

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 microbiological purity and antibacterial characteristics of six Nigerian herbal remedies with the stated efficiency of healing all manners of microbial infections were evaluated. 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. As a result, additional investigations on clinical efficacy trials, safety, toxicity, and affordability analyses must be initiated as soon as possible in order to proceed to the final step of synthesizing precursor molecules for novel effective antimicrobials.

Key words: antimicrobial susceptibility, bacteria isolated, herbal drugs, *Bacillus subtilis*

INTRODUCTION

Background of the study

“Both emerging and developed nations are seeing a rise in the use of herbal medicine, a type of complementary and alternative medicine” (Czech et al., 2011). According to a World Health Organization (WHO) assessment, 70–80% of the world's population, especially in developing nations, relies on

alternative medicines, primarily those derived from herbal sources, for their basic treatment. Traditional medicine has been cited by WHO as one of the most reliable ways to ensure that everyone in the world has access to health care. In order to achieve its objective of providing affordable and culturally appropriate healthcare for the entire world's population, WHO has promoted member states' responsible use of traditional plant-based medicines and created technical guidelines for the evaluation of herbal medicine (Twari, et al., 2019). According to Afolabi & Afolabi (2013), a medicinal plant is any plant that includes an active component that is employed therapeutically in one or more of its organs or that contains building blocks for the production of effective pharmaceuticals. Consider the research conducted by Afam-Ezeaku et al. in 2022, which demonstrated the effectiveness of mango (*Mangifera indica*) stem extracts in the treatment of toothaches. According to Doughari (2012), "a medicinal plant is one that has compounds in one or more of its organs that can be utilized therapeutically or that act as precursors for the semi-synthesis of chemo-pharmaceuticals. The secondary metabolites alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenics, tannins, resins, lactones, and volatile oils that are naturally produced and accumulated by medicinal plants include these. The parts of plants that are employed in the formulations of herbal medicine include leaves, roots, rhizomes, stems, barks, flowers, fruits, grains, or seeds. These parts of the plant include chemical components that are used to treat and control ailments". Researchers have discovered, according to Motaleb et al. (2011), that people all around the world frequently employ the same or closely

related plants to treat the same ailments. According to the WHO, 80% of the world's population uses herbal medicines, at least in part for primary healthcare.

The phytochemical components of all herbal medications are responsible for their pharmacological activity. Naturally occurring phytochemicals that contain defense mechanisms and protect against numerous diseases can be found in medicinal plants, leaves, vegetables, and roots. phytochemicals effective enough against microorganisms

A substance that kills or prevents the growth of germs like bacteria, fungus, protozoa, or viruses is known as an antibiotic. Antibiotics are chemicals created by microorganisms that either kill or stop the development of other microorganisms. While antivirals are specifically used to treat viral infections, antibiotics are typically used to treat bacterial infections. Antifungal medications are used to treat fungus infections, but some of its side effects can be fatal if taken improperly.

Antibiotics derived from various microorganisms are currently used to treat a variety of human diseases, so action must be taken to control their use. New drugs, either synthetic or natural, must be developed, and for a long time, plants have been a valuable source of natural products for maintaining health. India has a long history of using medicinal plants to create pharmaceuticals. The World Health Organization (WHO) defines a medicinal plant as any plant that contains

compounds that can be utilized therapeutically or that serve as a precursor to new, semi-synthetic chemo-pharmaceuticals.

For many years, evaluating medicinal plant materials for their therapeutic potential has represented a possible source of fresh, strong medications. Man has reaped enormous benefits from employing medicinal plants to manage disease since they are generally safer, more cost-effective, and occasionally have superior therapeutic value than manufactured pharmaceuticals. In order to give information that will be useful, further scientific inspection of the bioactivity of medicinal plants has become necessary as more of them are discovered.

