A STUDY ON ANTIBACTERIAL ACTIVITIES OF ESSENTIAL OILS FROM MOST COMMONLY USED SPICES IN ZANZIBAR AGAINST MILK SPOILAGE BACTERIA

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ABSTRACT

Milk spoilage is a problem in different parts of Tanzania especially in areas with limited cooling storage facilities, poor hygiene and lack of preservatives. On the other hand, spices and herbs which are primarily used to impart flavour and aroma in food, contain essential oils with antimicrobial activity against wide range of food spoilage bacteria. The main objective of this study was to assess the antimicrobial activity of essential oils from the most commonly used spices and herbs in Zanzibar against milk spoilage bacteria. Questionnaires, focus group discussions (FGDs) and direct observations were used to assess the knowledge on the spices and herbs added in milk at the households' level. Steam distillation using a Clevenger system was used to extract essential oils from spices and herbs. The extracts were screened for antimicrobial activity and subsequently, minimum inhibitory concentrations (MICs) were determined by microdilution method. Results demonstrated that 96.4% ($P < 0.05$) of the respondents use spices and herbs in milk to add flavour, colour and for other uses such as traditional medicine. The most commonly used spices and herbs added in milk are cardamom, cinnamon, ginger and lemon grass ($P<0.05$). All the extracts demonstrated antimicrobial activity against *Streptococcus thermophillus, Lactobacillus plantarum* and *Escherichia coli*. The MICs ranged between 0.0039 and 0.1250 µg/µl. However there was no significant difference ($P > 0.05$) among all the extracts against the tested microbial strains. Further studies are recommended on other spices and herbs to investigate whether the inhibitory activities are due to bactericidal or bacteriostatic properties. Moreover, this study opens the avenue for possibilities of using essential oils from spices and herbs as a means of milk preservation and hence to overcome the income loss and food insecurity caused by milk loss through spoilage microorganisms.

DECLARATION

I, AMINA AHMED, 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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(MSc. Candidate)

The declaration above is confirmed by

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Prof. R. H. Mdegela Date

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DEDICATION

I dedicate this work to the late Dr. Joseph Magadula who was my co supervisor, all spice users all around the world and to my family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collection
Eo(s)	Essential oil(s)
Fig	Figure
FGDs	Focus Group Discussions
ITM	Institute of Traditional Medicine
MIC	Minimum Inhibitory Concentration
Mls	Millilitres
MUHAS	Muhimbili University of Health and Allied Sciences
n	Sample size
NCCLS	National Committee for Clinical Laboratory Standards
NISCIR	National Institute of Science Communication and Information Resources
NIN	National Institute of Nutrition
NIT	Nitrotetrazoleum
SPSS	Statistical Package for the Social Science
SUA	Sokoine University of Agriculture
USDA	U.S Department of Agriculture

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Milk is an important source of nutrients to human and animals. As a complete diet food, milk is meant to be the first and the only food for the offspring of mammals (Pandey and Singh, 2011). Being highly nutritious food, milk serves as an ideal medium for the growth and multiplication of various microorganisms (Shobana and Naidu, 2000). Milk spoilage is a problem in many developing countries due to limited cooling storage facilities and irregular supply of electricity (Lore *et al.,* 2005). Pasteurization is the best suggested way to overcome the problem of microbes in food products. On the other hand milk contains alkaline phosphatase, a natural enzyme with thermal death value of 4.8°C which is greater than that of the most heat-resistant non-spore-forming microbes commonly found in milk (Murthy *et al*., 1993). However, Gram-positive and rod shaped bacteria such as *Bacillus*, *Paenibacillus* and *Clostridium* genera (Thusitha *et al*., 2002; Murphy, 2010) develop resistant spores under high temperature. These spores can remain dormant for extended period of time until conditions become favourable to germinate into vegetative bacterial cells which are capable to initiate spoilage even in packed products. Some preservatives, such as potassium metabisulphite and sodium benzoate can be used to extend the shelf life of milk products such as a non-rennet cheese (*paneer*) spread (Dwivedi *et al*., 2014). On the other hand, consumers have been consistently concerned about possible adverse health effects caused by the use of the chemical additives in their foods. This perception has stimulated research interest in finding natural but effective food preservation method (Chen and Hoover, 2003).

Increased health consciousness among consumers on the nutritional and health benefits from consumption of natural products has resulted in highly acceptance of natural products (Srinivasan *et al*., 2001; Kumarasamy, 2002). Nevertheless, there are many natural plant extracts rich in several phytochemical compounds including antioxidants which play important roles in both food system and human body tissues (Jayasinghea *et al*., 2013). On the other hand, indigenous plants have been used in herbal medicine for curing various diseases for many centuries and of late there is highly acceptance of natural products (Srinivasan *et al*., 2001; Kumarasamy, 2002). Moreover, there are a number of spices and herbs that are routinely used in food and have antimicrobial activity against human, food or plant pathogens (Rakshit and Ramalingam, 2010). Spices and herbs, contain essential oils which render strong antiviral, antifungal and antimicrobial activities. These essential oils contain compounds which are integrated in mouthwash, medicine and anti-acids (Bhowmik *et al*., 2012). Vanilla (*Vanilla planifolia*) contains vanillin compound exhibiting inhibitory effects on *E. coli* and *Listeria innocua* (Fitzgerald *et al*., 2004). In addition, cardamom *(Elettaria cardamomum)* posses antibacterial activity against *Styphylococcus aureus, Escherichia coli* and *Salmonella typhimurium* (Ağaoğlu *et al*., 2005; Mahady, 2005).

However, application of essential oils as food preservatives requires detailed knowledge about their minimum inhibitory concentration (MIC), the range of target organisms, the mode of action, and the effect on food matrix components and their antimicrobial properties (Andrews, 2011). Therefore the objective of this work was to explore the antimicrobial activities and effectiveness of the essential oils from the most commonly used spices and herbs in Zanzibar.

1.2 Problem Statement and Justification

Milk being highly nutritious food with high water activity and poor hygiene during handling and storage, serves as an ideal medium for the growth and multiplication of spoilage microorganisms in areas with limited cooling storage facilities and irregular supply of electricity in Tanzania. On the other hand consumers are concerned about possible health effects from the use of chemical preservatives. Thus the core problem central to this study is high loss of milk due to spoilage microorganism leading to income loss and food insecurity. This prompted the assessment of potentials in essential oils from spices and herbs if used as an alternative to cooling storage facilities and chemical preservatives. It is hypothesized that essential oils can be used to inhibit or destroy spoilage microorganisms in milk and extend its shelf life, contribute to income generation and food security in Tanzania.

1.3 Objectives

1.3.1 Main objective

The main objective of this study was to assess the antibacterial activities of essential oils extracted from most commonly used spices in Zanzibar against milk spoilage bacteria.

1.3.2 Specific objectives

To achieve the main objective, the following specific objectives were proposed:

i. To assess the knowledge of people on use of spices and herbs as milk preservative in Zanzibar.
ii. To determine the antimicrobial activity of essential oils from the four spices and herbs against milk spoilage bacteria.
iii. To establish the Minimum Inhibitory Concentration (MIC) of essential oils from four spices and herbs against milk spoilage bacteria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Dairy Loss

Dairy loss is one of the major problems of the dairy industry in developing countries especially in Africa. FAO (2005), reported the highest loss in the informal milk chain is in Eastern Africa and the near East whereby over $90 million worth of milk get lost each year. In Kenya, Uganda and Tanzania, annual dairy losses added up to US$56 million for a year. In Kenya, around 95 million litres of milk was lost, at a value of around US$ 22.4 million per year. In Uganda, approximately 27% of all milk produced was lost: 6% wasted at the farm level, while 11% and 10% of production was either lost to spillage or spoilage during transport or marketing, respectively. Cumulative losses in Tanzania amounted to about 59.5 million litres of milk in a year, with annual loss of around $14.3 million. Losses occurring at the farm are attributed by unhygienic milk handling, poor milking procedures and spoilage due to lack of cooling facilities. Rejection of milk by consumers due to spoilage has been reported in Tanzania, though losses are not quantified (Lore *et al*., 2005).

