IDENTIFICATION AND CHARACTERIZATION OF THE MICROBIAL CONTAMINANTS OF HERBAL MEDICINES IN KENYA

BY

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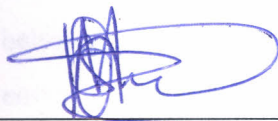
DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY

MARCH, 2011

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for a degree in any university

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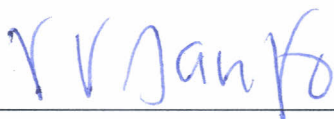This thesis has been presented for examination with our approval as university supervisors

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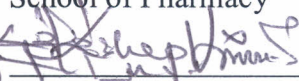
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Dedication

This work is dedicated to my mother Ruth Nyanchama Onyambu who always prayed and encouraged me, my father Paul Onyambu, my brothers and sisters, Wesley, Josphat, Dorcas, Lois and Gladys.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BP	British Pharmacopoeia
CFU	Colony Forming Units
DNA	Deoxy-ribonucleic Acid
EMB	Eosin Methylene Blue
EMEA	European Medicines Agency
GIT	Gastro-Intestinal Tract
GMP	Good Manufacturing Practices
MDRC	Mtishamba Drug Research Centre
MMW	Mortality and Morbidity Weekly
MR	Methyl Red
MSA	Mannitol Salt Agar
NA	Nutrient Agar
PCR	Polymerase Chain Reactions
rRNA	ribosome Ribonucleic Acid
SDA	Sabouraud's Dextrose Agar
STD	Sexually Transmitted Diseases
TAMC	Total Aerobic Microbial Count
TFTC	Too Few To Count
TMPAs	Traditional Medicine Practitioners Associations

TMTC	Too Many To Count
TVAMC	Total Viable Aerobic Microbial count
TYMC	Total yeast and mould count
USFDA	United States Food and Drug Agency
USP	United States Pharmacopoeia
UTI	Urinary Tract Infections
VP	Voges Proskauer
WHO	World Health Organization
XLD	Xylosy Lysine Desoxycholate agar

Abstract

With the ever increasing use of herbal medicines and the global expansion of the herbal medicines market, safety has become a concern for both health authorities and the public in many countries. This is because many contaminants and residues that may cause harm to the consumers have been reported. Many are natural such as naturally occurring radioxides, toxic metals, bacteria and fungi. Some arise from past and present use of agents or materials that pollute the environment and subsequently medicinal plants, such as emissions from factories or the residues of certain pesticides. Microbial contamination of herbal medicines can be influenced by environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the storage conditions of crude and processed medicinal-plant materials. For these reasons, there is currently a global danger to the health and well-being of the people. The World Health Organization, British Pharmacopoeia and the United States Pharmacopoeia have recommended tolerable microbial limits in non-sterile pharmaceutical products which include 10^7 cfu/ml bacteria and 10^5 cfu/ml fungi. These risks can be reduced by ensuring that there is enough surveillance of herbal medicines so that those with harmful contaminants and residues above recommended limits do not reach the public. This study therefore was aimed at identification and characterization of the microbial contaminants in herbal medicines sold in Kenyan herbal clinics, Chemists, supermarkets and streets. The microbial loads were established and the contaminants isolated and identified.

Thirty (30) herbal products collected by purposive random sampling were analyzed for their microbial contaminants. Microbial load analysis was done and interpreted based on the methods recorded by the USP and BP while isolation of pure cultures was done based on morphological differences where form, elevation, pigmentation and size were the major distinguishing factors for both fungal and bacterial contaminants. The pure isolates were then stocked in glycerol and stored in a freezer at -23 °C from where they were used for subsequent identifications. Gram-staining procedure was used to classify bacteria into Gram-negative and Gram-positive while microscopic and macroscopic observations formed the basis for fungal identification. Specific microbes were then characterized using differential and selective media and later confirmed using API 20 E strip following procedures by the manufacturer. Molecular characterization of the microbes that were not identified using the above techniques was done using the QiaAmp

procedures for DNA extraction, agarose gel analysis and cleaning of the PCR products. Sequencing was then done in Segolip (Ilri).

Fourteen (47 %) of the analyzed samples were powders, nine (30 %) liquids, five (16 %) tablets, one balm and capsule each (3 %). Blood purifiers, food supplements and antidiabetics formed the majority of herbal products in the market (50 %) while the rest claimed to manage blood pressure, STD and GIT problems among others. Ten (33 %) of the samples had a colony count of below 30 cfu/ml, these were interpreted as free from contamination. Twenty (67 %) of the samples however had bacterial counts in the range of 6.00×10^5 to 1.56×10^{10} cfu/ ml and fungal count in the range of 5.30×10^4 to 1.56×10^9 cfu/ ml. Of the 19 different types of bacteria identified, 13 (68 %) were Gram-negative rods while 6 (32 %) were Gram-positive rods and cocci.

The tests for specified microorganisms using differential and selective media confirmed the presence of *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Shigella* and *Candida* spp. in abundant growths. Microscopic and macroscopic examinations confirmed the presence of fungi i.e. *Rhizopus*, *Candida*, *Fusarium*, *Aspergillus*, *Torula*, *Penicillium* and *Alternaria* also in abundance. Biochemical tests by API 20 E confirmed the presence of *Enterobacter agglomerurans*, *Serratia marsensens*, *Klebsiella oxytocca*, *Chryseomonus luteola*, *Flavobacterium* spp and *Enterobacter cloacae*. Further genotypic analyzes of the isolates that were not identified and characterized by morphological and biochemical methods revealed the presence of *Kocuria rosea*, *Bacillus pumilus*, *Cronobacter sakazakii*, *Bacillus safensis*, *Bacillus subtilis* and *Bacillus flexus* some of the microbes isolated and characterized are pathogenic especially to immune-compromised individuals.

From this study, it is evident that herbal medicines sold in Kenya without control or regulations are contaminated with microbes which are potential pathogens hence pose a threat to patients. It is therefore recommended that a policy be established to enable regulation of these products. Herbalists should be trained to apply Good Manufacturing Practices, good harvesting practices and the safe handling and storage of herbal medicinal products. Further studies are recommended for herbal products to establish other contaminants and ways in which the contaminants can be reduced to recommended levels.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Background information.

Throughout history, man has utilized the plant kingdom for medicines. From the ancient Chinese and Egyptian papyrus writings, medicinal uses for plants were widely described. Indigenous cultures such as African and Native American used herbs in their healing rituals, while others developed traditional medical systems such as Ayurveda and Traditional Chinese Medicine in which herbal therapies were used. The use of herbal medicines started declining in favor of conventional drugs in the 19th century. This is the time when chemical analysis became available and scientists began to extract and modify active ingredients from plants. Modern medicine which emphasizes evidence-based therapy has also shown that man and plants are biologically inseparable (Griggs, 1981). With the increasing number of untreatable diseases and the various complications associated with conventional medicine, herbal medicines have gained popularity in both developed and developing countries (Adenike *et al.* 2007 and WHO, 2002 c). The World Health Organization (2000) estimates that 70-80% of the world's population use herbal medicine for their primary health needs. In Germany, about 600 - 700 plant-based medicines are available and are prescribed by some 70 % of German physicians. In the last 20 years in the United States, public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in herbal medicine use (Dos Santos-Neto *et al.* 2006, Gratus *et al.* 2009 and Hasan *et al.* 2009).

Echinacea (*Echinaea purpurea* and related species), St. John's wort (*Hypericum perforatum*), ginkgo (*Ginkgo biloba*), garlic (*Allium sativum*), saw palmetto (*Serenoa repens*), ginseng (*Panax ginseng*, or Asian ginseng; and *Panax quinquefolius*, or American ginseng) have been identified as the most widely used herbal medicines in the US (Lovera *et al.* 2007 and Manheimer *et al.* 2009). In addition, goldenseal (*Hydrastis canadensis*), valerian (*Valeriana officinalis*), chamomile (*Matricaria recutita*), feverfew (*Tanacetum parthenium*), ginger (*Zingiber officinale*), evening primrose (*Oenothera biennis*), and milk thistle (*Silybum marianum*) are also used.

In Kenya, *the conventional health care system is expensive and therefore provides for only a few*. The (WHO, 2002-2005) estimates that 70-90 % of Africa's rural population relies on traditional medicines to meet their health needs, thus the implication is that 70 % (approx. 28 million people) in Kenya are outside the national health care system and must rely on traditional forms of healthcare due to its perceived low cost and accessibility. Mwangi *et al*. (2005) further estimates that overall in Kenya, the doctor: patient ratio is 1:7142 but complementary medicine practitioner: patient ratio is 1:987 (urban area) and 1:378 (rural area). This necessitates assurance of sustainable availability, quality and the safety of herbal Medicine to ensure continued access to health care especially for rural communities. With regard to safety, quality and efficacy, potentially hazardous contaminants and residues in herbal medicines have been identified as toxic metals and non-metals, organic pollutants, mycotoxins and endotoxins, agrochemical residues, pesticide residues, solvents and biological contaminants. This has led to the establishment of internationally recognized regulatory guidelines to define the basic criteria for the evaluation of a number of these parameters in herbal medicines (WHO, 1998, 2002b,). WHO has further more published monographs for selected medicinal plants (WHO, 2002a). Microbial load evaluation is a major emphasis in these monographs. Tolerable limits have been set, beyond which the herbal medicines can be considered unsafe as human medicine (WHO, 1998 and 2000).

1.2 Definitions and description of Traditional Herbal Medicines.

According to the World Health Organization, Traditional Medicine can be defined as "the sum total of all knowledge and practices, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing". It highlights the practice, measures, ingredients and procedures that have enabled the African to alleviate his sufferings and cure himself (WHO, 1978). Herbal medicines form a major category of traditional medicines. These medicines derived from plants are also called phytomedicines, herbs and botanical drugs (Dnyaneshwar *et al.,* 2005). Phytomedicines are defined as preparations consisting of complex mixtures of one or more plant materials (Calixto, 2000). Herbs include botanical materials such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes and other plant parts. In addition to herbs, herbal materials include fresh juices,

gums, fixed oils, essential oils, resins and dry powders of herbs (WHO, 2000). Depending on the place (country or locality) herbal materials may be prepared or processed by various local procedures like steaming, roasting or fermentation. They may also be obtained by subjecting herbal drugs to processes such as extraction, distillation, expression, fractionation, purification, concentration or fermentation to give tinctures, extracts, expressed juices and processed exudates (Dnyaneshwar *et al.* 2005). Herbal teas consist exclusively of one or more herbal drugs intended for oral aqueous preparations by means of decoction, infusion or maceration (EMEA 2006). Botanical drugs are therefore herbal materials with supposed therapeutic benefits and intended for use as drugs (USFDA 2000).

1.3 Challenges in the use of herbal medicines.

The widespread and growing use of botanicals has, however, created public health concerns globally in terms of quality, safety and efficacy (Drew *et al.* 1997 and Straus *et al.* 2002). Because they are unregulated, herbal products are often mislabeled and may contain additives and contaminants that are not listed on the label. This has led to adulteration being identified as a challenge in herbal medicine. For example, over 40 drug adulterants not listed on labels were identified in Chinese herbal products. About 24 % of over 2600 traditional Chinese medicines sampled contained a therapeutic adulterant, with over half containing two or more adulterants including anti-inflammatory, analgesic, and diuretic agents. A sample of herbal creams used to manage eczema also contained unlabeled dexamethasone at pharmacologic doses (http://www.medscape.com/viewarticle/409518_5).

The second challenge is drug interaction. Very few cases of herb-herb or herb-drug interactions and their side effects have been reported. In the US, of the 1000 elderly people admitted to a hospital from the emergency department, it was found that 538 patients were exposed to 1087 drug-drug interactions; 30 patients experienced adverse effects as a consequence of these interactions (Doucet *et al.* 1996). A number of herbal products and effects in their interactions have been reported. Plausible cases of herb-drug interactions include: bleeding when warfarin is combined with ginkgo, garlic, dong quai (*Angelica sinensis*), or danshen (*Salvia miltiorrhiza*), (Chan *et al.* 1995, Wojcikowski *et al.* 2007, Cheng To 1999 and Yu *et al.*1997), mild serotonin syndrome in patients who mix St John's wort with serotonin-reuptake inhibitors; decreased bioavailability of digoxin, theophylline, cyclosporin, and phenprocoumon when these drugs are

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...ned with St John's wort (Cott *et al.*1998 and Ernst 1999), induction of mania in depressed ...s who mix antidepressants and *Panax ginseng*; exacerbation of extrapyramidal effects ...neuroleptic drugs and betel nut (*Areca catechu*) (Deahl 1989), increased risk of ...tension when tricyclic antidepressants are combined with yohimbine (*Pausinystalia* ...e); potentiation of oral and topical corticosteroids by liquorice (*Glycyrrhiza glabra*); ...sed blood concentrations of prednisolone when taken with the Chinese herbal product ...chai hu tang, sho-saiko-to" and decreased concentrations of phenytoin when combined ...the Ayurvedic syrup "shankhapushpi". Anthranoid-containing plants (including senna ...a senna] and cascara [*Rhamnus purshiana*]) and soluble fibres (including guar gum and ...um) can decrease the absorption of drugs (Taylor *et al.* 1995, Izzat *et al.* 1998, Izzo and ...2009 and Yang *et al.*2006). Despite the effects of herb-drug interactions, many patients ...alternative treatments do not disclose these to their conventional doctors. This was ...med in a survey conducted in the US where 18.5 % of the surveyed adults reported using ...than one herbal product or high dose vitamin with regular prescriptions while 60% of them ...disclosed to their doctors (Eisenberg *et al.* 1998).

...dition to the above challenges Kenya has no coordinating body for herbal medicine and its ...tioners who can act as a vehicle for growth of the sector and its effective integration into ...conomic and health sectors of the country. Institutions dealing with the various aspects of ...ional medicine and medicinal plants are uncoordinated in their approach and do not place ...ector as a high priority. This has led to influx of quacks or people with poor knowledge of ...actice but taking advantage of the situation. Numerous reports of such cases dominate the ...s with not much being done about it (Business Daily 2009; East African Standard 2009 and ...aily Nation 2006).

...mation acquisition, exchange and dissemination on traditional medicines and medicinal ...has been inadequate or not well documented and not properly packaged to enable ...ve utilization in the fields of conservation, production, safety and efficacy and ...ercialization. This has also contributed to an increasing loss of information from one ...tion to the next as the few remaining practitioners die without sharing the knowledge.

...nservation, the phenomenal international growth in demand for plant based or ...herapeutic treatments has led to the decline of many medicinal plant populations. This has

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been largely due to intensive harvesting that is not scientifically monitored and the use of unsustainable harvesting techniques for plants such as *Prunus africana*. Overexploitation and habitat loss are on the increase due to increasing human population and poverty. The financial compensation for the harvester is also too little and exploitative.

1.4 Herbal medicine practice and its regulation in Kenya

Estimates indicate that more than 1200 species of medicinal plants are used in Kenya from a flora of approximately 10,000 members (Kokwaro 1976). In a report (WHO 2005), Kenya was in a list of countries who were developing a national policy on herbal medicines. Currently though, there is no regulation, no national pharmacopoeia, no monographs, no manufacturing requirements and still no control mechanisms exists to ensure the implementation of any safety parameter of herbal medicines. The Pharmacy and Poisons Board responsible for the registration of all medicines in Kenya does not include herbal medicines in the essential drug list. In the year 2008, the Board started listing herbal medicines (Appendix 8) but still there are no clear post-market surveillance mechanisms. Herbal medicines are therefore sold without restriction.

The Ministry of Culture and Social Services regulates the practice of herbal medicine. Certificates of recognition are issued to herbalists by the Ministry's Department of Culture. In an effort to oversee the regulation of the practice, The National Traditional Health Practitioners Association (NATHEPA) was established by the Department of Culture. This Institutional Framework was endowed with the responsibility of spearheading the registration of Traditional Medicine Practitioners Associations (TMPA) for the effective regulation of traditional medicine in Kenya. It was also mandated to study the various Provincial Traditional Medicine Practitioners constitutions and originate a draft National TMPA constitution. Currently, the association lacks adequate capacity to regulate traditional medical practitioners and set standards for practice. Its leadership attributes this to lack of resources and personnel to sensitize its members and implement its work plan.

The Mitishamba Drug Research Centre (University of Nairobi) carries out phytochemical, pharmacological, toxicological and microbiological studies of traditional herbal remedies for human and veterinary use. The facility also offers consulting services and reports on traditional medicines and other complementary medicines, formulation and presentation of herbal remedies,

information on essential oils, their formulations and uses, consultation and dispensing of selected evidence-based herbal medicines. Herbalists who come to the Mitishamba Drug Research Center are issued with a certificate of recognition which enables them to register with the ministry. The center therefore documents information given by herbalist on details of the plants they use including the name (local names and botanical), part used, medicinal claim, preparation, dosage and administration details. For purposes of intellectual property rights, this information is kept confidential in their files. Issues about toxicity can be followed if the plant used is scientifically known to be harmful and they are advised about it. Table 1 shows the number of herbalists who visited the facility from the year 2002 to 2009 and their provincial locations. The number of herbalists who are officially recognized (Table 1) does not clearly reflect the true image on the ground. This is because there is an alarming increase in the number of herbal products sold in the streets, supermarkets and herbal clinics and the people who claim to be herbalists. But due to lack of a clear policy and guidelines as earlier noted, there are no mechanisms in place to follow up and arrest or prosecute those who are in the practice illegally. The need for a policy in herbal medicines is therefore long overdue. This will address the gaps discussed and will provide a sound basis for defining the role of herbal Medicine in national healthcare delivery and also ensure that the necessary regulatory and legal mechanisms are created to promote and maintain good practices in the industry.

