Potential of lemongrass (*Cymbopogon citratus*) essential oil for use on Bio-preservation of yoghurt

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Salisu Maiwada Abubakar and has not been presented anywhere for the award of degree or any certificate(s). All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this Dissertation and the subsequent write-up by

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APPROVAL

This Dissertation has been examined and approved for the award of Master Degree in Biotechnology

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Dedication

I dedicate this work to my respected parents Alhaji Tijjani Abdurrahman and Hajiya Khadija Tijjani who contributed immensely in my life. Thank you for believing in me.

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ABSTRACT

Microbial contamination at various stages of food chain is one of the major causes for dairy food spoilage that ultimately leads to waste, increasing food insecurity issues and substantial economic losses. Various synthetic chemical preservatives are being used to control microbial food spoilage and to extend product shelf life. These chemical products used have a lot of side effects on human health. The aim of this study is to investigate the bio-preservative potential of lemon grass essential oil (EO) on yoghurt. Physico-chemical, sensory and microbiological characteristics were studied. Lemon grass EO was extracted via steam distillation and was tested for phytochemical analysis. The oil was fractionated using column chromatography (Silica column) and the fractions obtained were tested for their potential antibacterial activity against some yoghurt spoilage bacteria. These spoilage bacteria were characterize using 16srRNA identification. The components of the effective fraction were identified by GC-MS and FTIR. Phytochemical screening of lemongrass EO showed the presence of tannins, flavonoids and terpenoids. Results for the total bacterial count (TBC) for yoghurt ranged from 110±2.31 $x10^6$ to 294.67±2.31 $x10^6$, total Lactobacillus count (LBC) ranged from 113.33±5.77 $x10^6$ to 240±20.00 $x10^6$ while at 1.0 and 2.0 µl/ml EO prevented the growth of fungi for up to 7 days. Citrobacter freundii and Acinetobacter baumanii are the two bacteria specie identified in this study. Results shows that sensory acceptability of yoghurt supplemented with lemongrass EO was higher than that of the control yoghurt prepared without EO. Yoghurt sample treated with 2µlml^{-1} was found to be most acceptable ($P<0.05$). The antibacterial activity was assessed for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method against Citrobacter freundii and Acinetobacter baumanii. MIC values ranged from 4.17±1.80 mg/ml to 16.67±7.22 mg/ml. The lowest MIC was recorded in fraction B 6.25±0.00 mg/ml for C. freundii. The chemical composition of fraction B analyzed by GC-MS and FTIR showed alkanes, aldehydes (citral 17.13% and geranial 7.57%), ketone, ester (1,2,3-propanetriyl ester 10.73%) and carboxylic acid (9-Octadecenoic acid 10.73% and Hexadecanoic acid 9.72%). These present findings suggested that addition of lemongrass essential oil could increase the shelf life of yoghurt for up to 7 days at room temperature and also inhibit some yoghurt spoilage bacteria.

CHAPTER ONE

INTRODUCTION

1.1 Background

Food safety is regarded as one of the key public health issue being discussed globally (Vergis *et al.,* 2015). Many food products are perishable by nature and require protection from spoilage during preparation, storage and distribution to give them a desirable shelf-life (Rasooli, 2007). Spoilage is a metabolic process that causes food to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be safe to eat, i.e. they may not cause illness because there are no pathogens or a toxin present, but changes in texture, smell, taste, or appearance cause them to be rejected (Rawat, 2015). The reasons for massive global food loss are diverse, but microbial spoilage, which affects organoleptic product quality (appearance, texture, taste, and aroma), plays a major role (Salas *et al*., 2017). The issue of food losses is of high importance in the efforts to combat hunger, raise income and improve food security in the world's poorest countries. Food losses have an impact on food security for poor people, on food quality and safety, on economic development (Rawat, 2015).

Yogurt is a well-known fermented dairy food, which is usually manufactured from cow's milk with or without the addition of some natural derivatives of milk, and possesses a gel structure that is the result of coagulation of the Milk protein by lactic acid produced by *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus bulgaricus* (*L. bulgaricus)* (Robinson, 2003). Yogurt is a coagulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. It has a smooth texture and a mildly sour and pleasant flavor. It is obtained from pasteurized or boiled milk soured by naturally occurring, or lactic acid fermenting bacteria i.e. *Lactobacillus bulgaricus and Streptococcus thermophilus* (Robinson, 2003).

