**ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM HERBAL MIXTURES SOLD AT ENUGU METROPOLIS**

**BY**

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**U14/NAS/MCB/058**

**DEPARTMENT OF MICROBIOLOGY,**

**FACULTY OF NATURAL ANDAPPLIED SCIENCES**

**GODFREY OKOYE UNIVERSITY**

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**TITLE PAGE**

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**A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY,**

**FACULTY OF NATURAL AND APPLIED SCIENCES.**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY**

**SUPERVISOR**

**MR S.O OKOLO**

**APPROVAL PAGE**

This project (isolation and identification of microorganism from herbal mixtures sold in Enugu metropolis), written under the direction of candidate project supervisor, and approved by the member of project committee, has been presented and accepted, by the department of microbiology, Godfrey Okoye University in partial fulfillment of the requirement of the award of Bachelor of Science (B.Sc) degree in microbiology.

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Project supervisor

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Dr. (Mrs.) Marian N. unachukwu Date

Head of Department Microbiology

**DEDICATION**

I dedicate this project to God almighty my creator, my strong pillar, my source of inspiration and strength in working on this project, I am indebted in you, thank you sir.

**ACKNOWLEDGEMENT**

The achievement of any work requires not only determination and hard work but also the corporation and assistance of others.

To God goes my whole hearted appreciation and thanks for giving me opportunity, courage and wisdom to bring this program to a successful end, for granting me the Opportunity to be among the living, up to this time of my life.

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**ABSTRACT**

The safety, efficacy and quality of herbal mixtures have been an important concern for health authorities and health professional, especially now there is increase in the use of herbal mixtures. This study was aimed at isolation and identification of microorganisms from some liquid herbal mixtures sold in Enugu metropolis, South East of Nigeria. A total of twenty samples of herbal mixture were selected at random, from herbal shops in Enugu metropolis and were analysed in Microbiology laboratory, Godfery Okoye University, samples were inoculated onto Nutrient agar, MaCkonkey agar and Blood agar plates, and incubated at 37oC for 24 hrs. Potatoe dextrose agar slants were also inoculated for the isolation of fungi. Viable bacterial counts of the samples were also performed using nutrient agar. The organisms isolated were identified using biochemical tests, and the following organisms were identified *Streptococcus* sp*, Escherichia coli, Staphylococcus* sp*, Proteus* sp*, Aspergillus* sp*, Rhizopus* sp*, Pseudomonas* sp *Bacillus* sp *Penicillum* sp *mucor* sp*.* the herbal medicine were highly contaminated and most of the organisms, isolated were gotten through poor handling, poor manufacturing of this herbal mixtures and raw materials used in preparing this herbal mixtures.

**CHAPTER ONE**

**1.0 INTRODUCTION**

**1.1 BACKGROUND OF STUDY**

Herbal medicines are naturally occurring plant derived substances that are used to treat illness with local or regional healing practices. And these products are complex mixtures of organic chemicals that may come from any raw or processed part of a plant.

Herbal medicine botanically is known as medicine or phytomedicine, is it the process of using plant seeds, Berries roots, leaves, barks, or flower for medicinal purposes, which many of them are believed to have medicinal properties which are used to treat minor illness and disturbances (Snezana, *et al*., 2012). They are promoted as natural and safe and are therefore the preferred choice. There herbal preparations are used to treat various types of aliment, including diaherea, urinary tract infection, typhoid fever and skin disease (Sofawora 1993).

The world health organization (WHO) defined traditional medicine (TM) as the total combination of knowledge and practices, whether explicable or not, used in diagnosing preventing or eliminating physical mental or social diseases(WHO, 2008) which may rely exclusively on past experience and observation, handed down from generation to generation verbally or otherwise.

Throughout history, all cultures have employed a variety of plant derived material for the prevention and treatment of disease (H.B *et al*., 1999) these herbal medicines have received official recognition worldwide by different health authorities (R.B,1983;O.Akarale 1987). In developing countries, as much as 80% of the indigenous population depends on traditional plants as their primary source of health care (R.B, 1983). In most African countries including Nigeria herbal medicine is recognized as an important component of health care system, especially among rural dwellers that constitute about 70% of the population (Esinione *et al*., 2002). Alternative medicine, such as herbal medicine are now gaining popularity, especially because of typically low side effect (Wilt *et al*., 2000) and high level of acceptance by patient (Ujam *et al*., 2013).

There appear to be an increase in the production of herbal medicine in the last decade, and there has been an upsurge in the circulation of herbal product in Nigeria (Oyetayo, 2008).With these increased usage, the safety, efficacy and quality of medicine have been an important concern for health authorities and health professional (Oluyege and Adelabu, 2010).Due to the increased widespread use of traditional medicine it has prompted the WHO to promote the integration of traditional medicine and complimentary or alternative medicine into the national health care system of some countries and to encourage the development of national policy and regulation as essential indicators of the level of integration of such medicine within the national healthcare system (WHO, 2011).

Since they are natural products all parts of the plants can be degraded by bacteria and fungi especially molds. Unscientific methods of cultivation and collection, inappropriate harvesting and cleaning, unsuitable transportation, prolonged drying and storages, inadequate hygiene of producers and congenital climatic condition renders the raw plant material prone to infestation and exposed them to many microbe contamination. Raw materials are most often degraded by microorganisms before harvesting during handling and after prolonged storage (Matthew, 1995; Kenneth 1989). The presence sufficient numbers of microbes can be harmful to consumers. As a result of fungal contamination, the risk of mycotoxin production, especially afflatoxin, should be taken into consideration in the manufacturing process because of the proven mutagenic, carcinogenic, tretratogenic, neurotoxic, nephrotoxic, immunosuppressive activities, (Reifei, 1988; Scimca, 1995; FAO, 2000; Hohler, 2000).

The microbial quantity of herbal drugs has to be coordinated with the regulation of the European pharmacopoeia 6th edition and regulation of medical safety of dietary ingredient. Despite several reports of fungal contamination and aflatoxins production in food stuff, limited research has be carried out on the microbial isolation and identification.

Herbal product purchased was analyzed, by isolating and identifying microbial contaminants. The microbial properties of some liquid herbal infective drugs produced and marketed in Enugu, south east Nigeria, the level of contamination was estimated and also identified. While isolation of pure culture was done based on morphology, difference where elevation forms, pigmentation and size were the major distinguishing factors for the major distinguishing factors for both fungal and bacterial contamination.

**1.2 AIM**

The overall aim of this work was to culture some selected herbal mixture sold by clinics, chemists, supermarkets, and streets in Enugu metropolis for microbial, contamination.

**1.3 OBJECTIVES**

1. To isolate microorganisms from the herbal preparations.
2. To identify the organisms.
3. To determine the bacterial load and fungal load of the preparation.
4. To compare the bacterial loads of the different preparations.