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

“In sub-Saharan Africa, traditional medicines (TMs) are an essential part of healthcare, and a stronger integration of traditional and biomedical health systems may be required. Nearly 70% of people still commonly receive healthcare through traditional healers or vendors in Nigeria, for example, despite increased access to biomedicine” (Aluko, 2015). “Understanding how and why TMs are used in Nigeria is essential due to their significance. The intricate dynamics of TM use in sub-Saharan Africa have already been brought up by others. Studies on the factors that influence the usage of TM have looked at cultural belief systems, the high cost and limited accessibility of biomedicine, disease understanding, safety concerns with biomedicine, and perceptions of TMs as being more effective” (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 medications include herbs, herbal ingredients, herbal preparations, and completed herbal goods that have actual plant parts, other plant components, or mixtures as active substances.**
- **Herbs are unprocessed plant parts that can be whole, broken up, or ground up, such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes, or other plant parts.**
- **Herbal materials include fresh juices, gums, fixed oils, essential oils, resins, and dry powders of herbs in addition to the actual herbs. These materials may be prepared locally in some nations by different methods such steaming, roasting, or stir-baking them with honey, alcoholic beverages, or other ingredients.**

Herbal preparations serve as the building blocks for completed herbal remedies and can comprise extracts, tinctures, fatty oils, or comminuted or powdered plant components. They are created through various physical or biological processes, including as extraction, fractionation, purification, and concentration.

Herbal preparations created from one or more herbs are known as finished herbal products. The phrase "combination herbal product"

can also be used when more than one herb is employed. Excipients may also be present in finished herbal products and herbal blends in addition to the active components. However, finished goods or mixtures containing isolated components from herbal materials or synthetic compounds with chemically defined active ingredients are not regarded as being 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

substances in herbal remedies that have therapeutic effect are referred to as active substances. If adequate analytical methods are available, the preparation of herbal medicines whose active ingredients have been identified should be standardized to contain a specific amount of the active ingredients.

The usage of plants for healing by humans can be traced back to the Neanderthal era (Forest, 2009). An growing percentage of individuals who normally do not disclose concurrent use to their providers are now using herbal medicine. Patients seek out herbal treatments for a variety of causes. Many patients who use herbs have chronic or incurable ailments, such as diabetes, cancer, and heart disease, which may be explained by a "sense of control, a mental comfort from taking action". "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 typically employ unpurified plant extracts with a variety of components. These are said to interact synergistically such that the combined effect of the herb as a whole is higher than the sum of its individual effects. Additionally, it is asserted that using whole herbs as opposed to separated active components (or "buffering") reduces toxicity. Although the amounts of the constituent substances in two samples of a particular herbal drug may differ, practitioners assert that this generally does not result in clinical issues. Although there is some experimental support for synergy and buffering in some whole plant preparations, it is unknown to what extent this holds true for all herbal products (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

“Plant substances known as phytochemicals are non-nutritive but have protective or disease-preventing qualities. They are organic chemical compounds that give plants their color and organoleptic qualities. They are non-essential nutrients, which means that the human body does not need them to maintain life” (Iwu et al., 1999).

The Flavonoids

A class of polyphenolic substances known as flavonoids is commonly present in the plant kingdom. There are currently roughly 3000 different types of flavonoids (Kuhnau, 2016). According to Caesarone et al. (1992), several are not hazardous to animals and some are frequently utilized in medicine to maintain capillary integrity. According to Robak et al. (1988), flavonoids also have anti-inflammatory, anti-hepatotoxic, and anti-ulcer properties. They also prevent the activity of enzymes like xanthine oxidase and aldose reductase. They have the capacity to scavenge free radicals and are strong antioxidants. According to Hertog et al. (1993), many contain

anti-allergic and antiviral properties, and some of them offer protection against cardiovascular mortality. They have been demonstrated to slow tumor growth in mice and stop the growth of several cancer cell lines in vitro.