Table 1. Herbalists who visited the Mitishamba Drug Research Centre between 2002 and 2009

Province	2002	2003	2004	2005	2006	2007	2008	2009	Total
Nairobi	12	8	6	7	17	12	24	24	110
Rift Valley	10	5	21	16	11	10	13	8	94
Central	6	23	19	10	16	11	10	12	107
Nyanza	0	2	6	1	5	0	5	3	22
Western	1	6	4	1	1	0	3	0	16
Eastern	4	1	1	4	2	6	2	1	21
Coast	0	1	1	1	2	1	1	0	7
North Eastern	0	0	0	1	0	0	0	0	1

Source: MDRC

1.5 Microbiological diversity and their importance

Microorganisms include bacteria, fungi (yeasts and moulds) protozoa, viruses and microscopic algae. Majority of microorganisms make great contributions to the wellbeing of man by helping to maintain balance of living organisms and chemicals in our environment. Marine and freshwater microbes form the basis of food chains in oceans, lakes and rivers. Soil microbes help in the breakdown of wastes and incorporation of nitrogen gas from the air into the organic compounds, recycling of chemical elements in the land and air. Bacteria and algae also play an important role in photosynthesis. Other uses include yeast which is used in bread, alcoholic beverages and cheese making, antibiotics and genetic engineering. Bacteria produce natural pesticides. Though most microorganisms cause no damage and form an important part of human ecology or normal flora, their presence in large numbers has been associated with disease to immune-compromised and weak individuals (Warren and Levinson 2004). Table 2 shows the normal flora and their anatomic locations. They can also be nonpathogenic in their usual

anatomic locations but turn pathogenic in other parts of the body. It is important to note that most of the infectious diseases on earth are caused by microorganisms. These diseases along with famine and wars have been ranked as the main challenges facing mankind and his progress on earth (Morens *et al*. 2004)

Table 2. Anatomic locations of Normal Flora

Member of normal flora	Anatomic location
Bacteroides spp.	Colon, throat, vagina
Candida albicans	Mouth, colon, vagina
Clostridium species	Colon
Corynebacterium species (diphtheroides)	Nasopharynx, skin, vagina
Enterococcus faecalis	Colon
Escherichia coli and other Coliforms	Colon, vagina, outer urethra
Gardnerella vaginalis	Vagina
Haemophillus spp.	Nasopharynx, conjunctiva
Lactobacillus spp.	Mouth, colon, vagina
Propionibacterium acnes	Skin
Neisseria spp.	Mouth, nasopharynx
Pseudomonas aeruginosa	Colon, skin
Staphylococcus aureas	Nose, skin
Staphylococcus epidermidis	Skin, nose, mouth, vagina, urethra
Viridans streptococci	Mouth, nasopharynx

Table from Warren and Levinson 2004

1.5.1 Microbial contaminants of herbal medicines

Medicinal plants have been associated with a broad range of microbial contaminants, mainly bacteria and fungi (Dnyaneshwar *et al*. 2005). The diversity of these microorganisms can be found either in or on the plant material. Occurrence of pathogens among microorganisms limits the use of these plants (Wolfang 2002). The pathogenic microbes render the plant material harmful either by the production of toxic substances or by transforming the chemicals in the plant into harmful substances. The common pathogenic bacteria on herbs are *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Staphylococcus aureus. Aspergillus spp.* Is a common fungal contaminant (Dnyaneshwar *et al*. 2005). The WHO and the British Pharmacopoeia have issued guidance for assessing the microbial load of herbal medicines and other pharmaceuticals (BP, 2007 and WHO, 1998). The guidelines give specific limits for each microbial contaminant.

1.5.2 Factors influencing microbial contamination

Microbial contamination of herbal medicines can be influenced by environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the storage conditions of crude and processed medicinal-plant materials (Dnyaneshwar *et al*. 2005). Further variation in microbial risk may occur due to the different stages of production. Processing factors also largely determine the microbial quality of the final product. For example, the application of hot water extraction in herbal teas and infusions significantly reduces microbial contamination because boiling water decreases the viable microbial count and also kills some pathogens. However, herbal medicines subjected to coldwater extraction like herbal maceration may host a large number of microbes and the extraction temperature provides better conditions for microbe multiplication (Esimone *et al*. 2007)

1.5.3 Previous work on microbial contamination

Studies have been conducted to determine the types of fungi and their toxins contaminating medicinal plants (Hitokoto, 1978). These include species of the genera: *Aspergillus, Fusarium, Penicillium, Mucor, Rhizopus, Cladosporium* and *Aureobasidium*. Bacteria of the genera *Bacillus, Micrococcus, Corynebacterium, Proteus, Escherichia, Streptococcus, Staphylococcus, Klebsiella, Listeria, Actinobactor, Citrobactor, Lactobacillus* and *Serratia* were isolated from herbal medicines in Nigeria (Adenike *et al.* 2007). Fungi of the genera *Torulopsis, Aspergillus, Penicillium, Candida, Hansenula, Saccharomyces, Actinomadura* and *Trichosporon* were isolated in the same study (Esimone *et al.* 2007). It was established that though most of the isolated microorganisms are not considered pathogenic they were present in large quantities. This study along with others has raised questions about the efficacy and safety of herbal medicinal products.

1.5.4 Principles of microbial identification

1.5.4.1 Staining

Microorganisms in their living state are very difficult to visualize. This is because they are not only minute but are also transparent and colorless when suspended in an aqueous medium. For studies, division into specific groups and diagnostic purposes, biological staining and light microscopy are major tools in microbial identification (Foles *et al.* 1994). A stain or dye can be defined chemically as an organic compound containing a benzene ring, a chromophore and auxochrome group (Cappuccino and Sherman 2004). In acidic (anionic) stains the chromogen exhibits a negative charge and therefore has a strong affinity for the positive constituents of the cell. Basic stains mostly used for bacterial staining are cationic, thus on ionization, the chromogen portion which exhibits a positive charge has a strong affinity for the negative constituents of the cell.

Staining techniques are further divided into simple and differential. The latter requires the use of three or more chemical reagents applied sequentially to a heat fixed smear. The primary stain which is the first reagent imparts its color to all cells while the second decolorizes though it may or may not remove the primary stain based on the chemical composition of the cellular components. The final reagent called the counterstain has a contrasting color to that of the

primary stain. The gram stain is the most important type of differential stain in bacteriology which divides bacterial cells into two major groups based on their cell difference (Preston and Morrel 1962). Gram positive cells have a thick peptidoglycan layer while gram-negative ones have a much thinner one surrounded by outer lipid containing layers. This is an essential tool for classification and characterization of these microorganisms.

In Gram staining, crystal violet is used as a primary stain and stains all cells purple. Gram's iodine is used as a mordant which is a substance that increases the cell's affinity for a stain. This happens by binding to the primary stain thus forming an insoluble complex. The complex intensifies the stain color and all cells appear purple-black at this point. Ethyl alcohol (95 %) a decolorizer serves as a protein dehydrating agent and as a lipid solvent. In Gram-negative cells, it increases the porosity of the cell wall by dissolving the lipids in the outer layers. This facilitates the release of the unbound crystal violet-iodine complex, leaving the cells colorless unstained. In Gram-positive cells, the much thicker peptidoglycan layer retains the crystal violet-iodine complex as the pores are made smaller due to the dehydrating effect of the alcohol thus cells remaining purple. Safranine, a counter stain lastly stains red those stains that have been decolorized. Gram-negative cells absorb the counter stain while gram-positive cells retain the purple color of the primary stain.

1.5.4.2 Differential and selective media

Selective media are used to isolate specific groups of bacteria by incorporating chemical substances that inhibit the growth of one type of bacteria while permitting the growth of another. Differential media distinguish among morphological and biochemically related groups of microbes by incorporating chemical compounds that on inoculation and incubation produce a characteristic change in the appearance of bacteria growth or the medium surrounding the colonies thus permit differentiation. Differences may also show up as colony size, media color, gas bubble formation and precipitate formation.

Mannitol salt agar (MSA) contains a high concentration of salt, 7.5 % NaCl which is inhibitory to the growth of most bacteria other than staphylococci. It also performs a differential function by having a carbohydrate mannitol which some staphylococci ferment, and phenol red, a pH indicator for detecting acid produced by mannitol fermenting *Staphylococci* which exhibit a

yellow zone surrounding their growth. Those that do not ferment mannitol will not produce color change.

MacConkey agar allows the isolation of Gram-negative bacteria. Its composition of crystal violet inhibits growth of Gram-positive bacteria. It also permits the differentiation of enteric bacteria on the basis of their ability to ferment lactose. Coliform *bacilli* produce acid as a result of lactose fermentation. They exhibit a red coloration on their surface. *Escherichia coli* produce greater quantities of acid thus the medium surrounding its growth also becomes red because of the action of the acid that precipitates the bile salts, followed by absorption of the neutral red.

Eosin- methylene blue agar (Levine) has lactose and the dyes eosin and methylene blue which permit the differentiation between enteric lactose fermentors and non-fermentors as well as identification of colon bacillus and E. *coli*. *Escherichia coli* colonies appear blue black with a metallic green sheen caused by the large amount of acid produced which precipitates the dyes to the growth surface. Other coliform bacteria including *Enterobacter aerogenes* produce thick mucoid, pink colonies. Enteric bacteria that do not ferment lactose produce colorless colonies which appear to take on the purple of the media. The media is also partially inhibitory to gram-positive organisms.

1.5.4.3 Biochemical identification

Biochemical characteristics play a major role for   bacterial identification. They include enzymes (catalase, oxidase, decarboxylase), fermentation of sugars, capacity to digest or metabolize complex polymers and sensitivity to drugs. Others include hydrogen sulphide production, indole test, phenylalanine deaminase test, nitrate reduction and methyl red test. Biochemical identification can be done by Rapid test whereby a biochemical system for the identification of *Enterobacteriacae* and other Gram negative bacteria consisting of plastic strips with 20 µl of dehydrated biochemical substrates is used to detect biochemical characteristics. The biochemical substrates are inoculated with pure cultures and suspended in physiological saline. After 12 hours, the 20 tests are converted to 7-9 digital profile (API 20 E system). The API Biochemical identification system was studied and evaluated by Michael *et al*. (1996) who found out that 94.9 % of the microbes were correctly identified while 13 % were assigned unacceptable or low discrimination profile. They termed the technique user friendly and accurate. The major

Biochemical reactions for identification of the enterobacteriaceae are discussed below and their chemical equations given.

1.5.4.3.1 Voges-Proskauer Reaction

Acetoin and butylene glycol are detected by oxidation to diacetyl at an alkaline pH, and the addition of α-naphthol which forms a red-colored complex with diacetyl. The production of acetoin and butylene glycol by glucose fermentation is an important biochemical property used for the identification of *Klebsiella, Enterobacter,* and *Serratia* spp.
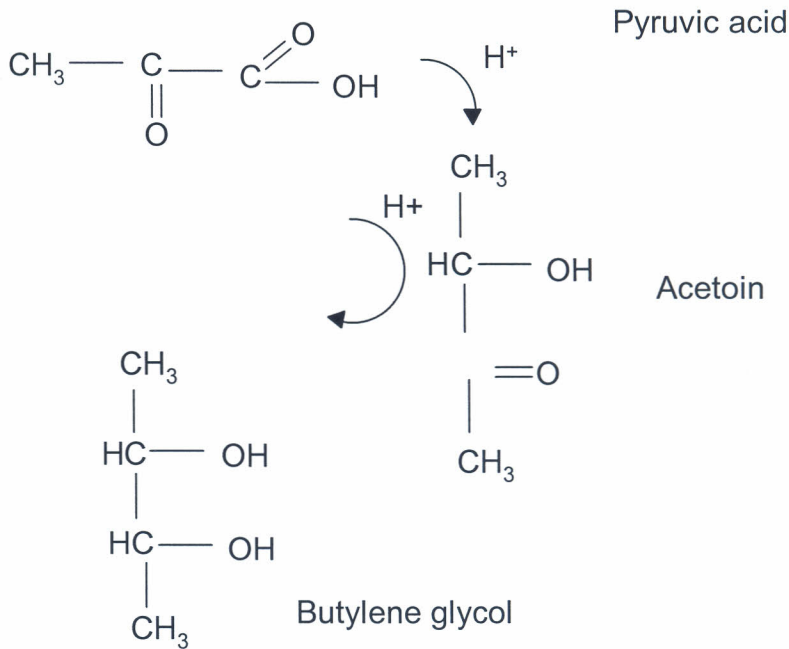
Figure 1. Reduction and condensation of pyruvic acid to acetoin and butylene glycol

1.5.4.3.2 Phenylalanine Deaminase Reactions

Enterobacteriaceae utilize amino acids in a variety of ways including deamination. Phenylalanine is an amino acid that forms the keto acid phenylpyruvic acid when deaminated. Phenylpyruvic acid is detected by addition of ferric chloride that forms an intensely dark olive-green colored complex when binding to phenylpyruvic acid. The deamination of phenylalanine is an important biochemical property of *Proteus, Morganella,* and *Providencia* spp.

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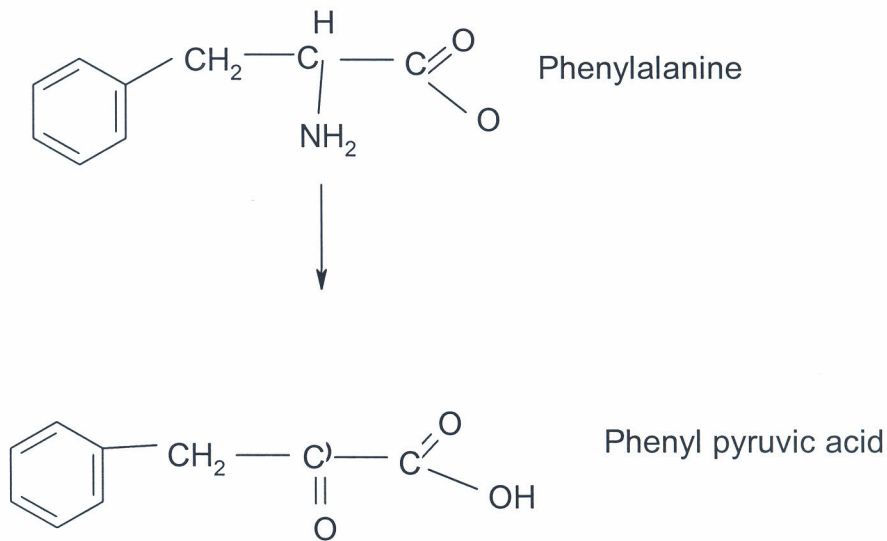
Figure 2. Deamination of Phenylalanine to phenyl pyruvic acid

1.5.4.3.3 Indole Reaction

Enterobacteriaceae that possess tryptophanase can utilize tryptophan by deamination and hydrolytic removal of the indole side chain. Free indole is detected by *p*-dimethylamino-benzaldehyde, whose aldehyde group reacts with indole forming a red-colored complex. Production of indole from tryptophan is an important biochemical property of *Escherichia coli*, Shigella, *Edwardsiella tarda*, *Klebsiella oxytoca*, and *Proteus vulgaris*.
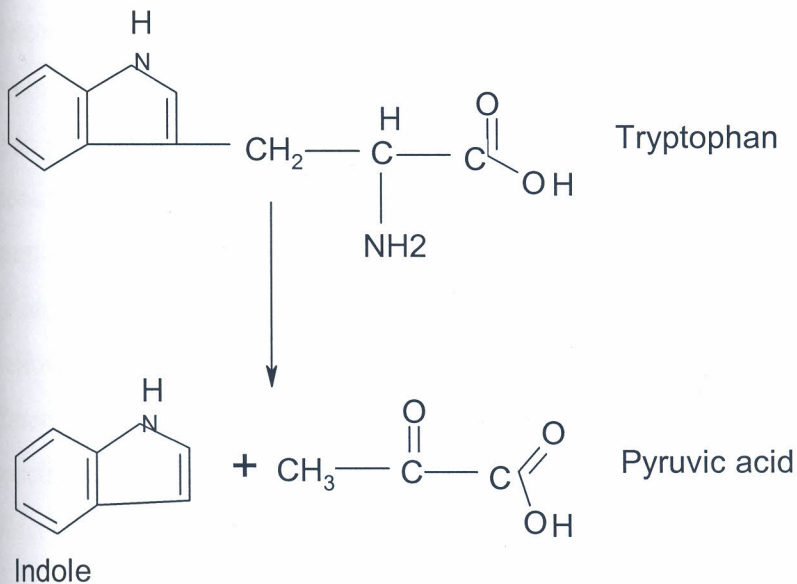
Figure 3. Removal of indole side chain from tryptophan

1.5.4.3.4 Citrate Utilization

Citrate is utilized by several of the *Enterobacteriaceae* as a single carbon source. To test this ability bacteria are incubated in medium that contains only citrate as a source of carbon. Ammonium phosphate is available as a nitrogen source. *Enterobacteriaceae* that can utilize citrate will extract nitrogen from ammonium phosphate releasing ammonia. Ammonia produces an alkaline pH shift, and the indicator bromthymol blue turns blue from its green color at neutral pH. Citrate utilization is a key biochemical property of *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Serratia* species.

H
|
H C— C OOH
|
C— C OOH
|
H C— C OOH $(NH_4)_3 PO_4$
H NH_3
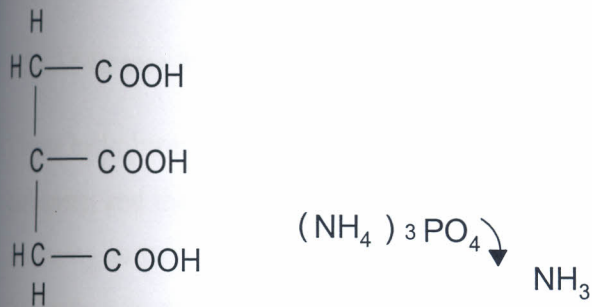
Figure 4. Release of Ammonia by nitrogen extraction in presence of citrate

1.5.4.3.5 Amino Acid Decarboxylation

Enterobacteriaceae contain decarboxylases with substrate specificity for amino acids, and are detected using Moeller decarboxylase broth overlayed with mineral oil for anaerobiosis. Moeller broth contains glucose for fermentation, peptone and beef extract, an amino acid, pyridoxal, and the pH indicator bromcresol purple. If an *Enterobacteriaceae* contains amino acid decarboxylase, amines produced by decarboxylase action cause an alkaline pH, and bromcresol purple turns purple. Lysine, ornithine, and arginine are utilized. A base broth without amino acid is included in which glucose fermentation acidifies the broth, turning the bromcresol purple yellow. Decarboxylation patterns are essential for the genus identification of *Klebsiella*, *Enterobacter*, *Escherichia*, and *Salmonella*. Decarboxylation patterns are also essential for the species identification of *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis*, and *Shigella sonnei.*

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$$NH_2 \text{---} (CH_2)_4 \text{---} \underset{\underset{\displaystyle NH_2}{|}}{\overset{\overset{\displaystyle H}{|}}{C}} \text{---} \overset{\overset{\displaystyle O}{\|}}{C} \text{---} OH \qquad \text{Lysine}$$

Decarboxylation

$$\underset{NH_2}{\overset{NH_2}{>}}(CH_2)_5 \quad + \quad CO_2$$

Cadeverine
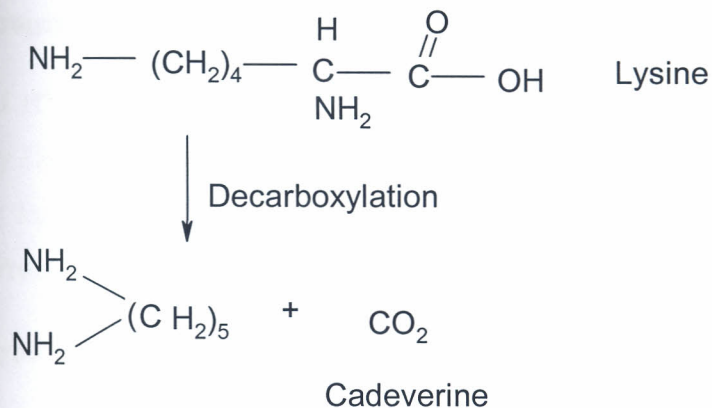
Figure 5. Lysine decarboxylation

1.5.4.3.6 Urease Reaction

Urease hydrolyzes urea releasing ammonia which alkalinizes the medium by forming ammonium carbonate, and the pH indicator phenol red becomes red. *Proteus*, *Morganella*, and *Providencia* are strong urease producers, *Klebsiella* a weak urease producer, and *Yersinia enterocolitica* frequently a urease producer.

