharmacokinetic characteristics of different classes of antibiotics may favour the development of resistance as well as dose regime (like insufficient dose, too short duration of treatment or long term use), active concentration or route of excretion of the drug.

i) The long term use of sub-MIC (sub-therapeutic doses) is regarded as one of the major factors responsible for development of resistance. This exerts a potent selective pressure for the emergence of resistant clones that already pre-existed in the bacterial population. The progressive emergence of insensitive bacteria and of acquired resistance in human clinical settings and the veterinary fields reflects the "tuning of these micro-organisms to antibiotic polluted" ecosystems.

ii) The amount of antibiotics used is also a selective force.

Antimicrobial susceptibility tests and resistance profile

There are three test methods (disk diffusion, broth dilution and agar dilution). Antimicrobial susceptibility testing methods that consistently provide reproducible and repeatable results is when followed correctly.

Disk diffusion - Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk. The diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. The diameter of this zone of inhibition around the antimicrobial disk is related to minimum inhibitory concentration (MIC) for that particular bacterium/antimicrobial combination; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its infusibility. Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Its main advantages are: low cost, ease in modifying test antimicrobial disks when required, can be used as a screening test against large numbers of isolates, can identify a subset of isolates for further testing by other methods, such as determination of MICs. Manual measurement of zones of inhibition may be time-consuming. Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly so that the zones of inhibition around antimicrobial discs in the disc diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally this can be accomplished if the discs are no closer than 24 mm from centre to centre, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar.

Broth and agar dilution methods- The aim of the broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the visible growth of the bacterium being tested (MIC, usually expressed in μg/ml or mg/litre). However, the MIC does not always represent an absolute value. The true MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution. Antimicrobial ranges should encompass both the interpretive criteria (susceptible, intermediate and resistant) for a specific bacterium/antibiotic combination and appropriate quality control reference organism (Firn, 2010).

MATERIALS AND METHOD

Materials

The materials and instruments to be use will include crucibles, Whatman filter paper, volumetric flasks, beakers, conical flasks, muslin cloth, oven, measuring cylinder, spatula, electric scale, Bunsen burner (stove), funnels, aluminum foils, test tubes, syringes, pipettes, cotton wools, petri dish and antibiotic disc.

Chemical and reagents

Agars, normal saline, methyl red, ethanol (alcohols), concentrated acetic acid, sulphuric acid, diluted ammonia, water, ferric chloride, potassium ferrocyanide, ethyl acetate, hydrochloric acid, petroleum ether, sodium hydroxide, potassium hydroxide (potassium permanganate). Hydrogen peroxide, sodium chloride, copper sulphate, sodium picarate, methyl red, cresol green, folin-ciocaltean reagent, folin-dennis reagent, Erichrome black and solechrome dark blue.

Collection of plant samples

A total number of six (6) herbal products namely (Goco herbal drug, Super 7, Dr. Aladin 7 keys herbal mixture, Betroth herbal drug, Bitters herbal mixture and Dansa herbal medicine) will be randomly purchased from different hawkers in Onitsha metropolis.

Some of the products had comparable batch numbers, date of production, expiry dates and National Agency for Food, Drug Administration and Control (NAFDAC) numbers on the products labels. None of the products will be expired prior to analysis at the. The physical attributes of the products will be studied and other information obtainable from the product labels will be recorded.

Method

a. **Preliminary Phytochemical Screening**

The extracts will be subjected to preliminary chemical screening for their presence or absence of active phytochemical constituents by the following methods:

Test for Alkaloids

The extracts were treated with dilute (10%) hydrochloric acid and filtered. The filtrates were treated with various alkaloidal reagents.

a. Mayer's test: The extracts were with Mayer's reagent (Potassium mercuric iodide). Appearance of cream colour indicates the presence of alkaloids in all extracts.

b. Dragendroff's test: The extracts were treated with the Dragendorff's reagent (Potassium bismuth iodide), the appearance of reddish brown precipitate indicates the presence of alkaloid in all extracts.

c. Hager's test: The extracts were treated with the Hager's reagent (Picric acid), the appearance of yellow colour precipitate indicates the presence of alkaloids in all extracts.

d. Wagner's test: The extracts were treated with the Wagner's reagent (Iodine solution) the appearance of brown colour precipitate indicates the presence of alkaloids in all extracts.

