PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF BACTERIA ISOLATED FROM HERBAL MEDICINES VENDED IN MOROGORO MUNICIPALITY, TANZANIA

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ABSTRACT

Herbal medicines are plant-derived materials used as therapeutic substances or dietary supplements. A cross-sectional study was conducted to estimate prevalence of bacteria and antimicrobial susceptibility of *Escherichia coli* and *Staphylococcus aureus* in herbal medicinal products (HMP) vended in Morogoro Municipality, Tanzania. Fifty samples of HMPs were purchased from vendors in six locations in Morogoro Municipality. The samples were analysed by using laboratory standard procedures such as total viable count, isolation and characterization of bacteria and agar disc diffusion method to determine antimicrobials susceptibility. Approximately 88% of HMP had significantly (P<0.05) higher total microbial counts than the recommended level of $\leq 10^3$cfu/ml for HMP intended for human consumption, $\leq 10^5$ cfu/ ml for pre-treated products and no *E. coli* or *Salmonella* spp. allowed to be present at any level by World Health Organization (WHO) and British Pharmacopeia standards. The results showed that 10% of HMP were contaminated with *E. coli* and 8% with *S. aureus*. Furthermore, *E. coli* and *S. aureus* isolated were susceptible to ciprofloxacillin (CIP) but resistant to nalidixic acid (NA), cefatoxime (CTX), oxacillin (OX), co-trimoxazole (SXT) and vancomycin (VA). In this study *E. coli* isolates showed more resistance against CTX, OX, VA and SXT while *S. aureus* had high resistance against NA. The study also observed unhygienic handling practices and low level of safety knowledge of the HMP vendors. This could predispose patients and other consumers of the products to the risk of infections with pathogenic and antimicrobial resistant bacteria. It is recommended that food scientists, health officers, extension officers and other stakeholders should play their roles of training and enforcing quality assurance through monitoring the production and distribution of HMP to safeguard consumers.

DECLARATION

I, **Jonas Daniel Kira**, do hereby declare to the Senate of Sokoine University of Agriculture that, this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

This work is dedicated to my parents Daniel Elisonguo and Anamenasia Daniel Silemu who laid the foundation of my education and taught me the values of resilience; to my sons Jimmy and Baraka and my lovely daughter Estergrace, for their endurance during the period of my study.

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LIST OF ABREVIATIONS AND SYMBOLS

%	Percent
&	And
±	Plus or Minus
∑	Summation
≤	Less than or equal to
≥	Greater than or equal to
®	Registered trade mark
°C	Degrees Celsius
°E	Degrees East of Greenwich
°S	Degrees South of Equator
µg	Microgram
AIDS	Acquired Immune- deficiency Syndrome
APH	American Public Health
API	Active Pharmaceutical Ingredient
AR	Antimicrobial Resistance
BA	Blood agar
BC	Before Christ
BP	British Pharmacopeia
BPW	Buffer Peptone Water
cfu/ml	Colony forming units per millimetre
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CPS	Coagulase positive Staphylococci
DM	Diabetes mellitus

DNA	Deoxyribonucleic acid
DNAse	*Deoxyribonuclease*
EAggEC	Enteroaggregative
EHEC	Enterohaemorrhagic
EPEC	Enteropathogenic
FBD	Food Borne Diseases
FBO	Faith Based Organization
FC	Faecal Coliform
GACP	Good Agriculture and Collection Practices
GAP	Good Agriculture Practice
GMP	Good Manufacturing Practices
HF	Health facility
HIV	Human Immune-deficiency Virus
HM	Herbal medicine
HMP	Herbal medicine product
IMViC	Indole, Methyl red, Vogues proskeur and Citrate
ISO	International Standards Organization
MHA	Muller Hilton Agar
MHB	Muller Hilton Broth
ml	Millilitre
MM	Modern Medicine
MSA	Mannitol salt agar
NA	Nutrient Agar
NB	Nutrient broth
NBS	National Bureau of Statistics
NPHC	National Population and Household Census

NPHC	National Population and Housing Census
NS	Normal saline
pH	Hydrogen ion concentration
PHC	Public Health Care
PMC	Pest Management Centre
PPE	Personal protective equipment
psi	Pounds per square inch
RTE	Ready to Eat
spp	Species
SPSS	Statistical Package for Social Sciences
SUA	Sokoine University of Agriculture
TBC	Total Bacterial Count
TCC	Total Coliform Count
TVBC	Total Viable Bacterial Count
TVC	Total Viable Count
TZS	Tanzania Standards
URT	United Republic of Tanzania
USA	United States of America
VRBG	Violet Red Bile Glucose Agar
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Herbal medicines (HM) are plant-derived materials or plant extracts which are used as therapeutic substances (Drew and Myers, 1997) and can also be used as dietary supplements. World Health Organization (WHO) (2007) defined HMP as botanical medicine or phytomedicine. WHO (2008) describes traditional HMP as herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients that used for prevention or treatment of different ailments. In the recent years, the usage of HMP in prevention and treatment of human diseases has potentially maintained its popularity worldwide (WHO, 2011).

In Tanzania, over the last few years, there has been a remarkable overwhelming increase of public awareness and usage of herbal medicinal products (HMP) in the treatments and/or prevention of diseases accompanied by a lot of advertisement in the newspapers, radios, billboards and television media (Kitula, 2007). The government recognizes HMP as it raises income of practitioners and for those who receive HMP get healed and become healthy and productive (Kitula, 2007; Temu *et al*., 2009). The contribution of HMP cannot be neglected in our society since they are easily accessible healthcare options for people with limited financial resources (WHO, 2007). Recently, traditional practitioners are also struggling to work on HMP that can cure the deadly HIV/AIDS epidemic in our nations (Kitula, 2007) and also to minimise microbial contamination.

The microbial contamination of HMP indicates the amount of microbes it has, a high level of contamination indicates low quality of packaging materials, storage, its handling

practices and more likely to cause adverse effects to consumers (WHO, 2011). There are adverse effects of varying severity, including deaths especially if the way they are conserved during exposition for sales provides conditions for microorganisms to grow and reach considerable levels of contamination (Temu *et al*., 2009). The presence of microbes cannot be detected organoleptically (seen, smelled or tasted) and microbial quality issues are of major importance to public health (WHO, 2000; Prescott *et al*., 2005).

Pathogenic microorganisms commonly isolated from HMP pose a serious threat to human health. Some of these pathogens include *Escherichia coli, Staphylococcus aureus, Salmonella spp., Listeria monocytogens, Bacillus spp., Mycobacterium spp., Campylobacter spp., Clostridium spp*., *Pseudomonas aeruginosa* and *Proteus spp.* (Foster, 2002; Prescott *et al*., 2005; Oleyege and Adelabu 2010, Keter and Mutiso, 2011; Meshack *et al*., 2013). HMP is known vehicle that serves as means of transmission of food-borne pathogens to humans.

Furthermore, apart from HMP being potential carrier of pathogens can also cause serious health risk to consumers due to their antimicrobial resistance. Report on global surveillance of antimicrobial resistance (AR) revealed that AR is no longer a prediction for the future; it is happening right now, across the world, and is putting at risk the ability to treat common infections in the community and hospitals (WHO, 2014). The development of AR is a natural phenomenon (WHO, 2014) however certain human actions accelerate the emergence and spread of AR leading to public health threat. There is inappropriate use of antimicrobial drugs in humans, agriculture and in animal husbandry (Kagashea *et al*., 2010; Nonga *et al*., 2010; Katakweba *et al*., 2012; WHO, 2014). Foster (2002), Prescott *et al*. (2005) and Esimone *et al*. (2007) also suggested that inappropriate control and prevention of plants bacterial and fungal-borne diseases by using antimicrobials favour the

emergence and selection of resistant strains. Presence of antimicrobial residues in HMP may have resulted from farmer failure to observe the withdrawal periods, incorrect dosage levels and illegal addition of antimicrobials into herbal drugs during preparations (Syit, 2008). Antimicrobial resistance can cause significant danger and suffering for children and adults who have common infections which were initially easily treated before by regular antibiotics (Cheesbrough, 2000; Prescott *et al*., 2005).

This study identified, quantified and established potential predisposing factors of some of common bacteria that found in HMP as contaminants. Furthermore, the study determined the susceptibility of the bacterial isolates to commonly used antimicrobials in human health practices. Therefore, information gathered from this study will be useful for Municipal council authorities and other public health stakeholders in the development of strategic plans towards regulating safe HMP.

1.2 Problem Statement and Justification of the Study

Consumption of HMP is increasingly becoming popular. Although modern medicine is well developed in most parts of the world a large number of people in developing countries especially in rural areas, where more than 80% of the population live still rely on HMP for Primary Health Care (PHC) (Kayombo, 1999). The HMP are often perceived as being natural and therefore safe, but they are not necessarily free from microbial and other contaminants which are due to factors such as adulteration, substitution, cross contamination, lack of standardization, inappropriate use of antimicrobials and poor handling practices and/or poor storages (Foster, 2002; Lau *et al.,* 2003; Prescott *et al*., 2005). Temu *et al*. (2009) found powder and liquid HMP sold in the open air markets in Dar es Salaam, Tanzania, being contaminated by pathogenic microbial agents that can pose risk of acquisition of Food Borne Diseases (FBD) to those taking these mixtures.

In spite of many studies conducted and documented on HMP still little information is known regarding bacteriological quality of HMP vended in open air markets in Morogoro municipality and elsewhere in Tanzania and so, assessment of prevalence of bacteria in HMP is essential to establish the degree of contamination and recommend corrective measures to safeguard consumers (Prescott *et al*., 2005; Esimone *et al*., 2007; Ruth 2013). On the other hand, antimicrobial residues cause bacterial resistance to common antimicrobials (Foster, 2002; WHO, 2014). Researchers elsewhere have reported increasing incidence of antimicrobials resistance amongst the commonly isolated bacteria from HMP (Foster, 2002; Esimone *et al*., 2007; Oleyege and Adelabu, 2010; Rahimi and Nayebpour, 2012). Apparently, there is a little information available on the prevalence and susceptibility of bacteria found in HMP in Tanzania, thus hindering the choice of appropriate antibiotics for treatment of commonly human bacteria-borne diseases.

1.3 Objectives

1.3.1 General objective

To determine prevalence and antimicrobial susceptibility of bacteria isolated in HMP vended in the open air markets in Morogoro municipality, Tanzania.

1.3.2 Specific objectives

i. To determine microbial load of HMP vended in Morogoro municipality;
ii. To investigate presence of *Escherichia coli* and *Staphylococcus aureus* in the HMP vended in Morogoro municipality;
iii. To establish antimicrobial susceptibility profile of *Escherichia coli* and *Staphylococcus aureus* isolates from HMP vended in Morogoro municipality.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of HMP

The history of using HMP intertwined with that of modern medicine (MM) and most of synthetic conventional medicines originated from plants (Janetzang, 1994; Adeleye *et al*., 2005). The HM is the ancient type of medicine and had history of dominant healing therapy throughout all cultures and people worldwide (Vickers *et al*., 2001; WH0, 2008; Keter and Mutiso, 2011). The use of HMP has history dating back to the very down of mankind and has been used for medicinal purposes long before recorded history (WHO, 2011). WHO (2008) reported HMP to be used in ancient Chinese and Egyptian papyrus writings that were described medicinal plants used 3000 years before Christ (BC). According to WHO (2008) and Keter and Mutiso (2011) people in different parts of the world tended to use the same plants for medicinal purposes and 25% of pharmaceutical drugs are derived from botanicals. The WHO estimates that 70% to 80% of the population in developing countries uses HMP (WHO, 2007). In sub-Saharan Africa, the traditional healers still play a major role in the provision of healthcare. This has been attributed in part to the availability, affordability, accessibility and the role of HMP to meet PHC demand in developing African countries (WHO, 2008; Ndhlala *et al*., 2011). Before cosmopolitan medicine, traditional medicine dominated medical system for millions of people in Africa but the arrival of the Europeans was a noticeable turning point in the history of ancient tradition and culture (Schulz *et al*., 2001).

The situation in Tanzania is not much different where communities in rural areas still rely on HMP (Kitula, 2007). The HMP is used as first aid measure before the patient is referred to modern Health Facilities (HF) due to lack of accessibility and affordability to modern

PHC (Temu *et al*., 2009). In many cases people would choose to combine both traditional HMP and MM, especially if they are afflicted with chronic diseases (Kitula, 2007). Myovella, V.I personal communication (2014) reported number of patients admitted in HF after been under course of HMP those failed to treat their conditions they were suffering. This implies that most of people chosen to use HMP before seek for modern PHC services.