Pasteurization has been used as a public health measure to eliminate or reduce the activities of spoilage and pathogenic microorganisms in milk (Thusitha *et al*., 2002). However *Bacillus* and *Streptococcus* species are likely to survive pasteurization due to their ability to form heat-resistant endospores. On the other hand, non-endospore-forming bacteria, including *Mycobacterium paratuberculosis*, *Listeria monocytogenes* and *Escherichia coli* serotype O157:H7 can also survive boiling at 63ºC for 15 minutes (Binderova and Rysanek, 1999; Grant *et al*., 1999).

2.2 Milk Spoilage

Bacterial succession is the main cause of milk spoilage. Some bacteria that survive pasteurization may gradually replace the original inhabitants through establishment of new species population. The succession of microbes in pasteurized milk follows the same sequence as observed in unpasteurized milk for *Streptococci*, *Lactobacilli*, Yeasts, Moulds and *Bacillus* species. *Streptococc*i and *Lactobacilli* ferment milk sugars resulting in increased acidity which favours the growth of moulds and yeast (Axtell, 2004). On the other hand, *Escherichia coli* is a Gram negative, non-spore forming rod shaped bacteria, a facultative anaerobe also ferments milk sugars to form lactic, acetic and formic acids which favour growth of other milk spoilage microorganisms (Doyle and Schoeni, 1984). Milk spoilage by these microorganisms result in unacceptable texture, odour, flavour and depletion in nutrients content of the milk.

2.3 Antimicrobial Compounds in Spices and Herbs

Selim (2010) signified that, the small quantities of volatile oils from spices and herbs added in foodstuffs without affecting organoleptic properties would reduce bacterial contamination. Phytochemical substances which are found in essential oils of spices such as cinnamon and cloves, act as mould inhibitor when added to bakery products as flavouring and aroma agents (Pandey and Singh, 2011; Paster *et al*., 1995). *Syzygium* species (Myrtaceae) which is traditionally used as anti-inflammatory remedy, is also capable of possessing antibacterial activity (Muruganadan *et al*., 2001).

2.3.1 Cardamom

Cardamom *Elettaria cardamomum* a member of Zingiberaceae family is primarily cultivated in Southern India, Sri Lanka, Guatemala and Tanzania. Normally, its darker seeds from a pod are used in cooking varieties of food at the household level (Rakshit and

Ramalingam, 2010). Its essential oil is used as a flavouring component in alcoholic and non-alcoholic beverages, frozen desserts, candies, baked goods, puddings, condiments, relishes, gravies, meat and meat products (Mahady *et al*., 2005; Rakshit and Ramalingam, 2010).

2.3.2 Cinnamon

Cinnamon, *Cinnamomum cassia* a member of Lauraceae family, contains important essential oil in leaves, fruits and barks. Cinnamon is used in medicine, pharmaceutical, perfumery, cosmetics and several other industries apart from its primary role in adding flavor in food and beverages (Rakshit and Ramalingam, 2010). Essential oil of cinnamon is also used as a rub to promote blood circulation in aromatherapy (Fabio *et al*., 2003, Chaudry and Tariq, 2006). Cinnamon essential oil contains both anti-fungal and anti-bacterial compounds that can be employed in prevention of food spoilage by microbes (Fabio *et al*., 2003).

2.3.3 Lemon grass

Lemon grass belongs to the section of Andropogan called *Cymbopogam* of the family Graminaceae (Gramineae or Poaceae). It is a very large genus which includes about 500 described species. *Cymbopogan citrates* and *C. flexuosus*, are only species which give essential oils when subjected to extraction process (NISCIR, 2005). The oil of lemon grass possess bactericidal and anti-fungal properties, which is comparable to penicillin in its effectiveness (Lutterodt *et al*., 1999). The vitamin C extract from lemon grass has been used in commercial drugs such as belladonna and jaborandi (Isam *et al*., 2009). Moreover, essential oils of lemon grass have been used in gallstone dissolving preparations (Elastal *et al.,* 2005). This use may be attributed by the characteristic diuretic effect of its essential oil (Stadtman, 1996).

2.3.4 Ginger

Ginger (*Zingiber officinale* Roscoe, Zingiberacae) is a medicinal plant that has been widely used in Chinese, Ayurvedic and Tibb-Unani herbal medicines all over the world. It has been used as natural remedy since ancient times for arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis (Ali *et al*., 2008). It is also believed to have anti-inflammatory and anti-allergy properties (Takeda *et al.*, 2007).

2.3 Importance of Spices

2.3.1 Food and beverages

Syzygium aromaticum, commonly known as cloves, and locally known as *kenepeli* and *kanumpari* by the Igala and Hausa ethnic groups of central and Northern Nigeria, respectively, is a common spice used in preservation of local beverages (Atawodi *et al*., 2011). Essential oils as natural preservatives, are useful in reduction and elimination of microbes, including experimentally inoculated *E. coli* O157:H7 (Selim, 2010). Devendra and Tanwar (2011) reported that, clove powder demonstrated higher microbial effect on chicken nuggets throughout the storage period when it was compared with control preparation.

2.3.2 Anti-cancer activity

Spices and herbs used in foodstuffs, are also beneficial in the reduction of the risks associated with cancer. Some spices such as clove and clove oil reduces the risk of lung, skin and digestive tract cancers (Schönfelder and Schönfelder, 2004).

2.3.3 Antioxidant

Antioxidants reduce oxidative stress, which cause different diseases such as diabetes, atherosclerosis and cancer. Extracts from spices inhibit oxidative rancidity and retard the development of off flavours in food products such as snacks and meat (Giese, 1994).

2.3.4 Painkiller

Many people in India have been using some spices in traditional medicine (USDA, 1997). People along the coastal zones including Zanzibar and Tanga, mix cloves with warm water or coconut oil as a remedy for relief from toothache and massage respectively (Bhowmik *et al*., 2012).

3.3.5 Digestion

Spices such as clove and its oil has been used in Chinese medicine in treatment of digestion problems, nausea and vomiting (Mahady *et al*., 2005; Takeda *et al*., 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

Baseline survey was conducted in Zanzibar assessing the people's knowledge on the use of spices and herbs in milk. According to the 2012 Population and Housing Census, the total population of Zanzibar was 1,303,569 of which 630,677 were males and 672,892 were females. Extraction of essential oils and antimicrobial tests were conducted at the Department of Natural Product Development and Formulations, Institute of Traditional Medicine at Muhimbili University of Health and Allied Sciences.

3.2 Study Design

A cross sectional study design involving direct observation, interviews and laboratory based experiments were conducted to establish the inventory of the most commonly used spices and amount of each spice or herb added in one litre of milk. The efficacy test of the four essential oils from mostly used spices was conducted on *S. thermophillus, L. plantarum* and *E. coli* ATCC 25922 using microdilution method.