Test for Cardiac Glycosides

a. **Keller-Killani test :** When a pinch of the extracts were dissolved in the Glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated Sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides in all extracts.

Test for Flavonoids

a. Shinoda's test: The extracts were dissolved in alcohol, to that one piece of magnesium followed by conc. Hydrochloric acid were added drop wise and heated. Appearance of magenta color shows the presence of flavonoids in all extracts.

b. Ferric Chloride test: To the extracts, few drops of neutral ferric chloride were added. Blackish red colour was observed in all extracts.

Test for Saponins

a. **Foam test:** The extracts were diluted to 20 ml with distilled water and shaken well in a graduated cylinder for 15 minutes. The formation of foam in the upper part of the test tube indicates the presence of saponins in all extracts

Test for Steroids

a. **Salkowski reaction:** To 2 ml of extract, added 2ml chloroform and 2 ml conc. H_2SO_4. Shaked well. Chloroform layer showed red color and acid layer showed greenish yellow fluorescence.

b. **Liebermann-Burchard test:** When the extracts were treated with concentrated sulphuric acid, few drops of glacial acetic acid, followed by the addition of acetic anhydride, absence of green colour indicates the absence of steroids in all extracts.

Test for Tannins

a. **Lead acetate solution**: When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins in all extracts.

b. Ferric Chloride Solution: When the extracts were treated with ferric chloride solution, NaOH, & AgBr Solution appearance of green colour precipitate indicates the presence of tannins in all extracts.

Test for phenol

Ferric chloride test: 2 drops of neutral ferric chloride solution was added to 1ml of diluted aqueous solution of the test sample. A greenish purple color indicates the presence of phenolic compounds.

Antimicrobial analysis

Test organisms

The test organisms used for this study will be a clinical isolates of*Escherichia coli, Staphylococcus spp, Klebsiellaspp, salmonella spp and Shigella spp*. .

Re-identification of the organisms

All the test organisms will be aseptically grown on 5 ml nutrient broth overnight at 37^{o}C and then subcultured onto MacConkey agar, nutrient agar and cystein lactose electrolyte deficient (CLED) medium plates to get pure cultures of the organisms. These plates will be incubated at 37^{o}C for 24 hours. Pure cultures of these isolates will be identified biochemically using standard microbiological conventional identification techniques.

Determination of the antimicrobial activity

The antimicrobial activities of drugs will be evaluated on the bacterial isolates using Agar well diffusion method. The extracts will be reconstituted into a concentration of 10 mg/ml. Four (4) holes or wells will be bored on Mueller Hinton agar plate using a 6 mm diameter sterile cork borer. A small portion of the plant extract (50 µl) at concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml will be filled in each of the four wells. The negative control culture plate will be filled with 50 µl of the solvents used for the extraction. Inoculated plates will be incubated at 37^{o}C for 24 hrs and Zones of inhibition will be recorded for each tested organism.

RESULT

A total of four bacteria (*Bacillus* spp*, E coli, Salmonella* spp *and Staphylococcus aureus*) and two fungi (*Aspergillus* spp and *Candida* spp) were isolated from the six herbal products. The distribution of the isolates in the herbal products is shown in Table 1.

Table 1: Microbial count of bacteria and fungi isolated from herbal drugs hawked in Onitsha.