**CHAPTER TWO**

**2.0 LITERATURE REVIEW**

**2.1 ORGANISM ISOLATED FROM HERBAL PREPARATIONS**

Herbal medicine is an integral part of ‘traditional medicine’, and Traditional medicine has a broad range of characteristic and element which earned it the working definition from the world health organization (Oreagba *et al.,* 2011). Traditional medicine are diverse health practices , approaches , knowledge and benefits that incorporate plant, animal or mineral based medicines , spiritual therapies, Manuel techniques, and exercises which are applied singularly or in combination to maintain well-being , as well as to treat, diagnosed or prevent illness (WHO,2008). Traditional herbalist uses various herbal preparations to treat various types of aliment including diaherea, urinary tract infection, typhoid fever and skin disease. (Sofawora, 1993). Alternative medicine, such as herbal medicine is now gaining popularity especially because of typically low side effect (wilt *et al*., 2000), low cost (Vander hoof, 2001), and high level of acceptance by patient (Ujam *et al*., 2013).

The growing, harvesting and manipulation methods usually applied cannot avoid microbial contamination of the plant materials which therefore reflects the environmental conditions as well as the specific hygiene during the diverse treatment (kneifel *et al.,* 2012).Raw materials are most often degraded by microorganisms before harvesting, during handling and after prolonged storage (Matthew 1995; Kenneth 1989). The presence of large numbers of microbes can be harmful for human consumption. Herbal medications are likely to be contaminated with a wide variety of other potentially pathogenic bacteria. In the scientific study conducted in Dares, Salam Tanzania, it was observed that liquid and powdered herbal drugs had high levels of bacterial contamination (Justin *et al*., 1998) Also in a study evaluated, the bacterial contamination of herbal medicinal preparation sourced from identified herbal remedies were contaminated with *Salmonella typhi* and *Shigella spp*, besides *Escherichia coli* and *staphylococcus aureus* (Abba *et al*., 2009). Isolation of a gram negative *P. aeruginosa* from herbal materials raises deep health concerns. Edaphic factors are the probable source of the isolate as the bacteria is primarily a soil bacterium, reflecting poor harvesting and cleaning of herbal materials. *Salmonella species* detected in some samples are causative agents of various infections like *salmonella* food poisoning which is a major problem in the world (Greenwood *et al*., 1997). The presence of the fungal contaminant shows the possibility of poor storage conditions. This is a serious contaminant since some common species of fungi produce toxins like aflatoxins. According to the WHO (Greenwood *et al*., 1997), aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts. The limits of microbial contamination are total aerobic bacteria 105 CFU/g, Yeast and Mould are 103 CFU/g (WHO 2000). However, none of the herbal suspensions exceeded the recommended total aerobic counts. The absence of contaminants may be due to hygienic packing or presence of bactericidal or bacteriostatic substance that would have killed possible microbial contaminants. Investigation of possible antimicrobial adulterants in the herbal suspensions is suggested. The isolation of the pathogens from herbal products in other parts of the world has been reported. Microbial load of some medicinal plants sold in local markets of Benin, Nigeria reported presence of *P.* *aeruginosa* and *B. subtilis* among others (Idu *et al*., 2011). In Kaduna Nigeria, studies indicated presence of pathogenic*.* . *Salmonella typhi* in 65.7% of herbal products analysed and *E. coli* in 58.7% of the samples analysed (Abbha *et al*., 2009). Evaluation of microbial quality of plant materials in Belgrade indicated the presence of *E. coli, Bacillus* and *Clostridia* species (Stevic *et al*., 2012). When evaluation of microbial and fungal contaminations of herbal products that was carried out in Ghana (Ahene *et al*., 2011), aflatoxins, nitrobacteria and *P. aeruginosa* among others were found to be present. In South Africa, studies have shown that herbal products are heavily contaminated with bacteria (Adeleye *et al*., 2005).Also in a research conducted in Bangladesh to assess the pathogenic proliferation in the locally available commercial herbal medicine , the pathogenic load was compared with the microbiological standard given by the British pharmacopoeia Out of 85 oral liquid samples, 2 were found to be highly contaminated with a total aerobic bacteria and fungi and some of the sample showed coliform but none of the sample was contaminated *salmonella spp* and *shigella spp* but with bacteria and fungi in some of the sample, and this suggest the fact that aseptic handling is necessary during processing of oral herbal medicines. The increasing widespread use of traditional medicine has prompted the W.H.O to promote the integration of traditional medicine and complimentary or alternative medicine into the national healthcare system of some countries and to encourage the development of national policy and regulation as essential indicators of the level of integration of such medicine within a national healthcare system (WHO 2011)The growing number of national traditional medicine research institutes in developing countries is also a sign of the growing importance of traditional medicine. Notable examples are found in Nigeria, china, India, Bangladesh, Madagascar and Vietnam (WHO 2000).

**2.2 HISTORY OF HERBAL MEDICINES**

The history of herbal medicine probably began with our most ancient ancestor hunter gatherers, who would have learned that eating certain herbs resulted in a palliative effect. Most likely this began with a marshmallow plant which has the effect of calming stomach upsets. Today’s herbal medicine is constantly evolving science and incorporates information we have over the centuries from herbal practitioners around the globe. The history of herbal medicine began with earliest man who was first written in the herbal record which was 2800 BC which was the pent’sao by shennong (also known a divine farmer). In 40 B.C, Hippocrates wrote the first herbal medicine record in Greek in 100 B.C the first illustrated herbal record was produced in Greece in 50 B.C; the Roman Empire began spreading information about herbal remedies throughout the empire as well as the plant used for various remedies. In 200 A.D. Galen, a herbal practitioner developed a classification system for remedies and illness. In 800 A.D, Monks helped spread knowledge about herbal medicine through their infirmaries at each monastery. During the 1100s Avicema a physician and Parisian scholar, wrote the canon of medicine in the 1800’s.The national association of medicinal herbalist was founded to help promote and defend the practice of herbal medicine. In 1941, the pharmacies and medicine act was passed, stripping herbal practitioners of the right to dispense medicinal herbs. In 1968, the medicine act was passed and restores practitioner’s right to dispense medicinal herbs. The British herbal medicine association was also founded and published the British herbal pharmacopoeia.

In 2000, the British government decreed that herbal medicine should undergo the same testing as convectional drugs. These herbal medicines would then be licensed