3.3 Sample Size

Fifty six willing respondents from selected households in Zanzibar were interviewed to assess their knowledge on the use of spices and herbs in milk. The antimicrobial test involved three extracts of essential oils on three milk spoilage bacteria namely *S. thermophillus, L. plantarum* and *E. coli* ATCC 25922 were performed due to limited time and resources.

3.4 Materials and Equipments

In preliminary survey materials used included questionnaires, notebooks, camera and audio recording devices. In laboratory works, material, reagents and equipments used were

ginger, cardamom, lemon grass, cinnamon, water, La clavenger, laboratory films, culture plates (96 well microplates with their lids), 96 racks with sterile tips, bacterial cultures, 70% ethanol, cotton wool, bacterial test culture broth and nitrotetrazoleum (NIT indicator). Others were gloves, masks, autoclave, incubator plant extracts, autoclave, analytical balance, coops, distilled water, flasks, petri dishes, vials, media (Nutrient broth and soy agar), cotton wool, swab sticks and methylated spirit.

3.5 Data Collection

3.5.1 Preliminary survey

This survey aimed at capturing the knowledge of respondents on the milk and commonly spices and herbs added in milk.

3.5.2 Sample collection

3.5.2.1 Spices collection and handling

Fresh spices (lemon grass, cinnamon, ginger and cardamom) were purchased from Kizimbani farms in Zanzibar and transported to the ITM laboratory in Dar es Salaam. To avoid oxidation and evaporation of volatile oils, the raw materials were packed well and stored in freezer. Pictures of the raw spices and herbs are depicted in Fig. 1 below.

Ginger Lemongrass Cinnamon Cardamom

Figure 1: Pictures of raw spices whose essential oils were used in bioassay

3.5.2.2 Extraction of Essential Oils from four commonly used spices

The four most commonly used spices in milk (Table 4) were processed, weighed (Table 9) and subject for essential oils extraction using steam distillation by Clevenger apparatus (Fig. 2). To get maximum yield, temperature and time were controlled. Essential oil extracts were aseptically collected, stored in sealed vials and subsequently taken to the microbiology laboratory for the efficacy testing against *S. thermophillus, L. plantarum* and *E. coli* ATCC 25922.

3.5.2.3 Bacterial strains collection

Cultures of milk spoilage microorganisms named *Lactobacillus plantarum* and *Streptococcus thermophillus* were obtained from Food Science and Technology laboratory at SUA Morogoro while *E.coli* ATCC 25922 was obtained from ITM.

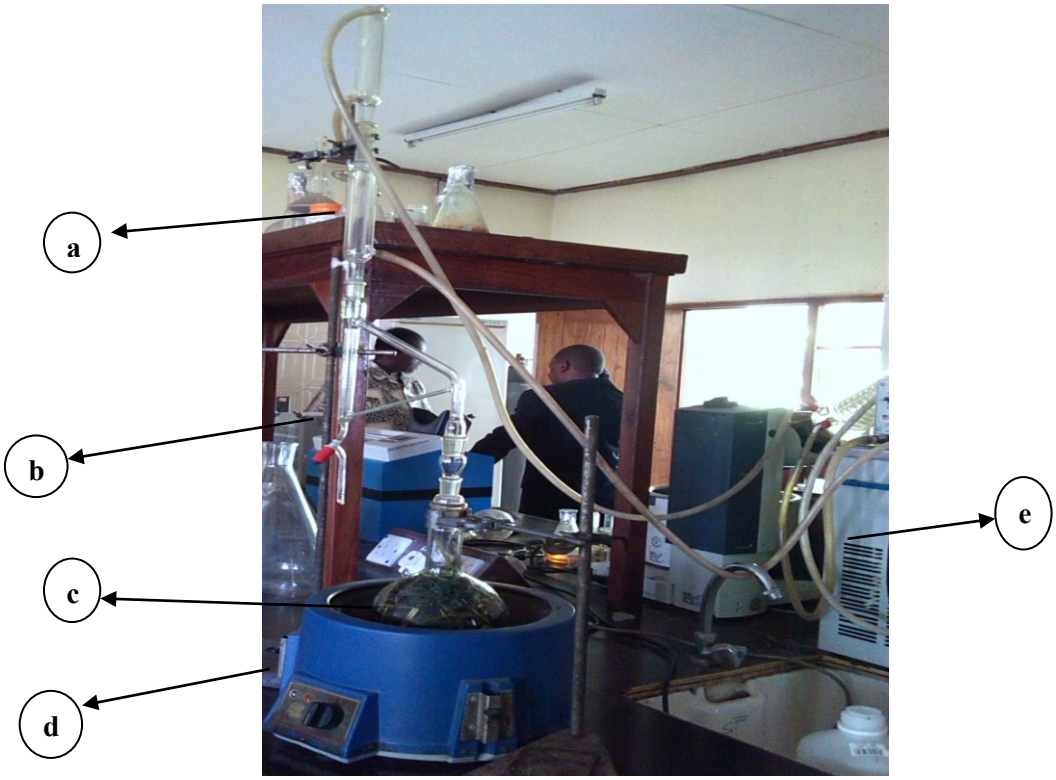

Figure 2: Set up of steam distillation of essential oils using Clevenger apparatus

Key: a - condenser, b – oil collector, c – round bottom flask, d – heater and e – water chiller

3.6 Multiplication of Bacteria Cells

Sample of each oil extract was cultured prior to bioassay. The aim was to test whether there was microbial contamination that would interfere with results at the later stage. Strains of *S. thermophillus, L. plantarum* and *E. coli* ATCC 25922 were sub-cultured in nutrient broth and soy agar. Thereafter, soy agar and nutrient broth manufactured by Hi-media in India were prepared accordingly and sterilised at 121ºC for 15 minutes. To activate the bacteria cells, each 5 mL of nutrient broth into test tubes was inoculated with bacteria strains for three hours. About 30 mL of agar was poured into each petri dish and left to solidify. Thereafter 0.5 mL of bacteria suspension was poured onto plates with agar and spread over by using sterile swab sticks. The petri dishes were labelled accordingly and incubated overnight.

3.7 Efficacy Test of Essential Oils on Bacteria Strains

3.7.1 Overview of the efficacy test

To achieve the efficacy test of the extracts, microdilution method was employed using gentamycin and soy peptone broth as positive and negative controls respectively. A two-fold serial dilution method with some modifications was carried out to determine the antimicrobial activities of the essential oils. Each strain of the bacterial suspensions was prepared in a sterile normal saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard. About 0.5µL of inocula was taken with sterile tips and transferred into 96 – well microplates with soy peptone broth (growth control), soy peptone broth with gentamycin (positive control) and essential oils (treatment). The mixture was subsequently incubated at 35°C for overnight, the presence of turbidity with reference to 2% NIT standards indicated growth of microorganisms. The minimum inhibitory concentration (MIC) was recorded as the last well in a row where there was no colour change as described by Andrews (2011) and NCCLS (2000).

3.7.2 Preparation of media and indicator preparation

Soy peptone broth was prepared according to manufacturer's instructions and number of bacterial strains to be tested. Distilled water, prepared media and all the equipments to be used in bioassay were autoclaved at 121ºC for 15 minutes.