Urease reaction

$$\underset{NH2}{\overset{NH2}{>}}C=O \searrow \quad Urea$$

$$CO_2 + NH_3$$

$$\Big\downarrow H_2O$$

$$(NH_4)_2 CO_3$$
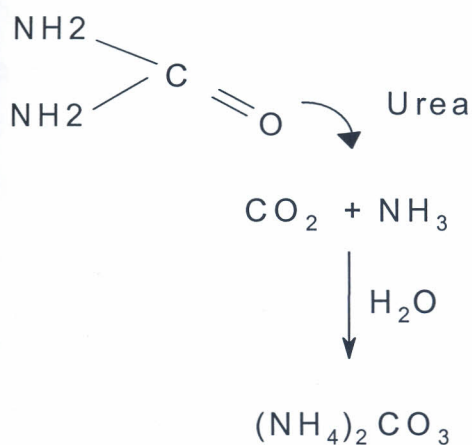
Figure 6. Hydrolysis of Urea to ammonium carbonate

Urease-Producing *Enterobacteriaceae include Proteus Morganella, Providencia rettgeri, Klebsiella pneumonia, Klebsiella oxytoca, Enterobacter cloacae, Yersinia enterocolitica*

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1.5.4.3.7 Hydrogen sulfide (H₂S)

In presence of H^+ and a sulfur source (sodium thiosulfate, sulfur-containing amino acids and proteins) many *Enterobacteriaceae* produce the colorless gas H_2S. For detection of H_2S a heavy-metal (iron or lead) compound is present that reacts with H_2S to form black-colored ferrous sulfide. H_2S-Producing *Enterobacteriaceae Salmonella*, *Edwardsiella*, *Citrobacter*, *Proteus*

1.5.4.4 Genotypic and molecular identification

This method involves examining the genetic material of the organisms. The methods have revolutionized bacterial identification and classification because of their speed and accuracy. They include polymerase chain reactions (PCR), nucleic acid probes and plasmid fingerprinting. In PCR, sequence specific primers are used in the amplification of DNA or RNA of specific pathogens. The presence of the appropriate amplified PCR product confirms the presence of the organisms. The determination of a small amount of DNA sequence can be used for microbial identification. The most common sequence used for microbe identification is DNA sequence of the 16S rRNA gene (Drancourt *et al*. 2000). Polymerase Chain Reactions are used to amplify the 16S rRNA gene and the sequence determined. Ribosomal RNA is a major component for ribosome and ribosomes have the same function in protein synthesis in all cells. Information obtained from the sequences is analyzed and compared with other known sequences in databases using Basic Local Alignment Search Tool, BLAST (Andrew, 2000). Computer analysis of 16S rRNA sequence has revealed the presence of signature sequences, short oligonucleotides unique to certain groups of organisms and useful in their identification.

1.5.4.4.1 Polymerase Chain Reactions (PCR)

The polymerase chain reaction, or PCR, is a powerful method for obtaining millions of duplicates of a DNA fragment from (theoretically) just one original copy. The reaction begins with a solution containing the DNA from a cell. The goal is to amplify a specific region, the target DNA. The solution contains Taq polymerase. *Taq* is a type of heat-stable DNA polymerase derived from a species of bacteria living in hot springs. Because *Taq* polymerase continues to function normally at high temperatures, using it allows researchers to separate the DNA strands without destroying the polymerase. The solution also contains primers which are short, single-stranded DNA molecules and deoxynucleoside triphosphates, or dNTPs. The PCR

cycle, which is typically, repeated about 35 times. Temperature changes are automated by PCR machines. In the first step, denaturation, the solution is first heated to nearly boiling at 95 °C. The heat breaks the hydrogen bonds between the two DNA strands and allows them to separate. Despite the intense heat, the *Taq* polymerase remains active. In the second step, primer annealing, the temperature is dropped to around 60 °C. The exact temperature depends on the length and base composition of the primers. At this relatively low temperature, the primers can form hydrogen bonds with the single-stranded DNA. Two primer types are created, each one complementary in sequence to one of the two ends of the target DNA. To make the primers, the sequences at the ends of the target DNA must be known. In the third and final step, extension, the temperature is increased to 72 °C. This is the optimal temperature at which *Taq* polymerase functions. The primers are essential in this process, because they provide free 3' hydroxyl groups, to which the polymerase can add additional dNTPs. Each new dNTP that joins the growing strand is complementary to the nucleotide in the opposite strand.

At the end of this first cycle, there are two DNA copies instead of the one original copy. In cycle 2 the process repeats, so that there are now four double-stranded copies. The process continues, doubling the number of target DNA copies with each cycle. Doubling occurs because each newly synthesized segment of DNA serves as a template in the subsequent cycle. More and more of the fragments consist of just the target DNA.
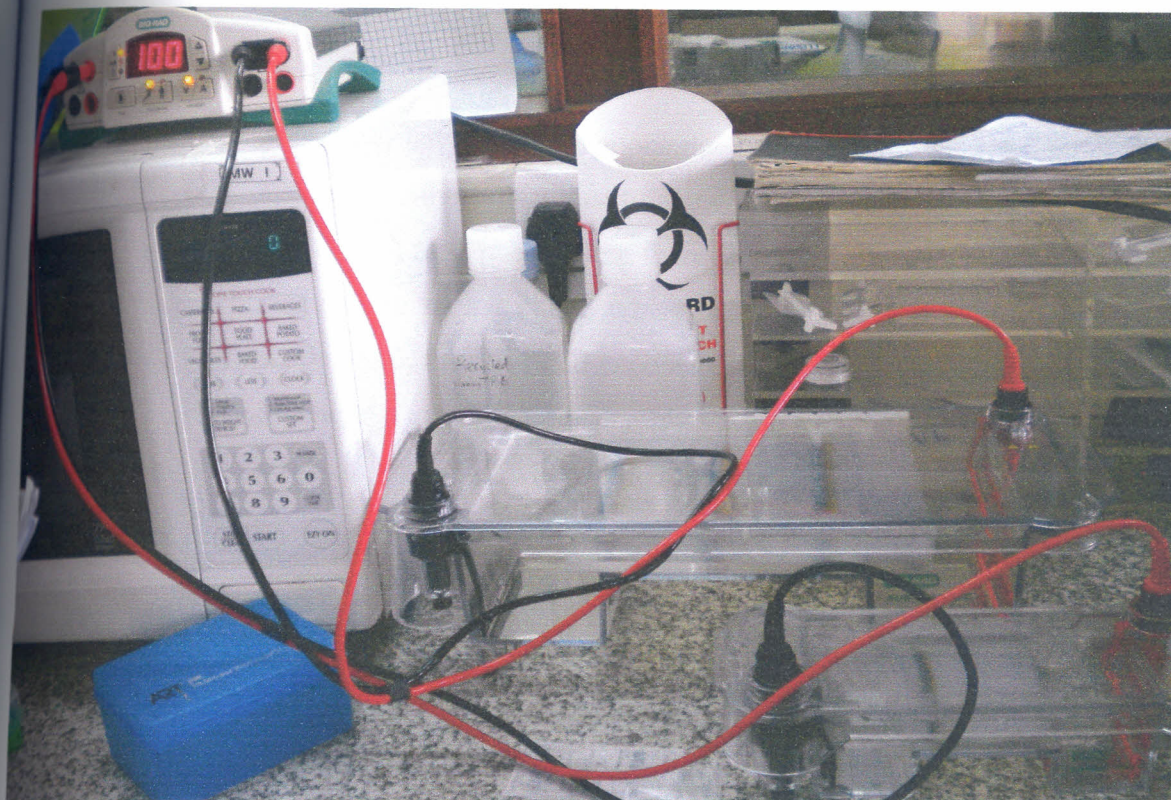
Figure 7. Agarose gel Electrophoresis set up. A demonstration of DNA migration during electrophoresis at 100 mAMP.

The DNA is negatively charged and therefore migrates towards the positively charged (red) anode (Figure 7). Generally gels are run at approximately 80 - 100 mAmp. A setting any higher will cause the buffer to heat up to the point that it begins to melt the gel. Tracking dyes present in the loading buffer migrate along with the DNA, with a separation that leaves one migrating behind, and one migrating ahead of the sample. Bromophenol blue and xylene cyanol dyes used and in this set up migrated at similar rates to DNA fragments and were able to help in monitoring the migration distance.

1.5.5 Microbial growth conditions and phases

Cappuccino and Sherman (2004) have documented that enteric bacteria are capable of survival at a broad range of pH which is characteristic of their environment. The specific pH range for bacteria is between 4 and 9 with the optimum being 6.5 to 7.5. Fungi, moulds and yeasts prefer acidic environments with optimum activities at a pH of 4 to 6. Adjustment of the pH for medium is frequently done to about 7.0. This is advantageous to the growth of microorganisms. The use

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of buffers like buffered peptone water in sample preparation retards shifts in pH which can be detrimental to the growth of microorganisms. The optimal temperatures range from 30-37 °C for bacteria and 20-25 °C for fungi.

Cappuccino and Sherman (2004) have further demonstrated 4 phases in which bacterial growth is divided (Figure 8). In lag phase, the cells adjust to a new environment with rapid biosynthesis of enzymes in preparation for the next phase. Here there is no cell division thus no increase in numbers but cells increase in size. In log phase, where bacterial stocking is recommended and attainable at an absorbance of 0.06, there is rapid exponential increase in population. On average, it last for 6 to 12 h (overnight). At the stationery phase, the population is maintained at its maximum level for a period of time. Growth slows down and the number of microbial deaths balances the number of new cells hence population stabilizes. The metabolic activities of individual surviving cells also slow down. Finally in the decline or death phase, the microbes die at a rapid and uniform rate because of the depletion of nutrients and build up of metabolic wastes.
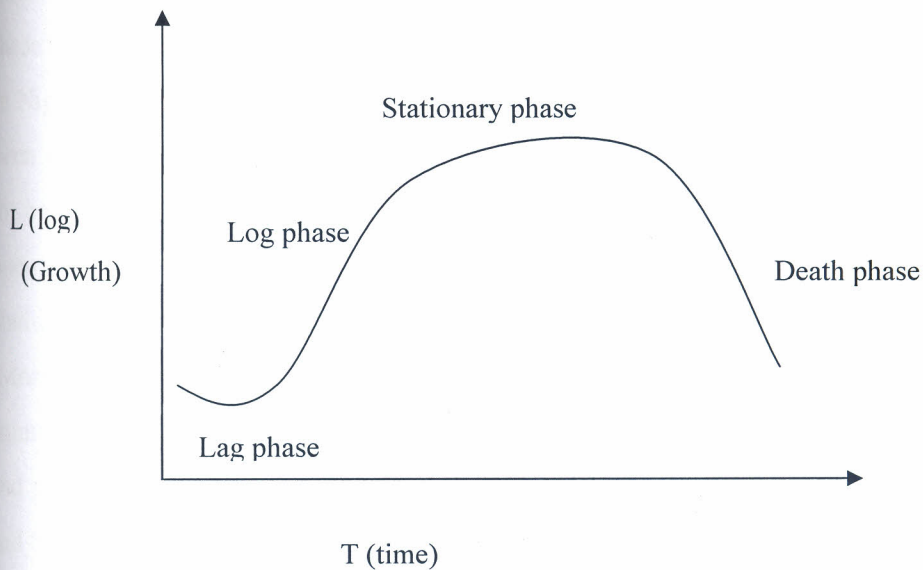
Figure 8. Microbial growth phases

1.6 Study justification

Herbal medicines have become a topic of increasing global importance having both economic and health implications. Individuals with serious illnesses such as HIV/AIDS often seek this form of healthcare besides the conventional one. A study in the US reported that 22 % of patients attending an HIV clinic used medicinal herbs as a form of treatment (Klassher *et al*. 1991). A similar study with AIDS patients indicated that 26 % used herbal medications, while 37 % used dietary supplements in addition to antiretroviral therapy (Duggan *et al*. 2001). Individuals use herbal medicines and supplements to support their immune system, treat or prevent specific opportunistic infections and also relief side effects from conventional antiviral medications (Greger *et al*. 2001, Zhang 1995 and Hanna 1998). Reports regarding side effects, sale of adulterated products and misleading therapeutic claims are on the rise (Marcus *et al*. 2002). The presence of raw animal tissues as ingredients in a number of dietary supplements and their potential risks was reported by Norton (2000). Other evaluations have indicated either the herb was missing from a supplement or found in very small amounts (Cupp 1999). Potential harmful interactions between herbs and drugs have also been reported (Drew 1997). Lack of regulation for herbal products and supplements therefore poses another danger which is contamination by microorganisms. It is however surprising that only a few studies have been conducted to asses these threats. Both fungal and bacterial contaminants have been detected in commercial products in Nigeria (Halt 1998 and Adenike *et al*. 2007). Evidence of human contamination of herbs has been documented where the source of a hepatitis E infection in a Japanese man was determined to be a Chinese herbal medicine (Ishikawa *et al*. 1995). Though many countries have documented studies on microbial contamination of herbal medicinal products, Kenya has no such studies despite cases of contamination and aflatoxin poisoning being reported in foods (Morbidity and Mortality Weekly 2004). India, China, Australia and Britain among other countries have already taken steps in quality control of herbal medicines by establishing policies and publishing herbal pharmacopoeias (Benzi *et al*. 1997, Briggs, 2002, GMP for Indians, 2002 and Starling, 2003). A study to evaluate samples of commercialized herbal medicinal products in the Kenyan herbal clinics, supermarkets and streets for bacterial and fungal contaminants is therefore important to provide the scientific data needed to instill quality control measures. There are no policies for herbal medicines in Kenya. Such medicines in Kenya are sold without restriction or regulation. The investigation of the microbiological quality of herbal medicines in

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Kenya is overdue. Such investigations will help contain the dangers associated with herbal medicines and restore the confidence of those Kenyans who resort to these products for their health care. Data from such investigations can also be the basis for the formation and implementation of herbal medicine policy in Kenya and can also be used by regulatory bodies like the Pharmacy and Poisons Board.

1.7 Objectives of the study

1.7.1 General objective

To identify and characterize microbial contaminants of herbal medicines in Kenya

1.7.2 Specific objectives

1. To determine the level of bacterial and fungal load of the herbal preparations.
2. To isolate and identify the contaminants based on their morphological and biochemical characteristics.
3. To characterize the bacterial contaminants by genotypic identification

CHAPTER TWO: METHODOLOGY

2.1 Sampling design

Samples for this study were collected by purposive random sampling technique. Five provinces were identified and selected for sampling based on the number of herbalists recorded to be practicing in the provinces and accessibility of the researcher to these areas. These were Nairobi, Central, Rift valley, Nyanza and Western which have the largest number of herbal practitioners in the order they appear. Twenty samples obtained from herbal clinics, supermarkets and street vendors were sampled in the first phase of this study with more samples being drawn from Nairobi City. A further sampling of products listed in the Pharmacy and Poisons Board was done. A list of 54 herbal medicines manufactured and marketed by different pharmaceutical companies in Kenya was obtained from the Pharmacy and Poisons Board (appendix 8). Ten samples from the list were randomly obtained from Pharmacy outlets in Nairobi based on the products that were widely advertised and stocked in the chemists. In total 30 samples from both liquid and powder formulations were subjected to analysis for this study

2.2 Equipment used for the study

A weighing balance, Mettler Toledo XP504 (Mettler Toledo inc. Polaris Parkway Colombus, USA) was used for weighing media and samples in grams; it was calibrated to 3 d.p. A Spectrophotometer, spectronic 20D+ colorimeter (Cole-Parmer Instrument Company, Vernon hills, Illinois USA) was used to measure absorbance of the cultures in broth. Micropipettes along with sterile tips, Pipetman (Gilson inc. Middletown, USA) were used for accurate measurement of samples to be inoculated or cultured from 200-1000ul. A refrigerator, LG (Digitalmax Co. ltd Uiwang-city Kyeonggi-Do, South Korea) was used for storage of culture stocks and isolates up to -20 °C. A steam autoclave, All American 25 X-2 (Alfa medica Co. Hempstead New York, USA) was used for sterilizing media, plates and test tubes at 15 lbs 121 °C for 15 minutes and decontamination at same temperature and pressure for 30 minutes. Incubator WTB Binder (Wolf Laboratories Ltd. Pocklington U.K) was used for incubation of bacteria for growth at 33-37 °C and fungal cultures at 23-25 °C. A light microscope, Leica BME 13395 H2X (SEO enterprises inc. Lakeland Florida, USA) was used for examining stained microorganisms in slides and 1.5 ml Eppendorf tubes were used for stocking pure isolates awaiting identification. A nanodrop

2000 C spectrophotometer (Thermo scientific Inc. Wilmington, USA) was used to measure to concentration of DNA before sequencing while a Gene Amp PCR system Thermal Cycler (Applied Biosystems, California, USA) was used to amplify the bacteria DNA. A centrifuge (Biofuge Pico, Osterode Germany) was used to centrifuge during DNA extraction. A shaking water bath (Amerex Instruments Inc. Lafayette, CA.USA) was used for the incubation of cultures that needed vigorous shaking. Isolation, inoculation and transfer of cultures and DNA sample was done in a Biosafety cabinet (The Baker Company, Sanford USA) to avoid cross contamination which might give false results.

Standard Good Laboratory Practice for microbiology was observed e.g. protective clothing which included laboratory coats designated for sterility room, cap, gloves, and dust mask. The working area was disinfected before and after each procedure by wiping with a piece of cotton wool soaked in 70 % alcohol. Hands were thoroughly washed with soap and disinfectant before leaving the microbiology laboratory. Movement from the sterility room was restricted until the procedures were over.

2.3 Media preparation

The specific media supplied in powder form (Himedia Laboratories Pvt, India) was weighed, prepared and reconstituted in distilled water. Each plate was estimated to carry 25 ml of media. Nutrient agar (digest of animal tissue 5.00, beef extract 1.50, yeast extract 1.50, sodium chloride 5.00, agar 15.00 g/l) and final pH (at 25 °C) 7.4 ± 0.2 was prepared by suspending 28.0 g in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely then sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Nutrient broth (with the same ingredients except agar) was prepared by suspending 13.0 g in 1000 ml distilled water and heated to dissolve the medium completely then sterilized as above.

Sabouraud Dextrose Agar (SDA) (mycological peptone 10.00, dextrose 40.00, agar 15.00 g/l) and final pH at 25 °C 5.6 ±0.2, was prepared by suspending 65.0 g in 1000 ml distilled water. Heat to boil to dissolve medium completely then sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. Sabouraud Dextrose Broth (SDB) (all the ingredients as SDA except Agar) was prepared by suspending 30 g in 1000 ml distilled water, then heating to dissolve completely and finally sterilized as above. Buffered peptone water (proteose peptone 10.00, sodium chloride

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5.00, disodium phosphate, and anhydrous 1.50 g/l) and final pH 7.2 was prepared by suspending 20 g in 1000 ml distilled water. Then dispensing 10 ml aliquots to test tubes and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. MacConkey Agar (peptic digest of animal tissue 1.50, casein enzymatic hydrolase 1.50, pancreatic digest of gelatin 17.00, lactose, 10.00, bile salts 1.50, sodium chloride 5.00, crystal violet 0.001, neutral red 0.03, agar   15.00 g/l) and the final pH 7.1 it was then prepared by suspending 51.5 g in1000 ml distilled water. Heat to boiling with gentle swirling to dissolve the agar completely then sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min, finally cooled to 50 °C and poured into sterile Petri-plates. The surface of the medium should be dry when inoculated.