According to Kitula (2007) the pharmacopeia of HMP in Tanzania is colossal and many medicinal plants are known only by their local names. Table 1 shows commonly indigenous sold medicinal plants in different parts of Tanzania.

Table 1: Plant species commonly used to prepare vended herbal medicinal products (HMP) in different parts of Tanzania

Species	Local name (s)	Part used	Use/Treatment
Warburgia salutaris	Paperbark tree and Altarara (Masai)	Bark	Malaria, colds, diarrhoea, and pain
Olea europaea	Brown olive, Msenefu (Chagga)	Bark	Used as a bottle sterilizer and round worm repellent for both animals and human
Lannea schweinfurthii	Mlungulungu	Root	Hernia stomach ulcers, Diabetes Mellitus (DM), stomach problems in pregnant women
Salvadora persica	Toothbrush tree or O'remiti (Maasai)	Stem	Used as toothbrushes *(mswaki* sticks)
Lonchocarpus capassa	Mapagola, Kababu, Apple ring acacia and Winterthorn	Stem and root	Impotency, bilharzias, and hookworm
Grewia bicolour	Mkone or mkole (Swahili)	Bark and root	Colds, stomach problems, snake bites and syphilis
Parinari curatellifolia	Mobola-plum or cork tree	Root	Epilepsy

Source: (FAO, 1993)

2.2 Practices and Safety Knowledge of HMP Vendors

Food safety has been defined by Ruth (2013) as probability of not suffering from some hazards associated with consuming a specific food; Likewise HMP should be free from hazards that are associated with consumption. Some authors recognized that the conditions under which HMP vendors operate are often unacceptable for the purposes of exposition and selling HMP (Keter and Mutiso, 2011; Ruth, 2013).

The HM vendors are often poor, uneducated, show little concern toward the safe handling of HMP and food safety knowledge and practices (WHO, 2007). Studies by WHO (1996), FAO (2013) and Ruth (2013) have observed vendors having poor food safety and handling practices. Socio-demographic characteristic such as age, and gender have been reported to play no role in food and/or HMP safety knowledge of vendors (WHO, 2007; Temu *et al*., 2009; Soares *et al*., 2012; Ruth, 2013). Ruth (2013) reported three main factors for occurrence of food poisoning with regard to vendors being knowledge, attitude and practice. Therefore knowledge and practice shaped by habits and other perceptions that result from socio-cultural and economic influences (Ruth, 2013).

2.3 Sources of HMP Microbial Contaminants

According to Temu *et al*. (2009) two most likely sources of contamination of herbal preparations are the use of untreated water and exposure of HMP to polluted environments that include dust and other particulate matters. Oleyege and Adelabu (2010) reported contamination of HMP collected from open air markets with faecal coliforms (*E. coli, Salmonella* spp.) and *S. aureus* at the levels above WHO limits (Table 2); and mentioned major sources of microbial contaminants in HMP were environments, poor quality of the raw materials, packaging components, untreated water for preparation and unhygienic handling practices. According to British Pharmacopeia (BP) (2007) and WHO (2007) the

presence of *E. coli, Salmonella spp* and *S. aureus* or any one of them indicates poor quality of production and harvesting practices and handling by personnel who are infected with pathogenic bacteria during harvest/collection, post-harvest processing and manufacturing process. Generally, microbial limits for HMP that are intended for human consumption are considered as same as for food stuffs (Table 2).

Table 2: Permissible limits of Microbial load and pathogenic microorganisms in HMP

S/N	Microbial load	Permissible limits as per WHO		
		Contamination in the crude plant materials	Plant materials that has been pre-treated	Other material for internal use
1.	Total viable aerobic counts	-	10^7cfug^{-1}	Less 10^3cfug^{-1}
2.	*E. coli*	10^5 cfug^{-1}	10^4 cfug^{-1}	10 cfug^{-1}
3.	Total *Enterobacteriaceae*	-	10^4 cfug^{-1}	10^3 cfug^{-1}
4.	*Salmonella spp*.	-	None	None
5.	*Pseudomonas aureginosa*	Absent	Absent	Absent
6.	*Coliforms*	Absent	Absent	Absent
7.	*S. aureus*	-	10^4 cfug-^{1}	10^2 cfug-^{1}

Source: (WHO, 2007).

2.4 Faecal coliforms (FC)

According to Rahimi and Nayebpour (2012), FC are pretty specialized types of bacteria which are dominated by *E. coli*. The FC is subset group of coliform bacteria that have ability to thrive in healthy human intestine and pass out in high numbers through faecal materials. Presence of FC in Read to Eat (RTE) food or HMP indicates potential

contamination of food with faecal materials (WH0, 2013). BP (2007) and WHO (2007) recommended that no *Salmonella* spp. *or E. coli* strain is allowed to be present in RTE products.

2.5 *Escherichia coli*

E. coli are Gram-negative, facultative anaerobic, rod-shaped bacteria which are considered as the normal bowel flora of different species of mammals and birds (Farrokh *et al*., 2012). *E. coli* is a group of harmless bacteria that are most often used as indicator organisms for faecal contamination and breaches in hygiene. *E. coli* clones have acquired virulence factors that have allowed them to adapt to new niches and in some cases to cause serious disease (Rahimi and Nayebpour, 2012). The pathogenic *E. coli* are divided into six groups on the basis of their virulence properties such as enterotoxigenic causative agent of diarrhoea in humans, pigs, sheep, goats, cattle, dogs and horses; enteropathogenic (EPEC) causative agent of diarrhoea in humans, rabbits, dogs, cats and horses; enteroinvasive found only in humans; verotoxigenic found in pigs, cattle, dogs and cats; enterohaemorrhagic (EHEC) found in human, cattle, and goats and enteroaggregative *E. coli* (EAggEC) which found only in human (Bonkoungou *et al*., 2013). The presence of *E. coli* in HMP indicates potential faecal contamination due to poor personal hygiene and sanitation (WHO, 2013).

2.6 *Staphylococcus aureus*

According to Cheesbrough (2000) *Staphylococcus aureus* is a Gram positive *coccus*, resistant to heat, drying and radiation. Its strains can be pathogenic and relatively non-pathogenic. They produce disease when the bacteria get into the body through contaminated RTE materials or through broken skin especially under the hair follicles (Prescott *et al*., 2005). They produce some enzymes such as *coagulase* (bound and free

coagulases) which clots plasma and coats the bacterial cell to probably prevent phagocytoses. *Hyaluronidase* (also known as spreading factor) breaks down *hyaluronic* acid and helps in spreading of *S. aureus*. The bacteria also produce DNAse (*deoxyribonuclease*), an enzyme that breaks down the DNA, lipase to digest lipids, *staphylokinase* to dissolve fibrin and aid in spread, and beta-lactamase for drug resistance (Cheesbrough, 2000). These enzymes are implicated with *Staphylococcal* invasiveness and many extracellular substances some of which are heat stable enterotoxins that render the food dangerous even though it appears normal (Prescott *et al*., 2005). Once bacteria have produced toxin, the HMP can be extensively and properly treated, killing the bacteria without destroying the toxin. Many of their toxins are gene–based that is carried on plasmids (Cheesbrough, 2000). The presence of *S. aureus* in HMP indicates poor quality of raw materials, poor harvesting and personal hygiene and handling by personnel infected with bacteria during manufacturing process (WHO, 2007; FAO, 2013).

2.7 Influence of Different Preparation Techniques on the Microbial Quality of HMPs

The preparation of HMP commonly involves steps in which raw materials are subjected to unfavourable conditions to survival of microorganisms. The following are some of production processes and their influence on the microbial load.

2.7.1 Drying process

According to Rocha *et al.* (2011) drying is the most common and fundamental method for post-harvest preservation of medicinal plants because it allows quick conservation of the medicinal qualities of the plant material in an uncomplicated manner. This process may also facilitate the marketing of medicinal plants, because drying results in reduction of the volume and weight of the plant with positive consequences for transport and storage. Medicinal plants can be dried in a number of ways namely; in the open air (shaded from

direct sunlight); placed in thin layers on drying frames, wire-screened buildings; by direct sunlight, if appropriate in drying ovens and solar driers; by indirect fire; baking; lyophilisation; microwave; or infrared devices (Martins *et al.,* 2001). When possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents. In the case of natural drying in the open air, efforts should be made to achieve uniform drying of medicinal plant materials and to avoid microbial contamination and their growth (WHO, 2003; WHO, 2007; FAO, 2013). Drying at high temperature decreases the total aerobic microbial count in herbs, lowering of water activity to the level required for preventing growth of microorganisms and quality of heat sensitive ingredients in HMP diminish on high temperature (Kulshrestha *et al*., 2008; FAO, 2013).

2.7.2 Extraction methods

The drugs which are subjected to cold water extraction may host a considerable amount of microbes, and if extraction procedure is carried out at ambient temperature usually enables microbial multiplication (Kneifel *et al.*, 2002). According to Kneifel *et al.* (2002) and Martins *et al*. (2001) application of hot water in extraction compensates for microbiological contaminations, since boiling water reduces the viable counts by several log units and inactivates possible pathogens.

2.7.3 Storage and packaging of HMP

According to Martins *et al*. (2001) and Fennel *et al.* (2004) prolonged storage of HMP in poorly ventilated storehouses usually increases moisture content in the bulk due to heat exchange capacity, rendering herbs more susceptible to bacteria growth and toxin production. Some of microbes are regarded as normal flora on the raw plants and they have ability to survive in dried HMP (WHO, 2007). Efunyoye (1996) reported occurrence of pathogenic bacteria like *B. cereus*, *Aeromonas hydrophila, Shigella* spp., *Enterobacter*

agglomerans, *E. cloacae*, *Vibrio fluvialis*, *Pasteurella multocida*, *S. epidermidis*, *Acinetobacter iwoffii*, *Klebsiella* spp., *B. subtilis, S. aureus* and *Pseudomonas aeruginosa* in HMP which were stored for sale in markets. This showed that herbal drugs were hazardous for human health if not stored in proper conditions. WHO (2007) recommends that fresh medicinal plant materials should be stored at appropriate low temperatures, ideally at 2 - 8°C; frozen products should be stored at less than -20°C. Processed HM should be packaged into impermeable materials as quickly as possible to prevent contamination of the product and to protect against unnecessary exposure to potential pest attacks (Martins *et al*., 2001).

2.7.4 Good Agriculture and Collection Practices of HMP

Good agriculture and collection practices (GACP) are specific principles and methods that should be applied to primary production and during herbs collection process, to create safe and wholesome HMP for the consumers (WHO, 2003). Appropriate and consistent quality of HMP can be attained by establishing GACP systems from primary production to final product (FAO, 2013). According to WHO (2003), primary production, collections, preparations and processing of HMP have direct influences on the quality of the Active Pharmaceutical Ingredients (API) and microbial quality. FAO (2013) suggested that all HMP must be grown and handled properly after collection in order to meet good manufacturing practices (GMP) quality standards. GACP is an essential practice for ensuring that herbal raw materials are accurately identified and not adulterated with contaminants. Therefore, GACP is mandatory to be practiced in the whole chain of HMP production to ensure quality and safety so as to safeguard the health of consumers (WHO, 2007).

2.8 Health and Economic Impact of Unsafe HMP

Food safety is an essential public health issue for all countries. FBD due to microbial pathogens, biotoxins, allergens, and chemical contaminants in HMP represent serious threats to the health of thousands of millions of people (WHO, 2007). Serious outbreaks of FBD have been documented on every continent in the past decades, illustrating both the public health and social significance of these diseases. HMP consumers everywhere view FBD outbreaks with ever-increasing concern. Outbreaks are likely, however, to be only the most visible aspect of a much broader, more persistent problem. FBD not only significantly affect people's health and well-being, but they also have economic consequences for individuals, families, communities, businesses and countries. These diseases impose a substantial burden on health-care systems and markedly reduce economic productivity. Poor people tend to live from day to day, and loss of income due to food borne illness perpetuates the cycle of poverty (FAO, 2013).

The cost of FBD is estimated to exceed $5 billion per year in the United States (Foegeding *et al*., 1994). Economic burden on people in India affected by an outbreak of *Staphylococcus aureus* food poisoning was found to be higher than in case of a similar outbreak in the US (Sudhakar *et al.,* 1988).