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

The chemical elements known as phenols, phenols, or polyphenolics (or polyphenol extracts) are widely distributed as natural color pigments that give fruits of plants their color. The enzyme phenylalanine ammonia lyase (PAL) is primarily responsible for converting phenylalanine into phenolic compounds in plants. They serve a variety of purposes and are crucial to plants. The control of human pathogenic diseases may benefit most from their use in plant defense against pathogens and herbivore

predators (Puupponen- Pimiä et al., 2008). They are divided into three groups: phenolic acids, flavonoid polyphenols (catechins, flavonones, xanthenes), and non-flavonoid polyphenols. The phenolic chemicals found in plant flora that are most frequently found include caffeic acid and chlorogenic acid, both of which are known to trigger allergic reactions. “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

“In general, glycosides are defined as the condensation products of sugars (including polysaccharides) with a wide variety of organic hydroxy (occasionally thiol) compounds (always monohydrate in character), with the requirement that the hemiacetal entity of the carbohydrate must essentially participate in the condensation. Glycosides are phytoconstituents that are colorless, crystalline, hydrogen- and oxygen-containing (some also contain nitrogen and sulfur), water-soluble, and present in cell sap. Chemically, glycosides are composed of an aglycone or genin and a carbohydrate (glucose)” (Kar, 2007). Aglycones are represented by ethanol, glycerol, or phenol. Because they react in a

neutral manner, glycosides are easily hydrolyzed into their component parts by ferments or mineral acids. Based on the type of sugar component, the chemical makeup of the aglycone, or the pharmacological effect, glycosides are categorized.

Saponins

The steroid or triterpenoid glycosides known as saponins can be found in a variety of diets. They can produce foams in aqueous solutions, have a harsh taste, and hemolyze red blood cells. They have a reputation for impairing swine and poultry growth performance. Their capacity to combine with sterols, particularly those found in animal cell membranes, appears to be a factor in their ant nutritional qualities. The gut mucosa appears to become more permeable as a result. In comparison to other monogastric animals, poultry are more susceptible to saponins. Alfalfa meal contains high levels of saponin, compared to low levels in other legumes such soy beans, rapeseed, and other kinds of peas. Because they are only present at legal levels in monogastric animals, saponins are generally of little concern.

One of the most common and chemically diverse categories of natural products is terpenes. They are flammable unsaturated hydrocarbons that are typically found in liquid form in oleoresins, resins, and essential oils. According to the number of carbon atoms, terpenoids are hydrocarbons of plant origin with the general formula $(C_5H_8)_n$

and are categorized as mono-, di-, tri-, and sesquiterpenoids. Terpinen-4-ol, thujone, camphor, eugenol, and menthol are a few examples of commonly significant monoterpenes (Griebel et al., 1995). Steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal, or cytotoxic effects are included in the triterpenes (C30). including monoterpenes, common triterpenes including amyrins, ursolic acid, and oleanic acid sesquiterpene (C15) play a significant role in numerous 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

Antibiotics, antiviral, antifungal, anti-protozoan, and other antimicrobials come in many different varieties. Both natural and manufactured sources of antibiotics are available, and they are used to treat bacterial infections. Phenyl propanoids (like chloramphenicol), polyketides (like tetracycline), aminoglycosides (like streptomycin and gentamycin), macrolides (like erythromycin), glycopeptides (like vancomycin), and second-generation -lactams (like cephalosporins) are a few examples of drugs with a natural origin. Sulphonamides, quinolones, and oxazolidinones are examples of compounds from synthetic sources. According to Nascimento *et al.* (2000), the majority of antibiotics work by inhibiting either protein synthesis or the bacterial cell wall.

The quinolones that prevent DNA synthesis and the sulphonamides that prevent the production of metabolites necessary for DNA synthesis are the exceptions (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

The primary antibacterial components in plants are phenolic compounds. Phenolic compounds are well known for their antibacterial activities despite the fact that the precise mechanism by which they exert their antimicrobial actions is unclear. Some of the antibacterial activity may be explained by phenolic compounds' capacity to change the permeability of microbial cells, allowing the loss of macromolecules from the inside of the cell (Kar, 2007). Another possibility is that phenolic chemicals disrupt membrane function and interact with membrane proteins, resulting in

structural and functional changes to the membrane. When phenolic compounds are combined, they can have synergistic antibacterial actions and improve the antimicrobial response compared to the reaction of each component alone (Firn, 2010). Furthermore, the impact of phenolic compounds can vary depending on concentration; at low concentration, phenols affect enzyme activity while at high concentrations they cause protein denaturation. It has been reported that the antimicrobial activity of isothiocyanates 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, Fenicol, Lincosamides, Macrolides, Streptogramins, Pleuromutilins, Tetracyclines)
- v) Inhibit respiration that is antagonism of metabolic pathways