**2.3 SOME HERBAL MEDICINES IN USE IN NIGERIA AND THEIR APPLICATIONS.**

Few herbal remedies have conclusively demonstrated any positive effect on humans, possibly due to inadequate testing (Ernst 2007). Many of the studies cited refer to animal model investigations or in-vitro assays and therefore cannot provide more than weak supportive evidence. However, examples of medicinal plants in use in other parts of the world and in Nigeria in particular that have demonstrable some interesting pharmacological results include: *Aloe Vera*. It is traditionally used for the healing of burns and wounds (Maenthaisong *et al*., 2007). A systematic review (from 1999) states that the efficacy of aloe Vera in promoting wound healing is unclear, while a later review (from 2007) concludes that the cumulative evidence supports the use of aloe Vera for the healing of first to second degree burns (Ernst 2007; Vogler and Ernst, 1999). Boophone **(***Boophone disticha***)** this highly toxic plant has been used in South African traditional medicine for treatment of mental illness (Stafford *et al*., 2008). Research demonstrated in vitro and in vivo effect against depression (Pedersen *et al*., 2008; Sandager *et al*., 2005; Neergaard *et al*., 2009). Alligator pepper, *Aframomism melegueta.* K. Schum (Zingiberaceae) Local names: Yoruba- oburo ata. Ata ire, Urhobo- Erhie, Hausa - chitta, gyan’dammar yaji. The fruits, seeds, leaves are used as stimulant, and as remedy against cold. Calendula (*Calendula officinalis*) is used traditionally for abdominal cramps and constipation (Gordon 1998). In animal research an aqueous-ethanol extract of *Calendula officenalis* flowers was shown to have both spasmolyticand spasmogenic effects, thus providinga scientific rationale for this traditional use(Bashir *et al*., 2006).Goat weed (*Ageratum conyzoides* L(Compositae) Local name: Yoruba – ime-esu,imi-ewure, Ibo- akwukwo-nwa osi nake,Urhobo- Ikpamak. The whole plant leaves andseeds are used in herbal formula. The juicefrom fresh plant is used for dressing wounds,ulcers, and craw craw and as a remedy for inflammation.A decoction of the root is a remedyfor abdominal pains and the raw root ischewed for digestive disorders.Garlic (*Allium sativum*) L.Liliaceae, Hausa-Tafarnwa, The bulbs and leaves parts isused in ethno medicine. It has diuretic propertiesand is given in fevers, coughs, flatulence,disorders of the nervous system. It has beenused as a remedy for asthma and hoarsenessof the chest. The bulb juice is used as a broadspectrum antibiotic against fungi and bacteria.It may also lower total cholesterol levels(Ackerman *et al*., 2001).Echinacea (*Echinacea angustifolia, Echinacea pallida*, *Echinacea purpurea*) extractsis used for the treatment of rhinoviruscolds (Shah *et al*., 2007).Feverfew (*Chrysanthemum parthenium*) issometimes used to treat migraine headaches(Shrivastava  *et al*., 2007). Although many reviewsof Feverfew studies show no or unclearefficacy, a more recent RTC showed favorableresults (Silberstein 2005). Feverfew is notrecommended for pregnant women as it maybe dangerous to the fetus (Yao *et al*., 2006;Modi and Lowder 2006).Gawo (*Faidherbia albida*), a traditional herbalmedicine in West Africa, has shown promisein experimental animal tests (Tijani *et al*.,2008). German Chamomile (*Matricaria chamomilla*)has demonstrated antispasmodic, anxiolytic,anti-inflammatory and some anti mutagenicand cholesterol-lowering effects in animalresearch (McKay *et al.,* 2006). In vitrochamomile has demonstrated moderate antimicrobialand antioxidant properties and significantant platelet activity, as well as preliminaryresults against cancer. Essential oil ofchamomile was shown to be a promising antiviralagent against herpes simplex virus type 2HSV-2) in vitro (Koch *et al*., 2008). Ginger (*Zingiber officinale*), administered in 250 mg capsules for four days, and effectively decreased nausea and vomiting of pregnancy in a human clinical trial used for colds, toothaches, asthma, rheumatism, piles and headaches. The ripe fruit is given as laxative. Seeds boiled with milk are believed to be powerful abortificient and remedy for diabetes. Grapefruit (*Naringenin*) components may prevent obesity. Green tea (*Camelia sinensis*) components may inhibit growth of breast cancer cells and may heal scars faster (Belguise *et al.*, 2007; Zhang *et al*., 2006). Honey may reduce cholesterol and wound healing (Al Walili 2004). Lemon grass (*Cymbopogon citratus*), Local name: Isoko- eghu. When administered daily, the aqueous extract of the fresh leaf, has lowered total cholesterol and fasting plasma glucose levels in rats, as well as increasing HDL cholesterol levels. Lemon grass administration had no effect on triglyceride levels (Adeneye and Agbaje 2007). *Morinda citrifolia* (*noni*) is used in the Pacific and Caribbean islands for the treatment of inflammation and pain (Pande *et al.,* 2005). Human studies indicate potential cancer preventive effects (Wang *et al.,* 2009). Black cumin (*Nigella sativa)* has demonstrated analgesic properties in mice. The mechanism for this effect, however, is unclear. In vitro studies support antibacterial, antifungal, anticancer, anti-inflammatory and immune modulating effects (Hajhashemi *et al*., 2004). Pawpaw (*Carica papaya* L Caricaceae) local name: Hausa- gwanda, Ibo- okwulu ezi, Yoruba- ibepe, sigun, gbegbere is used as insecticide, use for wound dressing (Regnault *et* *al*., 2004). Peppermint oil is used in Nigerian ethno medicine as remedy against irritable bowel syndrome (Capello *et al*., 2007). Pomegranate contains the highest percentage of ellagitannins of any commonly consumed juice. Punicalagin, an ellagitannin unique to pomegranate, is the highest molecular weight polyphenol known. Ellagitannins are metabolized into urolithins by gut flora, and have been shown to inhibit cancer cell growth in mice (Heber 2008). *Rauvolfia serpentina*, high risk of toxicity if improperly used extensively for sleeplessness, anxiety and high blood pressure and has been widely used in Nigeria in the management of psychiatric problems.

Rose hips – Small scale studies indicate that hips from Rosa canina may provide benefits in

The treatment of osteoarthritis. *Saw Palmetto* can be used for (high blood pressure) BPH. The fat soluble extract of this berry has become a leading natural treatment for BPH. This extract when used regularly, has been shown to help keep symptoms of BPH in check (Schneider *et al.,* 1995). Shiitake mushrooms (*Lentinus edodes*) are edible mushrooms that have been reported to have health benefits, including cancer preventing properties (Fang *et al*., 2006). In laboratory research a shiitake extract has inhibited the growth of tumor cells through induction of apoptosis. Both a water extract and fresh juice of shiitake have demonstrated antimicrobial activity against pathogenic bacteria and fungi (Hearst *et al*., 2009). St. John's wort has yielded positive results, proving more effective than a placebo for the treatment of mild to moderate depression in some clinical trials (Kuznetsov *et al*., 2005). A subsequent, large, controlled trial, however, found St. John's wort to be no better than a placebo in treating depression (Gaster and Holroyd 2000). However, more recent trials have shown positive results (Davidson or positive trends that failed significance. A 2004 meta-analysis concluded that the positive results can be explained by publication bias but later analyses have been more favorable. The Cochrane Database cautions that the data on St. John's wort for depression are conflicting and ambiguous. Stinging nettle in some clinical studies effective for benign prostatic hyperplasia and the pain associated with osteoarthritis. In-vitro tests show anti inflammatory action. In a rodent model, stinging nettle reduced LDL cholesterol and total cholesterol. In another rodent study it reduced platelet aggregation. Umckaloabo (*Pelargonium sidoides*): an extract of this plant showed efficacy in the treatment of acute bronchitis in a controlled trial and is approved for this use in Germany. Willow bark (*Salix alba*) can be used for a variety of anti-inflammatory and antimicrobial purposes due to presence of salicylic acid and tannins. Has been in use for approximately. 6000 yrs and was described in the 1st century AD by Dioscorides (Mahdi *et al.*, 2006). Cam wood BaphianilidaLatal (Papilionaceae) local name: Yoruba-owiwi, irosun, Hausa- majiga, Urhobo- orhua. In urhobo land a paste of its leaves is applied to the lower portion of the abdomen of pregnant women to prevent miscarriage. Bitter leaf **(***Vernonia amygdalina***,** L (Compositae) local name: Yoruba- ewuro, Hausa-shiwaka, Urhobo- olugbo. A decoction of the leaves is used for stomach pains. Also used for skin infections, as an antipyretic, laxative and anti diabetic. Ginkgo (*Ginkgo biloba*) has been used in traditional medicine to treat circulatory disorders and enhance memory. Although not all studies agree, ginkgo may be especially effective in treating dementia (including Alzheimer's disease) and intermittent claudication (poor circulation in the legs). It also shows promise for enhancing memory in older adults. Laboratory studies have shown that ginkgo improves blood circulation by dilating blood vessels and reducing the stickiness of blood platelets. By the same token, this means ginkgo may also increase the effect of some blood-thinning medications, including aspirin. Kava kava (*Piper methysticum*) is said to elevate mood, well-being, and contentment, and produce a feeling of relaxation. Several studies have found that kava may be useful in the treatment of anxiety, insomnia, and related nervous disorders. However, there is serious concern that kava may cause liver damage. It's not clear whether the kava itself caused liver damage in a few people or whether it was taking kava in combination with other drugs or herbs. It's also not clear whether kava is dangerous at previously recommended doses, or only at higher doses. Some countries have taken kava off the market. It remains available in the United States, but the Food and Drug Administration (FDA) issued a consumer advisory in March of 2002 regarding the "rare" but potential risk of liver failure associated with kava-containing products.