2.8.1 Prevention and control of microbial contaminants in HMP

According to WHO (2007), prevention and control of microbial quality is the process whereby microorganisms are prevented from getting into HMP from human carriers by general improvements in water supplies, public health education, environmental and personal hygiene. Pathogenic bacteria from primary materials can be controlled through GACP and those from environmental and packaging materials can be prevented by manufacturers and vendors adhering to principles of environmental cleanliness and

general hygiene practices (WHO, 2007). Generally, the HMP should be manufactured in compliance with GACP from the starting material onwards; the herbal preparation should be manufactured in compliance with GMP (Foster, 2002). Therefore, according to Foster (2002), WHO (2007) and FAO (2013) microbial quality control can be well achievable during cultivation, harvesting, storage, processing, packaging and transportation, at these stages possibility of reducing the microbial bio-burden is higher compared to finished HMP.

2.9 Antimicrobial Resistance (AR)

Microbial resistance to antimicrobials usually mediated through resistance gene-coded bacterial plasmids (Cheesbrough, 2000). Plasmids are self-replicating extra-chromosomal DNA molecules found in Gram negative and Gram positive bacteria. These plasmids called R-plasmids harbour a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds which include antibiotics, heavy metals, mutagenic agents such as formaldehyde (Cheesbrough, 2000; Foster, 2002). According to Foster (2002) and Katakweba (2014) resistant bacteria strains may develop almost anywhere particularly in pressurized environment containing previously non-resistant bacteria strains such environments include HMP. The HMP have been previously implicated as a pool for such contaminations (Esimone *et al.,* 2007).

Over the last two decades, development of antimicrobial resistance resulting from agricultural use of antimicrobials that could impact on the treatment of diseases affecting the human population that require antibiotic intervention has become a significant global public health concern (Oliver *et al*., 2011). According to WHO (2014), global report for antimicrobial resistance surveillance that involved *E. coli, K. pneumoniae* and *S. aureus* showed that, the proportion resistance to commonly used specified antibacterial drugs

exceeded 50% in many settings. Nonga *et al*. (2010) and Katakweba *et al.* (2012) reported antimicrobial resistance happening due to extensive use and misuse of antibiotic drugs in veterinary and human health practices.

2.9.1 Detection of AR in the population and HMP samples

Guidelines for standard methods of broth and agar disc diffusion susceptibility and molecular testing has been developed (CLSI, 2008). These methods describe all aspects of the testing procedure including media specifications, bacterial inoculums, solvents and diluents for each antimicrobial, incubation time and temperature and interpretive criteria for categorical analyses (CLSI, 2008; CLSI, 2012). The validity of results can be objectively evaluated by testing quality control organisms along with the isolates under investigation (CLSI, 2008). The selection of quality control organisms is based on the drugs tested and each drug should have a corresponding organism with a quality control range including the concentrations tested. Commonly used quality control organisms for testing non-fastidious aerobic bacteria include *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (CLSI 2008). Bacterial isolation is carried out and the isolates are subjected to a panel of antibiotics and detection of resistant bacteria (CLSI 2012).

2.9.2 Phenotypic methods of detecting AR

Both quantitative and qualitative tests have been described for phenotyping bacteria for AR (Adeleke and Omafuvbe, 2011). Quantitative tests include disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test (Adeleke and Omafuvbe, 2011). The tests are inhibitory rather than killing of bacteria and the end results are reported as susceptible, intermediate and resistant. Qualitative Results are reported as minimal inhibitory concentration in μg/ml or mg/l (Adeleke and

Omafuvbe, 2011). Of these, the disk diffusion and the broth microdilution tests are the most commonly used in both human and veterinary medicine. Disk diffusion method is used to determine the antimicrobial agent sensitivity profiles of the bacteria. The minimum inhibitory concentration is measured and recorded as whether the organism is susceptible (S), intermediately susceptible (I) and resistant (R) to the antibiotics (Adeleke and Omafuvbe, 2011; CLSI, 2008; Prescott *et al*., 2005).

2.9.3 Effects of AR in human health

Resistance to antimicrobial agents (AR) has resulted in morbidity and mortality from treatment failures and increased health care costs (WHO, 2014). The effects of antimicrobial resistance in animals and humans were also highlighted by Kanyeka (2014) to be causes of treatment failure to commonly human diseases, increases of food borne illness, greater mortality and morbidity and increased of health care costs due to hospitalization and expensive drug choices. Hence, indiscriminate use of antimicrobials in agriculture increases the possibility of antimicrobial resistant bacteria that may be transferred to humans through consumption of HMP infected with resistant strains and exposed consumers to various chronic bacterial diseases (Prescott *et al*., 2005; Kanyeka, 2014; WHO, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

This study was conducted in Morogoro Municipality in Morogoro Region, Tanzania from November 2014 to June 2015. The municipality lies along longitudes 35.6^{o}E to 37.4^{o}E of the Greenwich Meridian and latitudes 5.7°S to 6.5^{o}S of Equator (Fig. 1). It is located about 195 kilometres to the west of Dar es Salaam, the commercial capital city of Tanzania. The municipal is situated at the foot of Uluguru mountain whose peak is about 1 600 feet above sea level. The human population of Morogoro Municipality was 315 866 of which 151 700 are males and 164 166 are females (NBS, 2012). The population density of the municipality was about 1,095 people per square kilometre. There were 77 040 households, with an average of four people per household (NBS, 2012). The selection of Morogoro Municipality, as study area, was made base on convenience of accessibility from the laboratory (because of limited resources allocated for this present study), and it generally represents population HM vendors in urban areas of Tanzania.

Morogoro Municipality residents receive their PHC services from health facilities owned by the government of Tanzania, individuals, Faith Based Organisations (FBO) and others from HMP vendors. The ratio of people to health facility was 7,421:1; about 30% of people live more than 5 km away from the nearest health centre. There were two hospitals and the ratio of bed to patient was 1:1,000 and one physician per 23 188 people (UN-HABITAT, 2009).

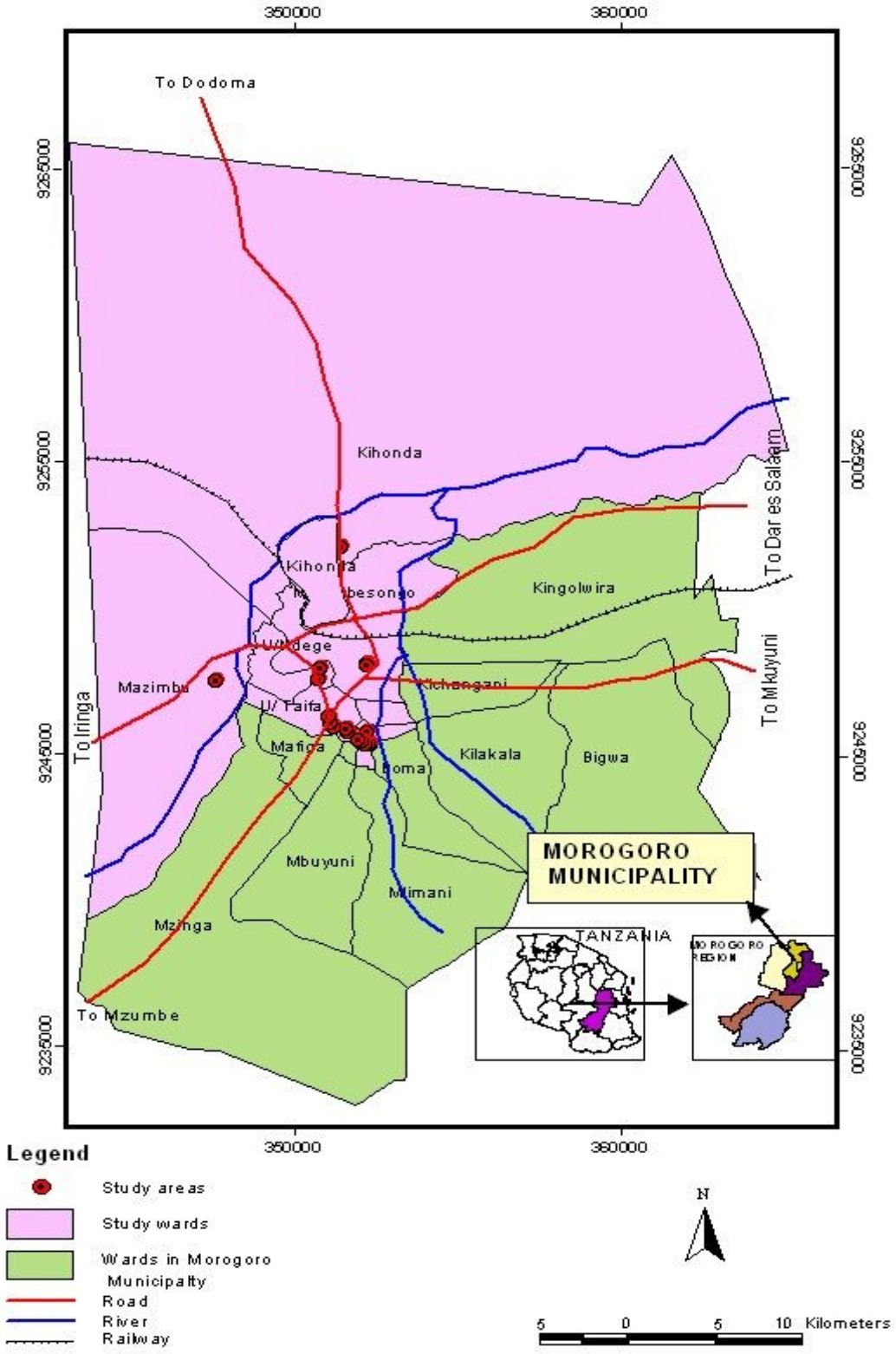

Figure 1: A map of Morogoro Municipality showing study areas

3.2 Study Design

A cross-sectional descriptive study was conducted that involved purchasing of HMP for laboratory analyses. In addition, a checklist (Appendix 1) was used to collect information on HMP handling practices, packaging materials, water used for preparation and state of the product collected from HMP vendors. Demographic information and information on HMP safety training of the vendors were gathered. The checklist contained three main categories of information to be collected: (i) The HMP vending environment (sanitary status) (ii) Personal hygiene practices – wearing of personal protective equipment (PPE) and presence of hand washing facilities. (iii) Conditions for storage and packaging materials (cool/refrigeration storage for liquid HMP, suitability of containers and other packs used). Absence or presence of each component was recorded.

3.3 Ethical Consideration of the Study

Research permit was provided by the Vice Chancellor of Sokoine University of Agriculture. The permission to conduct the study was approved by Morogoro Municipality authority and the consent was signed by study participants before starting the study (Appendix 2). All the information obtained from participants and the laboratory results obtained after HMP samples analysis were kept under the custody of the researcher as confidential and publication of the research results will adhere to publication ethics.

3.4 Sample Size and Sampling Techniques

3.4.1 Sample size determination

A formula by Kothari (2004) for unknown population (i.e. $n = Z^2SD^2/e^2$) was used to calculate the sample size for this study. Where Z, is the estimated standard variation at 95% Confidence interval (CI) which was considered the point of the normal distribution corresponding to the level of significance (Z=1.96). Standard deviation (SD) was

estimated at 0.205 or 20.5% (Waiganjo, 2013) and e is the estimated error and was considered at 0.05 or 5%. Therefore, the sample size ‘n’ was calculated as:

$$n = \frac{(1.96)^2 \times (0.2)^2}{(0.05)^2} = 61.45 \text{ (Approximately 62)} \ldots\ldots(1)$$

Note that, because of financial constraints, availability of vendors and limited time allocated for this present study only 50 samples were used.

3.4.2 Sampling techniques

Fifty samples were purchased randomly from vendors around Morogoro Municipality. The HMP samples were collected from 24 different vendors, approximately eight samples from each of six sites. Samples purchased from HMP vendors included the following sites: Mji Mkuu (Morogoro main market), Kihonda, Mafisa (Msamvu bus terminal), Mazimbu, Mawenzi market and Sabasaba market (Fig.1). The vending sites were selected based on the population of HMP vendors and preliminary visits made by the researcher. The vendors were blinded on the actual reasons for purchasing the medicines to avoid bias and encourage cooperation.

3.5 Sample Collection for Laboratory Analysis

Approximately, 10 g or 10 ml for each product were purchased and packed aseptically in sterile zipped polythene bags and plastic bottles in case of liquid samples. All samples were coded for identification purposes and stored in a cool box with ice packs during field work before being shipped to Pest Management Centre (PMC) laboratories at Sokoine University of Agriculture (SUA) for bacteriological analyses. The laboratory analysis of the samples was conducted on the same day.