The microbial spoilage of foods is due to the biochemical activity of microorganisms as they grow in food causing changes in the food's appearance, odor, texture, or taste. Since food borne pathogens do not typically give an organoleptic indication of their presence, the organoleptic changes caused by spoilage microorganism serve as a warning to the consumer that the food could be unsafe for consumption, thus, protecting millions of people from food borne illness

(Amin, 2012). Spoilage bacteria may originate on the farm from the environment or milking equipment or in processing plants from equipment, employees, or the air. Troublesome spoilage microorganisms include aerobic psychrotrophic Gram-negative bacteria, yeasts, molds, hetero fermentative lactobacil, and spore-forming bacteria (Ledenbach & Marshall, 2009). Psychrotrophic bacteria can produce large amounts of extracellular hydrolytic enzymes, and the extent of recontamination of pasteurized fluid milk products with these bacteria is a major determinant of their shelf life (Ledenbach and Marshall, 2009). *Staphylococcus aureus* is generally regarded as a food borne pathogen causing spoilage and intoxication due to the production of heat stable enterotoxins (Amin, 2012).

Improving foods shelf-life can have great economic impact in reducing losses attributed to spoilage and allowing products to reach distant and new markets. Food preservation is a continuous fight against microorganisms spoiling the food or making it unsafe. Until now, approaches to seek improved food safety have relied on chemical preservatives, antibiotics or on the application of more drastic physical treatments (e.g. high temperatures or refrigeration). Nevertheless, these methods have many drawbacks (Amin, 2012). Currently, there is a strong debate about the safety aspects of chemical preservatives due to impairment/reduction of the nutritional value of food, episodes of adverse food reactions, cardio-vascular disease, many carcinogenic and teratogenic attributes as well as residual toxicity. Processing at high temperatures extensively damages the organoleptic, nutritional and physicochemical properties of food. Refrigerators are either expensive to maintain or means for their maintenance (electricity) are lacking and this method of preservation makes the yoghurt product prone to microbial and other sources of contamination (Ben Jemaa *et al*., 2017; Amin, 2012).

However, the increasing negative consumer perception of synthetic additives and the worldwide growing problem of allergies, is causing the food industry to search for more effective preservation strategies (Pellegrini *et al.,* 2018). As a result, there is a necessity to find a novel and safe natural preservative agents to extend shelf-life of foods. An alternative to synthetic chemical preservatives is represented by the employment of essential oils (EOs) which are commonly employed in foods as aromatizing and flavoring agents, these plant volatile fractions

can be exploited by the food industry for their antimicrobial and antioxidant properties (Pellegrini *et al.,* 2018).

Cymbopogon citratus is a perennial grass plant belonging to the family Gramineae lemongrass, comprising approximately 500 genus and 8000 herb species (Olayemi *et al.,* 2017). The prefix 'lemon' owes to its typical lemon like odor, which is mainly due to the presence of citral, a cyclic monoterpene - a major constituent of essential oils of Cymbopogon species as well as citrus fruits. Its high citral composition has made it important for several chemical syntheses (Olorunnisola *et al.,* 2014). Similarly, several investigations carried out on different lemongrass extracts showed other important therapeutic potentials such as anti-cancer, anti-hypertensive and anti-mutagenicity. Others include non-toxic properties, anti-diabetic, anti-oxidant, anxiolytic and anti-fungi (Olorunnisola *et al.,* 2014).

Essential oils (also known as volatile or ethereal oils) are complex mixtures of volatile compounds produced by living organisms or from plant materials like flowers, bud, seeds, leaves, wood, fruits, roots, twigs and barks (Vergis *et al*., 2013). They can be obtained from plant materials by several methods, steam distillation, expression, and so on. Among all methods, for example, steam distillation method has been widely used, especially for commercial scale production (Tongnuanchan and Benjakul, 2014). Essential oils are considered to be secondary metabolites and important for plant defense as they often possess antimicrobial properties (Hyldgaard *et al*., 2012). They are proven to have antiviral, antimycotic, antiparasitic, antioxidant and insecticidal properties in addition to the antibacterial action. The phenolic components are responsible for the antibacterial properties of essential oils (vergis *et al*., 2013).

Although the primary use of essential oils in food industry is flavoring, they represent an interesting source of natural antimicrobials for food preservation (Hyldgaard *et al.,* 2012). However, application of essential oils as food preservatives requires detailed knowledge about their properties, i.e., the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), the range of target organisms, the mode of action, the effect of food matrix components on their antimicrobial properties as well as sensory quality of the food. The

antimicrobial activity of EOs can be completely associated with their active constituents (Hyldgaard *et al.,* 2012).

1.2 Statement of Problem

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Yoghurt 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 (Moh *et al.,* 2017). Artificial chemical preservatives such as propionic acid, sodium benzoate, and sorbic acid are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances, has led researchers to examine the possibility of using natural additives (Zhang *et al.,* 2015)..