SAMPLE	Total viable count(x 10^4cfu/g)	Total coliform count (x 10^4cfu/g)	Total fungi count (x 10^4cfu/g)
Goco	$2.10^c \pm 0.11$	$1.80^b \pm 0.40$	$3.19^c \pm 0.30$
Super 7	$2.70^a \pm 0.16$	$1.92^a \pm 0.03$	$5.20^a \pm 0.02$
Dr. Aladin	$1.75^d \pm 0.01$	$1.40^d \pm 1.00$	$3.00^d \pm 0.10$
Beetroth	$0.90^e \pm 1.00$	$1.70^c \pm 0.10$	$3.04^d \pm 0.00$
Dansa	$2.00^b \pm 1.00$	$0.30^e \pm 0.01$	$4.10^b \pm 0.01$
Bitters	$0.10^f \pm 0.05$	0.00 ± 0.00	$2.00^e \pm 0.01$

*Values are mean scores± Standard deviation of triplicate

*Data in the same column bearing different superscript differ significantly ($p < 0.05$).

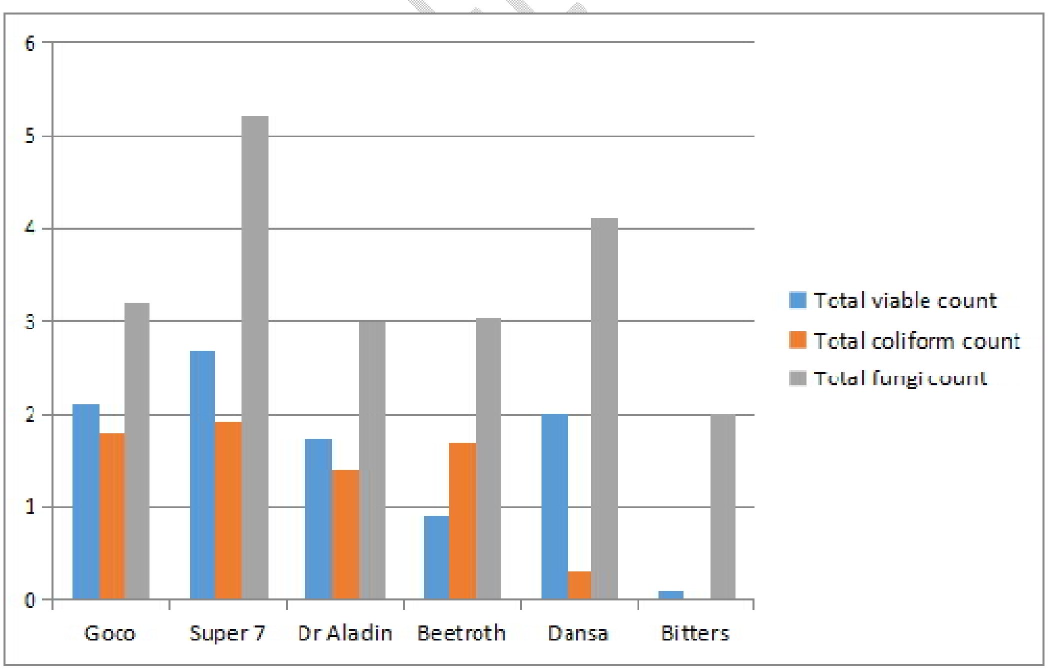

Fig.1: Microbial count of bacteria and fungi isolated from herbal drugs hawked in Onitsha.

Table 2: Microorganisms isolated from Nigeria herbal preparations

Microbial isolates	*S. typhi*	*E. coli*	*S. aureus*	*Candida albicans*	*Aspergillusspp*	*Bacillus spp*
Goco	+	−	+	−	+	−
Super 7	+	−	+	−	+	+
DrAladin	+	−	+	−	+	−
Beetroth	+	−	+	−	+	+
Dansa	+	−	+	−	+	−
Bitters	+	−	+	−	+	−

+: present, −: absent

Table 3: Qualitative phytochemical constituents of herbal drugs hawked in Onitsha.