3.6 Sample Preparation

The sample containers were opened under aseptic conditions and 10 g aliquot(s) placed into a sterile container by using wooden tongue depressors for powders and sterile pipette was used to transfer 10 ml of liquid herbal product into 90 ml of sterile normal saline. Then 1 ml of the prepared homogenate inoculum obtained by vortexing was transferred into a test tube containing 9 ml of sterile physiological saline. The procedure was repeated up to ten dilutions and in the last dilution 1 ml of inoculum was discarded.

3.7 Determination of Prevalence and Intensity of Bacteria in HMP

3.7 1 Laboratory media and reagents preparation

The media and reagents used for laboratory analyses were prepared according to manufacturer's instructions. Descriptions for preparation and handling of media and reagents are as detailed below:

3.7.2 Nutrient Agar (NA)

The medium NA (DIFCO Laboratories, USA Lot 132932XG) composed of 5 g/l Bacto peptone, 3 g/l Bacto Beef extract, 15 g/l Bacteriological agar and final pH of 6.8 ± 0.2 at 25°C. Twenty three grams of the powdered medium were dissolved into 1000 ml of distilled water, mixed well until the mixture was uniformly mixed. Then the mixed solution was heated with gentle agitation and boiled until completely dissolved. The medium solution was sterilized in the autoclave at 15 psi; 121°C for 15 minutes then allowed to cool to 45^{o}C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility. All of the stock prepared media were stored under refrigeration temperature for future use.

3.7.3 Buffered peptone water (BPW)

The BPW powder (Oxoid® Ltd., Basingstoke, Hampshire, England, CM0509, and Lot 1442805) composed of 10 g/l Peptone, 5 g/l Sodium chloride, 3.5 g/l Di-sodium phosphate and 1.5 g/l Potassium di-hydrogen phosphate and final pH 7.2 ± 0.2 at 25°C. Twenty grams of the powdered medium were added into 1000 ml of distilled water. The culture medium was mixed well and each 10 ml were dispensed into capped test tubes. Then, the test tubes were sterilized by autoclaving at 15 psi, 121°C for 15 minutes and cooled to 25°C before use. All of the stock prepared media were stored under refrigeration temperature for future use.

3.7.4 Violet Red Bile Glucose (VRBG) agar

The VRBG medium (Oxoid® Ltd., Basingstoke, Hampshire, England, CM0485, Lot 1437851) composed of 7 g/l Peptone, 5 g/l Sodium chloride, 1.5 g/l Bile salts No. 3, 10 g/l Glucose, 3 g/l Yeast extract, 0.03 g/l Neutral red, 0.002 g/l Crystal violet, 12 g/l Agar and final of pH 7.4 ± 0.2 at 25°C. Thirty eight and half grams (38.5 g) of the powdered medium were suspended into 1000 ml of distilled water. The medium was boiled for 60 seconds with frequent agitation to dissolve completely. There was no further sterilization. Then, the media was mixed well and placed into water bath set at 48ºC for use within 3 hours from preparation time.

3.7.5 MacConkey agar

The medium (Oxoid® Ltd., Basingstoke, Hampshire, England, CM0007, Lot 1367351) composed of 20 g/l Peptone, 10 g/l Lactose, 5 g/l Bile salts, 5 g/l Sodium chloride, 0.075 g/l Neutral red, 12 g/l Agar and final of pH 7.4 ± 0.2 at 25°C. Fifty grams of the powdered medium were suspended into 1000ml of distilled water. The medium was boiled to dissolve completely followed by sterilization by autoclaving at 15 psi, 121°C for 15

minutes and cooled to ≤ 45°C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility.

3.7.6 Coagulase plasma

Rabbit Coagulase Plasma (Himedia Laboratories Pvt Ltd, Mumbai 40086 India, Ref. FD 248- 1VL, lyophilized 10 x 15 ml rabbit plasma with 0.15% ethylene di-amine tetra-acetate and 0.85% sodium chloride, contains dry natural rubber). The medium was prepared by reconstituting the powder with 5 ml of sterile distilled water followed by gentle shaking. Then, each 0.2 ml of the media was dispensed into test tubes for coagulase test.

3.7.7 Mueller-Hinton Agar (MHA)

The MHA (Oxoid® Ltd., Basingstoke, Hampshire, England, and CM0405 Lot 1009158) composed of 300 g/l Beef, dehydrated infusion, 17.5 g/l Casein hydrolysate, 1.5 g/l Starch, 17 g/l Agar and final pH of 7.3 ± 0.1 at 25°C. Thirty eight (38) grams of the powdered medium were suspended into 1000 ml of distilled water, mixed well and brought to boil to dissolve the medium completely. Then, the medium was sterilized by autoclaving at 15 psi, 121°C for 15 minutes, cooled to below 45°C and poured into sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility.

3.7.8 Mannitol Salt Agar (MSA)

The MSA ("Nissui" Ltd., Japan, code 05236, lot 639007) is composed of 10.1g/l protease peptone, 1.000 g/l Beef extract, 71.3 g/l Sodium chloride, 10g/l Dimannitol, 0.025g/l Phenol red and 13.8g/l Bacteriological agar and final pH of 7.4 ±0.2 at 25°C. A total of

111 g of the powdered medium were dissolved into 1000 ml of distilled water, mixed well until the mixture is uniform. The media solution was sterilized in the autoclave at 15 psi; 121°C for 15 minutes then allowed to cool to 45^{o}C and poured onto sterile Petri dishes. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility.

3.7.9 Blood Agar (BA)

The BA (Oxoid® Ltd., Basingstoke, Hampshire, England, CM0271 Lot 1037003) is composed of 15.0 g/l protease peptone, 2.5 q/l liver digest, 5.0 g/l Yeast extract, 5.0 Sodium chloride and 12.0 g/l Bacteriological agar and final pH of 7.4 ±0.2 at 25°C. Forty (40) grams of the powdered medium were dissolved into 1000 ml of distilled water, mixed well and left on the bench to stand until the mixture is uniform. The medium solution was sterilized in the autoclave at 15 psi, 121°C for 15 minutes then allowed to cool to 45^{o}C, then mixed with the whole blood 10% of total volume of horse blood and poured onto sterile Petri dishes. The reconstitution and mixing was performed in a flask with 2.5 times the volume of medium to ensure adequate aeration of blood. The plates were left at room temperature for two hours for the media to solidify then put upside down in the incubator for 24 hours at 37°C to check for sterility. All of the stock prepared media were stored under refrigeration temperature for future use.

3.7.10 Normal saline solution

The solution was prepared by dissolving 8.5 g of Sodium chloride (Sigma-Aldrich, Co., USA, Catalogue S5886, Lot SLBC3215V) into 1000 ml of sterile distilled water, mixed well and sterilized by autoclaving at 15 psi, 121°C for 15 minutes and cooled to below 45°C, the solution was ready for use.

3.8 Laboratory Analysis of HMP

There were two kinds of laboratory analyses performed to the HMP samples. The first was analysis for prevalence and intensity of bacteria in HMP which involved establishing the TVC and isolation of *E. coli and S. aureus.* The second analysis was to establish antimicrobial susceptibility of bacteria isolated from HMP, with focus on *E. coli* and *S. aureus*.

3.8.1 Determination of total viable counts

The samples for colony counts were serially diluted in sterile physiological normal saline from 10^{-1} to 10^{-10}. Using sterile pipette 1 ml of each dilution was inoculated into each plate containing NA. The plates were incubated at 37°C for 24 hours (±3 hours). Colony counts were done using marker pen and reported as colony forming units (cfu)/g or (cfu)/ ml (ISO, 2013) (Fig. 2).

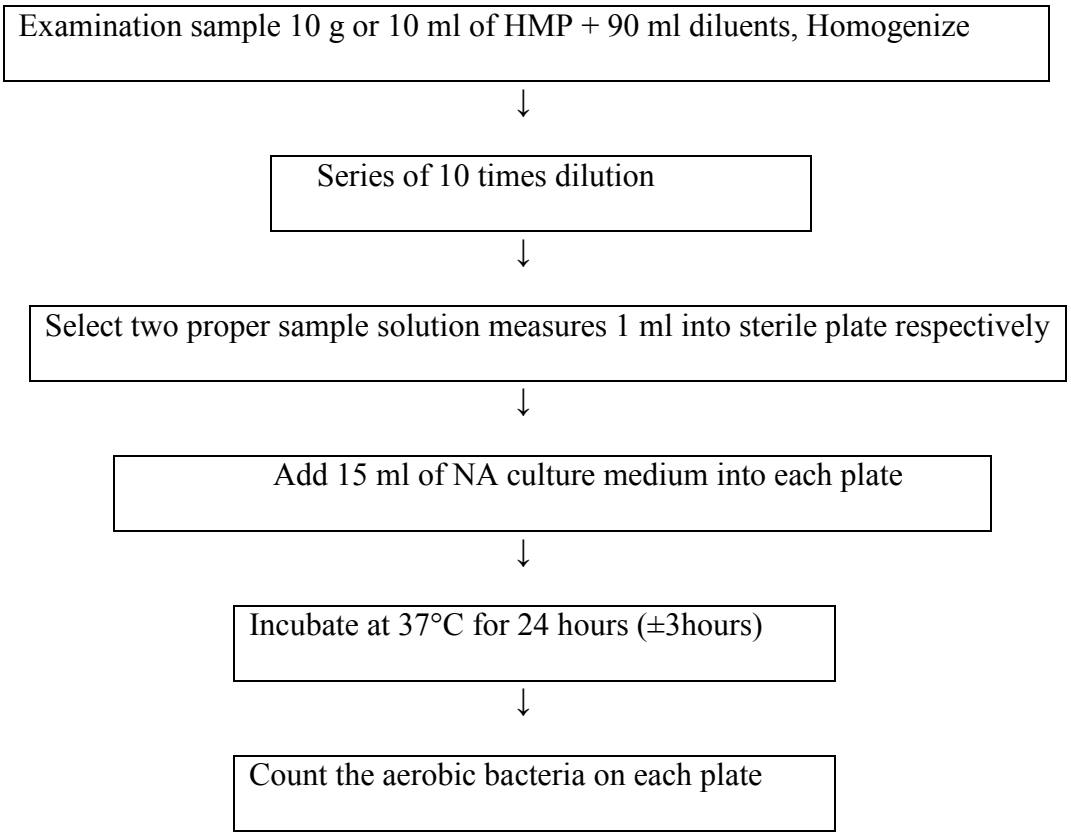

Figure 2: The examination procedures of TVC in HMP

3.8.1.1 Microbial colonies calculation

The countable microbial colonies from two consecutive plates of each sample were converted into colony forming units per millilitre or gram (cfu/ml or cfu/g) using an internationally accepted formula (ISO, 2013)**:**

$$N = \frac{\sum C}{V \times (n_1 + n_2) \times d} \quad \text{.....(2)}$$

Where; N = number of microbial colonies counted, C = sum of colonies identified on two consecutive dilution steps, where at least one contained 10 colonies, V = volume of inoculum on each plate in ml and d = dilution rate corresponding to the first dilution selected (the initial suspension is a dilution), n_1 = number of plates for first critical dilution and n_2 = number of plates for the second critical dilution selected (ISO, 2013).

3.8.2 Isolation and identification of faecal coliforms

The dilutions were prepared for initial suspensions as described for TVC in Fig. 2 above. Thereafter, about 10 ml of VRBG agar at 44ºC- 47ºC was poured into two Petri dishes. Using a sterile pipette 0.1 ml of the test sample was transferred from the last dilution to each of the Petri dishes. The inoculum and the media were carefully mixed by rotating the Petri dishes and allowed to solidify at room temperature. After solidification, a covering layer of about 5 ml of the VRBG agar was added onto Petri dishes to prevent spreading growth and to achieve semi-anaerobic conditions. The contents were allowed to solidify again. Thereafter, the plates were inverted and incubated at 37ºC for 24 hours. After incubation period, the plates were examined for typical and atypical colonies of coliforms. Typical colonies were pink to red or purple, with or without precipitation haloes or colourless mucoid colonies, with a diameter of 0.5 mm to 3.0 mm. By using a new sterile pipette for each dilution, the procedure was repeated as above with further dilutions up to

the first dilution and for the remaining test samples, only consecutive critical dilution steps were chosen for the inoculation on plates.

3.8.2 Isolation of *Escherichia coli*

Isolation and identification of *E. coli* was done by sub culturing of a single colony from primary culture in section 3. 8. 2. The presumptive isolates of *E. coli* were sub-cultured from VRBG agar plates and then restreak into MacConkey agar plates to obtain pure colonies for *E. coli*. The plates were inverted and incubated at 44.5ºC for 24 to 48 hours. Presumptive E. coli colonies were identified to species level by Gram staining and (IMViC) tests (indole, methyl red, Voges Proskauer and citrate) were carried out to confirm the presence of *E. coli* (Benson, 2002).