**2.4 W.H.O APPROVAL OF HERBAL MEDINCES**

The world health organization (WHO) noted that inapproiate use of traditional medicines or practice can have negative of dangerous effects and advised that further researcher are needed to ascertain the efficacy and safety of several medicinal plants and practice used in traditional medicine system. In order to meet the growing needs and challenges which have arisen due to widespread use of traditional medicine, world organization has developed some strategies to tackle them, these strategies are:

* Increasing availability and affordable of traditional medicines.
* Integrating relevant traditional medicine with national health care system by developing and implementing national medicine policies and programmes.
* Promoting the safety, efficacy if traditional medicine therapy as providing guidance and regulating and quality assurance standards.
* Promoting sound of traditional medicine by providers and consumers.
* Documentation of traditional medicine and remedies.

**2.5 COMMON MICROBIAL CONTAMINANTS ASSOCIATED WITH MEDICINAL PLANTS USED IN PRODUCING HERBAL MEDICINE**

From studies conducted it is an evidence that herbal medicine without control or regulations can be contaminated with microorganism which are potential pathogens hence pose a threat to patient (Gansanjo 2011).Herbal preparations in the developing countries are produced through unhygienic conditions. Many contaminants and residues that may cause harm have been reported. Many are natural such as naturally occurring radioxides, toxic metals, bacteria and fungi. The growing, harvesting and manipulation methods usually applied cannot avoid microbial contamination of the plant material which therefore reflects the environmental conditions as well as the specific hygiene during the diverse treatments (kneifel *et al.*, 2002). Biological contamination refers to impurities in medicinal herbs, their preparations and products, and may involve living microbes such as bacteria and their spores, yeasts and moulds, viruses, protozoa, insects (Their eggs and larvae), and other organisms. However, products of microbial metabolism such as toxins, low-molecular-weight metabolites from moulds are important chemical contaminants (kosalec *et al*., 2009). The main microbial contaminations of plant materials used to prepare herbal medicine in general are attributed to total aerobic mesophilic *Enterobacteria*, yeasts and moulds (kneifel *et al.*, 2002). The presence of higher numbers of spores’ of bacteria could be explained by the fact that some of these organisms (e.g. *Bacillus* and *Clostridium* sp.) produce spores which are resistant to harsh processing, elevated heat and dry conditions. Therefore, they can survive for a long time on the product in a dormant state*. Bacillus cereus* and *Clostridium perfringens* are recognized as having potential pathogenicity and have been incriminated in food poisoning (Kunene *et al*., 1999). Although bacterial endospores and fungal spores can be regarded as the two dominating groups of contaminants associated with medicinal plants, a broad diversity of bacterial, fungal cells and viruses can be found either in or on the plant material (kneifel *et al.*, 2002). *B. cereus* and *C. perfringens* were isolated from chamomile and other herbs (Martins *et al*., 2001). Although *Enterobacteria* can be found in nature, this family possesses some indicative value towards faecal contamination. The presence of *Enterobacteria* and *Escherichia coli* reflect the situation regarding faecal contamination (Ezeh *et al*., 2001). Together with the group of coliform, it can be taken as an indicator for undesirable hygiene conditions, although this conclusion has to be related to the magnitude of viable count measured (kneifel *et al.*, 2002). *Staphylococcus aureus* is not common contaminant of this type of plant material and relatively rarely found. However, contamination could provide amount of enterotoxin produced by *S. aureus*, depending on the specific nature of the individual (Kosalec *et al*., 2009). Herbal medications are likely to be contaminated with a wide variety of others potentially pathogenic bacteria. In a study which was evaluated, the bacterial contamination of powdered herbal medicinal preparations sourced from identified herbal retail outlets in different parts of Kaduna, Nigeria, the results showed that a number of herbal remedies were contaminated with *Salmonella typhi* and *Shigella spp*., besides *E. coli* and *S. aureus* (Abba *et al*., 2009). In addition, the presence of pathogenic bacteria likes *bacillus cereus*, *Aeromonas hydrophila*, *Shigella* sp., *Enterobacter agglomerans*, *E. cloacae*, *Vibrio fluvialis*, *Pasteurella multocida*, *S. epidermidis, Acinetobacter iwoffii,* *Klebsiella* sp*, B. subtilis* ,*Pseudomonas aeruginosa,* and *fungi Rhizopus stolonifer* alsowere observed to be present in plant samples analyzed recently (Alwakeel 2008; Idu *et al*., 2011).Because they are widespread in the atmosphere, moulds are common natural contaminantsof medicinal herbs. It is known that, under favourable conditions, some fungi can synthesizetoxic metabolites – mycotoxins. Among the known mycotoxins, the most toxic one is aflatoxinsynthesised by species of *A. flavus* and *A. parasiticus*, and a minor number of other fungi(Kulshrestha *et al*., 2011). Contamination by *A. flavus*, the most famous aflatoxin producer, is common inmedicinal plant and herbal tea (Halt, 1998). *Aspergillus flavus* colonization does not necessarily reduce yield,but causes economic losses by contaminating with aflatoxin (Amaike *et al*., 2011). In a study of 91 medicinalherb samples in Brazil (Bugno *et al*., 2005). It was found that 50 % of aerial part samples were contaminatedwith fungi. Samples of medicinal plants were evaluated (Bugno *et al*., 2006), for the fungal contamination,and results indicated that predominant mycoflora (89.9% of the isolates) corresponded togenera *Aspergillus* and *Penicillium*, which are extremely important from the mycotoxicologicalstandpoint. The fungal contamination of powdered herbal medicinal preparationssourced from some herbal retail outlets in some parts of Nigeria was evaluated (Anyanwu, 2010) andthe results showed that all of the herbal preparations had the presence of fungal contaminantswith predominance of *Aspergillus* sp. and *Penicillium* sp*,* but *Mucor* sp., *Candida* sp*, Trichosporium* sp., also were found. The fungal deterioration adversely affects theChemical composition of the raw materials and thereby decreases the medicinal potency ofherbal drugs (Kumar *et al*., 2009).The risk of the presence of microorganisms in a plant product depends on this finality of theuse, its nature and its potential damage that may be caused to the consumers. Consideringnatural flora, current production conditions and the need to warrant the quality and thesafety of these products, monographs establish a maximum fungal contamination limit forproducts that contain raw material of natural origin (Bugno *et al*., 2006). Although high fungal loads may beaccepted due to the natural origin of those products, they indicate the potential for spoilageand mycotoxigenesis. Further studies are recommended for herbal products to establish other contaminants and ways in which the contaminants can be reduced to recommended levels. The microbial loads should be established and the contaminants isolated and identified.