EMB Agar Levine (peptic digest of animal tissue 10.00, dipotassium phosphate 2.00, lactose 10.00, eosin 0.40, methylne blue 0.065, agar 15.00 g/l) and final pH 7.1. It was prepared by suspending 37.5 g in 1000 ml distilled water then heat to boiling to dissolve the medium completely and finally sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. Overheating was avoided. Cooled to 50 °C and shook the medium in order to oxidize the methylene blue (restore the blue color) before dispensing to sterile petridishes.

Salmonella Shigella Agar (SSA) peptic digest of animal tissue 5.00, beef extract 5.00, lactose 10.00, bile salts mixture 8.50, Sodium citrate 10.00, Sodium thiosulphate 8.50, Ferric citrate 1.00, Brilliant green 0.00033, Neutral red 0.025, agar 15.00 g/l) and final pH 7.0 , then prepared by suspending 63.0 g in 1000 ml distilled water. It was heat to boiling with frequent agitation to dissolve the medium completely but was not autoclaved. Overheating may destroy the selectivity of the medium, it was then cooled to about 50 °C, mixed well and poured into sterile petridishes.

Xylose-Lysine Deoxycholate Agar (XLD) agar used for selective isolation and enumeration of *Salmonella* serotype *typhi* and *Salmonella* species with ingredients (yeast extract 3.00, L-lysine 5.00, lactose 7.50,   sucrose 7.50, xylose 3.50, sodium chloride 5.00, sodium deoxychocolate 2.50, sodium thiosulphate 6.80, ferric ammonium citrate 0.80, phenol red 0.08, agar 15.00 g/l) final pH 7.4, it was   suspended in 56.68 g in 1000 ml distilled water then heat with frequent agitation until the medium boils because autoclaving and overheating is not recommended for this media, it was   transferred immediately to a water bath at 50 °C   then after cooling poured into sterile Petri-dishes.

Mannitol Salt Agar is used for selective isolation of pathogenic *Staphylococcus aureas* and has proteose peptone 10.00, beef extract 1.00, sodium chloride 75.00, D. mannitol 10.00, phenol red 0.025, agar 15.00 g/l, final pH 7.4 prepared by suspending 111.0 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely then sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

All the above media were tested for viability using *Bacillus pumilis*, NC08241, *Staphylococcus aureus*, NC07447, *Pseudomonas aeruginosa*, NC12924, *Saccharomyces cerevisiae*, NC010716, *Candida albicans*, NC10400, *Aspergillus niger*, NCPF3179. These are standard microorganisms supplied in the laboratory. All the media supported growth as per the requirement and were therefore recommended for use in this study.

2.4 Sample preparation

All samples were prepared based on their solubility in fats or water. In water soluble samples, 1 g or ml of the herbal product being examined was dissolved in 10 ml buffered peptone water pH 7.0. Further serial tenfold dilutions were prepared using the same diluents. For fatty products, 1 g or 1 ml of the sample was homogenized with 0.5 ml of polysorbate 20 and heated to 40 °C in a water bath then 10 ml of buffered peptone water was added. This was mixed carefully while maintaining the above temperature for 15 min. Further tenfold serial dilutions were prepared using the same diluents.

2.4.1 Sample examination

All samples were analyzed using plate-count method specifically surface-spread or spread-plate technique. Using Petri-dishes 9 cm in diameter, 25 ml of molten nutrient agar for bacterial cultivations and same volume of sabouraud's dextrose agar for fungal cultivation was poured to each petridish at 45 °C and allowed to solidify. One ml of the sample prepared as section 2.4 was pipetted over the surface of the medium then spread using a sterile bent glass rod. Atleast 2 petridishes were used for each medium and each level of dilution. The plates were then incubated in an inverted position at 33 °C for bacterial media and 23 °C for fungal media. Observation and recording of results was done after 48 hours for bacteria and 72 hours for fungus. A mechanical colony counter was used to count the number of colonies. Dilutions that yielded between 30 and 300 colonies were counted (statistically valid, USP and BP recommendations). Plates with more

than 300 colonies were designated as too many to count (TNTC), while those below 30 were designated as too few to count (TFTC). The number of organisms per ml of original culture was calculated by multiplying the number of colonies counted by the reciprocal of the dilution factor divided by the innoculum which was usually 1ml (Cappuccino and Sherman, 2004). The limits were further interpreted as recommended in the pharmacopeia (BP 2007)

2.4.2 Isolation of pure cultures

Based on morphologic differences, colonies were isolated from their axenic cultures where form, elevation, pigmentation and size were the major distinguishing characteristics. Petri-dishes were divided to quadrants and Sub culturing done by the streak plate technique where an inoculating loop was sterilized by holding it in the flame until it became red hot. It was then allowed to cool for 10 sec. Then holding the plate with the stock culture on the palm of one hand, slightly raised its cover by using the thumb and the little finger, and then carefully touched the surface where growth was exhibited. The loop was then drawn lightly over the hardened surface in one quadrant of the other Petri dish in a straight or zigzag line. The needle was reflamed again before being used to transfer another colony to another quadrant. In the case of culture transfer to broth medium, the loop was shaken slightly to dislodge the organisms.

2.4.3 Glycerol stocking and storage of pure isolates.

Colonies isolated to nutrient broth were incubated in a shaking water bath overnight and the absorbance of broth was read in a spectrophotometer at 600 nm. They were ready for harvesting at absorbance of 0.06. Using a sterile pipette, the isolates in broth were pipetted to 1.5 ml Eppendorf tubes at the ratio of 70:30, glycerol to broth respectively. Each isolate was stocked in duplicate and labeled with the code given to the isolate during isolation which included the 2 letter codes from the sample they were isolated, and a number given in the order of isolation. The isolation date was also labeled and the temperature at which storage was done. All the isolates were stored in a freezer at -23 °C. The isolates at this temperature were then used in the subsequent identifications.

Viability of the isolates was done after every one month to confirm if they were still alive and functional. Fungal and bacterial isolates were streaked to SDA and NA, respectively and incubated under conditions stated. Growth in the agar for each isolate confirmed viability.

2.5. Identification

Colony morphology observations formed a major identifying criterion for fungus. The characteristics observed included; form (circular, irregular, spreading), elevation (flat, slightly raised or markedly raised), pigmentation (red, white, pink, colorless), size (pinpoint, small, medium, large) and texture.

2.5.1 Gram Stain and Microscopy

The Gram stain procedure modified by Preston and Morel (1962) was used in the microscopic identification of bacterial contaminants. The reagents used were; Methylene blue alkaline solution or Loffler's reagent (Fine Chemicals Ltd New Delhi, India), Safranine (biological stain) Rankem limited New Delhi, Malachite green powder. Chemo quip Limited Nairobi, Grams iodine (chemo quip limited Nairobi Kenya) and Ethanol, absolute analytical grade, (ACS Charlie, European Union). All prepared as indicated in reagent preparation section.

A clean microscope slide was labeled with the code of the unknown organism using a marking pencil. One loopful of bacteria culture was applied on the slide using an inoculating loop. The smear was then heat fixed on the slide by passing it over a flame of the Bunsen burner. These procedures were done under sterile conditions to avoid any contamination.

The slide with the bacterial smear was held at the edge using a cloth peg over a staining basin. It was then flooded with crystal violet the primary stain for 1minute. The stain was washed off with tap water before applying Gram's iodine for another one minute. The Gram's iodine was washed off with tap water before adding 95 % ethyl alcohol drop by drop until the alcohol was clear. The smear was counterstained with safranin for 45 sec before it was gently washed off with tap water. The smear was then blot dried before viewing it under a microscope on the oil immersion objective lens.

2.5.2 Test for specific microbes using differential and selective media

2.5.2.1 *Staphylococcus aureus*

A pure culture from Nutrient agar was sub-cultured on a plate of mannitol salt agar freshly prepared as per the manufacturer's details. It was then incubated at 33 °C for 72 h. growth of

white colonies surrounded by yellow zones indicated the presence of *Staphylococcus aureus* and was confirmed by gram staining and other biochemical reactions.

2.5.2.2 *Escherichia coli*

A pure culture from Nutrient agar was inoculated into Eosin Methylene Blue agar. The innoculum was then incubated at 33 °C for 72 h. Colonies with dark centers and greenish metallic sheen indicated the presence of lactose fermenters. This was confirmed to be E. coli by use of biochemical reactions.

2.5.2.3 *Salmonella* spp.

A pure culture from Nutrient agar was inoculated into Xylose Lysine Desoxycholate agar. It was then incubated at 33 °C for 48 h. growth of well developed red colonies with black centers was considered to be the presence of *salmonella* spp. They were further subjected to biochemical tests for confirmation.

2.5.2.4 *Klebsiella pneumoniae*

A pure culture from nutrient agar was inoculated into MacConkey agar. It was then incubated at 33 °C for 72 h. growth of round mucoid colonies with colorless edges was considered to be the presence of *Klebsiella pneumonia* which were further subjected to biochemical tests for confirmation.

2.5.2.5 *Enterobacter aerogenes*

A pure culture from nutrient agar was inoculated into Eosin Methyhlene Blue. It was then incubated at 33 °C for 72 h. Growth of pinkish large mucoid colonies was considered to be the presence of *Enterobacter aerogene*. Further biochemical tests were done for confirmation.

2.5.2.6 *Shigella* spp.

A pure culture from nutrient agar was inoculated into *Shigella Salmonella* agar. It was then incubated at 33 °C for 72 h. growth of mucoid colonies with black centers was considered to be the presence of *Shigella*spp. A further subjection to biochemical tests was done for confirmation.

2.5.2.7 *Candida spp.*

A pure culture isolated in Sabouraud's Dextrose broth was inoculated into Sabouraud's dextrose Agar. It was then incubated at 25 °C for 5 days. Growth of small round moist, white to colorless colonies with even edges was considered to be the presence of Candida spp. Further microscopic studies were done for confirmation.

2.5.3 Bacteria identification by API 20 E

The API 20 E strip was prepared by distributing 1 ml of distilled water into the honeycombed wells of the tray to create a humid atmosphere. The isolate code was labeled on the elongated flap of the tray. The strip was then removed from its packaging and placed in the incubation box. The inoculum was prepared by using a tube containing 5 ml of 0.85 % Nacl. A well isolated colony (18 h old) from an isolation plate was added to the tube using a pipette and carefully emulsified to achieve a homogenous bacterial suspension which was used immediately after preparation as recommended by the manufacturer

With a pipette both tube and cupule of the tests CIT, VP and GEL were filled with the bacterial suspension while only the tube was filled for other tests. Anaerobiosis was created in the tests ADH, LDC, ODC, H$_2$S and URE by overlaying with mineral oil. The incubation box was then closed and incubated at 37 °C for 24 h.

2.5.3.1 Reading the strip

After the incubation period, the strip was read by referring color changes in Table 3. Some tests which required the addition of reagents were also done. TDA test was done by adding 1 drop of TDA reagent then color changes observed (Table 3). IND test was done by adding 1 drop of JAMES reagent then observing color changes. VP test was done by adding 1 drop of VP 1 and VP 2 reagents then waited for 10 min and recorded the observations. The oxidase test was done by using oxidase discs (Himedia laboratories, India) to detect oxidase production by microorganisms. This was done by touching and spreading a well-isolated colony on oxidase disks then reaction observed within 10 sec. at room temperature (25 °C) change in color was observed and if none after 60 sec. it was considered negative.

Identification was obtained with a numerical profile which was determined using a result sheet given along with the API 20 E kit. In the sheet, the tests are separated into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests. The oxidase reaction constituted the 21st test and had a value of 4 if positive. Identification was then performed with the analytical profile index by looking up at the numerical profile in the list of profile (KEMRI CMR)

Table 3. Interpretation of Biochemical results.

Tests	Active ingredients	Qty (mg/cup)	Reactions/ Enzymes	Results	
				Negative	**Positive**
NPG	2-nitrophenile-βD-galactopyranoside	0.223	β-galactosidase (ortho NitroPhenyl-βD-galactopyranosidase)	colorless	yellow (1)
DH	L-arginine	1.9	Arginine DiHydrolase	yellow	red/orange (2)
DC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red/ orange(2)
DC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red/orange(2)
IT	trisodium citrate	0.756	CITrate utilization	pale-green/yellow	blue-green/ blue (3)
S	sodium thiosulphate	0.075	H_2S production	colorless/ grayish	black deposit/ thin line
RE	Urea	0.76	UREase	yellow	Red/ orange (2)
DA	L-tryptophane	0.38	Tryptophane DeAminase	TDA/ immediate	
				yellow	reddish brown
D	L-tryptophane	0.19	INDole production	JAMES/immediate	
				colorless, pale-green/yellow	pink
P	Sodium pyruvate	1.9	Acetoin production (voges proskauer)	VP 1 + VP 2 / 10 min	
				colorless	Pink/red (5)
EL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment

LU	D-glucose	1.9	Fermentation / oxidation (GLUcose) (4)	Blue / blue-green	Yellow / yellowish-green
AN	D-mannitol	1.9	Fermentation / oxidation (MANnitol) (4)	Blue / blue-green	Yellow
O	Inositol	1.9	Fermentation / oxidation (INOsitol) (4)	Blue / blue-green	Yellow
OR	D-sorbitol	1.9	Fermentation / oxidation (SORbitol) (4)	Blue / blue-green	Yellow
HA	L-rhamnose	1.9	Fermentation / oxidation (RHAmnose0 (4)	Blue / blue-green	Yellow
AC	D-sucrose	1.9	Fermentation / oxidation (SACcharose) (4)	Blue / blue-green	Yellow
EL	D-melibiose	1.9	Fermentation / oxidation (MELibiose (4)	Blue / blue-green	Yellow
MY	Amygdalin	0.57	Fermentation / oxidation (AMYgdalin) (4)	Blue / blue-green	Yellow
RA	L-arabinose	1.9	Fermentation / oxidation (ARAbinose) (4)	Blue / blue-green	Yellow
OX	Oxidase	0.18	Cytochrome- Oxidase	No color change	Deep purple blue

(1) A very pale yellow should also be considered positive.

(2) An orange color after 36-48 h incubation must be considered negative.

(3) Reading made in the cupule (aerobic).

(4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.

(5) A slightly pink color after 10 minutes should be considered negative.

Table copied from biomerieux http://www.biomeriux.com

2.5.4 Molecular identification of bacteria

Isolation of genomic DNA from bacterial plate cultures was done by removing bacteria from culture plate with an inoculation loop and suspending in 180 µl of buffer ATL (Lysis buffer supplied in the QIAamp DNA mini kit) by vigorous stirring Isolation of genomic DNA from bacterial suspension cultures was done by pipeting 1 ml of bacterial culture into a 1.5 ml micro centrifuge tube, and centrifuging for 5 min at 5000 × g (7500 rpm) after adding buffer ATL to a

total volume of 180 µl the protocol on DNA extraction was followed. Isolation of genomic DNA from gram-positive bacteria was done by Pelleting bacteria by centrifugation for 10 min at 5000 ×g (7500 rpm) then suspending bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/ml lysozyme or 200 µg/ml lysostaphin; 20 mM Tris.HCL, pH 8.0; 2 mM EDTA; 1.2 % Triton). It was then incubated for 30 min at 37 °C. Then added 20 µl proteinase K and 200 µl buffer AL then Mixed by vortexing. It was then incubated at 56 °C for 30 min and then for a further 15 min at 95 °C. Extended incubation at 95 °C can lead to some DNA degradation. Centrifugation was done for 30 sec then added up to 25 mg to a 1.5 ml micro centrifuge tube containing no more than 80 µl PBS. The sample was homogenized by vortexing and 100 µl buffer ATL was added before proceeding with the protocol below on DNA purification.

2.5.4.1 DNA Extraction and quantification

After the treatments above DNA purification was done by adding 20 µl of proteinase K, mixed by vortexing, and incubated at 56 °C for 10 min until the tissue was completely lysed. Vortexing was done occasionally during incubation to disperse the sample. Centrifuging for 1.5 ml micro centrifuge tube was done to remove drops from the inside of the lid. Then 4 µl RNase A (100 mg/ml) was added and mixed by pulse-vortexing for 15 s, then incubated for 2 min at room temperature. Centrifugation of the 1.5 ml micro centrifuge tube was done to remove drops from inside the lid before adding 200 µl buffer AL to the sample. It was again mixed by pulse-vortexing for 15 sec, and incubated at 70 °C for 10 min. the 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from inside the lid. Then 200 µl of ethanol (100 %) was added to the sample, and mixed by pulse-vortexing for 15 sec. After mixing, briefly centrifuged the 1.5 ml micro centrifuge tube to remove drops from inside the lid. Carefully applied the mixture above (including the precipitate) to the QIAamp mini spin column (in a 2 ml collection tube) without wetting the rim, closed the cap, and centrifuged at 6000 × g (8000 rpm) for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate discarded. The QIAamp mini spin column was carefully opened and 500 µl Buffer AW1 (wash buffer) added without wetting the rim then closed the cap, and centrifuged at 6000 × g (8000 rpm) for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate discarded. The QIAamp mini spin column was carefully opened and 500 µl Buffer AW2 (wash buffer) added without wetting the

rim, closed the cap and centrifuged at full speed (20,000 × g; 14,000 rpm) for 3 min. the column was placed in a new 2 ml collection tube and the old collection tube with filtrate was discarded. It was then centrifuged at full speed for 1 min to eliminate the chance of possible buffer AW2 carryover. The QIAamp mini spin column was placed in a clean 1.5 ml micro centrifuge tube and the one containing the filtrate discarded. The column was carefully opened and 200 µl buffer AE (elution buffer) was added. It was then incubated at room temperature for 1 min, and then centrifuged at 6000 × g 8000 rpm for 1 min. The above step was repeated and the final product kept in a freezer at -23 °C. After extracting DNA, quantification was done by Nanodrop 2000C Spectrophotometer (Thermo Scientific Inc., Wilmington, USA) to determine the concentration and purity of the isolated DNA.