3.8.3.1 Confirmation of *E. coli*

Gram staining and biochemical reactions (indole tests) were performed to isolated colony from NA plates to confirm the presence of *E. coli* in the tested samples. Parallel with the test samples, a control strain of *Escherichia coli* (ATCC® 25922) was used.

3.8.3.1.1 Gram staining technique for *E. coli* and *S. aureus*

The Gram staining of the bacterial colony was done on a sterile glass slide as described by Cheesbrough, (2000). A drop of normal saline was placed on a glass slide and a loop full of well-isolated bacteria colony was added. A smear was made dried in air and fixed by gently flaming. A fixed smear was covered with crystal violet stain for about two minutes then, rapidly washed with slowly running tap water and again the smear was covered with Lugol's iodine for about two minutes and washed again with tap water. Thereafter, acetone-alcohol was used to decolorize the fixed smear and washed for the third time. Then, the fixed smear was covered with counter (stain neutral red) that stayed for about

two minutes then washed off with running tap water. The slide with smear was placed on a draining rack for the smear to dry. A drop of oil immersion was added on the smear and examined under the light microscope with 100× objective to visualize the morphology of the bacteria.

3.8.3.1.2 Indole test

Peptone water was prepared and about 3 ml of it was dispensed in bijou bottles using a sterile pipette. Then, fresh sterile loops were used to pick a well-isolated colony of bacteria and inoculated into bijou bottles. Thereafter, the bottles were incubated at 37ºC for 48 hours. After incubation 0.5 ml of Kovac's Indole Reagent (Loba Chemie Pvt. Ltd, Lot LM01131303) was added to the inoculated bijou bottles. The bottles were subjected to gentle shaking and examined for red colour in the surface layer within 10 minutes (Cheesbrough, 2000). In Fig. 5 a red ring on top of the tubes indicated indole positive reaction confirming presence of *E. coli*.

3.8.4 Isolation and identification of *S. aureus*

According to Abba *et al*. (2009) 1 g or 1 ml of the sample was suspended into 25 ml of peptone water in sterile McCartney bottle and incubated for 18 hrs at 37°C. Isolation of the *S. aureus* was achieved by streaking the pre-enriched culture from the peptone water onto selective differential agar plates of freshly prepared MSA; a selective and differential medium used for the isolation of pathogenic staphylococci. The plates were incubated at 37°C for 24 hours under aerobic conditions. Colonies showing golden yellow colour or colourless were presumed to be *Staphylococcus* spp. On MSA, pathogenic *S. aureus* produces small colonies surrounded by yellow zones as a result of mannitol sugar fermentation. These colonies were sub-cultured onto NA for purification and masking the effect of acid produced during fermentation of MSA to the biochemical tests. The colonies

selected from NA were subjected to Gram stain, to check the morphology and staining characteristics. The gram positive *cocci* organisms were subjected to catalase test to differentiate between *Staphylococcus* spp. and *Streptococcus* spp. Catalase positive colonies were further subjected to slide and tube coagulase test for the confirmation of *S. aureus* (Cheesbrough, 2000).

3.8.4.1 Confirmation of Coagulase Positive *Staphylococcus* (CPS)

Confirmation of CPS was done by sub-culturing selected typical and/or atypical colonies from NA for both Haemolysis and Coagulase test as biochemical test for confirmation of *S. aureus.*

3.8.4.1.1 Haemolysis test

The Horse Blood agar plates were inoculated with colonies from NA to determine the haemolytic reactions. Using a sterile loop an isolated colony from each cultured plate was inoculated and stabbed on single space on the BA plate. A control culture was stabbed at the same time. The plates were inverted and incubated at 37ºC for 24 hours, after which the plates were examined for haemolysis and compared with the controls. A β-haemolysis was confirmatory for *Staphylococcus* spp.

3.8.4.1.2 Coagulase test

The Rabbit Plasma was used for the test. From incubated test tube containing NA broth, 0.1 ml of each test culture was aseptically added to 0.2 ml of the rabbit plasma in eppendorf tube and incubated at 37ºC for 4–6 hours. The tubes were examined for clotting and if the test was negative, tubes were re-examined again at 24 hours of incubation. The test was considered to be positive if the clot occupied more than half of the original volume of the liquid. Parallel with test samples, controls were also tested. As a positive

control, 0.1 ml of sterile NA broth inoculated with a known *S. aureus* was added to 0.2 ml of rabbit plasma in test tube and incubated. Also, for a negative control 0.1 ml of sterile NA broth was added to 0.2 ml of rabbit plasma and incubated without inoculation.

3.8.5 Determination of antimicrobial susceptibility

Evaluation of antimicrobial susceptibility for isolated bacteria was performed on MHA by agar disc diffusion method according to CLSI (2012). *S. aureus* and *E. coli* were inoculated into MHB and incubated at 37^{o}C for 24 hours. Each isolate was inoculated in a Petri dish containing MHA and overlaid with antimicrobial discs with amoxicillin (20µg), ciprofloxacin (5µg), gentamycin (10 µg), cefatoxime (30 µg), nalidixic acid (30 µg), oxacillin (1 µg), co-trimoxazole (1.25/23.75 µg) and vancomycin (30 µg). Thereafter, the plates were incubated under aerobic conditions at 37°C for 24 hours. After incubation period, the plates were examined for zones of inhibition around the discs. Diameters of inhibition zones around the discs were measured in millimetres using a metal calliper or transparent ruler, and the results were recorded and classified as resistant (R), intermediate (I) and sensitive (S) according to CLSI, (2012). The isolates of *E. coli* and *S. aureus* that were resistant to three or more of the eight classes of antimicrobial agent used in this study were defined as having multiple antimicrobial resistances. Standard reference strains of *S. aureus* (ATCC 25922) and *E. coli* (ATCC 25923) were used as quality control organisms in antibiotic susceptibility determination.

3.9 Data Analysis

Laboratory results were summarized and stored into MS-Excel spread sheets. Data collected by checklist were analysed by using SPSS (2007) version 16.0. Descriptive statistics particularly percentages, means and counts from responses were used to determine distributions and magnitudes of variables. A student t- test was used to find

statistical differences between the WHO limits and sample microbial counts. One way analysis of variance (ANOVA) was adopted to compare differences in means of TVC in six wards

CHAPTER FOUR

4.0 RESULTS

4.1 Sources of HMP Samples for Laboratory Analysis

A total of 50 samples were examined from 24 different HMP vendors in six selected wards of Morogoro municipality. Table 3 shows the distribution and forms of HMP samples collected for laboratory analyses.

Table 3: Distribution and forms of HMP samples collected during the study in six selected sites in Morogoro municipality, Tanzania, 2014/15

Ward/site	Number of vendors (n)	Number of samples per site	Form of HMP	
			Powder samples	Liquid samples
Kihonda	3	8	6	2
Msamvu main bus terminal	3	7	5	2
Morogoro main market	5	11	9	2
Sabasaba market	5	11	7	4
Mawenzi Market	4	6	4	2
Mazimbu	4	7	6	1
Total	**24**	**50**	**37**	**13**

4.2 Socio- demographic Information of HMP Vendors

The socio-demographic information of 24 vendors in the six HMP vending sites is presented in Table 4 where most of the vendors were males. Whilst more than half (58.31%) of the vendors had some primary school education, approximately 33.3% had not attended any formal training. The median age of the vendors was 54.5 years, ranging

from 15 to 70 years old. Most of the vendors had not received any training on quality assurance and safety of HMPs.

Table 4: Socio-demographic characteristics of HMP vendors involved in the study

Characteristics assessed	Category	Number of respondents (n) (%)
Gender	Male	15 (62.50)
	Female	9 (37.50)
Age	10–29	6 (25.00)
	30 – 49	4 (16.67)
	50– 69	9 (37.50)
	70+	5 (20.83)
Level of education	No formal education	8 (33.33)
	Standard I – VII	14 (58.31)
	Secondary education	2 (8.33)
	College education	0 (0.00)
Attended training on safety of HM	Yes	2 (8.30)
	No	22 (91.70)

4.3 Environmental Factors that Could Predispose HMP to Microbial Contamination

It was observed that location of vending sites and some traditional methods of handling HMP could lead to contamination from environments, equipment and handlers (Table 5). Nevertheless, statistical associations were not analysed because of small sample size. Only approximately 20.8% of vendors sold their HMP in reasonably clean well planned places (Table 5). Generally, it was observed that most HMP vending sites were dirty and unsuitable for marketing.

Table 5: Environmental factors that could predispose HMP to microbial contamination

Factor	Category	Number and percentage (n) (%)
Possibility of HMP to encounter cross-contamination*	High	9 (37.5)
	Moderately	15 (62.5)
	Low	0 (0.0)
Presence of any hand washing facilities or disinfectant and does it minimize contamination	Yes	1 (4.2)
	No	23 (95.8)
Presence of any waste disposal facility	Yes	5 (20.8)
	No	19 (79.2)
Presence of contaminant materials such as food wastes and dusts around the vending site	Yes	15 (62.5)
	No	9 (37.5)
HMP protected from sources of contamination	Yes	7 (29.2)
	No	17 (70.8)
Location of the vending site	Near to disposal pits	1 (4.2)
	Slums	2 (8.3)
	Crowded area	16 (66.7)
	Well arranged	5 (20.8)

* A site was classified as poor if HMPs were displayed on ground and there were dirty materials and there were high possibilities of cross contamination, moderately if displayed on table and there were few of dirty materials around, that can get into HMP and effectively if displayed on table or shelves, low possibilities of dust or any dirty materials get into the HMP.

4.4 Handling Practices that Could Predispose HMP to Microbial Contamination

It was observed from this study that most of HMP handlers could cause microbial contamination of their HMP during handling. Based on the observation made to vendors, it

was found that, approximately 95.8% of the HMP vendors did not wear any personal protective equipment (PPE). Other practices were identified and thought to likely facilitate contamination of HMP vended as shown in Table 6 and well displayed in Fig. 3. These include poor personal hygiene, lack of safety knowledge on expiry dates of the medicines, and reuse of unsafe packaging materials and containers.

Figure 3: Practices that may predispose HMP to contamination. Note that, vendors are re-using packaging materials (A) and (B) handling their products in the open air markets on the ground, that can be a source of contaminants in HMP.

Table 6: Practices that could predispose HMP to microbial contamination at vending sites

Practice	Observations (score rates)	Number of respondents (n) (%)
Personal hygiene:		
General cleanness of the vendors	Good	(16) 66.7
	Poor	(8) 33.3
Vendor wearing PPE	No	(23) 95.8
	Partial	(1) 4.2
Package and storage of HMP:		
Manufacturer	Own	(14) 58.3
	Import	(10) 41.7
Ability of the packaging material to prevent contamination	Yes	(7) 29.2
	No	(17) 70.8
HMP packed in sealed containers	Yes	(7) 29.2
	No	(17) 70.8
Availability of refrigeration facility for liquid HMP	Yes	(0) 0.0
	No	(24) 100
Raw HM separated from processed products	Yes	(12) 50.0
	No	(12) 50.0
Ways of determining the expiry dates	Colour changes	(1) 4.2
	Sensory evaluations	(1) 4.2
	Date	(3)12.5
	Unknown	(19)79.2

4.5 Microbiological Quality of HMP Vended in Morogoro municipality

4.5.1 Total Viable Counts (TVC)

A total of 50 HMP samples purchased from vendors in Morogoro municipality were cultured for total TVC in NA (Appendix 3)**.** It was found that 44 (88%) of the samples had microbial contamination. The counts ranged from 9.09×10^4 to 1.64×10^8cfu/g or ml. Mean TVC for liquid and powder HMP were 1.4×10^7cfu/ml and 9.26×10^5cfu/g respectively, which is higher than recommended level of $\leq 10^3$cfu/ml for material that are intended for human consumption and $\leq 10^5$cfu/ ml for pre-treated material that will undergo further treatments (BP, 2007; WHO, 2007). The overall results indicated that all contaminated samples had higher TVC than the recommended level of 10^3cfu/ml for herbal medicines intended for human consumption by WHO/BP international standards for HMPs. Comparison of TVC between six wards showed that more HMPs from Kihonda had more microbial load with mean of $(2.12 \pm 2.04) \times 10^7$ cfu/ml. However, the variation in bacteriologic parameters between these six wards was not statistically significant (p= 1.035).