Antibacterial resistance

A microorganism's capacity to survive antibiotic actions is known as resistance. Through random mutation and natural selection, antibiotic resistance may develop. Another way to engineer it is to put a population of bacteria under evolutionary stress. After a gene is produced, bacteria can use plasmid exchange to horizontally (between members of the same species) transfer the genetic information. A bacteria is referred to as multi-resistant or, more colloquially, a superbug if it possesses many resistance genes. As a result of the antibiotics' effect against the pathogen, only those harmful bacteria that have undergone a mutation that allows them to survive will be able to proliferate (Firm, 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 is the process of releasing a predetermined concentration of an antimicrobial agent from disks, tablets, or strips into a solid culture medium that has already been seeded with the chosen inoculum separated from a pure culture. The foundation of disk diffusion is the identification of an inhibitory zone that is inversely correlated with the bacterial susceptibility to the antibiotic contained in the disk. A gradient of the antibiotic is produced by the antimicrobial agent's diffusion into the seeded culture media. The zone of inhibition is defined as the point at which the concentration of the antibiotic is so low that it can no longer prevent the growth of the test bacterium. 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 diffusibility. 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 goal of the broth and agar dilution procedures is to find the minimum inhibitory concentration (MIC, typically expressed in g/ml or mg/litre) of the assayed antibiotic that suppresses the observable growth of the bacterium being tested. The MIC does not, however, always denote an absolute value. The real MIC lies between the test concentration with the lowest inhibitory effect on bacterial growth and the subsequent test concentration with the lowest inhibitory effect. As a result, one dilution may be thought of as an intrinsic variation in MIC determinations made using a dilution series. Antimicrobial ranges should include the relevant quality control reference organism in addition to the interpretation criteria (susceptible, intermediate, and resistant) for a given bacterium/antibiotic combination (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. Dragendorff'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*, *Klebsiella spp*, *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°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°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 μ l) 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 μ l of the solvents used for the extraction. Inoculated plates will be incubated at 37°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 ⁴ cfu/g)	Total coliform count (x 10 ⁴ cfu/g)	Total fungi count (x 10 ⁴ cfu/g)
Goco	2.10 ^c ± 0.11	1.80 ^b ± 0.40	3.19 ^c ± 0.30
Super 7	2.70 ^a ± 0.16	1.92 ^a ± 0.03	5.20 ^a ± 0.02
Dr. Aladin	1.75 ^d ± 0.01	1.40 ^d ± 1.00	3.00 ^d ± 0.10
Beetroth	0.90 ^e ± 1.00	1.70 ^c ± 0.10	3.04 ^d ± 0.00
Dansa	2.00 ^b ± 1.00	0.30 ^e ± 0.01	4.10 ^b ± 0.01
Bitters	0.10 ^f ± 0.05	0.00± 0.00	2.00 ^e ± 0.01