1.3 Justification

The inhibition of the growth and activity of microorganisms is one of the main purposes of the use of chemical preservatives (Olaniran *et al*., 2015). Due to the lack of household refrigeration facilities and poor electricity supply in rural areas of Nigeria and other parts of West Africa, there are several attempts to increase the shelf life of yoghurt. Common examples include use of antibiotics such as natamycin, and various chemical preservatives such as propionic acid, sodium benzoate, and sorbic acid in the preservation of yoghurt. The increasing demand for safe foods, with less chemical additives, has increased the interest in replacing these compounds with natural products, which do not injure the host or the environment (Olaniran *et al*., 2015). Chemical products used for conserving food have a lot of side effects on human health. Carcinogenic, teratogenic, allergic and high toxic effects are the most important problems of these chemical additives (AL Kassaa *et al*., 2016). Essential oils often possess antimicrobial properties (Hyldgaard *et al*., 2012). They are proven to have antiviral, antimycotic, antiparasitic, antioxidant and insecticidal properties in addition to the antibacterial action. As such, the use of

natural antimicrobials to control spoilage and pathogenic microorganisms is gaining a renewed interest (Zhang *et al.*, 2015).

1.4 Aim of the study

The aim of this study is to investigate the potential of lemongrass (*Cymbopogon citratus*) essential oil for use on Bio-preservation of yoghurt.

1.5 Objectives of the study

To achieve the main objective, the following specific objectives were outline

1. Extraction of Lemongrass essential oil.
2. Phytochemical screening of essential oil.
3. To characterize essential oil from Lemongrass.
4. To isolate and characterize the yoghurt spoilage bacteria using 16srRNA identification.
5. To assess the bio-preservative potential of the lemongrass essential oil on yoghurt.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bio-preservation

Bio-preservation is a technique of extending the shelf life of food by using natural or controlled microbiota or antimicrobials (Singh, 2018; Ananou *et al.,* 2007). Yoghurt is highly perishable due to high nutritional content, moisture and neutral pH. It requires proper preservation to maintain quality and safety. Failing which leads to human illnesses and disease outbreaks. These food borne illnesses are serious and costly public health concern worldwide (Singh, 2018). In the USA, acute gastroenteritis affects 250 to 350 million people annually, and an estimated 22% to 30% of these cases are thought to be foodborne diseases with the main foods implicated including meat, poultry, eggs, seafood, and dairy products (Mead *et al.,* 1999). Several bacterial pathogens including *Salmonella*, *Campylobacter jejuni*, *Escherichia coli 0157:H7*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum* are found associated with such outbreaks (Nath *et al.,* 2014).

Preservation of food by a suitable means is the key of food quality and safety (Singh, 2018). Until now, approaches to seek improved food safety have relied on the search for more efficient chemical preservatives or on the application of more drastic physical treatments (e.g. high temperatures). Nevertheless, these types of solutions have many drawbacks; the proven toxicity of many of the commonest chemical preservatives (e.g. nitrites), the alteration of the organoleptic and nutritional properties of foods, and especially recent consumer trends in purchasing and consumption, with demands for safe but minimally processed products without additives (Ananou *et al.,* 2007).

2.1.1 Characteristic of Bio-preservatives used in food processing industry

The following requirements should be fulfilled by any bio-preservative to be used commercially

1. The bio-preservative to be used should not be toxic.
2. It should be accepted by recognized authorities.
3. It should be economical to the industries using it.

4. The product in which the bio-preservative is being used should not be affected by it, i.e. bio-preservative should not show any deleterious effect toward the organoleptic properties of that product.
5. When used at relatively low concentrations it should show effect.
6. The bio-preservative should be sufficiently stable if being stored.

(Gautam and Sharma, 2009).

2.2 Yoghurt

Yoghurt is a widely known dairy product with high consumption worldwide mainly because of the beneficial impact on consumer's health (Moh *et al.,* 2017). They are regarded as ready to drink foods commonly taken for energy production and for health, all over the world, especially in Nigeria (Taiwo *et al.,* 2018). It can also be consumed as a drink beverage to quench thirst. This product blend is rich in protein and improves healthy living (Taiwo *et al.,* 2018). It is a balanced food which contains virtually almost all the nutrients present in milk and in more absolvable form. There is a wide range of flavors available to spice it. Due to its nutritional, organoleptic and probiotic qualities, it is a popular drink that is in high demand (De *et al.,* 2014).
In circumstances of poor manufacturing practices and storage conditions, spoilage of the product occurs within a relatively short time. The deterioration of yoghurt is the result of changes in its physical, chemical and organoleptic/sensorial characteristics, making it unacceptable for human consumption (Mataragas *et al.,* 2011).

In the early years of milk fermentation, milk was simply allowed to ferment by its normal microbiota, but the actual process was not completely understood (Omola *et al.,* 2014). Cultures could be maintained by inoculating fresh milk with fermented milk. Today, Yogurt is obtained by lactic acid fermentation of milk through the activity of a starter culture of two bacteria, *Streptococcus thermophilus* and *Lactobacillus delbruckii sub-specie bulgaris* to decrease the pH of the milk and produce many different fermented milk products (Corrieu and Beal, 2016; Omola *et al.,* 2014). These microorganisms play important roles in the production of yogurt, from milk product through acidification and synthesis of aromatic compounds (Serra *et al*., 2009). This lactic acid fermentation of milk also serves as a means of prolonging the shelf of the nutrients in milk (Taiwo *et al.,* 2018).