**2.6 DECONTAMINATION OF PLANT MATERIALS USED IN HERBAL MEDICINE.**

Attempts have always been made to decontaminate and preserve these medicinal plants so as to get more safe, natural and potent medicines. The number of methods has been tried for decontamination such as heat treatment, UV irradiation and fumigation. However, volatility and heat sensitivity of the delicate flavour and aroma components of the medicinal plants do not permit the use of heat treatment (Gupta *et al*., 2011). Low penetration power of UV radiations makes this irradiation method unsuitable (Gupta *et al*., 2011). Fumigation with gaseous ethylene oxide brings down the microbial burden but this method is now prohibited or restricted in many countries due to the carcinogenic nature of one of its residue in treated medicinal plants (Kim *et al*., 2000; Satomi *et al*., 2005). Various disinfectant technologies have been suggested which include electromagnetic radiations, photodynamic pulsing, ultra high pressure and carbon-dioxides (CO2) treatment (Gupta *et al*., 2011). Gamma irradiation is now getting recognition throughout the world as a phytosanitary treatment of herbal materials. It improves the hygienic quality of various herbal materials and reduces the losses due to microbial contamination and insect damage (Farkas, 1998). Besides, it is a fast, safe, convenient, eco-friendly method which reduces the reliance on chemical fumigants and preservatives currently used by industries. The chances of recontamination are also reduced, as it can be done after packaging (Khattak *et al*., 2009). Some studies showed that the exposition of plant samples to different doses of gamma radiation can result in reduction in total bacterial counts and also indicated that the microbial load could be decreased by increasing the radiation-absorbed dose. These studies indicate that gamma irradiation is an effective treatment for microbial decontamination of medicinal plants (Gupta *et al*., 2011; Khattak 2012; Aquino *et al*., 2010). Certain plants contain natural barriers and antimicrobial substances which exert typical inhibitory effects on microbial growth and stability. It has been estimated that around 1400 herbs and spices many possess antimicrobial agents of different chemical nature as oils, peptides, liquid and organic extracts (Kneifel *et al*., 2002). Some medicinal herbs contain essential oils which act as natural antimicrobials and may inhibit mould development and mycotoxin production (Kosalec *et al*., 2009). Different studies have demonstrated the effectiveness of antimicrobials and their effective compounds to control or inhibit the growth of pathogenic and spoilage microorganisms (Negi 2012; Wang *et al*., 2009).

**2.7 MORE ON HERBAL MEDICINES**

Herbal medicine is practiced today in countries around the world. Each government determines the extent to which practitioners in Europe for example are practiced, if a medical consultation is given the herb which was considered drugs. Herbs are purchased over the countries, and they are considered herbal supplement in the United States which herbalist cannot prescribe drugs legally unless they also happen to be medical doctors, herbs can only be sold as herbal supplement, and these supplement can be found in stores locally and online. The FDA (Federal drug agency) does not approve most of these herbs because herbs they are not considered medication. Currently, there is an ongoing dispute between certified medical professionals and herbalists. Some people believe that herbal medicine is outdated and those who practice it, should be shutdown other people believe that perhaps may be healthier than synthetic drugs. In either case, there are people trying to meet the two camps in the middle, the doctors of naturopathy. Hopefully there will be a meeting of the treatment program of patients who don’t respond well to traditional medical procedures after all; many drugs used by respected members of the medical community are created from plants. From the time being, companies selling herbal supplements are experiencing a boom in sales. People are trying to live healthier lives and eliminating chemical from their diet the only question is whether or not herbalists will finally be treated as respected members of their community or forced out of business by conventional medical practioners (Charlotte, 2009). Modern medical science certainly comes with a high price tag, and pharmaceutically are no exception. One reason why herbal medicine is becoming more popular recently is because people simply can’t afford to pay for their medication month after month after month. A systematic review published in evidence based complementary and alternative medicine evaluated whether or not natural health product provides a cost effective choice in the treatment of disease. Researcher found that natural health products show evidence of cost effectiveness in other areas of modern medicine, but the preliminary data suggest that herbal products are more affordable than pharmaceuticals. Herbal products, such as herbal extracts essential oils and herbal teas are available in most health food and even grocery stores, so you don’t have to see doctors to get prescriptions before purchasing them. This certainly makes it easier to obtain herbal product and avoid additional health care costs. Herbs are classified as dietary supplement so they can be produced, sold and marketed without going through the FDA although this make it easier to purchase and use these beneficial products. It is our job as consumers to choose among the competitors make sure to read the ingredient and labels carefully before using a reputable and trustworthy company hat verifies the product is 100% pure grade. Herbs are used for the treatment of chronic and acute condition and various ailments, including major health concern like cardiovascular disease, prostrate problems, depression, inflammation and weakened immune system herbs are used around the world to treat conditions and disease, and many studies prove their efficacy in effect of the 177drugs approved worldwide for the treatment of cancer, more than 70 % are based on natural product or chemical limitation of natural product. There are numerous advantages and advantages and herbal medicine, anyone considering using herbal medicine to treat health conditions should speak with qualified health professional. Most herbal medicine are well tolerated by patient, with fewer unintended consequences than pharmaceutical drugs herbs typically have fewer side effect than traditional medicine, and may be safer to use over time. Herbal medicine tends to be more effective for long standing health complaints that don’t respond well to traditional medicine. one example is the herbs and alternative remedies used to treat arthritis (vioxx, a well-known prescription drug used to treat arthritis was recalled due to increased risk of cardio vascular complications alternative treatment for arthritis, on the other hand, have few side effects such treatment include dietary changes like adding simple herbs eliminating vegetables from the nightshade family and reducing white sugar consumption. Another advantage to herbal medicine is cost. Herbs cost much less than prescription medications research, testing, and marketing and considerably to the cost of prescription medicines. Herbs tend to be inexpensive compared to drugs. Yet another advantage of herbal medicine is their availability. Herbs are available without a prescription you can grow some simple herbs such as peppermint and chamomite, at home in some remote part of the world, herbs maybe the only treatment available to the majority of people (Kathleen, 2016) Herbs are not without disadvantages and herbal medicine is not appropriates in all situation there are few of the disadvantage s to consider. Modern medicine treats sudden and serious illness and accidents much more effectively than herbal or alternative treatments. An herbalist would not be able to treat serious trauma, such as a broken legs nor would he be able to heal appendicitis or a heart attack as effectively as a conventional doctor using modern diagnosis tests, surgery and drugs. Another disadvantages of herbal medicine is the very real risk of doing yourself harm through self-dosing with herbs while you can argue that the same thing can happen with medication, such as accidentally overdosing on cold remedies, many herbs do not come with instruction or package inserts. There’s a very real risk of over dosing. Harvesting herbs in the wild is risky, if not foolhardy, yet some people try to identify and pick wild herbs. They run a very real risk of poisoning themselves if they don’t correctly identify the herb, or if they use the wrong part of the plant. Herbal treatment can interact with medication, nearly all herbs come with some warming and such as valerian and st.johnswort can interact with prescription medication like antidepressants. It is important to discuss your medication and herbal supplements with your doctor to avoid dangerous interactions. Because herbal products are not tightly regulated, consumers also run the risk of buying inferior quality herbs the quality of herbal product may vary among batches, brand or manufacturer. This can make it much more difficult to prescribe the proper dose of an herb (Kathleen 2016). Herbal drugs appear relatively safe but there is limited human research or prospective data concern adverse effect and herbal drug interactions they are however less potent than their pure drug equivalent because they contain a mixture of many photochemical in small quantities even so herbal product are to totally free of risk and therefore it is necessary to see them with discretion. Herbal rugs aim to returning the body to a taste of natural balance so that it can star healing itself. (W H O 2000). Herbs need to be screened scientifically to ascertain their safety and efficacy. Traditional medicinal system is associated with the natural derived preparations for the treatment of various diseases. It explores the utilization of herbs, metals and minerals for medicinal purposes (Virupaksha *et al*., 2011). Herbal medications are widely believed to be beneficial, and the popularity and availability of the traditional remedies have generated concerns regarding the safety, efficacy and responsibility of practitioners using traditional remedies, Herbal products can be purchased without a prescription and might not recognize any potential hazards in an inferior product, The herbs are combined with metals that facilitate in assimilation and delivery of the ingredients into the human body (Gasser *et al*., 2009; Sarkar *et al*., 2010),Despite the poor risk-benefit analysis for herbs, it may be reasonable to use certain herbs for patients who have conditions where there are no known effective treatments, or when standard therapies have not been tolerated or have failed to lead to improvements (Chaudhary 2011; Rotblatt *et al*., 2002).