2.5.4.2 PCR Using *Taq* DNA polymerase

Before starting, a 10 × PCR buffer, dNTP mix, 25 mM $Mgcl_2$, forward primer, F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') and Reverse primer R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3') were thawed at room temperature and placed on ice. A master mix was then prepared as shown in Table 4. The master mix was thoroughly mixed and 50 µl volumes dispensed into PCR tubes. The template DNA (sample) was then added to tubes 2 µl each. A thermal cycler (GeneAmp PCR systems 9700, Applied Biosystems USA) was then programmed as seen in Table 5. Steps 2-5 were repeated in 30 cycles

Table 4. PCR master mix

Component	Volume/reaction in µl	Final × 10
H_2o (nuclease free)	34.75	340µl
Buffer	5	50 µl
$Mgcl_2$	2	20 µl
dNTPs	1	10 µl
Taq polymerase	0.25	2.5 µl
Primer F27	2.5	25 µl
Primer R 1525	2.5	25 µl

Table 5. Thermo cycler programming

Step	Temperature (° C)	Process	Duration
1	95	Initial denaturation	3 min
2	95	Denaturation	30 sec
3	53	Annealing	30 sec
4	72	Extension	1 min
5	72	Final extension	7 min
6	4	Hold	hold

2.5.4.3 Agarose gel electrophoresis

A 1 % agarose gel was prepared by suspending 1 g of agarose gel in 100 ml of TAE buffer (prepared as outlined in reagent preparation) then heating to boil then left to cool up to 60 °C then cast after adding 1ul of Ethidium bromide. After gelling (solidifying), it was slowly positioned in the electrophoresis chamber which was then flooded with a 1× TAE buffer. 1ul of a 10× loading dye was then carefully pipetted to the wells after removing the comb and then starting by loading a 1 kb ladder which was the standard, 1 µl of each sample was loaded to the wells. It was then left to run at 100 volts (Bio-rad, powerpac basic USA) for 30 min after which it was visualized under the UV (Alpha Imager, South Africa).

2.5.4.4 PCR products purification for sequencing

For sequencing purposes, the PCR product must be cleaned. Special buffers provided by QIAgen kit were optimized for efficient DNA recovery and removal of contaminants in each specific PCR applications. DNA adsorbs to the silica membrane in the presence of high concentrations of salts while contaminants pass through the column. Impurities are efficiently washed away and the pure DNA is eluted with a buffer.

The DNA fragments form PCR was cleaned using the QIAqick PCR Purification Kit protocol. 100 µl of buffer PB (to allow binding) was added to 20 µl of the PCR sample and mixed well. A QIAquick spin column was placed in a 2 ml collection tube and the sample applied to the column then it was centrifuged for 1 min at 8000 rpm. The flow through was then discarded and 0.75 ml buffer PE (washes salts away) added. It was then centrifuged for 1 min at 8000 rpm. The flow through was discarded then centrifuging again done for 1 min. this was meant to remove residual ethanol from buffer PE. The QIaquick column was placed in a clean 1.5 ml micro centrifuge tube and then DNA was eluted by adding 50 µl Buffer EB (10 mM Tris.cl, pH 8.5) to the centre of the membrane and centrifuging the column for 1 min. The purified DNA was then analyzed on a gel by adding 6 µl of the loading dye to 20 µl of the purified DNA. The solution was then mixed by pipetting up and down before loading the gel. The loading dye contains 3 marker dyes (Bromophenol blue, xylene cyanol and orange G) that facilitate estimation of DNA migration distance and optimization of the agarose gel run time (Set up in Figure 7).

CHAPTER THREE: RESULTS AND DISCUSSION

3.1 Sample characteristics

Fourteen samples (47 %) were powders, nine (30 %) liquids, five (16 %) tablets, one balm and capsule each (3 %). Herbal products claimed to treat diabetes, purify blood and food supplements were the majority in the market (50 %). The rest were herbal medicines claimed to be for the management of blood pressure, sexually transmitted diseases, gastro-intestinal problems, viral diseases and pain. Twenty samples (67 %) were acidic with a pH range of 4.6-6.9, while ten samples (33 %) had a near neutral to alkaline pH ranging from 7.0-7.7. Liquids were however found to have the lowest average pH of 6.0 followed by powders (pH 6.0). Tablets balm and capsules had near optimum pH (Table 6).

Table 6. Sample characteristics

Dosage	Sample I.D	Packaging	Therapeutic Claim	pH
Powders	BP	Plastic paper bag	Treatment of High blood pressure	6.4
	CS	100 g Plastic container	Treats STD's and related diseases	5.6
	DF	100 g Plastic container	Antidiabetic	6.0
	GI	Plastic container	Antidiabetic	5.6
	GS	Plastic container	Antidiabetic	6.3
	HB	Plastic container	Antidiabetic	5.9
	KC	Plastic container	Antiviral	6.5
	KD	Small plastic paper bag	Antidiabetic	6.5
	MB	100 g plastic container	Blood purifier	5.3
	MC	Small plastic bottle	Antiviral	6.9
	MS	Small plastic bottle	GIT problems	5.1

	MW	Well-sealed plastic p. bag	STD's	6.1
	NP	Plastic container	Blood purifier	5.3
	ND	Bottle with sealed cup	Expectorant	7.0
Liquids	VH	Small well-sealed bottle	Vitamin supplement	7.1
	ES	Well sealed bottle in a box	Food supplement	7.4
	GT	Small bottle in a box	Food supplement	7.3
	KH	Plastic bottle	Blood purifier	4.8
	KM	100 ml plastic bottle	Cough syrup	4.6
	NE	Small plastic bottle	Antidiabetic	5.5
	RB	25 mg bottle	Pain-killer	7.1
	ST	Small plastic bottle	Blood purifier	5.3
	US	Small white plastic bottle	GIT problems	4.8
Tablets	AD	2 tablets in a sachet	Antidiabetic	5.6
	BS	Aluminium strips	Pain-killer	7.3
	CV	In plastic tubes	Peptic disorders	7.7
	MH	2 tablets in blister pack	Aphrodisiac	5.8
	TK	In blister pack	Eczema	7.4
Balm	EM	25 g plastic bottle	Decongestant	7.4
Capsules	MZ	In blister pack	Vitamin supplement	7.4

Though the liquid and tablet formulations were well packaged, powder formulations especially those obtained from street herbalists/ vendors were not permanently packaged. This means one large container held the bulk product which was then dispensed in portions to customers and in small paper bags. This may have contributed to the high microbial load witnessed in powder

formulations (Table 7) which ranged from 1.19×10^6 cfu/ ml to 1.56×10^{10} cfu/ ml. One tablet though well packaged in a blister pack had a microbial load of 1.50×10^{10} cfu/ ml. This may have been contaminated prior to packaging as there was no point of microbial entry after the seal. The same applied to liquid formulations whose microbial load ranged from 3.28×10^7 cfu/ ml to 1.82×10^9 cfu/ ml. They may also have been exposed to the contaminants prior to packaging.

Based on the pH levels determined, one sample (KM) had the lowest pH (4.6). The bacterial and fungal counts for the sample were observed to be equally low. This suggests that higher pH favored contaminants of herbal preparations. This as well agrees with the observation made by Lekaminara (1999) that bacterial growth is optimal at pH 5-8.5. Despite 99 % of the samples obtained from herbal clinics, street vendors and supermarkets lacking satisfactory labeling, sample KM had all the details including dosage, therapeutic value, manufacturing and expiry dates, storage conditions, indications, contraindications, active ingredients, excipients, manufacturing company and addresses etc. This sample was obtained from a herbal clinic but had addresses indicating that it was imported. The same details were available in all the products sampled from the listed herbal products in the Pharmacy and Poison's Board register. The microbial load for the listed herbal products was below 100 cfu/ ml. This means when cultured, none of the products had a colony count of above 30. These products are subjected to post-market surveillance by the Pharmacy and Poisons Board and the industries which manufacture and or market them are always inspected. Though the board does not have the correct guidelines for registration of these drugs, they are regulated like the other essential drugs which is the reason for their quality and safety as far as microbial contamination is concerned

3.2 Microbial load

Ten (33 %) of the samples had a microbial load below 100. This means none had a colony count of above 30 cfu/ ml. A total of 3 (10 %) of the samples contained an average total aerobic microbial count range of 3.6×10^5 to 6.50×10^5 cfu/ ml. One (3 %) of the samples had a total aerobic microbial count and total yeast and moulds count of 6.00×10^5 cfu/ ml and 5.30×10^4 cfu/ml respectively. Whereas 2 (7 %) of the samples were bacterial compliant (Table 7) their fungal load was still higher than the stipulated limits (BP 2007, USP-NF 2009). Fifteen (50 %) of the samples had a microbial load ranging from 2.21×10^7 to 1.56×10^{10} cfu/ml.

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Table 7. Microbial load of samples

Sample ID	Av. TAMC (cfu/ml)	Av. TYMC (cfu/ml)	TVAMC (cfu/ml)	Compliance
AD	3.00×10^6	5.20×10^6	8.20×10^6	Not compliant
ND	0	0	0	Compliant
VH	0	0	0	Compliant
ES	0	0	0	Compliant
GT	0	0	0	Compliant
RB	0	0	0	Compliant
BS	0	0	0	Compliant
CV	0	0	0	Compliant
TK	0	0	0	Compliant
EM	0	0	0	Compliant
MZ	0	0	0	Compliant
BP	2.61×10^7	2.04×10^7	4.65×10^7	Not compliant
CS	1.21×10^7	1.00×10^7	2.21×10^7	Not compliant
DF	2.50×10^7	1.84×10^6	2.68×10^7	Not compliant
GI	2.08×10^7	2.23×10^7	4.31×10^7	Not compliant
GS	1.56×10^{10}	1.07×10^7	1.56×10^{10}	Not compliant
HB	1.03×10^{10}	8.1×10^8	1.11×10^{10}	Not compliant
KC	1.01×10^7	2.05×10^7	3.06×10^7	Not compliant
KD	1.56×10^9	1.48×10^7	1.57×10^9	Not compliant
KH	2.14×10^7	1.14×10^7	3.28×10^7	Not compliant
KM	6.00×10^5	5.30×10^4	6.53×10^6	Compliant
MB	6.50×10^5	5.40×10^5	1.19×10^6	Bacterial complia

MC	2.50×10^7	6.80×10^7	9.30×10^7	Not compliant
MH	1.50×10^{10}	5.40×10^6	1.50×10^{10}	Not compliant
MS	2.50×10^7	7.10×10^6	3.21×10^7	Not compliant
MW	2.94×10^7	2.63×10^7	5.57×10^7	Not compliant
NE	1.80×10^9	1.56×10^9	1.82×10^9	Not compliant
NP	3.6×10^6	5.00×10^5	4.10×10^6	Bacteria complia
ST	2.73×10^7	2.07×10^7	4.80×10^7	Not compliant
US	1.66×10^7	1.86×10^7	3.52×10^7	Not compliant

Legend

AV. **TAMC** = Average Total Aerobic Microbial Count

AV. **TYMC** = Average Total Yeast and Moulds Count

TVAMC = Total Viable Aerobic microbial Count

The limits of bacterial and fungal contamination given in the British Pharmacopoeia (2007) are: Total Aerobic microbial Count (TAMC) not more than 10^7 bacteria and Total yeast and Mould Count (TYMC) not more than 10^5 fungi for herbal products to which boiling water is added before use. TAMC not more than 10^5 bacteria and TYMC not more than 10^4 fungi for herbal products to which boiling water is not added before use. For all the above, *Escherichia coli* and *Salmonella* should not be present.

The herbal products under study especially those from herbal clinics, supermarkets and street vendors did not meet the above specifications. The samples were contaminated to varying degrees with pathogenic bacteria and fungi. Ten (33%) of the 30 samples were however free from microbial contamination, but twenty (67 %) had bacterial count in the range of 6.00×10^5 to 1.56×10^{10} cfu/ ml and fungal count in the range of 5.30×10^4 to 1.56×10^9 cfu/ ml

3.3 Gram staining and microscopic characteristics

Of the 19 different species of bacteria isolated and identified in this study (Table 8), 13 (68 %) were Gram-negative rods while 6 (32 %) were Gram-positive rods and cocci.

Table 8. Microscopic and Gram's characteristics of identified bacteria

Bacterial species	Shape	Arrangements	Gram reaction	Motility
Klebsiella pneumonia	Straight rods	Singles, pairs	G-ve	Non-motile
Chryseomonas luteola	Short rods	Pairs	G-ve	Motile
Klebsiella oxytoca	Straight rods	Singles and pairs	G-ve	Non-motile
Enterobacter cloacae	Rods	Singles, pairs	G-ve	Motile
Serratia marcescens	Short rods	pairs	G-ve	Motile
Escherichia coli	Straight rods, cocobacilliary	Singles/ pairs	G-ve	Non-motile
Enterobacter agglomerulans	Short rods	Singles/ pairs	G-ve	Motile
Flavobacterium spp.	Straight rods	Singles/ pairs	G-ve	Non-motile
Pseudomonas aeruginosa	Straight and slightly curved rods	Singles	g-ve	Motile
Shigella spp.	Short rods	Singles, clustered	G-ve	Non-motile
Salmonella spp.	Straight rods	Paired	G-ve	Motile
Enterobacter aerogenes	Short rods	Singles, pairs, clustered	G-ve	Motile
Bacillus pumilus	rods	Singles, pairs	G+ve	Motile
Kocuria rosea	Cocci	Pairs, tetrads	G+ve	Motile
Cronobacter sakazakii	Rods	Sinlges, pairs	G-ve	Motile
Bacillus safensis	Rods	Singles, pairs	G+ve	Motile
Bacillus flexus	Rods	Singles, pairs	G+ve	Motile

Bacillus subtillis	rods	Singles, pairs	G+ve	Motile
Staphylococcus aureus	Cocci	Singles, pairs and irregular clusters	G+ve	Non-motile

3.4 Biochemical Characterization by differential, selective media and API 20 E

The test for specified microbes carried using differential and selective media showed growth of *Staphylococcus aureus* in MSA (Appendix 1), *Klebsiella pneumonia* in MacConkey (Appendix 4), *Escherichia coli* (Appendix 3) and *Enterobacter aerogenes* in EMB (Appendix 2), *Salmonella* spp. in XLD and *Shebelle* spp. in SSA, while *Candida* spp., *Aspergillums* spp. (Appendix 5), *Rhizopus* spp (Appendix 6), *Fusarium* spp. *Torula* spp. And *Alternaria* spp. (Appendix 7) grew in SDA. There was no growth in Cetrimide agar ruling out the presence of *Pseudomonas* spp. This microbe was however diagnosed by API 20 E (Figure 9).
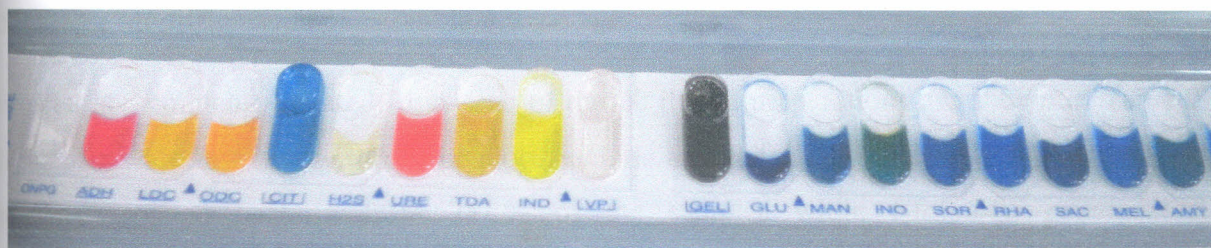
Figure 9. API strip Color changes for the identification of *Pseudomonas aeruginosa* (sample NE042)

In addition to the above microbes being confirmed by API 20 E, *Chryseomonas luteola*, *Klebsiella oxytoca*, *Enterobacetr cloacae*, *Serratia marsencens*, *Enterobacter agglomerulans* and *flavobacterium* spp were also identified.

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Table 9. Numerical profiles of the microbes identified by API 20 E.

Isolate	Numerical profile	Identity
AD224	1211773	*Klebsiella pneumonia*
AD226	3203000	*Chryseomonas luteola*
BP229	1047573	*Klebsiella oxytoca*
BP231	7305573	*Enterobacter cloacae*
DF157	7215773	*Klebsiella pneumonia*
MH024	7317777	None
KM002	5106720	*Serratia marsencens*
KM014	7255773	*Klebsiella oxytoca*
MH018	1004572	*Escherichia coli*
MH030	1247173	*Enterobacter agglomerulans*
MH033	1212004	*Flavobacterium spp.*
US201	7355773	None
NE042	2212004	*Pseudomonas aeruginosa*
NE058	0044400	*Shigella spp.*
KM001	7317577	None
US202	2176304	*Salmonella spp.*
US204	5005673	*Enterobacter aerogenes*

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3.5 Positive identifications per sample

Microbial contaminants isolated from each of the 20 herbal preparations are shown in Table 9. All samples were invested with one or more bacteria and fungi. *Escherichia coli* was the most frequently isolated bacteria from 75 % of the samples while *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Staphylococcus aureus* followed in 70 %, 60 % and 45 % of the samples respectively. *Salmonella* spp was isolated in 40 % of the samples while *Shigella* spp was in 20 %. The rest of the bacteria were isolated from less than 10 % of the samples. *Candida* spp formed the major fungal isolate from 50 % of the samples followed by *Fusarium* and *Aspergillus* species which were in 35 and 25 percents of the samples respectively. *Rhizopium* spp was isolated from 20 % of the samples while the rest of the fungal species were isolated from less than 10 % of the samples.

Table 10. Microbes identified in each sample

Sample	Microbe identified
AD	*Klebsiella pneumonia, Chryseomonas luteola, Aspergillus spp.*
BP	*Klebsiella oxytoca, Enterobacter cloacae, Candida spp.*
CS	*Staphylococcus aureus, E. coli, Rhizopium spp. Fusarium spp., Bacillus subtillis*
DF	*Staphylococcus aureus, Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Salmonella spp*
GI	*Enterobacter aerogenes, Klebsiella pneumonia, E. coli, Aspergillus spp. Fusarium*
GS	*E. coli, Klebsiella pneumonia, enterobacter aerogenes, Fusarium spp. Aspergillus spp. Rhizopus spp., Baccillus safensis*
HB	*Staphylococcus aureus, Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Salmonella spp, Candida spp. Aspergillus spp, torula spp, Bacillus flexus*
KC	*Salmonella spp. Enterobacter aerogenes, Candida spp.*
KD	*Staphylococcus aureus, E. coli, Candida spp. Fusarium*
KH	*Klebsiella pneumonia, Enterobacter aerogenes, E. coli, Candida spp. Torula spp*
KM	*Staphylococcus aureus, Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Salmonella spp. Shigella spp. Candida spp. Serratia marsenses, kocuria rosea*
MB	*Klebsiella pneumonia, E. coli, Fusarium*
MC	*Klebsiella pneumonia, E. coli, Rhizopus spp. Candida spp. Penicillium spp, Torula spp.*
MH	*Klebsiella pneumonia, E. coli, E. aerogenes, salmonella spp, Shigella spp, Candida spp, Enterobacter agglomerurans, Flavobacterium spp, Bacillus pimillis, enterobacter cloacae*
MS	*Staphylococcus aureus, Klebsiella pneumonia, Enterobacter aerogenes, Salmonella spp. Rhizopus spp, Penicillium spp.*
MW	*Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Fusarium spp.*
NE	*Staphylococcus aureus, Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Salmonella spp, Shigella spp, Candida spp. Pseudomonas aeruginosa., Bacillus pimillis*
NP	*Staphylococcus aureus, Salmonella, Aspergillus spp. Fusarium spp.*

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| ST | *E. coli, Candida spp. Penicillium spp.* |
| US | *Staphylococcus aureus, Klebsiella pneumonia, E. coli, Enterobacter aerogenes, Salmonella spp, Shigella spp, Enterobacter sakazakii* |

The level of contamination of herbal medicinal preparations by pathogenic Gram negative bacteria was high. Fifteen (75 %) of the herbal preparations obtained from herbal clinics, supermarkets and street vendors were contaminated by *Escherichia coli* which is a Gram-negative intestinal bacterium and is an indicator of fecal contamination. Fourteen (70 %) were contaminated by *Klebsiella pneumoniae* and 12 (60 %) by *Enterobacter aerogenes*, while 8 (40 %) were contaminated with *Salmonella* spp, 9 (45 %) by *Staphylococcus aureus* and 4 (20 %) by *Shigella* app. The products did not meet the standards further prescribed by the international pharmacopoeia on the total viable aerobic microbial count (TVAMC) which is the sum of fungal and bacterial load (International Ph. WHO, 2003). A Gram stain procedure revealed that 62 % of the samples were Gram-negative bacteria while the rest (38 %) were Gram positive. Gram negative organisms which were the majority of isolates in this study are pathogenic and majorities are associated with fecal contamination (Adenike *et al.* 2007). *Staphylococcus aureas* isolated and identified in this study is a member of the normal flora (Table 1). The Gram-positive cocci which can reside in the human body without causing any harm have however been associated with a number of complications especially to immune-compromised individuals. It produces proteins that disable the immune system destroying tissues (Gladwin and Trattler 2004). It also releases exotoxins which cause gastroenteritis, toxic shock syndrome and scalded skin syndrome. It has also been associated with pneumonia, osteomyelitis, acute bacterial endocarditis, septic arthritis and urinary tract infections (Warren and Levinson 2004)

Chrysomonas luteola belongs to bacteria normally found in the soil and water environments. Though it is not normally associated with human or animal pathogenicity, few cases of infections have been reported especially in patients with prosthetic heart valves (Ratogi 1998). *Enterobacter cloacae* also found in the soil has been identified as an important plant-growth promoting rhizobacteria especially in root colonization (English *et al.* 2009). *Favobacterium* spp. A genus of gram negative bacteria also found in the soil and fresh water environments has not been identified as a human pathogen but has been associated with disease in fish

http://en.wikipedea.org/wiki/flavobacterium).   In   the   same   group   of   gram-negative enterobacteriaceae, *Enterobacter agglomerurans* is mostly identified as frequent in animal houses using straw as bedding material, also a source of airborne endotoxins in animal housing (Zucker *et al*.1998).