*Values are mean scores± Standard deviation of triplicate

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

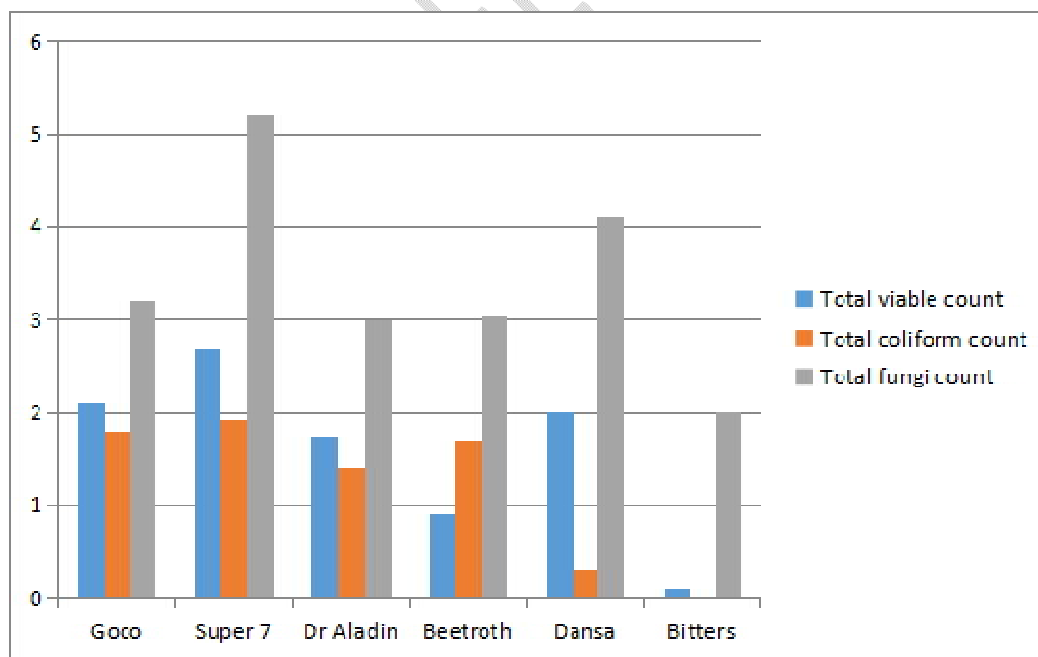


Fig.1: Microbial count of bacteria and fungi isolated from herbal drugs hawked in Onitsha.

Table 2: Microorganisms isolated from Nigeria herbal preparations

Microbial isolates	<i>S. typhi</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>Candida albicans</i>	<i>Aspergillus</i> spp	<i>Bacillus</i> 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	<i>S. typhi</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>Candida albicans</i>	<i>Aspergillus spp</i>	<i>Bacillus spp</i>
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

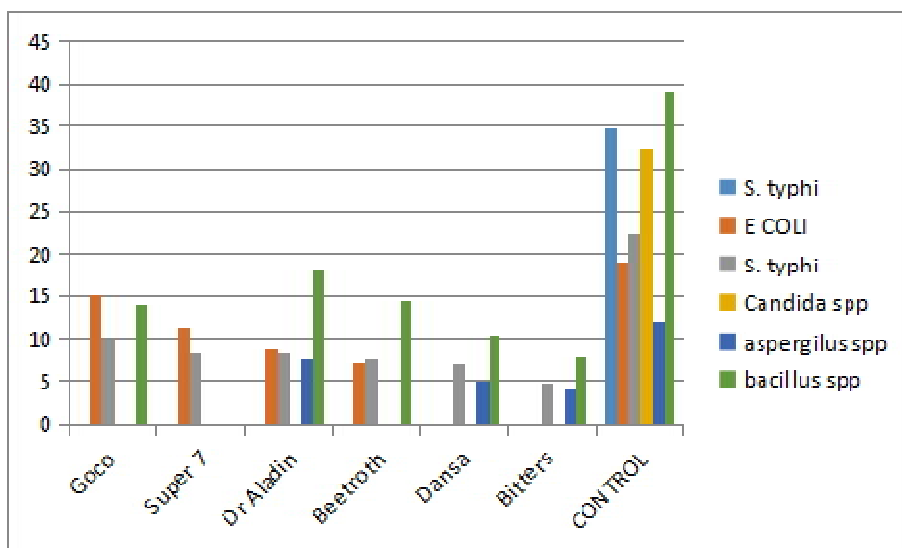


Fig. 2: Mean Zones of Inhibition (mm) of herbal drugs on some selected organisms.

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
<i>S. typhi</i>	9.50	1.30
<i>E. coli</i>	2.90	0.30
<i>S. aureus</i>	0.30	1.00
<i>B. subtilis</i>	0.50	1.00
<i>C. albicans</i>	5.00	10.00

<i>A. niger</i>	10.20	0.30
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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 permissible 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.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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