**CHAPTER THREE**

**3.0 MATERIALS AND METHODS**

**3.1 SAMPLE COLLECTION**

A total number of thirty different herbal mixtures were purchased from different part of Enugu metropolis. The samples were stored in the refrigerator at 4oC before the analysis. They were analysed in microbiology laboratory, Godfery Okoye University, thinker’s corners Enugu state.

**TABLE: 1 SAMPLE CODE AND LOCATION FROM WHERE SAMPLES WERE COLLECTED**

|  |  |
| --- | --- |
| SAMPLE CODE | LOCATION |
| A | ABAKPA |
| B | ABAKPA |
| C  | ABAKPA |
| D | ABAKPA |
| E | ABAKPA |
| F | EMENE |
|  G | EMENE |
| H | EMENE |
| I | EMENE |
| J | EMENE |
| K | GARRIKI MARKET |
| L | GARRIKI MARKET |
|  M | GARRIKI MARKET |
| N | GARRIKI MARKET |
| O | GARRIKI MARKET |
| P | OLD PART MARKET |
| Q | OLD PART MARKET |
| R | OLD PART MARKET |
| S | OLD PART MARKET |
| T  | OLD PART MARKET |

**3.2 PREPARATION OF MEDIA**

Media used for the work were nutrient agar, blood agar, macconkey agar, and saboriod dextrose agar for the isolation of bacteria and fungi respectively, all media used were prepared according to the manufacturer’s instructions.

**3.3 INOCULATION OF THE MEDIA FOR BACTERIAL ISOLATION**

The herbal mixtures were mixed and inoculated into culture plate, with the help of wire loop. Also 2ml of the samples were added to 5ml of peptone water. They were all incubated at 37oC for 24hours, (overnight). The peptone water culture were later subcultured onto the solid nutrient agar media and incubated at 37oC for 24hours

**3.4 INOCULATION OF THE MEDIA FOR FUNGI ISOLATION**

The herbal mixtures were inoculated into tubes containing potatoes dextrose agar with the help of sterile swab sticks and straight wire loop. The tubes were incubated at room temperature for seven (7) days.

**3.5 IDENTIFICATION OF FUNGI ISOLATES**

The growth pattern, pigmentation and size of colonies were recorded at the incubation period to aid identification of the organisms.

**3.5.1 USE SLIDE CULTURE METHOD**

The isolate was identified using cultural characteristics and morphology. With the help of sterile petrisdishes, sterile filter paper was placed in each of the petridishes and 1ml of distilled water was added into the petridish as sterile U shape glass rod was placed in each of the petridish. With the help of inoculating needle, a cube like shape of already prepared SDA was cut, four days to 1 week fungal growth was smeared by the four sides of the SDA using a sterile wire loop and a sterile cover slip was placed on the inoculums in the petridish. The plate was then covered and kept at room temperature for 4 to 7days as growth was observed daily before examination.

**3.5.2 CELL MORPHOLOGY OF FUNGI ISOLATE**

A drop of lactophenol (LP) was placed on a clean microscopic slide The cover slip from the glass culture was gently removed and placed in the drop of lactophenol (LP) and also a drop of LP was dropped on the slide from the old culture as the media cultured on the slide was gently removed a sterile cover slip was placed on the slide and observe microscopically. It was first view at X10 to focus the lens well then X40 to get a clearer view.

**3.6 EXAMINATION OF THE PLATE CULTURES**

After the incubation plate, were read and the colonial morphology of the isolates were described. The tubes for fungal cultures were also were examined for growth and description of the fungal isolates.

**3.7 BIOCHEMICAL TESTS**

Identification test were carried out according to the method of cow and steel. The isolates were first gram stained and various biochemical tests from the key cited by Aneja, K.R from the bergys Manuel of determinative bacteriology the test performed were:

1. Oxidase test
2. Catalase test
3. Citrate utilization test
4. Indole test
5. Coagulase test
6. Motility test
7. Urease test
	* 1. **METHOD OF GRAM STAINING**

Slide was placed with heat fixed smear on staining tray.

Smear was gently flooded with crystal violet and was left for 1minute.

The slide was slightly tilt and gently rinse with tap water or distilled water using a wash bottle.

The smear was gently flooded with gram’s iodine and let stand for 1 minute.

The slide was slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.

It was Decolorized using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5-10 seconds until the alcohol runs almost clear. Be careful not to over- decolorize.

It was immediately rinsed with water.

Gently flood with safranin to counter-stain and let stand for 30 seconds.

The slide was tilt slightly and gently rinse with tap water or distilled water using a wash bottle.

It was Blot-dry the slide with paper.

Viewed the smear using light microscope under oil immersion.

**3.7.2 CATALASE TEST**

Transferred small amount of bacterial colony to a surface of clean dry glass slide using a loop or sterile wood stick.

Placed a drop of 3% H2O2 on to the slide and mix.

Positive: Evolution of oxygen (within 5-10seconds) as evidenced by bubbling.

Negative: No bubbles or a few scattered bubbles.

**3.7.3 COAGULASE**

Placed a drop of normal saline on each end of a slide or on two or on two separate slides.

With the aid of wire loop, emulsify a portion of the isolated colony in each drop to make two thick suspensions.