Klebsiella pneumonia, a Gram negative lactose fermenting bacterium found in the normal flora of the mouth is ranked second from *E. coli* for urinary tract infections in older people and also an opportunistic infection of people with weakened immunity. It is also a common cause of pneumonia. *Pseudomonas aeruginosa* also a widespread microbe in soil, water and plants is pathogenic to humans and a common cause of nosocomical infections such as pneumonia and also UTI. Though rare to healthy individuals, it is life threatening to immuno-compromised people (http://emedicine.medscape.com/article/226748)

Escherichia coli, *Enterobacter aerogenes* and *Klebsiella oxytoca* also isolated and identified in this study are equally associated with infections such as diarrhea, UTI and pneumonia. *Salmonella* is the causative agent of enterocolitis and typhoid fever.

The fungus *Penicillium* spp also identified in the study are commonly considered as contaminants for food products but also associated with infections including pneumonia particularly in immunocompromised individuals. *Penicillium* was isolated from solids and liquid herbal products in a study by Esimone *et al*. (2007). Its isolation in this study therefore indicates that it is a common contaminant. In addition, these fungi produce mycotoxins, including ochratoxin A which is nephrotoxic and carcinogenic produced by *penicillium verrucosum* (Burge 1989). *Aspergillus* spp also identified has mycotoxins which are carcinogenic such as aflatoxins produced by *Apergillus flavus*. Some are considered opportunistic pathogens and have been associated with pulmonary infections (www.doctorfungus .org/the fungi/aspergillus). *Aspergillus* was isolated by Esimone *et al* (2007) in a study to identify contaminants in herbal medicines. Its isolation here therefore confirms Esimones findings. *Fusarium* is also a fungus common in soils and plants. Though most of its species are harmless saprobes and abundant members of the soil microbial community, it has been associated with mycotoxins at lower temperatures; it can also cause allergies and asthma in immunocompromised individuals (WHO, 1999-09-01). *Rhizopus* spp. is frequently found in house dust, soil, fruits, nuts and seeds. Exposure to large numbers of this fungus has been reported as cause of respiratory complications. It is also an opportunistic

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pathogen for immunocompromised individuals. People with diabetes, malnutrition and severe burns are also at risk (Barrat *et al*. 1999). Torula, yeast though with a lot of beneficial commercial applications can be pathogenic in cases of compromised immunity.

The fungus *Candida* spp. also isolated and identified in this study is the cause of many infections. *Candida albicans* causes mouth, skin, vaginal and penile infections. In rare occasions, it has been traced in the blood stream causing infections to vital organs especially in leukemia and Aids related cases (www.healthscout.com/ency/6813/main.html)

This study agrees with Esimone *et al.*, 2007 and Dnyaneshwar *et al.*, 2005 who found *Klebsiella pneumonia, Staphylococcus aureus, Escherichia coli, Salmonella spp.*, and *Pseudomonas aeruginosa* as major bacterial contaminants of herbal medicines sold and marketed in Nigeria. The same fungus isolated in large quantities in this study including *Aspergillus spp., Fusarium spp, Penicillium* and *Rhizopus spp*. were identified as major contaminants of herbal medicines.

3.6 Molecular characterization

3.6.1 Gel analysis

After electrophoresis DNA was seen as bands when visualized under the UV Machine fitted with a Camera. Each band represents DNA from bacterial samples. The one with multiple bands (M) is the standard DNA molecular weight marker (Figure10)
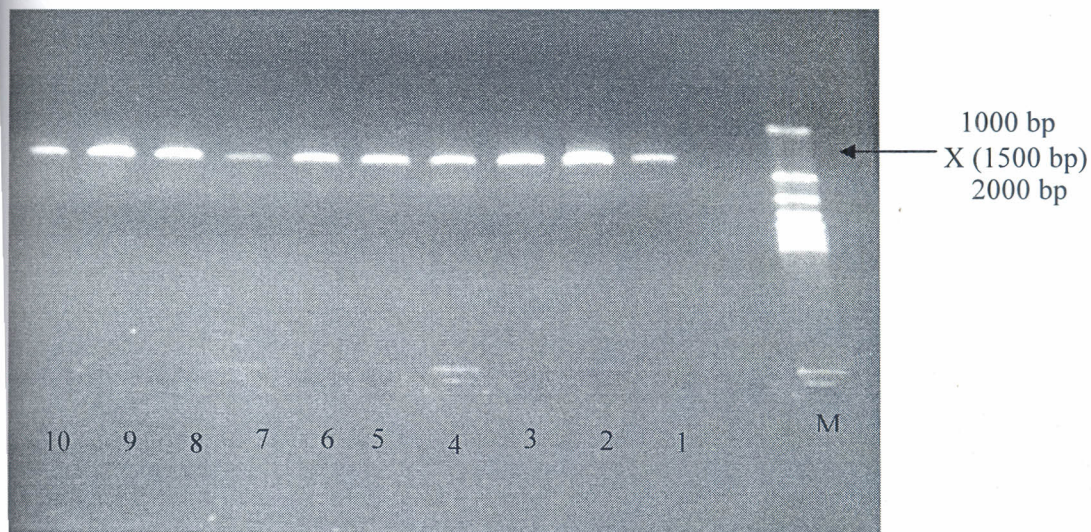
Figure 10. Agarose gel electrophoresis analysis of DNA.

The unknown samples (1-10) were estimated by visual comparison with the standard 1 kb ladder (M) to be 1.5 kb or 1500 bp (X). Our gene of interest (16SrDNA) is estimated to be 1500 bp and was therefore assumed to be present. 16SRDNA gene is a commonly used tool for identifying bacteria because DNA analysis is considered more reliable than traditional characterizations which depended upon phenotypic traits like Gram reactions, bacillus or coccus and others. It is also relatively short (1.5 kb), making it faster to sequence than many other unique bacterial genes. Since our samples (Figure 10) appeared as sinlge bands on an agarose gel, it was considered clean and not contaminated. This formed the basis for cleaning the PCR product for sequencing and not the agarose gel.
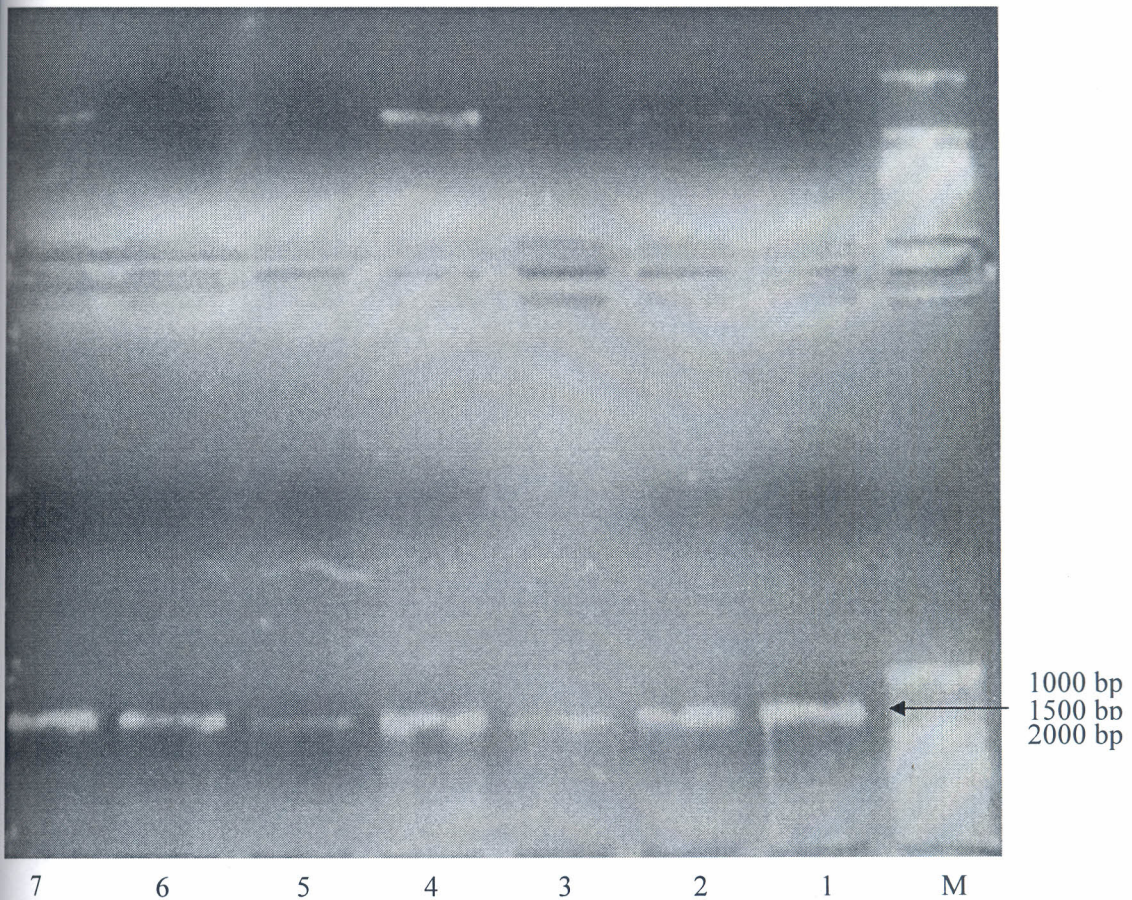
Figure 11. DNA bands in 1 % agarose gel

After purifying the PCR product, DNA was confirmed present when run on an ethidium bromide 1% agarose gel (Figure 11). Bands (numbered 1-7) were visible and compared against the 1 kb

ladder (M), they were approximately 1500 bp which is the estimated length of our target gene (16SrRNA).

Table 11. Bacterial deoxyribonucleic acid quantification

Sample ID	Conc. (ng/µl	A260	A280	A260/A280	260/230	Conc. Factor (ng/µl)
MH029	40.16	0.803	0.429	1.87	1.95	50
KM001	33.49	0.67	0.359	1.87	1.84	50
NE060	26.35	0.527	0.275	1.91	1.86	50
US201	30.9	0.618	0.338	1.83	1.74	50
MH024	25.89	0.518	0.268	1.93	1.82	50
MH034	40.21	0.804	0.416	1.93	1.81	50
KM008	13.04	0.261	0.142	1.83	1.16	50
KM013	23.34	0.467	0.26	1.79	1.73	50
CS168	15.07	0.301	0.17	1.78	1.37	50

Nucleic acids have an absorption maximum at 260 nm. Because most samples contain contaminants such as proteins and single stranded DNA that absorb maximally at 280 nm, the equation A 260/ A280 is used to calculate DNA in presence of contaminates. The higher the ratio, the more pure the DNA samples. Since ratios between 1.7 and 2.0 are acceptable, all the samples examined were considered pure because they were within this range (Table 11).

3.6.2 Sequence determination

In order to determine the nucleotide sequences of the unknown bacteria, Primers used were complementary to the sequence on both sides of the segments that were amplified. The Forward primer F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') and Reverse primer R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3') were also used for sequencing. They gave gene fragments of various sizes from each template. Each gene sequence fragment was used to

assemble a single sequence contigue using the contig assembly program (CAP) of the BioEdith suite (Hall, 1999). Fragments and contigs for sample KM001 and US201 are shown in figure 612 and 13 respectively while fragments and contigs for other samples (identified in table 12) are shown in Appendix 9.

Figure 12. Gene fragments and contig for isolate KM001 (*Kocuria rosea*)

>KM001_F27
CGAATGAGGGGCCTAACTGCAGTCGACGATGTTGCCCAGCTTGCTGGGCGGATTATGGCGAACGG
GTGAGTAATACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGG
ATACTACCTCTTGCCGCATGGTGGGTGGTGGAAAGGATTTTACTGGTTTTGTTTGGGCTCACTGCCT
ATCGGCTCGTTGGTGCGGTAACTGGTCGCCGAGGCAACGACTGGTAGCCGGCCTGAGAGGGTGAC
CGGCTGAAATGGGAATGACCACGGTGCAAACTCCTACCGGACAAACTCCGGGGAAAAGTGCCCCAT
TGGGCGGCTTCCTGATTGGACGACACCCTGAGAGAGATGACGCCCTTCAGGGTAGAAAAGCTCTTT
CATCATGGAAGTATGGAGAGAGACAGTACCTGGGAAAGAAGCGCCAGCTACCTACCTGGCCGCAGC
CTCGGAAAACGTCACGGCGTAAGTGTTGCCCGGAATTATTGGTCGTAAAGAGGTCGTAAGCGGTTT
GCCGCGTCTGTGGTGAAAGCCCGAGGCTCAGCCGCGTTCTCTGCTCTGAGGACGGAGACA

>*KM001_R1492*
AGTTCAACCTTACGAGGCTCATCCACAGGGTTAGGCCACCGGCTTCGGGTGTTACCAACTTTCGTGA
CTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACT
AGCGACTCCGACTTCATGAGGTCGAGTTGCAGACCTCAATCCGAACTGAGACCGGCTTTTTGGGATT
AGCTCCACCTCACAGTATCGCAACCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATA
AGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCTATGAGTC
CCCACCATCACGTGCTGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCCACCAGCCCCGAAGGGAAACCCCATCTCT
GGGGCGGTCCGGTGAATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCT
CCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCG
GGGCACTTAATGCGTTAGCTACGGCGCGGAAAACGTGGAATGTCCCCCACACCTAGTGCCCAACGT
TTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGT
AACAGGCACCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACA
CCAGGAAATTCCAGTCTCCCCTACTGCACTCTAGTCTGCCCGTACCCCCTGCAGACCCGGGGGGTTG
AGCCCCGGGCCTTTTCAACAGTCAGACGACGACAAACCGCCCTACGTATCTCCTTACGCCCAATTGA
TTTCCCGGATAACGCTTTGCGCCATAACGTATTTACCGCGGGCTGACTTGGCACGTCACGTTAGCCC
TGGCGCTTTCCTTTCTGACAGTACCGATCCAACTCTTCGCCTTTCTTTCCCCTTACTGAAAGTAATGT
TACACACCCGATAGATCCTGTTCATTCCCCCTTT

>Contig-2
CGAATGAGGGGCCTAACTGCAGTCGACGATGTTGCCCAGCTTGCTGGGCGGATTATGGCGAACGG
GTGAGTAATACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGG
ATACTACCTCTTGCCGCATGGTGGGTGGTGGAAAGGATTTTACTGGTTTTGTTTGGGCTCACTGCCT
ATCGGCTCGTTGGTGCGGTAACTGGTCGCCGAGGCAACGACTGGTAGCCGGCCTGAGAGGGTGAC
CGGCTGAAATGGGAATGACCACGGTGCAAACTCCTACCGGACAAACTCCGGGGAAAAGTGCCCCAT
TGGGCGGCTTCCTGATTGGACGACACCCTGAAAGAGGGAATGAACAGCATCTATCAGGGTATGAAA
CA*GCTACTTTCAGTAAGGGGAAAGAAAGGAGAAGAGTTGGATCAGTACCTGGCAGAAAGGAAAGCG*
CCAGGGCTAACGTGACCTGGCCAAGTCAGCCCGCGGTAAATACGTCACGGCGCAAAGCGTTACCC
GGGAAATCAATTGGGCGTAAAGAGATACGTAAGGCGGTTTGCCGTCGTCTGACTGGTGAAAAGGCC
CGAGGCTCAACCGCGTTCTCGGCTCTGCAGGGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGAC
TGGAATTTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGT
CTCTGGTGCCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTA
ACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG

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Contig-4

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GGGTGGGCCTACATGCAGTCGAGCGGTACACAGGCAGCTTGCTCCTTTGCTAAAAGCGGGACAG
TGTGAATTGTGTCTGGAACTGTGTGTGGTGGAGGGATATCTCCTGTGAAACGCTATCTCTTACCCC
TATCGTCTTCTCACCCCAGTGTGGGACCTTCTCGCCTCTCGTCCCCTCATATGTCCCCATATGGTAT
ATCTTATGTGGGGTGAAAAGTCTCCTCTGCGACAATCTCCCTATGTGCTGAGAGGAGAACCCCCCC
ACTGTGGCTGTGACACGGTCCCCACACCCCCAGGGGGGGGCCACTGGGGAAAATTTTGCACTGTG
GGCGAGCCTGTTGTGCCATCCATGTCCCGGCGTGTAAGAAGAAGCCTCTTCGGATGTGTAAGATAC
TTTTCCAGCGAAGAAAGAGGTGTTGTGTGTTAATAATCCACACCAATTGACGATTACTCGCACAAAA
AGCCACCCGCGTATAACCCCGCGACAGCAGCCGCGGGAAATACACGAGGGGTGCAAAGCGTTAA
TCGCAAATTACTGGGCGCAAAGCGCACACAGGCGGTTTGTGTTAACTCAGATGTGAAATCCCCGCG
GTCTCAACCTGTGGAACTGCATTTTAGAAACTGGCAAGCTTGAGTCTCTCATAGAGGGGGAGTAGAA
TCTCCAGTGTGTAGCGGAGAAATGCATAGAGATCTGTGAGAGAATACCGGTGGCGAGAGGCGGCCC
CCCTGTGACAAAAACAGTGACGCTCAGGTGCGAAAGCGTGGGGAGCAACACAGAATTAGATACCCC
TGTGTAGTCCACGCGCCTAAACGATGTCGACTCGTGGAGGTTGTGCCCTTGAGACGTGTGCTTCCG
AGAGCTAACGCGTTAAGTCGACCGCCTGTGGGAGTACAGCGCCGCAAGGATAAAACTCAAATGAAT
TGTCACGGGGCCCCCGCACAAGCGCTGGGAGAACGTGGTGTTAATATCGATGTGCAACGCGAAGAA
CCTCTACCTGTGGCTCGACATCTCAGAGAATCCCTCTCGCAGATGATGCGGAGAGTGCCTTCCTCG
GGAGACTCTTGAGACACGAGTGCCTGCCGATGGTGCTGTCGTCCACAGCCTCGTCTGTTGAGGAAA
TGTGGTGGGTATGAAGTCCCGCCGACAGACAGCAGGCGACAACCACCTGTATCCTTTGTTGCCAGC
ACATAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACC
TCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA
TGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACGTCGGAGACGCTAT
CACTTTGGAC
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3.6.3 Phylogenetic analysis