4.5.2 Prevalence and intensity of coliform bacteria

A total of 50 HMP samples were cultured for coliforms count, identification and isolation of *E. coli*. Out of those 21 (47.73%) had positive growth of coliforms with a mean growth of $2.15 \times 10^5 \pm 3.9 \times 10^4$ cfu/ ml ranging from 2.2×10^4 to 1.0×10^6 cfu / ml. The mean coliform count established was significantly higher than international standards for HMP ($P = 0.045$) (BP, 2007; WHO, 2007).

4.5.3 Specific bacteria isolated from HMP samples

In this study the prevalence rates of *E. coli* and *S. Aureus* were 5/50 and 4/50, respectively. Figure 4, 5 and 6 displays laboratory results for cultured plates and confirmation tests for

E. coli and *S. aureus*, respectively. From the results it was noted that the liquid HMP were more contaminated with *E. coli* and *S. aureus* than powder products. The variation between these two forms was statistically significant (*P*= 0.016). Other bacteria isolated were *Enterobacter* spp., *Bacillus* spp., *Staphylococcus epidermidis*, *Klebseilla pneumonia* and *Pseudomonas aeuroginosa*.

4.5.3.1 Results of Gram staining and biochemical test for *E coli* and *S aureus* isolates

4.5.3.1.1 Gram stains

Plates (**A)** and (**B)** in Fig. 4 show Gram negative bacteria appeared as rods with pale to dark red colour and Gram positive bacteria appeared *cocci* in shape with pale to dark purple colour. The plates were used to make preliminary confirmation tests for *E coli* and *S. aureus* isolates.

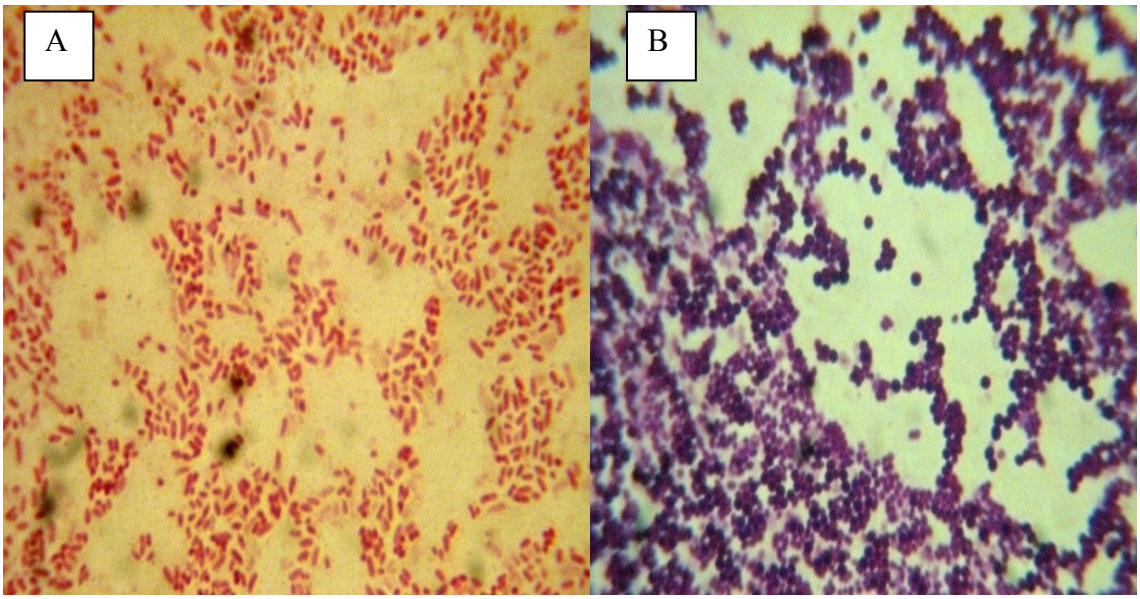

Figure 4: Plate (A) shows Gram negative rods of *E. coli* while plate (B) shows Gram positive cocci of *S. aureus* in smear of presumptive colonies of bacteria cultured from HMP, as seen using the 100× oil objectives

4.5.3.1.2 Results of biochemical analysis

Figure 5 below shows plate of MacConkey agar with presumptive colonies of *E. coli* (i) whilst (ii) is Kova's indole test performed to confirm the presence of *E. coli* in HMP

samples. Figure 6 displays positive growth colonies of *Staphylococci* spp. which characterized by golden yellow colour in cultured plates of MSA media (a) and (b) coagulase test for *S. aureus* isolated from HMP, where the test tubes show coagulase positive tests that confirm the presence of *S. aureus* in the tested samples.

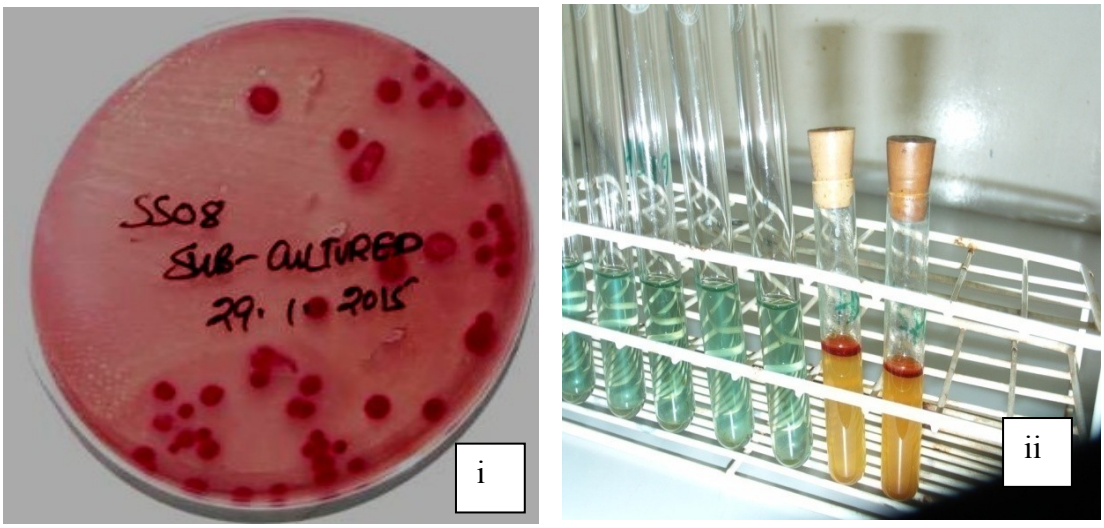

Figure 5: Plate of MacConkey agar with typical colonies of *E. coli* (i) and plate (ii) is the confirmatory test for *E. coli* isolated from HMP. Note that, the test tubes with red ring covers on the top after addition of Kova's indole reagent were positive for *E. coli*.

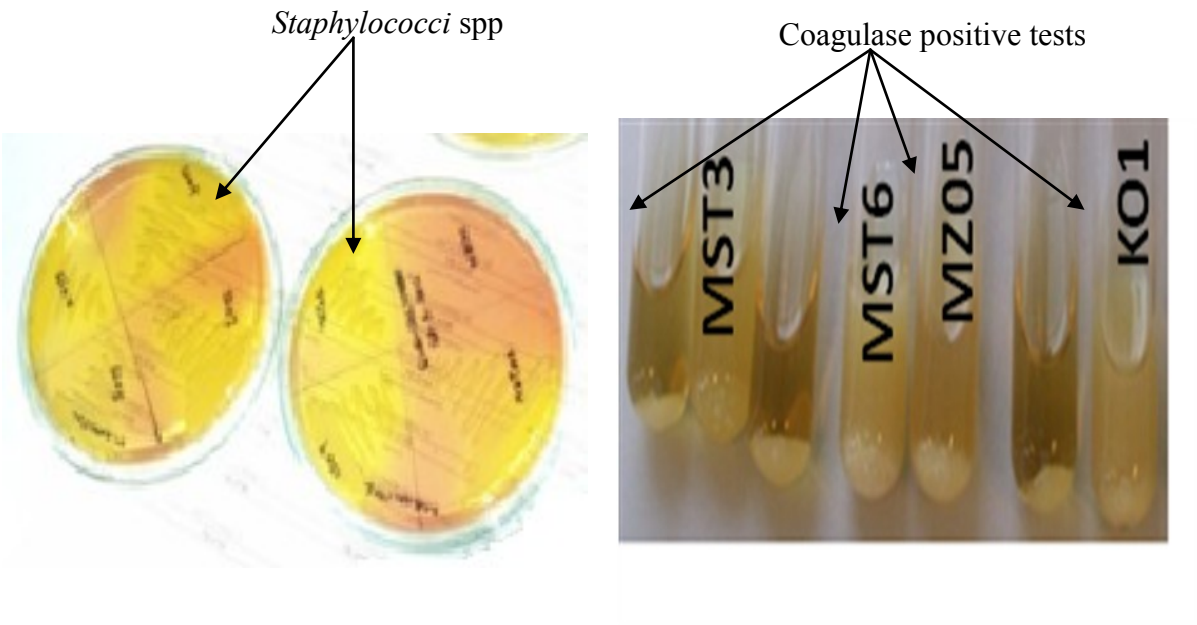

Figure 6: Plates of MSA with positive growth of *Staphylococci* spp. the colonies characterized by golden yellow colour and the right are test tubes for coagulase test, where MST6, MZO5 and K01 show coagulase positive tests, whilst MST3 shows coagulase negative test.

4.6 Antimicrobial Susceptibility test for *E. coli* and *S. aureus* Isolates from HMP

4.6.1 Antibiotics susceptibility profile of *E. coli* isolates from HMP

Five *E. coli* isolates from HMP samples were tested for susceptibility to eight (8) different antibiotics, which showed resistance levels as detailed in Table 7. Higher resistance was observed in oxacillin (OX), vancomycin (VA), cefatoxime (CTX) and co-trimoxazole (SXT). There was no resistance in ciprofloxacillin (CIP) and nalidixic acid (NA) while low resistance was observed for amoxicillin-clavulanic acid (AMC) and gentamycin (CN).

Table 7: Antibiotics susceptibility profile of *E. coli* isolates from HMP vended in Morogoro municipality

Name of antibiotic	Number of bacteria tested	Number of resistance bacteria (%)	Number of intermediate bacteria (%)	Number of susceptible bacteria (%)
AMC	5	1 (20)	1(20)	3 (60)
CIP	5	0 (0.0)	0 (00)	5 (100)
CN	5	1 (20)	1 (20)	3 (60)
CTX	5	4 (80)	0 (0.0)	1 (20)
NA	5	0 (0.0)	0 (00)	5 (0.0)
OX	5	5(100)	0 (0.0)	0 (0.0)
SXT	5	2 (40)	0 (0.0)	3 (60)
VA	5	4 (80)	1(20)	0 (0.0)
Total	**40**	**17 (42.5)**	**11 (27.5)**	**12 (30)**

Key: AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, CN = gentamycin, CTX = cefatoxime, NA = nalidixic acid, OX= oxacillin, SXT = co-trimoxazole, VA = vancomycin.

4.6.2 Antibiotics susceptibility profile of *S. aureus* isolated from HMP

Four *E. coli* isolates from HMP samples were tested for susceptibility to eight (8) different antibiotics, which showed resistance levels as detailed in Table 8**.** All four *S. aureus* isolated in this study showed resistance to NA while two were resistant to VA and one to CTX. On the other hand, all the isolates were susceptible to the rest of antimicrobials tested.

Table 8: Antibiotics susceptibility profile of *S. aureus* isolates from HMP vended in Morogoro municipality

Name of antibiotic	Number of bacteria tested	Resistance bacteria (%)	Intermediate bacteria (%)	Susceptible bacteria (%)
AMC	4	0 (0.0)	0 (0.0)	4 (100)
CIP	4	0 (0.0)	1 (25)	3 (75)
CN	4	0 (0.0)	1 (25)	3 (75)
CTX	4	1 (25)	2 (50)	1 (25)
NA	4	4 (100)	0 (0.0)	0 (0.0)
OX	4	0 (0.0)	0 (0.0)	4 (100)
SXT	4	0 (0.0)	0 (0.0)	4 (100)
VA	4	2 (50)	1 (25)	1 (25)
Total	**32**	**7 (21.9)**	**5 (15.6)**	**20 (62.5)**

Key: AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, CN = gentamycin, CTX = cefatoxime, NA = nalidixic acid, OX= oxacillin, SXT = co-trimoxazole, VA = vancomycin.