PHYTOCHEMICALS	Goco	Super 7	DrAladin	Beetroth	Dansa	Bitters
SAPONIN	+++	++	+++	-	+	++
FLAVONOID	-	-	+++	-	-	+++
ALKALOID	++	++	+	+++	+++	+++
TANNIN	+	+++	++	++	+++	+++
STEROIDS	-	-	+	-	++	+
TERPENIODS	++	++	-	-	+	+
GLYCOSIDES	+	+	-	-	-	-
CARBOHYDRATES	+	+	+	-	-	-
PROTEIN	-	-	-	-	-	-
ANTHROCYNIN	+++	+++	-	-	+	-
PHENOL	+++	+++	+	+++	+++	+
OIL AND RESIN	-	-	-	-	-	-
REDUCING SUGAR	-	+	-	-	+	-

Key

+++ = Present in high concentration

++ = Present in moderate concentration

++ + = Slightly or sparingly present

- = Absent.

4.1 Antimicrobial

The result of the antimicrobial screening test showed clear zones of inhibition around the impregnated discs of each of the three concentrations of both the herbal extracts and commercial antibiotics which were inoculated on the two isolates. The mean zones of inhibition for the herbal extracts are shown in Tables 3. The presence of the zones of inhibition is a clear evidence of the antimicrobial properties of the *herbal remedied*. The Minimum Inhibitory Concentration (MIC) of the extract was also determined from the results of the antimicrobial test. This is shown in Table 4.

Table 4: Mean Zones of Inhibition (mm) of herbal drugs on some selected organisms.

EXTRACT	*S. typhi*	*E. coli*	*S. aureus*	*Candida albicans*	*Aspergillus spp*	*Bacillus spp*
Goco	0.00+ 0.00	15.17+ 0.00	10.00+ 0.00	0.00+ 0.00	0.00+ 0.00	13.87+ 0.77
Super 7	0.00+ 0.00	11.30+ 0.00	8.53+ 0.00	0.00+ 0.00	0.00+ 0.00	0.00+ 0.00
DrAladin	0.00+ 0.00	9.00+ 0.00	8.40+ 0.00	0.00+ 0.00	7.80+ 0.00	18.03+ 0.05
Beetroth	0.00+ 0.00	7.20+ 0.00	7.57+ 0.00	0.00+ 0.00	5.30+ 0.00	14.37+ 0.10
Dansa	0.00+ 0.00	0.00+ 0.00	7.00+ 0.00	0.00+ 0.00	5.00+ 0.00	10.51+ 0.20
Bitters	0.00+ 0.00	0.00+ 0.00	4.67+ 0.00	0.00+ 0.00	4.00+ 0.00	8.03+ 0.50
CONTROL	34.83+ 1.00	19.1+ 1.15	22.33+ 0.57	32.271.36	12.00+ 0.00	38.93+ 1.00

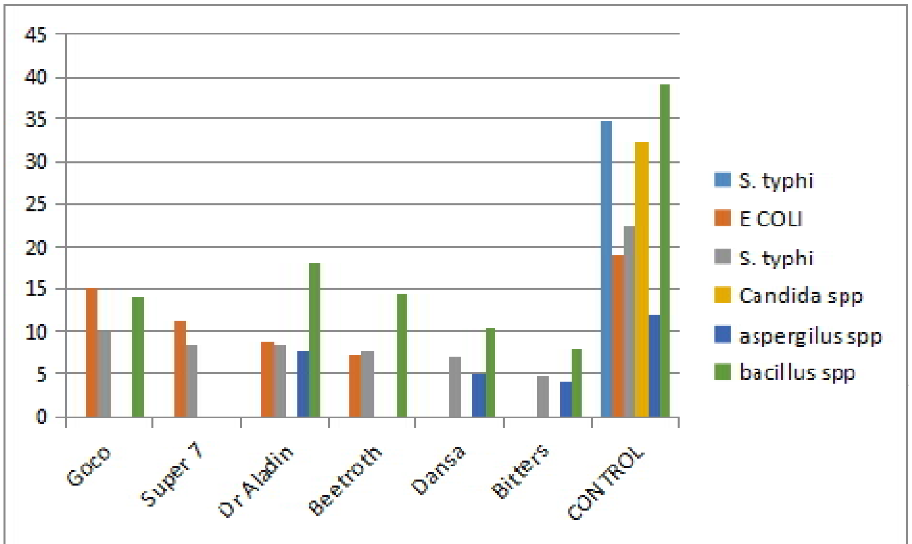

Fig. 2: Mean Zones of Inhibition (mm) of herbal drugs on some selected organisms.