3.7.3 The Antimicrobial activity tests

Fifty microliters of the broth were drawn into each well of sera culture plate followed by addition of 50 µL of the essential oil in each first well of the row. Two fold serial dilution was made from the first well to the last well in the row and the last 50 µL was discarded. Thereafter, 50 µL of the test organisms were added into each of the wells. To increase validity, test of all the treatments were performed in duplicate. The plates were covered, marked and incubated at 37ºC for overnight. Two hours before reading the results, 20 µL of the indicator (0.2% NIT) were added in culture plate and incubated for observing visible purple colouration (Figure 3). The MIC was recorded as the last well in a row where there was no colour change (Fig. 3) below by cycling the corresponding value in MIC score sheet (Table 1).

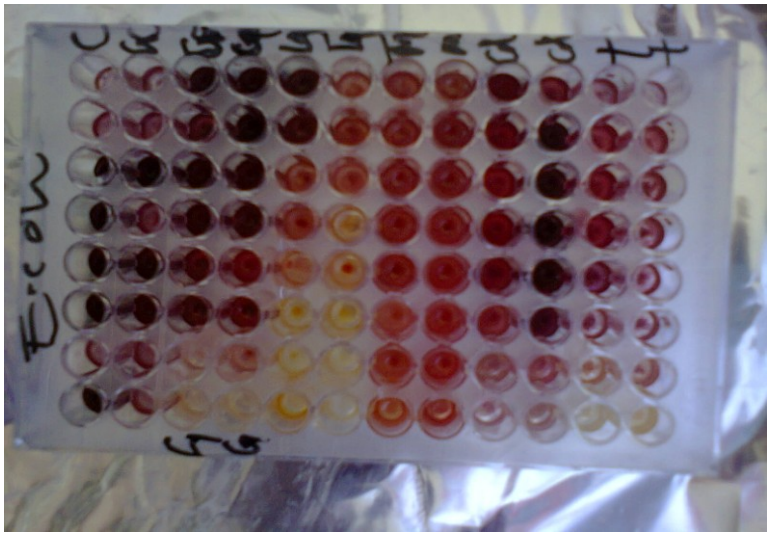

Figure 3: A picture of plate culture with nitrotetrazoleum (NIT)

Table 1: Antimicrobial Score Sheet in µg per µl

	A	B	C	D	E	F	G	H
1 +VE	10.0000	5.0000	2.5000	1.2500	0.6250	0.3125	0.1562	0.0781
2+VE	10.0000	5.0000	2.5000	1.2500	0.6250	0.3125	0.1562	0.0781
3 GC	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
4 GC	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
5 E1	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
6 E1	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
7 E2	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
8 E2	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
9 E3	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
10 E3	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
11 –VE	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019
12 –VE	0.2500	0.1250	0.0625	0.0312	0.0156	0.0078	0.0039	0.0019

Key: +VE control = Gentamycin, GC = Growth Control, E1 – En = sample extract of essential oils and -VE control = Media control

3.8 Data Management and Analysis

3.8.1 Knowledge assessment data

Data for the knowledge assessment were verified, coded and entered in Statistical Package for Social Sciences (SPSS) version 16.1 and Microsoft excel 2010 software. Analysis for proportions was achieved by using SPSS while graphs were generated by Microsoft excel database. To compare the proportions (%) of respondents between categories, chi square test was used at 95% confidence interval (CI) at $P < 0.05$.

3.8.2 Laboratory Data

The laboratory data were analysed by using MIC standard values (Table 2) as described by Aligiannis *et al*. (2001) and Sartoratto *et al*. (2004). To compare the means of MICs of essential oils between and within the tested milk spoilage bacteria strains, *p*-value was calculated and compared at critical probability of $P < 0.05$ at 95% CI using Two-Way ANOVA.

Table 2: MIC Standard Value Classification

MIC (µg/ µl)	Classification of antimicrobial activity of drugs
0.05 – 0.5	Strong antimicrobial activity
0.6 – 1.5	Moderate antimicrobial activity
MIC > 1.5	Weak antimicrobial activity

Source: Aligiannis *et al.* (2001); Sartoretto *et al*. (2004)

CHAPTER FOUR

4.0 RESULTS

4.1 Preliminary survey

4.1.1 Demographic data

Among the 56 interviewed respondents, 32 (57.1%) were females and 24 (42.9%) males ($P > 0.05$), where by 55.4 percent of both of them originated from Unguja. The age of the most respondents (61%) was in the range of 21 – 30 years. The detailed distribution of demographic data of the sample population is given in Table 3.

Table 3: Demographic data (n = 56)

Factor	Variable	Proportions (%)	*P value*
Sex			
	Male	42.9	
	Female	57.1	0.2482
Age			
	15-20	19.6	
	21-30	60.7	
	31-40	14.3	
	40 and above	5.4	
Level of education			
	Primary	5.4	
	Secondary	44.6	
	High school	3.6	
	Vocational	3.6	
	Certificate	26.8	
	Diploma	8.9	
	Degree	5.4	
	Masters	1.8	
Occupation			
	House wife	1.8	
	Peasant	10.7	
	Business Man/Woman	1.8	
	Student	62.5	
	Employee	23.2	
Origin			
	Pemba	35.7	
	Unguja	55.4	
	Others	7.1	
Marital status			
	Single	67.9	
	Married	28.6	
	Widowed	1.8	

4.1.2 The use of spices and herbs in milk

Most of the respondents (96.4%) (*P=0.0001*) declared using spices and herbs in milk while only 2 (3.6%) were not. The majority (68.5%) had an experience of using spices and herbs since their childhood (Table 4).

Table 4: Information on spices use in milk

Factor	Variable	n	Proportion of respondents (%)	*P value*
Awareness of spice use in milk		56		
	Aware		96.4	
	Not aware		3.6	0.0001
Source of information on spice use in milk		54		
	Parents		68.5	
	School / college		11.1	
	Traditional healers		7.4	
	Nobody		5.5	
	Friends		3.7	
	Relatives		3.7	
	Others Fellow villagers		1.8	
	Others		1.8	
Users of spices/ herbs in milk		56		
	Yes		96.4	
	No		3.6	0.0001
Place where spices are obtained		54		
	Markets /shop		85.2	
	Farms / gardens		14.8	0.0097

Among the 54 respondents, the majority reported using cardamom (96.3%) and cinnamon (88.5%) followed distantly by ginger (57.7%), lemon grass (26.6%) and other spices in

milk (*P <0.05*) (Table 5). The majority (82.1%) preferred ground spices/ herbs while the rest preferred whole or fresh. The amount added in one litre of milk ranged from a half to one and a half teaspoon which is equivalent to 4.6 - 8.6 g (Table 6).