Added a drop of human or rabbit plasma to one of the suspensions and mix gently.

Looked for clumping of the organisms within 10 seconds.

No plasma is added is to the second suspension to differentiate any granular appearance of the organism from true Coagulase clumping.

**3.7.4 CITRATE TEST**

Inoculated Simmons citrate agar lightly on the slant by touching the top of a needle to a colony that is 18 to 24 hours old.

Incubated at 350c to 370c for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.

Observed the development of blue colour denoting alkalinization.

Positive: colour change (Prussian blue).

Negative: no colour change.

**3.7.5 INDOLE TEST**

Took sterilized test tubes containing 4ml of tryptophan broth.

Inoculated the tube aseptically by taking the growth from 18 to 24 hours culture.

Incubated the tube at 370c for 24 to 28 hours.

Added 0.5 ml of Kovac’s reagent to the broth culture.

Observed for the presence or absence of ring.

Positive: Formation of pink or red colour (cherry-red ring).

Negative: No colour change.

**3.7.6 VOGES PRAUSKEUR TEST**

Inoculated the test organism into the VP medium.

Incubated aerobically at 370c for 24 hours.

Following 24 hour of incubation, aliquot 2ml of the broth to a clean test tube.

Incubated the remaining broth for an additional 24 hours.

Added 6 drops of 5% alpha naphtol and mix well to aerate.

Added 2 drops of 40% KOH, and mix well to aerate.

Positive: Pink-red colouration surface within 30minutes (shake the tube vigorously during the 30 minute period).

Negative: No colour change.

**3.7.7 METHYL-RED TEST**

Inoculated two test tubes containing VP-MR broth with a pure culture of the organism under investigation.

Incubated at 35oc for 4 days.

Added 5 drops of MR indicate solution to the first tube (for VP test Barrit’s reagent to another tube).

Positive: Red colouration.

Negative: No colour change.

**3.7.8 OXIDASE TEST**

Filter papers were divided according to the number of test samples

Sample colonies were collected and smeared on the designated areas on the filter paper

Oxidase reagent was prepared by mixing 0.1grams of the reagent with 10ml of distilled water

The reagent was aseptically introduced to the smears on the filter paper

A change in colour of the smears from their original colour to a deep blue colour was checked for within the 10-15 seconds after the reagent was placed on the samples. Change in smear colour to blue within 10-15 seconds of the test signifies an Oxidase positive microorganism. No colour change signifies Oxidase negative

**3.7.9 UREASE TEST**

Urea hydrolysis broth medium was sterilized and inoculated with a loop-full of the pure isolated samples

The tubes were incubated at 35-37o C for 18-24 hours

Colour changes were observed on all growth medium. If the colour changed from light orange to magenta (pinkish red), the organism is urease positive (produces the enzyme Urease). If no colour change was detected, the sample organism is urease negative.

**3.7.10 MOTILITY TEST**

A semisolid agar medium was prepared (Nutrient agar) and sterilized at 121o C for 15 minutes in a test tube

Using a straight wire, the samples were collected and inoculated into the agar by a single straight stab down to the center of the tube, about half the depth of the medium. With the wire still in place, the tubes were plugged and covered

The media were incubated at 37o C overnight

After incubation, the agar samples were observed for growth in or around the test tube. A spread of growth away from the line of stab indicated motile organisms (Motility positive). Growth only found along the line of stab indicates a motility negative result (Non-motile organisms).

**3.8 PROCEDURE FOR VIABLE BACTERIAL COUNT**

Add 1ml of the herbal mixture with 15mls or molten nutrient agar (of about 50oc)

Mix properly, and pour the plates Allow to solidify in the bench Incubate at 37oc for 24hours

Examine the plates and count the colonies

**CHAPTER FOUR**

**4.0 RESULT**

 Isolation process is a procedure, of separating the mixture of colonies to a single colony. This process was done by using streaking method to obtain pure culture according to the morphological presentation of microorganism on petrisdishes containing nutrient agar, and macconkey agar are circular, irregular, punciform, smooth, raised, the colonies were small and big and creamy in colour, the morphological presentation of microorganism on petrisdishes containing blood agar are circular, irregular, smooth, raised, colony size, big and small and colonies are pink in colour. The morphological presentation of microorganism on agar slant containing potatoes dextrose agar are pigmentation , are optical characteristic , texture of cultures, amount of growth which is slight, moderate and large and it form which are filform (threadlike ) echinulate (pointed out growth)beaded (like bead) effuse (thin spread surface) arboresent (branched tree like growth) rhizoid (root like growth). Gram staining was conducted to identify if these organism are gram positive and negatives, shape are bacillus and cocci and arrangement are chain, single and pairs.

**TABLE: 1 COLONIES MORPHOLOGY OF THE ORGANISM ISOLATED ON NUTRIENT AGAR**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ISOLATE | SHAPE | ELEVATION | SURFACE | SIZE | PIGMENTATION |
| **A** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKISH |
| **B** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKISH |
| **C** | IRREGULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **D** | CIRCULAR | ENTIRE | RAISED | PINPOINT | MILKISH |
| **E** | IRREGULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **F** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKISH |
| **G** | CIRCULAR | ENTIRE | FLAT | PINPOINT | MILKISH |
| **H** | CIRCULAR | ENTIRE | FLAT | PINPOINT | MUCOID MILKY |
| **I** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY/ODOUR |
| **J** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKISH |
| **K** | CIRCULAR | ENTIRE | FLAT | PINPOINT | MILKISH |
| **L** | IRREGULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **M** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKISH |
| **N** | CIRCULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **O** | CIRCULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **P** | CIRCULAR | UNDULATE | FLAT | PINPOINT | MILKISH |
| **Q** | IRREGULAR | ENTIRE | FLAT | SMALL | MILKISH |
| **R** | IRREGULAR | ENTIRE | RAISED | LARGE | MILKISH |
| **S** | IRREGULAR  | ENTIRE | RAISED | LARGE | MILKISH |
| **T** | CIRCULAR | ENTIRE | FLAT | PINPOINT | MILKISH |

**TABLE: 2 COLONIES MORPHOLOGY OF THE ORGANISM ISOLATED ON MACKONKEY AGAR**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ISOLATE | SHAPE | ELEVATION | SURFACE | SIZE | PIGMENTAION |
| **A** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **B** | CIRCULAR | ENTIRE | FLAT | PINPOINT | LACTOSE FERMENTER |
| **C** | CIRCULAR | ENTIRE | FLAT | SMALL | MILKY |
| **D** | CIRCULAR | ENTIRE | FLAT | SMALL | LACTOSE FERMENTER |
| **E** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **F** | CIRCULAR | UNDULATE | FLAT | PINPOINT | LACTOSE FERMENTER |
| **G** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **H** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **I** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **J** | CIRCULAR | ENTIRE | FLAT | PINPOINT | LACTOSE FERMENTER |
| **K** | IRREGULAR | UNDULATE | FLAT | SMALL | LACTOSE FERMENTER |
| **L** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **M** | CIRCULAR | ENTIRE | RAISED | SMALL | LACTOSE FERMENTER |
| **N** | CIRCULAR | ENTIRE | RAISED | SMALL | MILKY |
| **O** | CIRCULAR | FLAT | ENTIRE | SMALL | LACTOSE FERMENTER |
| **P** | IRREGULAR | FLAT | ENTIRE | SMALL | MILKY |
| **Q** | CIRCULAR | RAISED | ENTIRE | SMALL | MILKY |
| **R** | IRREGULAR | CONVEX | ENTIRE | SMALL | LACTOSE FERMENTER |
| **S** | CIRCULAR | ENTIRE | FLAT | PINPOINT | LACTOSE FERMENTER |
| **T** | IRREGULAR | UNDULATE | FLAT | SMALL | LACTOSE FERMENTER |