Minimum Inhibitory Concentrations (MIC)

The table shows the Minimum Inhibitory Concentrations (MIC) of the test organisms which is the lowest concentration of the herbal extracts which can inhibit the growth of the organisms as determined from the zones of inhibition.

Table 5: Minimum Inhibitory Concentrations (MIC) of the herbal extracts on selected organisms.

Microorganisms	Herbal extract	Antibiotic
S. typhi	9.50	1.30
E. coli	2.90	0.30
S. aureus	0.30	1.00
B. subtilis	0.50	1.00
C. albicans	5.00	10.00

A. niger	10.20	0.30

Discussion

The phytochemical screening and quantitative estimation of the percentage crude yields of chemical constituents of the six (6) herbal drugs studied showed that they were rich in alkaloids, flavonoids, tannins, phenol and saponins but poor in protein, resin, steroid and terpenoid. They were known to show medicinal activity as well as exhibiting physiological activity. The absence of oil/resin and protein in all the six drug samples in the present study is in contrast with the opinion of Griebel *et al*. (1995) who noted that protein is one of the active constituents. Steroids and flavonoid were found to be present in only two (Dr. Aladin and Bitters) and this makes both drug more efficiency than others according to Sofowara 2014 who noted that steroidal compounds are of importance and interest in pharmacy due to their relationship with such compounds as sex hormones. This may be the reason the Dr. Aladin and Bitters are used by expectant mothers or breast feeding mothers to ensure their hormonal balance, since steroidal structure could serve as potent starting material in synthesis of these hormones.

The plants studied here can be seen as a potential source of useful drugs. Further studies are going on these plants in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds. The antimicrobial activities of these plants for the treatments of the diseases as claimed by traditional healers are also being investigated.

Most of the medicinal plants are prepared in open environment and unhygienic condition which gradually lead to contamination of enteric pathogens having public health importance. In the present study, from six herbal medicinal preparations, aerobic bacterial counts were obtained from all the samples, of which only one has permutable limit of bacterial count according to WHO standard. The other four samples were beyond WHO limit with minimum count of 4.6×10^{5} CFU/mL and maximum of 2.41×10^{9} CFU/g with mean count of 2.15×10^{8} CFU/g or mL. The total aerobic bacteria count in the present study is in agreement with the higher counts of aerobic bacteria found in herbal materials. On the contrary, the present study has higher aerobic bacteria count than the study conducted from herbal medicinal preparation by Adeleye et al. at Lagos, Nigeria [2014]. The reason why the total bacterial count in our study was higher may be due to the primitive ways of preparation of the plant products, poor environmental sanitation, and storage conditions. The present study showed bacterial isolates like *Bacillus*, *Escherichia*, *Pseudomonas*, *Salmonella*, *and Staphylococcus.*

The finding of coliforms like *Escherichia coli*, *and Salmonella* spp.is very important public health concern that needs urgent need of management of herbal medicinal products to insure their safety and quality issue.

One of the major shortcomings of herbal preparations in the developing countries is the unhygienic conditions under which they are produced. In the present study, it was observed that herbal preparation A and B are not sterile (Table1). Bacteria that are of health importance such as *Bacillus* species were isolated from the herbal products. The presence of *Bacillus* species may be as a result of inadequate heat processing, improper handling of products and contaminated processing equipment.

The study claimed that 60% of the six herbal preparation used in this study label complete antimicrobial remedy with 99.9% efficacy may not also be true. The results shows that herbal preparation has lower zones of inhibition. More so, a higher concentration of the product (1g/ml) was used before a slight antibacterial effect was observed.