The phylogenetic relationship between the sample isolates with other known bacteria was determined by searching the nucleotide databases at the National Center for Biotechnology Information (NCBI, USA). The Basic Local Alignment Search Tool (Altschul et al., 1990) finds regions of local similarity between sequences to other sequences in the database and calculates the statistical significance of the matches. The database sequence with high similarity to each of the contigs of our unknown sample isolates were identified as shown (Table 12)

Table 12. Identities of microbes with similar sequences to isolates

Isolate ID	Contig No.	No. Bases	Identity
MH029	1	1120	*Bacillus pumilus*
KM001	2	1470	*Kocuria rosea*
S201	4	1533	*Cronobacter sakazakii*
MH024	6	1490	*Enterobacter spp.*
MH034	7	1467	*Enterobacter Cloacae*
KM008	8	547	*Bacillus safensis*
KM013	9	1494	*Bacillus flexus*
S168	11	1507	*Bacillus subtillis*

The sequences closely related to each of the isolates were fetched from the database and used to perform a multiple sequence alignment using the program MUSLE 3.6 (Edgar, 2004). The multiple sequence alignment was used to construct a neighbor joining phylogenetic tree which shows evolutionary relations of the microbes. MEGA 3 software (Kumar et al. 2004) was used in constructing the phylogenetic trees. From the tree (Figure 14) isolate KM001 Clusters together with *Kocuria rosea* indicating that it is very closely related to this species.
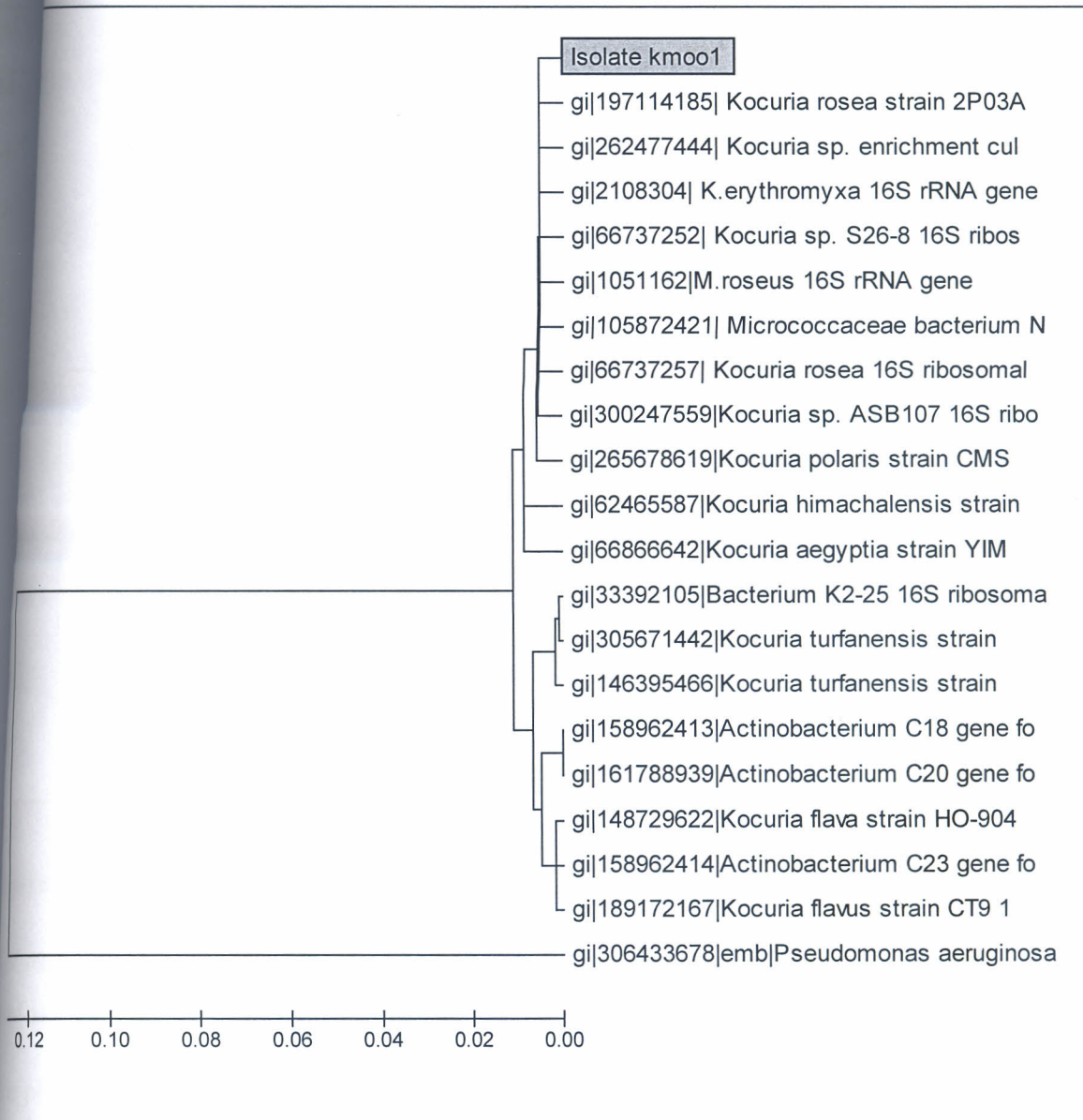
Figure 14. Phylogenetic tree of isolate KM001 based on 16SrRNA gene sequences.

The tree was constructed using the neighbor joining method and *Pseudomonas aeruginosa* was used as an out-group.
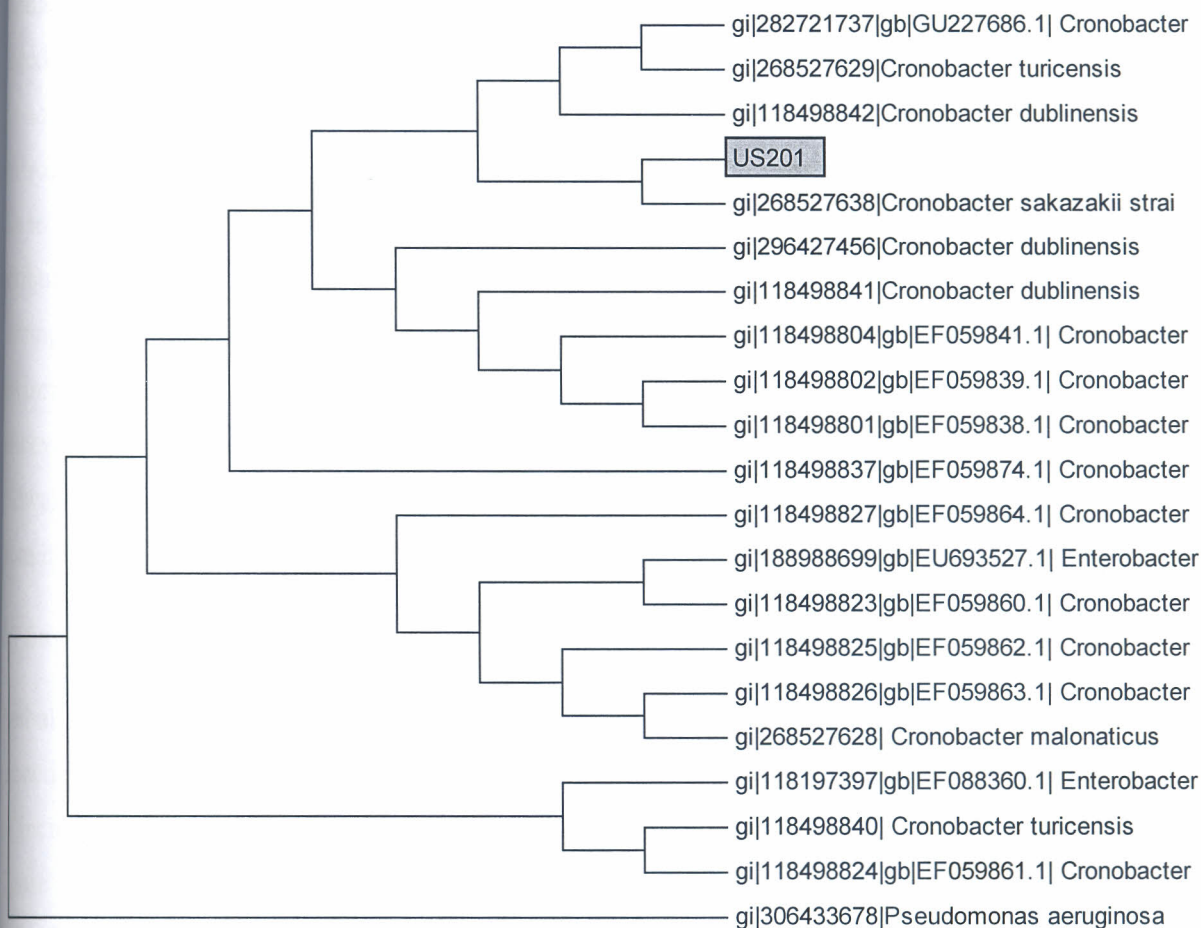
Figure 15. Phylogenetic tree of isolate US201 based on 16SrRNA gene sequence.

The tree was constructed using the neighbor joining method and *Pseudomonas aeruginosa* was used as an out-group.

The phylogenetic analyses of isolate KM001's 16SrRNA gene sequences have placed it within the genus *Kocuria*, the greatest sequence similarity being found with *Kocuria rosea* (Figure 8). *Kocuria* is a member of the micrococcaceae family. Though their role as human pathogens has not been clearly determined, Fevzi *et al.* (2004) reported *Kocuria rosea* causing catheter related bacterimia in patients. This microbe has not been reported before as a contaminant in herbal medicines and therefore its isolation and identification is of great significance. Though rare, it is found in the outer skin and therefore must have found its way to the herbal products due to poor handling and processing techniques.

Similarly, the phylogenetic analyses of the 16SrRNA gene sequences of isolate US201 has placed it within the genus *Cronobacter* with the greatest sequence similarity being found with *Cronobacter sakazakii*. This is a Gram-negative non-sporing rod which is widespread in the environment. It has been linked to incidences of infant meningitis and necrotizing enterocolitis due to contaminated infant formula (Carol *et al.* 2007). Its presence in herbal medicines can be attributed to incorrect handling. Isolates MH029, KM008, KM013 and CS168 were identified as coming from the genus *Bacillus* (Table 12). *Bacillus* organisms are common contaminants in the environment and majority of their bacteremia are not clinically significant. *Bacillus pumilus* has however been associated to a compound that has fungicidal activity against *Aspergillus* spp. (Edward *et al.*, 2003). Though many of the microbes reported in this study have been isolated from herbal medicines in the previous studies a few are new and were isolated for the first time.

3.7 Summary illustrations.

Herbal sample microbial analysis flow chart gives a step by step summarized diagrammatic procedure on how a sample was treated from the beginning to the end (Appendix 10). After 48 h of incubation, colony counts were done then colony morphology formed the basis of isolation. Those with similar characteristics were identified with the same code, stocked and later identified. The microscopic and macroscopic characteristics that lead to the identification of fungal contaminants in this study are summarized in Appendix 11 and biochemical identification of bacteria chart is also summarized in Appendix 12.

CHAPTER FOUR: CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The analyzed samples were powders (47 %), liquids (30 %), tablets (16 %), capsules and balm each (3 %). These products were claimed to treat a number of diseases including diabetes, high blood pressure, STI and GIT problems. Others were blood purifiers and food supplements. Majority of the samples (67 %) had a lower pH, while the rest (33 %) had near optimum pH.

The microbial load of the samples generally varied with 33 % having a colony count of below 30 cfu/ ml while 67 % had high bacterial and fungal counts ranging from 2.21×10^7 to 1.56×10^{10} cfu/ml. Though others complied with the selection criteria by having lower bacterial and fungal counts, they still did not qualify because they had higher levels of pathogenic microbes like *Escherichia coli* and *Staphylococcus aureus*.

Through microscopic observations and Gram-staining, 68 % of the bacteria were Gram-negative while 32 % were Gram-positive. Different species of fungi were also identified by microscopic and macroscopic observations. These included *Aspergillus* spp., *Candida* spp., *Rhizopium* spp., *Fusarium* spp., *Torula* spp. and *Alternaria* spp.

Through biochemical tests by differential, selective media and API 20 E profiling, 12 species of bacteria were identified. These were *Klebsiella pneumonia*, *Chryseomonas luteola*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Serratia marsences*, *Escherichia coli*, *Enterobacter agglomerulans*, *Flavobacterium* spp., *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella* spp. And *Enterobacter aerogenes*. *Escherichia coli* were the microbes isolated from most of the samples (75 %).

Molecular characterization of the isolates that were not identified from the previous techniques was done. The sequencing of the 16SrDNA gene showed closest relations of the isolates after phylogenetic analysis of their sequences. They were closely related to *Kocuria rosea*, *Cronobacter sakazakii*, *Bacillus pumilus*, *Bacillus safensis*, *Bacillus flexus* and *Bacillus subtilis*.

From the high numbers of contaminants in these preparations, it is evident that overall the examined herbal medicines are of poor microbiological quality. The presence of fungal contaminants especially *Aspergillus* and *Penicillium* is hazardous. These two have been

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associated with high level toxicity and have always been isolated from solid and crude herbal drugs (Esimone *et al.* 2007). This study has shown that a higher percentage of the herbal preparations examined were grossly contaminated and the microbial counts were generally higher than the accepted values for non-sterile pharmaceutical (BP 2007).

The presence of large numbers of selected pathogenic bacteria in the analyzed herbal medicinal products in this study may be due to the methods of their preparation or the equipment and materials in preparing the herbal medicines. Tap water which is mostly used in preparation of herbal medicines has also been associated with many coliform bacteria (Temu *et al.* 2009). Other possible sources of contaminants are the personnel that would introduce the bacteria when handling the raw materials during processing. The process of harvesting, drying storage, handling and the soil influence the bacteriological and fungal quality of raw materials which in turn affects the entire quality of the herbal preparation (Danladi *et al* 2009). Thus, manufacturers should ensure the highest quality of hygiene during the process as well as the lowest possible level of pathogenic organisms on their herbal preparations so as to maintain the correct quality, safety and efficacy of the final herbal preparation.

It may be concluded from this study therefore that most herbal preparations sold and marketed in Kenya are likely to be contaminated with a wide variety of potentially pathogenic bacteria and harmful fungus. This calls for thorough quality assurance enforcement and monitoring of production and distribution of herbal preparations throughout stages of production to the time they are in the market.

4.2 Recommendations

More studies to identify contaminants of herbal medicines need to be encouraged. Alongside reports of microbiological contamination, cases of adverse reactions of herbal therapies have been published in medical journals as noted earlier in the literature review. With increasing use and awareness of potential hazardous effects, these reports are liable to increase in frequency but with no systems in place for reporting such, it is difficult to determine the event rates. In the US, pharmacists, physicians, nurses and other health workers are encouraged to report adverse effects and product quality issues through the FDA special nutritionals adverse event monitoring system or the medwatch system or the USP practitioners reporting network (http://www.fda/oc/initives

ephedra/february2004/). Though not routinely done in Kenya, it is important for all health practitioners and the general public at large to report any adverse effects as this will mark the best starting point.

Interaction with herbalists in the areas studied during sample collection revealed poor knowledge in good storage and manufacturing practices of herbal medicines. Whereas a few practiced in clean well organized herbal clinics with shelves and good ventilation, others had no idea and kept their products in sacks stacked in dark stuffy rooms with little regard to bacterial, fungal and other forms of contamination. Most herbal medicine practitioners in Kenya are not educated. A study in Tanzania by Temu *et al.* (2009) revealed that 80 % of herbal medicine practitioners had received primary education, 19 % were illiterate while only 0.5 % had university education. Basic education for herbal practitioners on good manufacturing, good storage and good harvesting practices is therefore recommended, also change of attitude is necessary to accommodate those who would want to be in the practice but shy away due to their academic qualifications.

It is also recommended that careful phytochemical, pharmacological and toxicological studies be done on chosen herbal preparations as a starting point toward instituting ways that can be used to describe dosages in an informed way.

A policy in herbal medicine is a necessity in order for safety and efficacy of herbal medicines to be assured. The policy will address issues in a microbiological quality and will above all put up clear guidelines which will include post-market surveillance to oversee that all practitioners comply with the regulations. This will help us as a country to follow suit in what other countries like china have done hence incorporating herbal and traditional medicine as a whole to the national healthcare system.

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Appendix 1. *Staphylococcus aureus* **colonies in MSA**

MSA contains 7.5 % sodium chloride which discourages the growth of competing organisms thus select growth of staphylococcus. The medium also contains a PH indicator that changes color if the mannitol in it is fermented to acid. *S aureus* are therefore readily identified because they grow at high salt concentration and ferment mannitol to acid changing color to yellow.