Table 5: Commonly used spices in milk in Zanzibar (n = 54, *P=0.001*)

Common Name	**Swahili Name**	**Scientific Name (Species)**	**Family**	**Proportion of respondents (%)**	**Part Used**
Cardamom	*Hiliki*	*Elettaria cardamomum*	Zingiberaccae	96.3	Seed
Cinnamon	*Mdalasini*	*Cinnamomum zeylanicum*	Lauraceae	88.5	Bark
Ginger	*Tangawizi*	*Zingiber officinale*	Zingiberaceae	57.7	Rhizome
Lemon Grass	*Mchaichai*	*Cymbopogon citrates*	Gramineae	26.9	Leaf
Clove	*Karafuu*	*Eugenia caryophyllis* or *Syzgium aromaticum*	Myrtaccae	13.46	Flower bud
Turmeric	*Binzari*	*Curcuma longa*	Zingiberaceae	1.9	Rhizome
Vanilla	*Vanilla*	*Vanilla planifolia*		11.5	Pods
Basil	*Rihani*	*Ocimum basilicum*	Lamiaceae	1.9	Leaves
Star anise	*Nyota*	*Illicium Verum*	Magnolia	3.8	Seed and pods
Black pepper	*Piipili manga*	*Piper nigrum*	Piperaceae	1.9	Berries
Black tea	*Chai nyeusi*	*Camellia sinensis*	Theaceae	3.8	Leaves
Nutmeg	*Kungumanga*	*Myristica fragrans*	Myristicaceae	1.9	Seed

Table 6: Different amount of spices added in milk (n = 54)

Factor	Proportion of respondents (%)				*P* value
Weight in grams	Cardamom	Cinnamon	Ginger	Lemon grass	
0.5 - 4.5	7.4	11.1	1.8	1.8	
4.6 - 8.6	27.7	42.5	25.9	3.7	
8.7 - 12.7	14.8	12.9	14.8	3.7	
12.8 - 16.8	3.7	5.5	0.0	1.8	
Others	42.5	12.9	12.9	14.8	0.01

Most of the respondents (96.4%) had knowledge on the effects of spices and herbs in milk (Table 7).

Table 7: Knowledge on effects of spice and herbs use in milk (n = 56)

Factor	Variable	Proportion (%)
Awareness on desirable attributes of spices in milk		
	Yes	96.4
	No	3.6
Desirable effects		
	Mask animal smell	25.0
	Impart aroma	82.1
	Avoid gut bloating	5.4
	Extend milk shelf life	7.1
	Medicinal	16.1
	Increase appetite	17.9
	Enhance bone and/ or Muscles strength	10.7
	Enhance blood circulation	7.1
	Add nutrients	1.8

Results in Table 8 show that 75 percent of respondents had no experience on side effects from spices and herbs used in milk while 25 percent did.

Table 8: Adverse effects of added spices in milk (n=56)

Factor	Variable	Proportion (%)
Awareness of respondents regarding adverse effects		
	Yes	25.0
	No	75.0
Adverse effects		
	Cardamom causes sleep sickness in men	1.8
	Some cause lesion in gut example	1.8
	Appendicitis if cardamom is not well sieved	1.8
	High blood pressure	3.6
	If you put powdered cinnamon alone milk get curdle fast	1.8
	Increase body temperature	1.8
	Irritation in throat	3.6
	Remove natural flavour and aroma of milk	5.4
	Some contain antinutritional factors when used in excess	1.8
	Can cause nausea to some people	1.8
	Too much ginger cause milk to coagulate	1.8
	Too much ginger curdle milk	1.8
	Too much spice might spoil milk	1.8
	Too much spice may cause dizziness, milk bitterness e.g. ginger.	1.8

4.1.3 Households knowledge on milk spoilage

Out of 56 respondents, the majority reported microorganisms (73.2%) and poor storage (39.3%) are the major causes of milk spoilage (Figure 4).

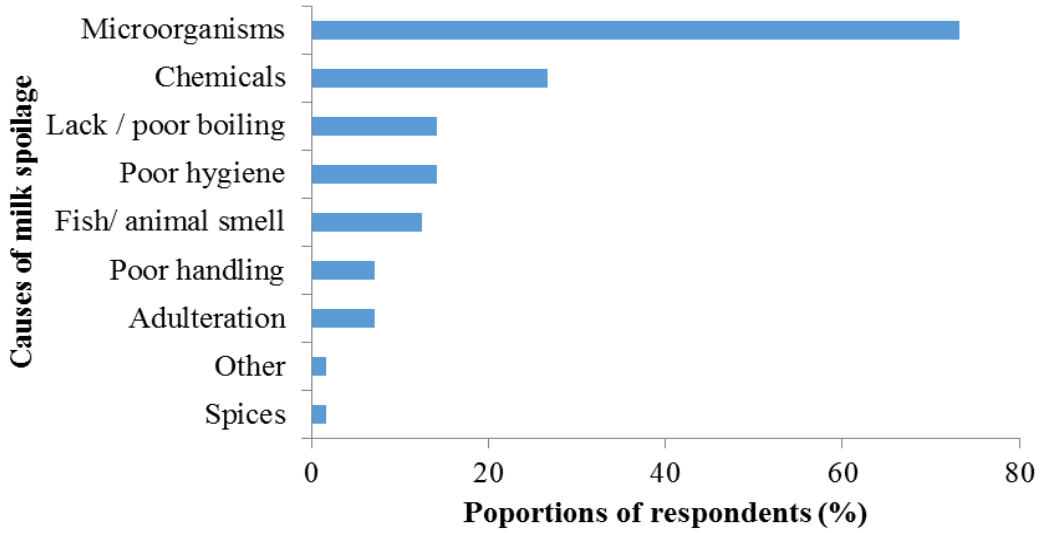

Figure 4: Causes of milk spoilage

Most of the households, used refrigeration (55.4%), boiling (42.9%), freezing (23.2%) and thermos (21.4%) to control milk spoilage (Table 9).

Table 9: Milk Spoilage (n = 56)

Factor	Variable	Proportion (%)
Awareness on microorganisms as source of milk spoilage		
	I know	73.2
	I don't know	26.8
Do you know any of them?		
	Yes	41.1
	No	58.9
Where did you get the information about it?		
	Public health officer	14.3
	Fellow villagers	1.8
	Relatives	1.8
	Friends	8.9
	Parents	37.5
	Media	12.5
	School/ College	28.6
	Others	3.6
Techniques of preventing milk from spoilage		
	Boiling and leave at room temperature	42.9
	Boiling and put in thermos	21.4
	Refrigerator	55.4
	Freezing	23.2
	Adding of spices	7.1
	Others	7.1

4.2 Extraction of Essential Oils

4.2.1 Observations

During extraction of cardamom and cinnamon oil, huge mass of foams were observed in the flask (Fig. 5). Milkish colour was observed in the collector during the extraction of cinnamon oil. The milkish colour disappeared after the extract was left to cool down.

Moreover, cinnamon oil formed emulsion with hydrosol which separated into layers after the extract was left to cool. The colour of cinnamon oil was slightly yellow, green-yellow for lemongrass, slightly yellow for ginger while cardamom was clear colourless (Fig. 6).

Figure 5: Foams observed during essential oils extraction

Lemon grass oils was characterised by sharp odour and higher irritability on skin while ginger oil was less irritative.

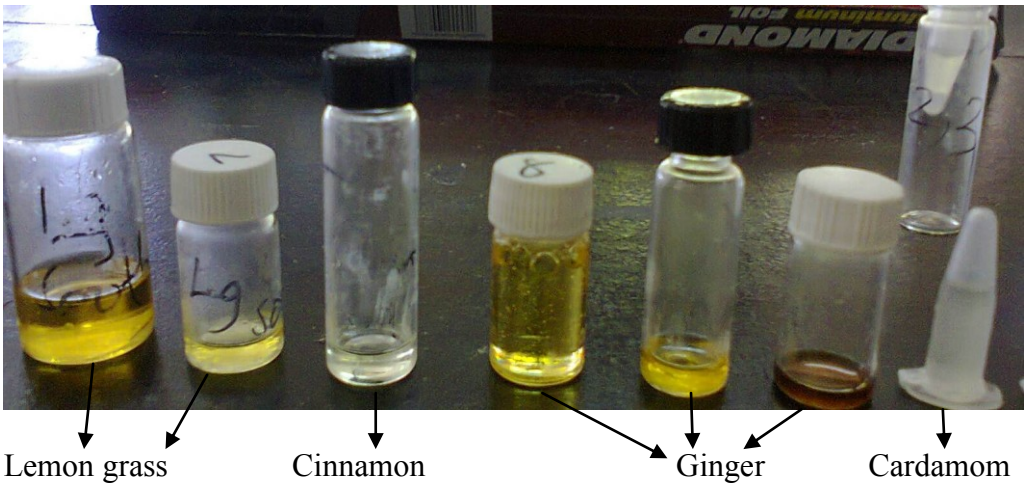

Figure 6: Essential oils extracted from spices and herbs

Cardamom seeds gave high yield (5.3%) compared to cinnamon (1.3%), lemon grass (0.4%) and ginger (0.4%) (Table 9).