4.6.3 General antimicrobial resistance patterns of *E. coli* and *S. aureus* tested

Generally, it was observed that *E. coli* and *S. aureus* were susceptible to ciprofloxacin but resistant to cefatoxime(CTX) and vancomycin (VA) with resistance level ranging from 20% to 100% (Fig .8). Furthermore, in this study Gram negative bacteria (*E. coli*) were susceptible to CIP and NA but showed more resistance against CTX, OX, SXT and VA. Gram positive bacteria (*S. aureus*) showed high resistance against NA and VA as displayed in Table 7 and 8 respectively.

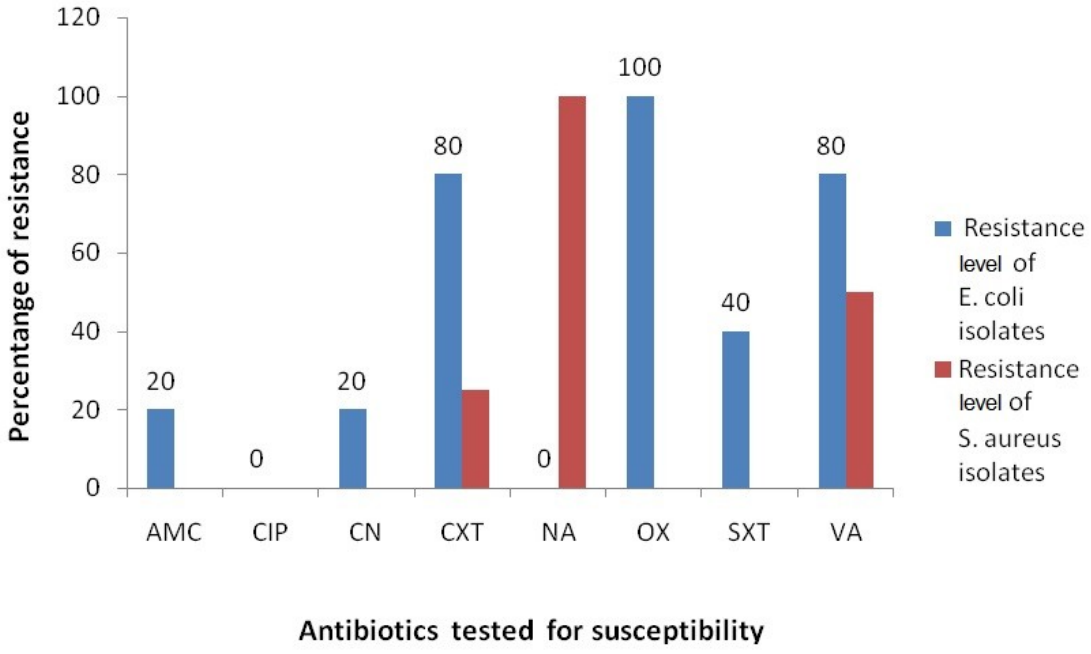

Figure 7: Resistance pattern for *E. coli* and *S. aureus* bacteria isolated from HMP samples collected from six sites in Morogoro municipality, Tanzania, 2014/15.

Key: AMC= amoxicillin-clavulanic acid, CIP = ciprofloxacillin, CN = gentamycin, CXT = cefatoxime, NA = nalidixic acid, OX= oxacillin, SXT = co-trimoxazole and VA = vancomycin.

CHAPTER FIVE

5.0 DISCUSSION

The general purpose of this study was to estimate prevalence and antimicrobial susceptibility of *E. coli* and *S. aureus* isolated from HMP vended in Morogoro Municipality, Tanzania. The study detected high prevalence of microbial contamination in HMP. The presence of microbes in HMP could be due to several factors observed, including traditional handling practices and exposition of products in a polluted environment. Other factors were use of contaminated raw materials, untreated water during preparations and packaging in unsterilized materials. Similar findings were reported in Dar es salaam, Tanzania by Temu *et al*. (2009) and elsewhere by Foster (2002), Oleyege and Adelabu (2010), Surekha (2011), Meshack *et al*. (2013) and Ruth (2013). Another study in agreements with this present study was carried out by Bisset (1994) who suggested that, HMP can be contaminated with microorganisms, pesticides, aflatoxins, radioactive substances and heavy metals during growth and storage. Also the present study revealed a high number of samples had higher TVC than the levels that are acceptable globally (BP, 2007; WHO, 2007). The presence of *E. coli* in some samples indicates faecal contamination whilst presence of *S. aureus* suggests poor storage, personal hygiene and handling practices by HMP vendors. Other bacteria found in HMP as contaminants were *Bacillus* spp. *Pseudomonas aureginosa*, *Enterobacters* spp. and *Klebseilla pneumoniae*. This implies that an important proportion of HMP vended in Morogoro municipality were contaminated by pathogenic microorganisms. This poses health risks to the consumers of the HMP.

This study further revealed that HMP business in Morogoro municipality was dominated by males and many vendors had not attended any formal education. Furthermore, the HMP

vendors had not received any training on quality assurance and safety of HMP. The finding in this present study concurs with studies carried out by Temu *et al*. (2009) and Ruth (2013) who reported most of HMP business in Dar es salaam, Tanzania and Haiti being dominated by men. A study conducted in the United States of America found that women demonstrated safer food preparation and handling techniques compared to men (Klontz *et al*., 1995). Although in the present study women were less educated than men, it is not known as to whether formal education is associated with handling practices. The high microbial contamination found in this study might be contributed by lack of safety knowledge and handling practices of HMP. This could not be analysed statistically given the small sample size, but might be due to the fact that most of HMP vendors do not undergo any training before launching their business.

Most HMP vendors were operating their businesses under unhygienic environments, including poor methods of handling their products. This could be among sources of microbial contamination. In addition, the HMP vended were not certified and did not have any product information label. This could lead to public health risks of consuming unauthorised as well as expired products. Studies by the FAO/WHO (2007), Abba *et al*. (2009), Temu *et al*. (2009), Oleyege and Adelabu (2010), Surekha (2011), Meshack *et al*. (2013) and Ruth (2013) reported contamination of HMP due to polluted environments, personnel involved in preparations, poor storage, lack of adequate portable water for extraction, lack of hand washing facilities, poor personal hygiene and use of poor packaging materials. These are similar to the findings of the present study. The HMP vendors should be given necessary training to safeguard consumers of these products.

The present study further found that most of the HMP had higher TVC than acceptable levels from the public health point (WHO, 2007; BP, 2007). This implies that most HMP sold in Morogoro municipality are not safe for human consumption from microbiological

point of view. Presence of high TVC in HMP indicates possible contamination from various factors such as raw materials; dirty equipment and packaging materials and/or handlers (Efunyoye, 1996; WHO, 2007; Abba *et al*., 2009; Temu *et al.,* 2009). Further studies are needed to assess HMP production to identify appropriate areas for intervention to safeguard health of HMP consumers

Coliforms bacteria were detected in (21) 47.73% of the HMP samples analysed. It is known that presence of coliforms in particular FC suggests faecal contamination, which is normally associated with uses of untreated water for preparations and poor hygienic practices in handling HMP. No coliform is allowed in any product intended for human consumption (BP, 2007; WHO, 2007). *E. coli* is a well-known enteropathogen and is the most common cause of childhood diarrhoea of bacterial origin (Bonkoungou *et al.*, 2013). *E. coli* is also used as a marker of faecal contamination (Jay, 1997). The results from present study are in line with another study in Kenya that reported 20.5% of HMPs use for oral cares having contamination of faecal coliform bacteria (Waiganjo, 2013). The observation cannot be ignored since there are several strains known to be highly pathogenic with a potential of causing illness to consumers. Some strains of *E. coli* are verocytotoxigenic like enterohaemorrhagic type of *E. coli* O157:H7 that are known to cause haemorrhagic colitis (John *et al*., 2001). *E. coli* O157:H7 is known to cause deadly diarrhoea in humans and consumption of contaminated food is reported to be among important routes of transmission of these pathogenic bacteria (Temu *et al*., 2009; Meshack *et al*., 2013). Therefore, isolation of *E. coli* in HMP is a serious public health fault and prompt intervention measures are needed to address the problem. Further studies are needed to characterise *E. coli* isolated from HMP to see if they are pathogenic strains.

Staphylococcus aureus has been previously reported to occur in HMP in Tanzania by Temu *et al.* (2009), in Kenya (Kaume *et al*., 2012; Meshack *et al*., 2013), in Nigeria (Abba *et al*., 2011; Oleyege and Adelabu, 2010) and in India by Surekha (2011). This bacterium species was also detected in the present study. Though *S. aureus* are defined as normal skin, mucous membranes of humans and animals flora it is an important cause of food poisoning following ingestion of preformed heat-resistant toxins. Lowy (1998) associated *S. aureus* with a number of complications especially to immune-compromised individuals. These bacteria are able to produce heat stable enterotoxins responsible for tissue damage and may release exotoxins which cause gastroenteritis (Cheesbrough, 2000; Prescott *et al*., 2005). Isolation of these contagious pathogens in HMP especially in developing countries is highly related to use of unhygienic equipment and poor personal hygiene practices in handling of HMP by most vendors. Education on the importance of GACP, GMP and personal hygiene should be provided to producers, vendors and consumers (Rocha *et al*., 2011).

Bacteria isolated in this study were resistant to some of the commonly used antimicrobials such as AMC, CXT, VA, OX, NA and SXT. These are frequently used in the community and in our indigenous health facilities. Studies conducted earlier by (Kagashea *et al*., 2010; Nonga *et al*., 2010; Katakweba *et al*., 2012 and Kanyeka, 2014) reported similar prevalence of bacterial resistance to commonly used antibiotics due to indiscriminate use of antimicrobials in human health and livestock practices. The development of resistance to bacteria isolated from HMP could be due to exposition of products in contaminated environments, inappropriate use of antimicrobials in treatment of plant and animal fungal and bacterial borne diseases and introduction of already developed resistant strains from personnel who were handling HMP. Foster (2002), Oliver *et al*. (2011), Esimone (2007) and Rahimi and Nayebpour (2012) reported resistance of bacteria isolates from herbs,

animal foods and humans due to inappropriate use of antimicrobials in agriculture for prevention and control of animal and plant bacteria-borne diseases, exposition of herbs in the polluted environment whereby pollutant agents such as heavy metals and mutagenic agents such as formalaldehaydes. Waiganjo (2013) also reported herbal materials used in management of oral health conditions by traditional practitioners in Nairobi, Kenya, having ability of preventing growth of bacteria. This implies that some of HMP have antimicrobial elements. The presence of antimicrobial in HMP increased the possibilities of bacteria to develop resistance mechanism against specific antimicrobials. Esimone *et al*. (2007) reported the ability of *S. aureus* strain isolated from HMP to produce penicillinase that was associated with resistance against penicillin containing antimicrobials. The high rate of resistance to antibacterial agents by strains isolated from herbal preparation indicates widespread antibiotics resistance among microorganisms from different sources (Esimone *et al*., 2007). Therefore, the presence of antibacterial resistant pathogens has an important public health implication especially in developing countries like Tanzania where there is widespread and uncontrolled use of antimicrobials for agriculture, animal husbandry and treatments of human bacterial-borne diseases (Kagashea *et al*., 2010; Katakweba *et al*., 2012). The present study did not analyse for any association between antimicrobial resistance and the intended use of the HMP. There is a need for further studies to determine actual active ingredients of the HMP and their association with intended uses of the products as well as their association with antibiotic resistance.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMEDATIONS

6.1 Conclusions

This study has shown that unregulated HMP marketed in Morogoro Municipality are highly contaminated with microbes, some of which are potentially pathogenic such as *E. coli* and *S. aureus*. The presence of microbes in HMP could be due to poor handling practices including exposition of products in a polluted environment, contaminated raw materials, poor manufacturing practices, use of untreated water during mixture preparations, contaminated packaging materials and low level of safety knowledge of HMP vendors. On the other hand, some of the commonly used antimicrobials have lost their potent against some of potentially pathogenic bacteria, which is likely due to indiscriminate use and presence of contaminants in HMP. Such unregulated medicinal products may facilitate transmissions of communicable diseases and increased difficulties in treatment of bacteria-borne infections which were initially easily treated by regular antimicrobials.