Conclusion

This study revealed that herbal preparation within Onitsha metropolis are not sterile and may serve as source of infection to the end users. Though, the herbal preparations contained some important phytochemicals and these phytochemical components may not be at an effective concentration to make the products efficacious in antagonizing the pathogenic activities of invading microorganisms. Multidrug-resistant (MDR) pathogens are a growing threat to human health and welfare. The presence of MDR infections, including those of *Staphylococcus aureus*, in both hospitals and communities, is disturbing to healthcare providers due to the difficulty in treating these infections, resulting in longer hospital stays and increased patient morbidity and mortality.

The present study on selected herbal medicines sold within Onitsha metropolis is found to demonstrate lesser antibacterial effects than 99% written in the label. Most of the tested traditional medicinal plant extracts have a promising antimicrobials effect on MDR bacteria. The current study seems to unravel further detailed investigations on the plant extracts showing appreciable antimicrobial responses against MDR pathogenic microbes of human; in terms of the (low risk of) toxicity, clinical efficacy trial (*in vivo* experiments), safety tests, and affordability analyses; are necessary to draw reliable conclusions. As the last lap of the journey towards the discovery of new and more efficient antibacterial agents from the extracts is green lighted by these later indicated tests and utterly essential

NOTE:

The study highlights the efficacy of "Herbal" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

REFERENCES

Abreu, N., Adewusi, E. A. and Afolayan, A. J. (2012). A review of natural products with hepatoprotective activity. *Journal of Medicinal Plants Research* Vol. 4(13), pp. 1318-1334.

Adepoju, G. K. (2005). The Attitudes and Perceptions of Urban and Rural Dwellers To Traditional Medical Practice in Nigeria: A Comparative Analysis', *International Journal of Gender and Health Studies Vol. 3, No. 1 and 2 190-201*.

Afolabi, F. and Afolabi, O. J. (2013). Phytochemicals Constituents of some Medicinal Plants in South west, Nigeria. *Journal of Applied Chemistry*, 4(1): 76-78.

Alarcon, C., Afolabi, A. and Akinmoladun, A. (1994). Antioxidant properties of *Myristica fragrans Houtt*. and its effect on selected organs of albino rats. *African Journal of Biotechnology*. Vol. 5 (13). pp. 1274- 1276.

Aluko, T. (2005). 'Trading in Traditional Medicine: The Challenges for Womanhood and the Health Care system in Nigeria', *International Journal of Gender and Health Studies Vol. 2, No 1 and 2: 47-54.*

Asenso-Okyere, H. (1996). 'The Behaviour towards Malaria care: A Multinomial Logit Approach', *Social Indicators Research 39:167-186.*

Babalola, K. A. (2009). An examination of the usage of herbal contraceptives and Abortifacients in Lagos state Nigeria. *Ethnobotanical leaflets* 13:140-146.

Batran, S. A., El-Gengaihi, S. E. and El-Shabrawya, O. A. (2006). Some toxicological studies of Momordicacharantia L. on albino rats in normal and alloxan diabetic rats. *Journal of Ethnopharmacology*.; 108: 236-242.

Cesarone, H., Afolayan, C. and Meyer, E. (1992). Studies of Nigerian medicinal plants I. Preliminary survey of plant alkaloids. *Journal of West African Science Association* 13 (1): 13-33

Czech, E., Kneifel, W. and Kopp, B. (2001). Microbiological Status of Commercially Available Medicinal Herbal Drugs- A Screening Study. *PlantaMedica.* 67: 263-269.

Doughari, J. H. (2012). Phytochemicals, Extraction Methods, Basic Structures and mode of Action as Potential Chemotherapeutic Agents, Phytochemicals - A Global Perspective of their role in Nutrition and Health. Dr. Venketeshwer Rao (Ed.). Intechopen Publishers.

Firn, C. (2010). Antibacterial activity of Crude Extract of *Azudirachitaindica*on *Salmonella typhi. World Journal of Biotechnology* 3(1): 347-351

Forest, T. (2009). 'Social and Economic Research: Principles and Methods', *AfricanInstitute for Applied Economics,* Enugu.