**TABLE: 3 VIABLE TOTAL COUNT ON NUTRIENT AGAR**

|  |  |
| --- | --- |
| SAMPLES CODE | BACTERIA COLONIE COUNT |
| A | **8** |
|  B | **59** |
| C | **20** |
| D | **42** |
| E | **43** |
| F | **32** |
| G | **28** |
| H | **76** |
| I | **4** |
| J | **3** |
| K | **56** |
| L | **21** |
| M | **40** |
| N | **44** |
| O | **20** |
| P | **58** |
| Q | **4** |
| R | **34** |
| S | **118** |
| T | **30** |

**TABLE: 4 COLONIES MORPHOLOGY OF THE ORGANISM ISOLATED ON POTATOES DEXTROSE AGAR**

|  |  |  |
| --- | --- | --- |
| **Samples** | **Slide culture****Colour** |  **Probable organisms** |
| A | Grey green | *Penicillum* sp |
| B | Black | *Aspergillus* sp |
| C | Blue Green | *Aspergillus* sp |
| D | Black(branched hypea) | *Rhizopus* sp |
| E | Grey green |  *Penicillum* sp |
| F | Black | *Aspergillus* sp |
| G | Black(branched hypea) | *Rhizopus* sp |
| H | Blue Green | *Aspergillus* sp |
| I | Milkish | *Mucor* sp |
| J | Blue Green | *Aspergillus* sp |
| K | Black | *Aspergillus* sp |
| L | Black(branched hypea) | *Rhizopus* sp |
| M | Black | *Aspergillus* sp |
| N | Grey green |  *Penicillum* sp |
| O | Black |  *Aspergillus* sp |
| P | Black |  *Aspergillus* sp |
| Q | Black(branched hypea) |  *Rhizopus* sp |
| R | White |  *Mucor* sp |
| S | Black(branched hypea) |  *Rhizopus* sp |
| T | Blue green |  *Aspergillus* sp |

.

**TABLE: 5 GRAMS STAINING REACTION**

|  |  |  |
| --- | --- | --- |
| **SAMPLES** | **GRAMS REACTIONS** | **ARRANGEMENT** |
| A | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| B | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| C | **GRAM POSITIVE ROD** | **CHAIN** |
| D | **GRAM POSITIVE ROD** | **CHAIN** |
| E | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| F | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| G | **GRAM NEGATIVE ROD** | **CHAIN** |
| H | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| I | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| J | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| K | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| L | **GRAM POSITIVE ROD** | **CLUSTERED** |
| M | **GRAM NEGATIVE ROD** | **CHAIN** |
| N | **GRAM POSITIVE COCCI** | **CHAIN** |
| O | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| P | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| Q | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| R | **GRAM POSITIVE COCCI** | **CLUSTERED** |
| S | **GRAM POSITIVE ROD** | **CHAIN** |
| T | **GRAM POSITIVE COCCI** | **CLUSTERED** |

Table 5 above shows the Gram reaction, of the bacterial isolates.

**TABLE 6: BIOCHEMICAL ANALYSIS**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Samples Isolates**  | **Gram** **Stain** | **Cat** | **Cog** | **Ind** | **VP** | **MR** | **Cit** | **Mot** | **Oxd** | **Probable****organism** |
| **A****B****D****G****T****S****I****J****K****L****Q****S****R**  | **Gram-positive cocci** | **+** | **+** | **\_** | **\_** | **\_** | **\_** | **\_** | **\_** | ***Staphylococcus aureus*** |
| **G**  | **Gram-negative rods** | **\_** | **\_** | **+** | **\_** | **\_** | **\_** | **+** | **\_** | ***Proteus* sp** |
| **M** | **Gram negative rods** | **\_** | **\_** | **+** | **\_** | **+** | **\_** | **+** | **\_** | ***E coli***  |
| **C****O****D****N** | **gram-positive rods** | **+** | **\_** | **\_** | **\_** | **+** | **\_** | **\_** | **\_** | ***Bacillus* sp** |

Table 6 above shows the biochemical reactions of all bacteria isolates used in identifying them. Catalase (Cat), Coagulase (Cog), Indole (Ind), Methyl red test (MR), Voges proskeur test (VP), Citrate utilization test (Cit), Motility test (Mot), Oxidase test (Oxd), and Gram staining were all tests carried out**.**

**CHAPTER FIVE**

**5.0 DISCUSSION**

This study aimed at isolation and identification of microorganisms from some liquid herbal mixtures sold in Enugu metropolis, south east of Nigeria. twenty samples of herbal mixture were selected at random, from herbal shop in Enugu metropolis and were analysed in microbiology laboratory, Godfery Okoye University. And all the herbal medicine were highly contaminated. “The presence of microbial contaminants in non sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patient taking the medicine” This facts that is supported by the article review on “rapid monitoring of microbial contamination “by Nakayima et al., (2005), Okunlola et al., (2007), The possible contaminated organisms were isolated from these product, are *E coli*, *staphylococcus aureus, bacillus* sp *,Aspergillus* sp *proteus* sp *Rhizopus, peniciliin,*. And the organisms isolated are the same with other finding results from other researchers that work on isolating microorganisms from herbal mistures. And this organisms may have occur, maybe a result of inadequate heat processing, improper handling of products and contamination processing equipment and supported by the work of (Frazier and Westhoff, 2003). Biochemical analysis of the products showed that some herbal products had so pathogenic microorganisms, which are very harmful and can bring toxin to the body, like Aspergillus for example cause toxin to the body and it is very harmful, Other finding have shown that apart from possible microbial degradation of the active constituents contained in the herbal preparations, the presence of these contaminating microorganism could constitute source of infection and serious health risk to the consumer of herbal preparation who were initially indicated (Mangram et al., 1999; Bowler et al., 2001).

* 1. **CONCLUSION**

the whole herbal medicine showed significant microbial growth all the herbal medicine bought from Enugu south east, Nigeria were highly contaminated with microorganism. Good manufacturing process have not be applied to their manufacturing of the herbal mixtures, and also raw materials used, for this production may have not been properly sterilize, which might lead to increased in the microbial content, and poor handing in preparing and packaging this herbal product .

**RECOMMENDATIONS**

* Herbal medicine practitioner should be educated on the need o produce a good and sterile herbal product, thereby reducing source of infection and serious health risk to the consumers taking it.
* Moreover, the regulatory agency, NAFDAC, should carry out more detailed and regular analysis on these herbal preparations to prevent the uninformed consumers from buying what may worsen their ailment.
* Validation of equipment cleaning procedures should be practiced in herbal products industries to prevent cross contaminations of drug products
* At every stage of processing, raw material, intermediate or printing material should be free from microbial and other contaminants.

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