6.2 Recommendations

Based on the findings of this study, it is recommended that:

i. Authorised food and drug agents should perform routine assessment of the quality of HMP vended and consumed by patients and other consumers in order to safeguard the health of general public from bacterial infections and other contaminants which might be caused by consumption of unsafe herbal products.

ii. Strict hygienic measures should be applied during HMP preparation and handling, achievable through formal training of producers especially farmers and vendors on good agriculture and collection practices and good manufacturing practices.

iii. Health officers, food scientists and extension officers and associated public health stakeholders should make periodic surveillance of HMP manufactures and vendors to create monitoring system on the practices and update on GACP and GMP. Also, educate public on effects of inappropriate use of antimicrobials for treatment of humans, animals and plant diseases.

iv. Moreover, there is a need for further research to establish chemical profile and potency, pattern of microbial contamination, and antimicrobial resistances for bacteria isolated from HMP countrywide so as to implement appropriate control measures.

v. The presence of some other pathogens such as *Bacillus anthracis*, *Clostridium perfringens, L. monocytogenes, Salmonella* spp. *and Vibrio cholera* have not been assessed in this study due to financial and time constraints, further studies should consider testing for these pathogens given their public health significance. Also it could be interesting to perform molecular characterization of isolated *E. coli* in this study to know if they are enteropathogenic strains.

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APPENDICES

Appendix 1: Check list used to assess possible factors for bacterial contamination in HMP vended in Morogoro Municipality, Tanzania, 2014/15

A). Information researcher supposed to inquire before procurement of HMP products.

Location: ……

Age: ……

Sex: …….

Education level: ……

Any training attended on HMP: ……..…….....

HMP code……

Common name of HMP.....Species name.....

What part of plant this Herbal product is coming from?

What type of illness/ailment normally treated or prevented by this HMP product?

Who is the manufacturer of this HMP?

How long this will take to Expiry from the time it was manufactured/ processed?

How can someone determine the expiry date?

How to process/ prepare the HMP before use?

Observational table for quality assurance (Tick the right option without asking)

Item	Options
Vending environment	
Does exposition of products setting minimize cross-contamination?	Effectively( ) Moderately( ) poorly( )
Is there any washing facilities or disinfectant? If Yes, does it minimize contamination?	Yes ( ) No ( ) Effectively( ) Moderately( ), poorly( )
Waste receiving receptacle present	Yes( ) No ( )
Presence of contaminant materials (Food wastes, dusts and others) around vending sites	Yes ( ) No ( ) if yes: Specify ----------)
Is the HMP protected from sources of contamination?	Yes ( ), No ( )
Locality of the vending	Sewer ( ) near to disposal pits ( ) slums ( ) crowded ( ), well arranged area ( )
Personal hygiene	
General cleanliness of the handler/hawkers?	Very good( ) Good( ) Poor( )
Vendor/ hawker has working uniform	Fully( ) Partial( ) None( )
Storage and package Materials	
Does the package material prevent contamination (safety and quality)	Yes( ) No ( )
HMP package in sealed container	Yes( ) No ( )
Is there refrigeration facilities	Yes ( ) No ( )
The raw HMP separated from processed products	Yes ( ) No ( )

Appendix 2: Volunteer Agreement Consent form for HMP Vendors

Title: Prevalence and antimicrobial susceptibility of bacteria isolated from herbal medicines vended in Morogoro municipality, Tanzania.

Principal Investigator: **Kira, Jonas Daniel**

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Email: jonasdkira@gmail.com Mobile phone: +255763516250

General information about research

The purpose of this study was to assess bacteriological quality and antimicrobial susceptibility of *E. coli* and *S aureus* bacteria isolated from HMP vended in Morogoro municipality, Tanzania.

Possible benefits, risks and discomforts

There are no direct benefits to be gained from this study immediately, neither are there any risks associated with it or payment that will be made direct to participants. The only inconvenience might come from the time you will spend some of you time to answer some questions that will be asked by investigator/enumerator during collection of samples for laboratory analyses. The sample(s) will be purchased at market price no sample will be requested for free from participants. The data from this study will be used only for the purpose of the study (Master dissertation).

Confidentiality

Your identity and your participation in this study will be treated strictly confidential. The information that we obtain from you will not be shared with anybody, except the study investigators. Your identity remains secret since your personal information will only be coded by a unique participant number. Your name will not appear in any reports or

publications resulting from this study. After the study is completed, you may request information about the study results.

Voluntary participation and right to leave the research

You will participate entirely voluntarily in this study. You have the right to refuse to participate in the study**.** You also have the right to stop your participation in the study at any time, even after you have signed this informed consent form. The withdrawal of your consent will not cause any disadvantage or loss of advantages/privileges

Contacts for additional information

Any questions or any further clarifications concerning the study can be directed to:

My research supervisors:

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   Morogoro- Tanzania

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I ---------- do hereby agree/ declare to participate in this study.

Name of Site ----------Signature----------Date----------

Appendix 3: Laboratory results for microbial analyses of HMP samples collected from 24 different HMP vendors in six sites of Morogoro Municipality, Tanzania, during a study, 2014/15.

S/N	Sample code	Form of THMP	TVC								Identification and isolation of E .coli				Identification of S. aureus			
			C1	C2	∑C	Volume of inoculum (V)	(N1+N2*0.2)	Dilution Factor (D)	V*(N1+N2 * 0.2)*D	∑C/V*(N1+N2 *0.2)*D (cfu/g or ml)	MacConkey Agar	Gram stain	Indole Tests	Citrate tests	MSA	Gram stain	BA (Haemolysis Test	Coagulase Tests
1	K01	Liquid	89	72	161	1	1.1	1.00E-04	1.10E-04	1.46E+06	+	+	_	+	+	_	_	_
2	K02	Powder	28	15	43	1	1.1	1.00E-04	1.10E-04	3.91E+05	+	_	_	+	_	_	_	_
3	K03	Powder	0	0	0	1	1.1	1.00E-04	1.10E-04	0.00E+00	NA	NA	NA	NA	NA	NA	NA	NA
4	K04	Powder	93	84	177	1	1.1	1.00E-04	1.10E-04	1.61E+06	_	_	_	_	_	_	_	_
5	K05	Powder	0	0	0	1	1.1	1.00E-04	1.10E-04	0.00E+00	NA	NA	NA	NA	NA	NA	NA	NA
6	K06	Liquid	91	73	164	1	1.1	1.00E-08	1.00E-06	1.64E+08	+	_	_	+	+	_	_	_
7	K07	Powder	100	92	192	1	1.1	1.00E-04	1.10E-04	1.75E+06	_	_	_	_	+	_	_	_
8	K08	Powder	35	29	64	1	1.1	1.00E-04	1.10E-04	5.82E+05	_	_	_	_	+	_	_	_
9	MBT 01	Powder	103	60	163	1	1.1	1.00E-04	1.10E-04	1.48E+06	_	_	_	_	+	_	_	_
10	MBT 02	Powder	99	72	171	1	1.1	1.00E-04	1.10E-04	1.55E+06	_	_	_	_	_	_	_	_
11	MBT03	Powder	87	47	134	1	1.1	1.00E-04	1.10E-04	1.22E+06	_	_	_	_	+	+	+	+
12	MBT04	Liquid	23	16	39	1	1.1	1.00E-04	1.10E-04	3.55E+05	_	_	_	_	_	_	_	_
13	MBT05	Powder	0	0	0	1	1.1	1.00E-04	1.10E-04	0.00E+00	NA	NA	NA	NA	NA	NA	NA	NA
14	MBT06	Liquid	67	55	122	1	1.1	1.00E-04	1.00E-05	1.22E+07	_	_	_	+	_	_	_	_
15	MBT07	Powder	43	34	77	1	1.1	1.00E-04	1.10E-04	7.00E+05	_	_	_	+	_	_	_	_
16	MM01	Powder	153	103	256	1	1.1	1.00E-04	1.10E-04	2.33E+06	_	_	_	_	_	_	_	_
17	MM02	Powder	81	19	100	1	1.1	1.00E-04	1.10E-04	9.09E+05	_	_	_	_	_	_	_	_
18	MM03	Powder	76	43	119	1	1.1	1.00E-04	1.10E-04	1.08E+06	_	_	_	_	_	_	_	_
19	MM04	Powder	87	69	156	1	1.1	1.00E-04	1.10E-04	1.42E+06	+	+	_	+	_	_	_	_
20	MM05	Liquid	65	61	126	1	1.1	1.00E-04	1.10E-04	1.15E+06	_	_	_	_	+	_	_	_
21	MM06	Liquid	15	9	24	1	1.1	1.00E-04	1.10E-04	2.18E+05	_	_	_	_	+	_	_	_
22	MST01	Powder	69	54	123	1	1.1	1.00E-04	1.10E-04	1.12E+06	_	_	_	_	_	_	_	_
23	MST02	Powder	221	109	330	1	1.1	1.00E-04	1.10E-04	3.00E+06	_	_	_	_	+	+	_	_
24	MST03	Liquid	70	43	113	1	1.1	1.00E-04	1.10E-04	1.03E+06	_	_	_	_	+	_	_	_
25	MST04	Powder	41	28	69	1	1.1	1.00E-04	1.10E-04	6.27E+05	_	_	_	_	+	+	+	_
26	MST05	Powder	37	35	72	1	1.1	1.00E-04	1.10E-04	6.55E+05	_	_	_	_	_	_	_	_
27	MST06	Powder	6	4	10	1	1.1	1.00E-04	1.10E-04	9.09E+04	_	_	_	_	_	_	_	_
28	MST07	Powder	37	35	72	1	1.1	1.00E-04	1.10E-04	6.55E+05	_	_	_	_	_	_	_	_
29	MST08	Powder	137	69	206	1	1.1	1.00E-04	1.10E-04	1.87E+06	+	_	_	+	_	_	_	_

30	MST09	Liquid	17	14	31	1	1.1	1.00E-04	1.10E-04	2.82E+05	+	_	_	+	_	_	_	_
31	MST10	Powder	16	12	28	1	1.1	1.00E-04	1.10E-04	2.55E+05	+	+	+	_	+	+	+	+
32	MST 11	Powder	32	24	56	1	1.1	1.00E-04	1.10E-04	5.09E+05	_	_	_	+	_	-	_	_
33	MZ01	Powder	62	50	112	1	1.1	1.00E-04	1.10E-04	1.02E+06	+	_	_	+	_	-	_	_
34	MZ02	Powder	25	23	48	1	1.1	1.00E-04	1.10E-04	4.36E+05	+	_	_	+	_	-	_	_
35	MZ03	Powder	20	17	37	1	1.1	1.00E-04	1.10E-04	3.36E+05	+	_	_	+	_	-	_	_
36	MZ04	Powder	35	28	63	1	1.1	1.00E-04	1.10E-04	5.73E+05	+	_	_	+	_	-	_	_
37	MZ05	Liquid	21	13	34	1	1.1	1.00E-04	1.10E-04	3.09E+05	+	+	+	_	+	+	+	+
38	MZ06	Powder	0	0	0	1	1.1	1.00E-04	1.10E-04	0.00E+00	NA	NA	NA	NA	NA	NA	NA	NA
39	MZ07	Powder	14	10	24	1	1.1	1.00E-04	1.10E-04	2.18E+05	_	_	_	+	_	-	_	_
40	SS01	Powder	19	17	36	1	1.1	1.00E-04	1.10E-04	3.27E+05	_	_	_	+	_	-	_	_
41	SS02	Powder	16	12	28	1	1.1	1.00E-04	1.10E-04	2.55E+05	_	_	_	+	_	-	_	_
42	SS03	Liquid	52	38	90	1	1.1	1.00E-04	1.10E-04	8.18E+05	_	_	_	+	_	-	_	_
43	SS04	Powder	74	63	137	1	1.1	1.00E-04	1.10E-04	1.25E+06	_	_	_	+	_	-	_	_
44	SS05	Liquid	143	125	268	1	1.1	1.00E-04	1.10E-04	2.44E+06	+	+	+	_	+	+	+	_
45	SS06	Powder	174	153	327	1	1.1	1.00E-04	1.10E-04	2.97E+06	+	+	_	+	+	_	_	_
46	SS07	Powder	130	86	216	1	1.1	1.00E-04	1.10E-04	1.96E+06	+	+	+	_	+	_	+	_
47	SS08	Powder	71	52	123	1	1.1	1.00E-04	1.10E-04	1.12E+06	+	+	+	_	+	+	_	_
48	SS09	Liquid	93	84	177	1	1.1	1.00E-04	1.10E-04	1.61E+06					+	+	+	+
49	SS10	Liquid	66	49	115	1	1.1	1.00E-04	1.10E-04	1.05E+06	NA	NA	NA	NA	NA	NA	NA	NA
50	SS11	Powder	0	0	0	1	1.1	1.00E-04	1.10E-04	0.00E+00	NA	NA	NA	NA	NA	NA	NA	NA