Griebel, D., Oniga, D., Oprean, I. and Tamas, R. M. (1995). Chemical composition of the essential oils of *Ocimumbasilicum* L. cultivated in Romania. *Farmacia*.;57:625–9

Harborne, H. B. (2013). Phytochemical method. *London: Chapman and Hall* 24(10): 675-680 .

Haristoy, X., Fahey, J. W., Scholtus, I. and Lozniewski, A. (2015). Evaluation of antimicrobial effect of several isothiocyanates on Helicobacter pylori. *Planta Medica* 71: 326-330.

Hertog, P., Trefil, P. and Stiborova, M. (1993). Flavonoids - Potent and versatile biologically active compounds interacting with cytochrome P450. *Chemico-Biol. Intern.* 139(1), 1-21.

Iwu, M. M., Awolola, B. and Okunola, M. O. (1999). *Handbook of African Medicinal Plants*. 1st Edn., CRC Press, Boca Raton, FL., ISBN-10: 084934266X, pp: 464.

Kar, K. (2007).Chemical composition and tannins in leaves of some browse plants from Delta (Central Nigeria) eaten by ruminants. Proc. Soc. Nutr. Physiol., 5:11-15.

Kuhnau, D. (2016). *Vernoniaamygdalina* hepatotoxic or hepatoprotective. Response from biochemical and toxicity studies in rats. *Afric. J. Biotech*. 5 (18): 1648 – 1651.

Kumar, A. (2004). 'Malaria: its Human Impact, Challenges, and Control Strategies in Nigeria', In focus Vol. 2 no.2.

Motaleb, B., Kumar, K. and Pandey, A. K. (2011). Chemistry and Biological Activities of Flavonoids: An Overview. *The Scientific World Journal*, 1-17.

Nascimento, G., Locatelli, P., Freitas, C. and Silva, G. (2000). Antibacterial Activity of Plant Extracts and Phytochemicals on Antibiotic resistant Bacteria. *Brazilian Journal of Microbiology*, Vol.31:247-256

Nwokocha, B. and Peter, F. (2011). Body requirements: In Nutritional Biochemistry. *University of Lagos Press,* Nigeria, pp. 11-13.

Puupponen-Pimia, R., Nohynek, L., Alakomi, H. L. and Oksman-Caldenteyl, K. M. (2008). Bioactive berry compounds novel tools against human pathogens. *Applied Microbiology Biotechnology*.

Robak, D., Jakhetia, M. L. and Bosisio, E. (1988). Pharmacological Activities of *Chelidoniummajus* L. Paparveraeae). *Pharmacologicalll Resources*.

Rocha, D. (2010), 'Malaria Prevention during pregnancy: Awareness and Factors contributing to disease occurrence among pregnant women in Gboko Metropolis, Benue State, Nigeria', *Journal of Medicine and Biomedical Sciences; ISBN 2078-0273*.

Samy, J., Feuerstein, I., Barrel, S. and Segal, R. (2006). The Antibacterial and Antispasmodic Activity of *Artemisia herba alba* Asso. II. Examination of Essential Oils from Various Chemotypes. *Pharmaceutical Biology*, Vol.25 (2): 89–96.).

Sarker, O. and Nahar, B. D. (2007). *Studies on the chemical composition and nutritive value of the fruits of African star apple*. M.Sc. Thesis, Univ. of Calabar, p. 67.

Singh, A. and Barret N. (2006). Efficacy of plant essential oils as antimicrobial agents against *Listeria monocytogenes*in hotdogs. LWT-Food Science and Technology.

Twari, B. K., Valdramidi, V. P., O'donnell, C. P., Muthukumarappan, K., Bourke, P. and Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry*.

Vickers, G. and Zollman, D. L. (2005). Molecular Epidemiology of Community and Health Care-Associated Methicillin Resistant Staphylococcus aureus in Manitoba, Canada. *Journal of Clinical Microbiology*.