Table 10: Yield of extracted essential oils

Spice	Weight used (g)	Extract in volume (ml)	Extract in weight (g)	Yield (%) in g
Cardamom	91.86	5.8	4.89	5.33
Cinnamon	522.64	8.6	6.55	1.25
Lemon grass	1432.01	1.8	5.48	0.38
Ginger	843.89	3.2	3.73	0.44

4.3 Bioassay

4.3.1 The antimicrobial and MIC tests

Gentamycin demonstrated higher MIC values on *L. plantarum* (2.5 µg/µL) and *S. thermophillus* (5 µg/µL). On the other hand, ginger oil demonstrated higher MIC value on *E. coli* (0.1250 µg/µL). However cardamom, lemon grass, cinnamon and ginger oils demonstrated lower MIC (< 0.5 µg/µL) in all tested bacterial strains (Table 11 and Fig. 7).

Table 11: Results of MIC values (µl/ µl)

Microorganism	+VE control	Cardamom	Cinnamon	Lemon grass	Ginger	Media Control	-VE control
L. plantarum	2.5000	0.0625	0.0625	0.0625	0.0156	No growth	Growth
E. coli ATCC 25922	0.3125	0.0625	0.0312	0.0078	0.1250	No growth	Growth
S. thermophillus	5.0000	0.0156	0.0039	0.0625	0.0039	No growth	Growth

P value for essential oils =0.1569 and *P value* for microorganisms = 0.5988

Results are from the two readings and there was no variation.

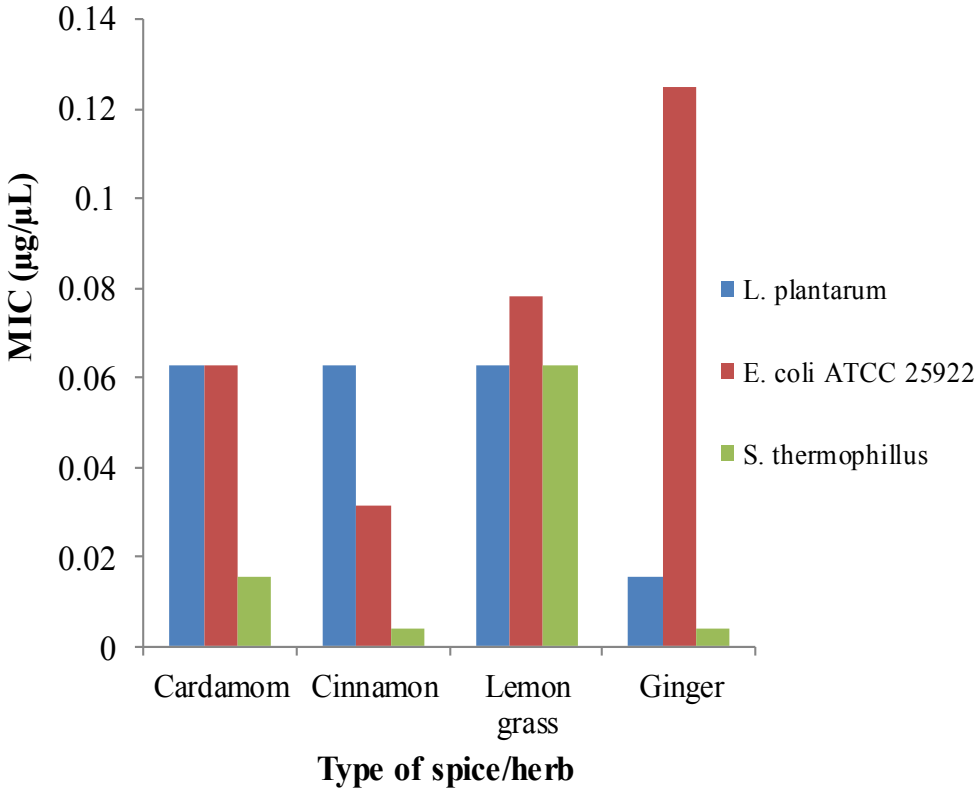

Figure 7: MIC values against milk spoilage bacterial strains

CHAPTER FIVE

5.0 DISCUSSION

5.1 Knowledge assessment

5.1.1 Characteristics of Respondents

Based on findings from the on preliminary survey, number of female participants was higher compared to male participants. In African societies, women are more informed about the appropriate methods of food handling and storage than men (Li-Cohen and Bruhn, 2002; NIN, 2006). However there was no statistical significant difference ($P > 0.05$) between female and male participants. On the other hand, most of the participants were younger with age ranged from 15 to 30 years (Table 3). According to Sudershan *et al*. (2007), most of the younger people are normally eager and willing to acquire new knowledge in different matters.

5.1.2 Knowledge on spices/ herbs use in milk

Zanzibar is known as a spice island in Africa, and there is much application of spices and herbs in several foodstuffs including milk, tea, meat, rice (*pilau)* and other traditional foods. Spices and herbs are normally added in foodstuffs such as milk to impart flavour, aroma and colour as well (Takeda *et al*., 2007). In Zanzibar there are many spices and herbs which are added in milk for different purpose including to impart flavour and for medicinal use. However the most commonly used spices mentioned by respondents are cardamom, cinnamon, ginger and lemon grass ($P < 0.05$). Most of the people acquire knowledge of spice and herbs use in milk from parents, friends, relatives, school/ college, traditional healers or media. On the other hand, some respondents and key informants in this study reported that some spices and herbs are used in milk as a natural remedy to gut bloating. In India, cardamom is used by traditional healer to treat cold, bronchitis, fevers,

inflammatory conditions of the oropharynx and liver complaints (Takeda *et al*., 2007). However, there are many reasons for adding spices and herbs in milk (Table 7) including extending its shelf life.

Most of the spice and herbs used by the households are purchased from markets and shops 87.5% ($P < 0.05$). However most of the spices and herbs available in market are cultivated in Zanzibar with exceptional to cardamom. Cardamom is less cultivated in Zanzibar, therefore huge amount is imported from India and thus why its price is higher compared to other spices and herbs which are sold in markets and shops. Moreover, Takeda *et al*. (2007) reported that cardamom is the "third most expensive" spice in the world after saffron and vanilla due to its higher production and pleasant scent.

5.1.3 Importance of spices and herbs in milk and their related health benefits

Apart from its direct purpose of adding flavour and aroma in milk, some households in Zanzibar use the mixture of milk, cardamom and honey as a natural remedy to help patients with higher blood pressure. In Indian, crushed cardamom seeds are normally boiled with milk and honey and used as an excellent remedy for impotence and depression (Takeda, 2000, 2004). Cardamom is also used in Chinese and Indian traditional medicine to improve digestion and to treat gastrointestinal problems such as diarrhoea and gas (Agaoglu, 2005; Mahady *et al*., 2005; Takeda *et al*., 2007). Cardamom is also employed in aromatherapy as a rub to promote blood circulation (Fabio *et al*., 2003, Chaudry and Tariq, 2006).