EMB is inhibitory to Gram-positive bacteria. It also has the dyes Eosin and Methylene blue which differentiates between enteric lactose fermentors and non-fermentors. The coliform bacteria *Enterobacter aerogenes* produce thick mucoid pink colonies

Appendix 3. *Escherichia. coli* **colonies in EMB**
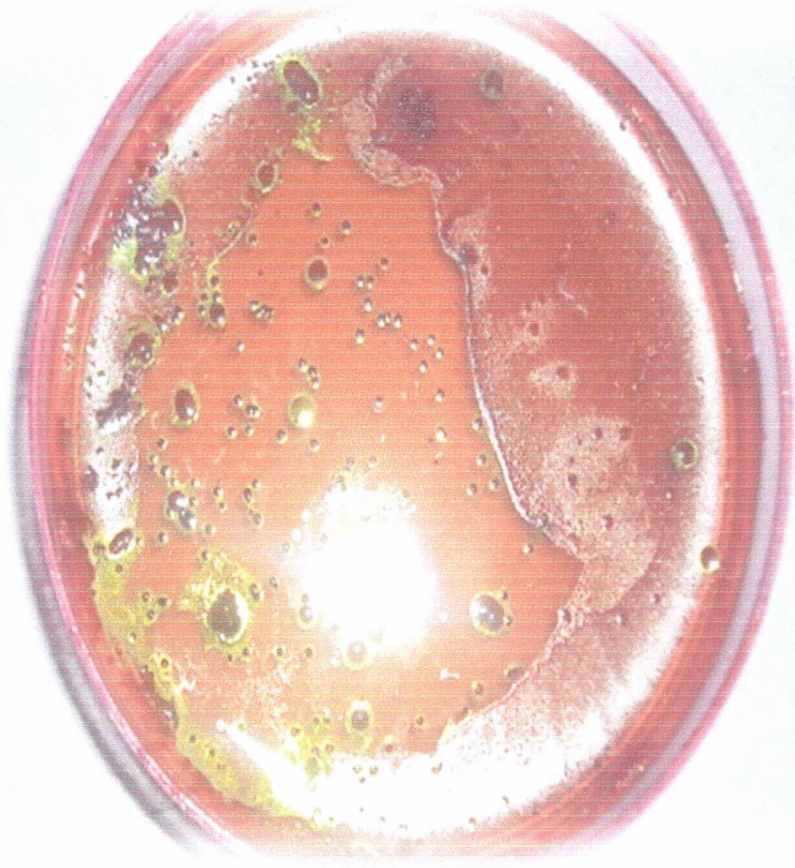
EMB is inhibitory to Gram-positive bacteria. It also has the dyes Eosin and Methylene blue which differentiates between enteric lactose fermentors and non-fermentors. E. coli appear blue black with a metallic green sheen caused by the large amount of acid produced which precipitates the dyes to the growth surface.
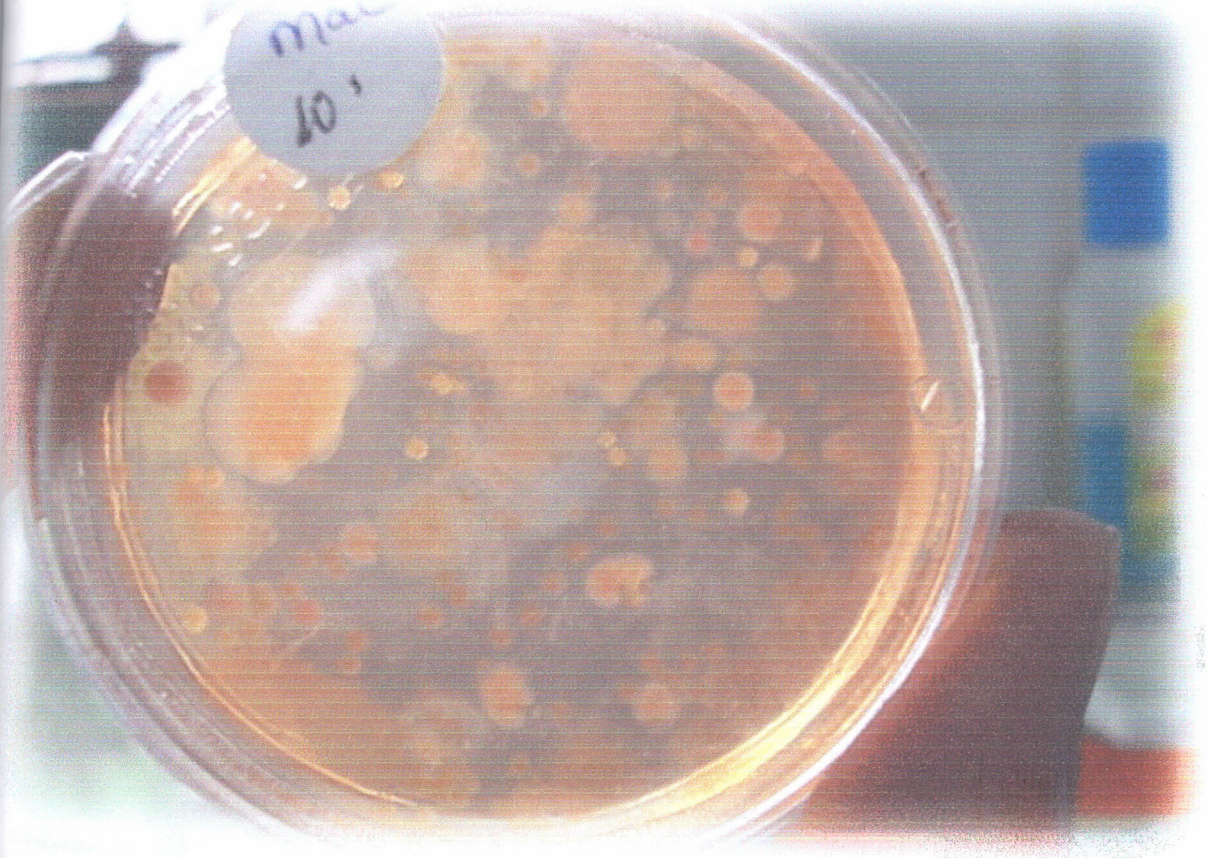
MacConkey agar is both differential and selective. It contains bile salts and crystal violet which inhibited the growth of Gram-positive bacteria. Because it also contains lactose, Gram-negative bacteria that grew on this disaccharide were non-lactose fermentors since they were colorless. Lactose fermentors grow red or pink colonies

Appendix 5. *Aspergillus* **spp. colonies in SDA**
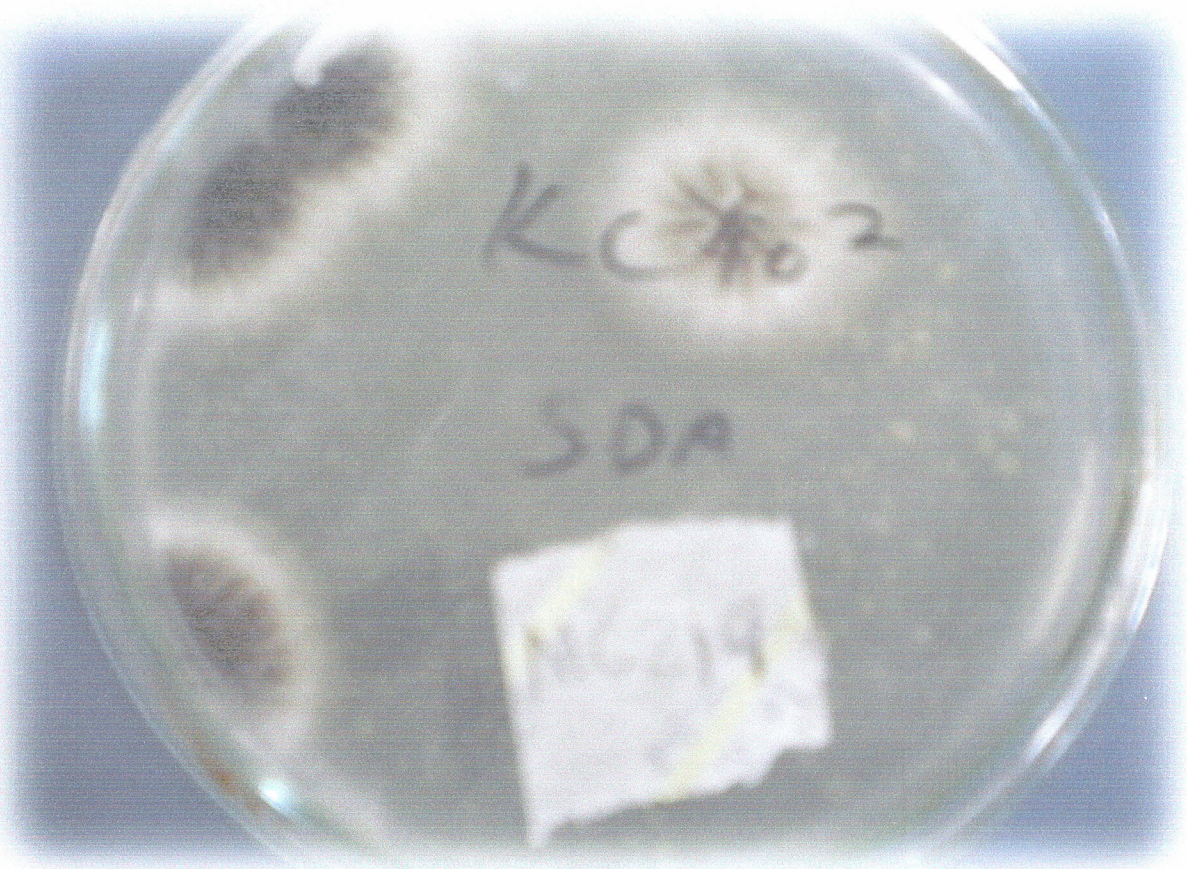
Aspergillus colonies are white and become black as culture matures.
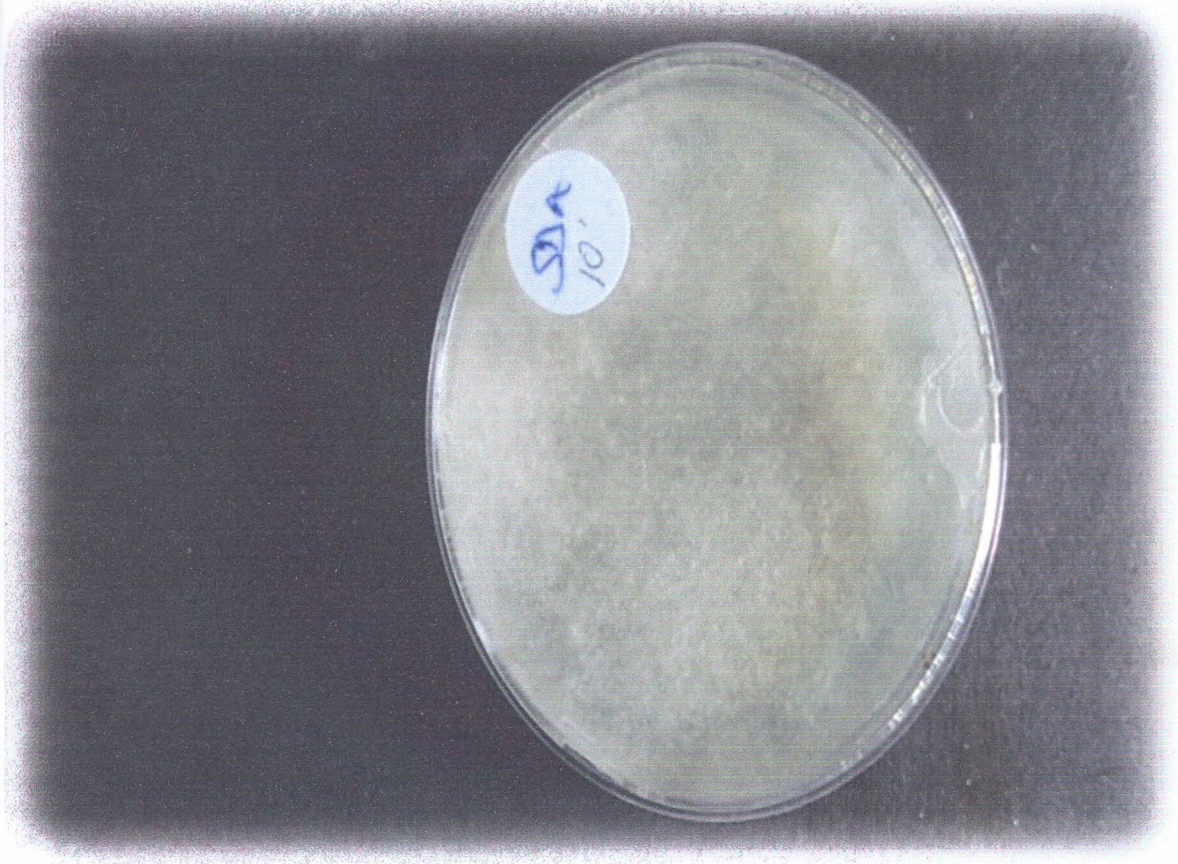
The fungi *Rhizopus* is rapidly growing white, cottony and swam over the entire plate

Appendix 8. Listed herbal Medicinal products

Name	Formulation	Therapeutic value
igomax forte	Tablets	Loss of libido
xtramunne	Tablets	Immune-booster
ravasil	Syrup	Cough
aluma	Spray	Topical analgesic
etasil	Lozenges	Antiseptic
aluma inhaler	Inhaler	Decongestant
ise-up and walk	Oral solution	Antimalarial
aluma gel	Gel	Decongestant
arbor	Powder	Expectorant
egeacid	Suspension	Anti-acid
Target bullet	Capsule	Health supplement
onadel jumor	Syrup	Ant-acid
ilver plus	Solution	Hemopoetic
Glikof	Surup	Cough
dobyul 2.0	Powder	Anti-cancer
Hypodea	Suspension	Immune-booster
Modul-8	Solution	Immunomodulant
Homkof	Tablets	Anti-tussive
Diarrago	Patches	Antidiarrhoal
Homkof	Syrup	Anti-tusive
Odmos	Lotion	Mosquito-repellent
Robb super	Ointment	Pain relieve
New advanced odmos	Cream	Mosquito repellent
Robb junior	Ointment	Pain
Moor balm	Balm	Irritant control, stimulant
Rob tarzan herbal	Ointment	Pain
C-vit daily	Effervescent tablets	Vitamin supplement
New pinkoo gripe	Oral solution	Anti-acid
Ojus	Syrup	Antidypepsia
Apem	Extract	Anti-diabetic,
Extrammune	Syrup	Immunomodulant anti-hypertensive
Vomiteb	Syrup	Antinosea
Emami-menthol plus	Balm	Decongestant
Alsarex	Tablets	Peptic disorders
Femipilc	Capsules	Nutraceutical
Imupsora	Tablets	Anti-psoriasis
Ps memory plus	Capsules	Nutraceutical
Takzema	Tablets	Anti-eczema

eximum	Ointment	Pain
eximum oil	Liquid	Pain
ol ache	Ointment	Painkiller
less-go	Capsules	Nutraceutical
ative diabetic complex	Capsules	Vitamins
lomz B	Capsules	Vitamins
ool freeze	Gel	Rube
Ierbogor herbal balm	Ointment	Balm
Dental life-tunic	Powder	Dental problems
Sugarita	Oral granules	Anticancer
Vab t herbal honey	Syrup	Antibacterial
Betasil peppermint	Lozenges	Colds
Herbal koosil	Lozenges	Colds
Restoplus	Granules	Herbal
Malarix	Globules	Antimalarial
Savir protein	Tablets	Immune booster

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Contig-1

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Contig-6

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>Contig-7

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81

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Appendix 10. Diagrammatic illustration of sample analysis

Herbal Samples innoculated by Spread plate

Incubation for 48 -72 hrs, Examined plates for each morphotype

Enumerated each colony with optimum counts (30 - 300)

Subcultured Each colony type

Anaerobi types not dealt with

Aerobic Microorganism colonies morphology studied, stocked according to differences

Stocked fungal colonies in 30% SD broth and 70% glycerol

Bacteria stocked in 30% nutrient broth and 70% glycerol

Flowchart 2 for identification

Flowchart 3 for Identification

Appendix 11. Microscopic and Macroscopic identification of Fungus

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                    ┌──────────────────────┐
                    │ Stocked fungal isolates│
                    │   streaked in SDA     │
                    └──────────────────────┘
            ┌────────────────┴────────────────┐
┌───────────────────────┐          ┌───────────────────────┐
│     Microscopic        │          │      Macroscopic       │
│     Examination        │          │      Examination       │
└───────────────────────┘          └───────────────────────┘
```

Microscopic Examination		Macroscopic Examination
Oval Spores, Nonseptate mycellium →	*Rhizopium* spp. ←	Rapid growing colonies, white colored, swarming the entire plate
Yeast like →	*Candida spp.* ←	Small, round, moist colonies with colorless edges
Oval conidia →	*Fusarium* spp. ←	Wholy white colonies changing to yellow
Single celled spores →	*Aspergillus* spp. ←	White to black colonies as culture matures
Oval cells →	*Tourna* spp. ←	Pink moist with unbroken even edges
Conidia in chains →	*Penicillium* spp. ←	Mature cultures greenish to blue-green

Appendix 12. Gram-negative bacteria identification Chart

- Aerobic gram negative rods on McConkey
  - Fermenting Lactose from McConckey
    - **Positive**
      - ONPG/Indole
        - **++**
          - E. coli, Klebsiela Oxyoca
            - Catalase, Urease, Oxydase
              - **+ - - -**
                - E. coli: Confirmed by API
              - **++++**
                - Klebsiela Oxytocyca: Confirmed by API
        - **+ -**
          - cloaca, Klebsiela pneumoniae, Flavobacterium
            - CIT, Urea, VP, Oxydase
              - **++ - -**
                - Flavobacterium spp.: API Confirmation
              - **+ - + -**
                - Enterobacter cloaca: API Confirmation
              - **+++ -**
                - Klebsiela pneumoniae: API confirmation
    - **Negative**
      - ONPG, Indole
        - **+ -**
          - Seratia, Yarsinia
            - Catalase, Urease, Oxidase, VP
              - **++ - +**
                - Seratia: API Confirmation
              - **+ - - -**
                - Yarsinia spp.: API Confirmation
        - **- -**
          - Pseudomonas aeruginosa: API Confirmation
        - **++**
          - Salmonela, Shigella
            - Catalase, Urease, Oxidase, VP
              - **+ - - +**
                - Shigella: API Confirmation
              - **- - - -**
                - Salmonela: API Confirmation

Appendix 13, Reagent and preparation

Reagents and preparation

Crystal violet (Huckers)

Solution A

Crystal violet (90% dye content)	2.0g
Ethyl alcohol (95%)	20.0ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80.0ml

Mixed solution A and B

Gram's iodine

Iodine	1.0g
Potassium iodide	2.0g
Distilled water	300.0ml

Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.0ml
Distilled water	5.0ml

Safranine

Safranine O	0.25ml
Ethyl alcohol (95%)	10.0ml
Distilled water	100.0ml

Ferric chloride reagent

Ferric chloride	10.0g
Distilled water	100.0ml

Kovac's reagent for detection of indole

P-Dimethylaminobenzaldehyde	5.0g
Amyl alcohol	75.0ml
Hydrochloric acid (concentrated)	25.0ml

Dissolved the p-Dimethylaminobenzaldehyde in the amyl alcohol and added the hydrochloric acid

Methyl red solution

Methyl red	0.1g
Ethyl alcohol	300.0g
Distilled water	200.0ml

Dissolved the methyl red in the 95% ethyl alcohol and diluted to 500ml with distilled water

Nitrate test for detection of nitrites

Solution A, sulfanilic acid	8.0g

Acetic acid, 5: 1 part glacial

Acetic acid to 2.5 parts

Distilled water	1000.0ml

Solution B, Alpha-naphthylamine

Alpha-naphthylamine	5.0g

Acetic acid, 5 N   1000.0ml

Sodium Chloride for suspending microorganisms

NaCl   0.85

Distilled water   100.0ml

1× TAE Buffer solution

Trizma base   4.8g

EDTA   0.372g

Deionized water   800ml

Adjusted to pH 8.0 using glacial acetic acid and made to 1000ml adding deionized water