Some households add cinnamon in milk to mask the animal smell. On the other hand, it was reported that addition of cinnamon in beverages such as milk, provides a lot of health

benefits such as improved blood circulation and male sex drive. It is believed that cinnamon is one of the natural products, which is an aphrodisiac spice (Takeda *et al*., 2007). The cinnamon cultivated in Zanzibar has a distinctive characteristic from that imported from India. It was reported that Indian cinnamon is sweeter compared to the one cultivated in Zanzibar, therefore Zanzibar's cinnamon is more preferred by traditional healers. It was experienced by one of the key informants that adding a half teaspoon of Zanzibar cinnamon in a glass of milk is sufficient enough to provide a relief to an individual suffering from gastric ulcer.

Ginger is a horticultural crop cultivated in Zanzibar throughout the year and marketed in a fresh and dried state. Ginger is a hot spice used in milk and other beverages as a tastemaker, a drug and an appetizer. It is also used to flavour sauces, curry dishes, confections, pickles, and ginger ale. In clinical trials, ginger has demonstrated stimulant effect in the intestines and saliva glands, digestive juices, and bile. It also tends to trigger the pumping action of the heart. Ginger is also regarded as aphrodisiacs spice in Asia (Takeda *et al*., 2007).

Lemon grass is used alone as a beverage or in a combination with other herbs such as tea leaves. In milk, lemon grass is used to impart its pleasant aroma and green colour. Lemon grass is used as a natural remedy through inhalation of the crushed grass in order to get relief from headache and flu or cold. It is also used as natural remedy in promoting male sex drive. Pharmacological investigations proved that, the essential oils of lemon grass demonstrates the antidepressant effect on the Central Nervous System (CNS) (Ayandele, 2007). Furthermore, the oil of lemon grass reported to contain male sex hormone (Gupta *et al*., 1993).

5.1.4 Adverse effects of spices in milk

Some participants reported on the adverse effects they experienced regarding the use of particular spice or herb for instance clove, cinnamon and ginger (Table 8). The aromatic oils of the clove have a stimulant and irritant effect. Over use of clove may result in increased blood circulation and slightly raised body temperature (Bhowmik *et al*., 2012). On the other hand, the usefulness of spice and herbs, differ from one individual to another, depending on experience and desired effect in the food product. Moreover, clove oils have been known to stimulate and disinfect the body, and stimulates both saliva flow and gastric juices (Bhowmik *et al*., 2012). However individuals suffering from gastric ulcers reported to avoid use of cloves in milk due to its relation to over secretion of gastric juice which induce pain immediately after use.

5.1.5 Knowledge of respondents on milk spoilage, handling and storage

Almost all of the respondents were aware of what caused milk spoilage depending on what they observed from home or learnt from school/ colleges. Respondents mentioned about poor storage and handling, lack or/ improper boiling, poor hygiene and contaminants from animal and chemicals such as adulterants, salts, acids from fruits and some spices. Lore *et al.* (2005) reported about milk spoilage in East Africa, the major causes of spoilage were associated with poor hygiene during storage and handling, use of poor technology, poor infrastructures and adulteration. The same was reported by FAO (2005) that most losses of the dairy products were due to a combination of poor production/handling practices and lack of technical knowledge.

Some respondents were aware of causes of milk spoilage of which, bacteria such as *Lactobacillus spp.*, *E. coli* and fungi were among the mentioned microorganisms. That awareness was acquired from relatives, friends, parents, media, school/ college and other sources including internet and extension officers. *Escherichia coli* is one of the most

pathogen of concern in affecting shelf life of milk at household level and food industries (Deak, 1996; Betts, 1999). From a clinical point of view, presence *E. coli* or its toxin in food is also capable of causing other infections. *E. coli* causes septicaemia (blood poisoning caused by bacteria or their toxins) and can infect the gall bladder, meninges, surgical wounds, skin lesions and the lungs, especially in debilitate and immunocompromised individuals (Cheesbrough, 2000). Moreover, microorganisms succeed one another as the chemical environments change. In both pasteurized and raw milk, the microbes themselves bring about these changes. The sequences of microbial growth start with *Streptococcus* followed by *Lactobacillus*, yeasts and moulds, and finally Bacillus (Goff, 2009).

5.2 Laboratory Work

5.2.1 Extraction of essential oils

The formation of foam during extraction of cardamom and cinnamon may be associated with the presence of phyto constituent bioactive components such as Saponin which have foaming characteristics (Eloff, 1998; Majorie, 1999). Some extracts including lemon grass oil is irritative on skin. The irritation effect is mostly due its lower pH values which range from 3 to 5 (Isam *et al*., 2009). Among all the extracts, cardamom had higher yield than the rest of extracts. This may be attributed by several factors including its natural pod which protects evaporation of the volatile oil. Storage condition is also reported to have influence on the yield of essential oils from the spices (Agaoglu, 2005).

5.2.2 Bioassay

5.2.2.1 The antimicrobial activity and MIC of the essential oils

All extracts demonstrated antimicrobial activity on all the tested bacteria strains. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the

antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2011). Gentamycin demonstrated strong antimicrobial activity (0.3125 µg/µl) only on *E. coli* while a weak activity was observed on *S. thermophillus* (2.5 µg/µl) and *L. plantarum* (5 µg/µl) (Table 11). On the other hand, all the essential oils demonstrated strong activity (MIC < 0.5 µg/µl) on all tested bacterial strains with exception to ginger oil. Ginger oil demonstrated a moderate activity against *E. coli* (Fig. 7). *Escherichia coli is* among the Gram negative bacteria which are more resistant to chemical and harsh conditions than Gram-positive bacteria (Street and Staros, 2014; Zaika, 1988). However there is no significant different among essential oils and the bacterial strains tested (*P> 0.05*). Ates *et al*. (2003) reported on the antibacterial activity of alcoholic extract of cinnamon against *Bacillus megaterium* and *Enterococcus faecalis*. Also Mahady *et al.* (2005) reported on the antibacterial effect of the essential oils from cardamom, *E. cardamomum* on *Escherichia coli*. Subsequently, Chaudry and Tariq (2006), reported on effectiveness of cinnamon essential oil on several bacteria strains. It was found that the oil was highly effective against *Streptococcus oralis*, *S. anginosus*, *S. intermedius*, *S. sanguis*, *Enterobacter aerogenes* and *Micrococcus roseus*. Lutterodt *et al*. (1999) reported on the bactericidal and anti-fungal effectiveness of lemon grass that was comparable to penicillin.

Essential oils can contain compounds such as saponins, tannins, alkaloids and flavonoids which are naturally occurring compounds in medicinal plants. These compounds possess bactericidal, pesticidal or fungicidal activities of the studied plant (Eloff, 1998; Majorie, 1999; Rios and Recio, 2005). For instance, the acidity nature of lemon grass oil provides a synergistic effect on antimicrobial activities of the bioactive components present in lemon grass (Hamza *et al*., 2009). However, the activity of essential oil as natural preservative, depend upon the type of spice, concentration of oil used, test medium, virulence factor or

resistance of microorganism to a given spice or herb and initial microbial load (Selim, 2010). Furthermore, the effect of the essential oil can be bactericidal in action (Hamza *et al*., 2009). Therefore, Minimal Bactericidal Concentration (MBC) test has to be determined to verify whether the exhibited efficacy is bactericidal or bacteriostatic as the MIC by itself, does not describe presence, type, or location of infection **(**Street and Staros, 2014).

To induce the antimicrobial activities in milk, the required amount of essential oils is at the range of 3.9 to 125 mls per one litre of milk. However the amount of spices and herbs mostly added in one litre of milk is between 4.6 to 12.7 g (Table 6) which is equivalent to 0.8018 to 5.7 x $10^{\wedge -3}$ mls as per essential oil yields. Therefore, the amount used to impart flavour in milk is not sufficient enough to give the antimicrobial activity shown. However, the further studies can be attempted to test for spices and herbs collected from different areas. Furthermore the synergistic from combined essential oils and combination of other treatment parameters such as time-temperature effect can be studied.

5.7 Limitations of the Study

Some people were reluctant to participate in the study due to lack of incentives and being busy with their economic and social activities. Due to limited time and fund, the study only focused on the antimicrobial effect of the single extract on three bacteria strains.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the findings of this study it is concluded that:

(a) Most of the people are not aware on the antimicrobial activities of spices and herbs which are added in milk.

(b) Spices and herbs play important roles in health apart from their primary use of adding flavour in milk. However there are some side effects reported to some spices such as clove and ginger.

(c) The spices and herbs which are added in milk for flavour, medicinal use and other uses, can also contribute to extend the shelf life of milk by inhibiting the growth of spoilage bacteria.

(d) All the essential oil extracts from cardamom, cinnamon, lemon grass and ginger demonstrated the strong antimicrobial activities in all tested isolates of milk spoilage bacterial strains with MICs range between 0.0039 to 0.1250 µg/µl. However, there was no statistical significant difference ($P>0.05$) among all the essential oils to all tested bacterial strains.

(e) Among the *L. plantarum, S. thermophillus* and *E. coli, E. coli* showed high value of MIC against ginger extract, however there was no significant difference ($P > 0.05$) with other extracts.

6.2 Recommendations

Based on the conclusions above, it is therefore recommended that:

(a) Communities should be mobilized to use spices and herbs in milk as can contribute to extend the shelf life of milk.

(b) Communities should be sensitized to use spices and herbs in milk and other foods since they are associated with several health benefits include improving blood circulation, muscle strength and reproductive health.

(c) Further toxicological studies are recommended in order to validate and authenticate the side effects mentioned.

(d) Extracts from essential oils and non-essential oil components from other spices and herbs are to be studied and identified for their chemical structures and antibacterial characteristics in order to expand the database of potential sources of natural milk preservatives.

(e) The study also recommends for further studies to elucidate the involvement of other bacteria and establish their sensitivity to different extracts of spices and herbs that can be added in milk.

(f) From the established MICs for each essential oil on a given microorganism, further attempts are required to test the effect of these oils whether are bactericidal or bacteriostatic.

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APPENDIX

Appendix 1: Questionnaire

Questionnaire no.....

I am AMINA AHMED from Sokoine University of Agriculture (SUA). I am doing a survey about uses of spices in milk. The survey is completely voluntary and your answers will be kept confidential and only used for the purpose of this study.

Study site..... Mob.....

Date of interview.....

Name of interviewee.....

Language used in interview.....

Section A: Demographic data

No	Question	Coding category
1.	Sex	1= male 2= female
2.	How old are you?	1= 15-20 2= 21-30 3= 31-40 4= > 40
3.	What is your highest level of education?	1 = none 2 = primary 3 =secondary 4 = high school 5 = vocational 6 = certificate 7 = diploma 8 = bachelor 9 = others specify.....
4.	What is your religion?	1= Christian 2= Muslim 3= others specify…….....
5.	What is your current occupation?	1=housewife 2= househusband 3= peasant 4= business 5= student 6= employee 7 = others specify……..
6.	What is your origin?	1 = Pemba 2 = Unguja 3 = Others specify…….
7.	What is your marital status?	1= single 2= married 3= widow/ widowed 4= divorced 5 = refused

Section B: Information about spices use in milk

No	Question	Coding category
8.1.	Are you aware that spices are used in milk? If "NO" skip Question 8.2	1=yes 2=no
8.2.	Where did you get the information about it?	1 = traditional healer 2 =fellow villagers 3 =relatives 4 =friends 5 =parents 6 =nobody 7 = school / college 8 = others specify……..
9. 1.	Do you use spices in milk? if YES, proceed Question 9.2	1= yes 2= no
9.2.	Mention the spices you use	
9.3.	Are the mentioned plants of spices available here?	1=yes 2=no 3=don't know
9.4.	Where do you get them?	1= farm 2= market 3= shop 4= others specify.....
9.5	Which part of the spice plant used is mostly used ?(circle more than one response)	1=leaves 2=stem 3=roots 4=barks 5=seeds 6= other specify.....
9.6	In which state is it used?	1= fresh 2= dried 3= powder 4= other specify.....
9.7	How much spice/ herb do you add in milk (approx. 1L)?	
10.1.	Do you know any desirable effects (advantages) of spices added in milk?(if no, skip Question 10 1.	1= yes 2= no 3 = don't know
10.2.	Which are those desirable effects?	
11.1.	Are there any adverse effects of using spices? (If no/ don't know skip Question 11.2.	1=yes 2=no 3=don't know
11.2.	Which are those undesirable effects?	

Section C: Effectiveness of spices in milk

No	Question	Coding category
12.	What do you think cause milk spoilage?	
12.1.	What about microorganisms?	
12.2.	Do you know anyone? If "YES" proceed Question 12.2.1.	1= yes 2= no
12.2.1	If YES, mention	
12.3.	Where did you get the information about it?	1= public health officer 2=fellow villagers 3=relatives 4=friends 5=parents 6= media 7= school / college 8 = others specify…….....
13.	How do you prevent milk from spoilage?	1= boiling and live at room temperature 2= boiling and put in thermos 3= refrigerator 4= freezing 5= others specify…….....
14. 14.1 14.2 14.3 14.4 14.5 14.6	How long the shelf life of milk in the following conditions? at room temperature without spices at room temperature with spices in thermos without spices in thermos with spices in thermos with spices in thermos without spices	1= < 6 hours 2= 6 - 12 hours 3= 12 -18 hours 4= 18 – 24 hours 5 = > 24 hours 6 = don't know 1= < 6 hours 2= 6 - 12 hours 3= 12 -18 hours 4= 18 – 24 hours 5 = > 24 hours 6 = don't know 1= < 8 hours 2= 8 - 16 hours 3= 16 -24 hours 4= 24 – 32 hours 5 = > 32 hours 6 = don't know 1= < 8 hours 2= 8 - 16 hours 3= 16 - 24 hours 4= 24 – 32 hours 5 = > 32 hours 6 = don't know 1= < 6 hours 2= 6 - 12 hours 3= 12 -18 hours 4= 18 – 24 hours 5 = > 24 hours 6 = don't know 1= < 6 hours 2= 6 - 12 hours 3= 12 -18 hours 4= 18 – 24 hours 5 = > 24 hours 6 = don't know

Section D: Spices storage

No	Question	Coding category
15	Are the mentioned (9.2) spices available throughout the year?	1= yes 2= no
15.1	If YES, justify	
16.2	If NO, justify	
17.	Do you store them? (if NO, skip Qn 25)	1= yes 2= no
18.1	How do you/ they store them?	

19. Those are all of the questions I had, do you have any comments that we have not discussed?

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Thank you for your cooperation