Lactic fermentation is a very old and well known principle. It comes from the discovery of letting raw milk being 'spoiled' by microbes and giving it a firmer texture and a sour taste, the Turks called it "yoğurt" and is the accepted terminology of the product today (Robinson, 2003). The process has been well refined over the years and is one of the most well-known and accepted method in the dairy industries around the world. The fermentation of milk consists of different metabolic pathways but is ultimately the conversion of lactose to lactic acid along with metabolites such as acetaldehyde, diacetyl and polysaccharides. Lactose is a disaccharide and the major carbohydrate and main source of energy for most of the bacteria growing in milk. The bacteria hydrolyse the lactose into galactose and glucose, the glucose in turn is converted to lactic acid either through homo- or heterofermentative action shown in equations below

Homofermentative: Lactose + $4H_3PO_4$ + 4 ADP → 4 lactic acid + 4 ATP + $3H_2O$

Heterofermentative: Lactose + 2 H_3PO_4 + 2 ADP → 2 lactic acid + 2 ethanol + $2CO_2$ + 2 ATP + H_2O

(Robinson, 2003)

The sensory properties of yogurts rely on three main characters:

(1) The composition of milk as raw material, which differs according to the milk source (e.g., cow, goat, or sheep; conventional or organic);

(2) The addition of ingredients that allow modifying the sensory properties (flavor, color, and texture) of the products, such as sweetening agents (sugar or other sweeteners for low-calorie products), flavoring agents (fruit aromas or vanilla) or fruits (small pieces enriched with sugar or jam), stabilizers (pectin, starch, or gelatin), or emulsifiers; and

(3) The technology employed for the manufacture, which may vary depending on the operations during milk pretreatment (fat and nonfat solid standardization, homogenization, or heat treatment) or yogurt post treatment (stirring, concentration, mixing, cooling, drying, or freezing).

(Corrieu and Beal, 2016).

2.3 Dairy loss

Dairy loss is one of the major problems of the dairy industry in developing countries especially in Africa (Moh *et al.,* 2017). Losses occurring at the farm are attributed by unhygienic milk handling, poor milking procedures and spoilage due to lack of cooling facilities. Pasteurization

has been used as a public health measure to eliminate or reduce the activities of spoilage and pathogenic microorganisms in milk. However *Bacillus* and *Streptococcus* species are likely to survive pasteurization due to their ability to form heat-resistant endospores (Rawat, 2015). 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. This makes microbes the main cause of milk spoilage (Salas *et al*., 2017).

2.3.1 Microbial Spoilage associated with diary foods

Microbial contamination can lead to food poisoning outbreaks and unsatisfactory products and this is an enormous economic problem worldwide (Moh *et al.*, 2017). Microbial contamination and foodborne microbial diseases constitute a large and growing public health concern. In fact, most countries with case-reporting systems have documented significant increases over the past few decades in foodborne microbial diseases incidence. Food safety challenges in Africa include unsafe water and poor environmental hygiene, weak foodborne disease surveillance, inability of small and medium scale producers to provide safe food, outdated food regulations, and inadequate law enforcement, as well as insufficient cooperation among stakeholders (Moh *et al.*, 2017).

Microbial succession is the main cause of milk spoilage (Salas *et al*., 2017). Food products are susceptible to spoilage due to their natural nutrient rich composition. This susceptibility does however depend on various factors, namely:

(i) The food matrix nature (living material or not, liquid or solid), its composition (nutrient content, solute type), and biological (e.g., its natural microbiota), physical, and chemical parameters (water activity, pH);

(ii) Management during harvesting of fruits and vegetables (maturity, handling) and raw material storage (temperature, hygrometry, and duration);

(iii) Technological processes applied during manufacture (e.g., heating, drying, salting, fermentation, preservative addition) including cleaning/disinfection steps; and

(iv) Storage conditions after manufacturing (type, atmosphere and extent of packaging, temperature, relative humidity) (Salas *et al*., 2017).

Microbial spoilage of food is known as rot, which manifests as loss of texture (soft rot), changes in color (black or grey) and often off odor. Chemical reactions that cause offensive sensory changes in foods are mediated by a variety of microbes that use food as a carbon and energy source (Gram *et al.*, 2002). These organisms include prokaryotes (bacteria), single-celled organisms lacking defined nuclei and other organelles, and eukaryotes, single-celled (yeasts) and multicellular (molds) organisms with nuclei and other organelles. Within a spoiling food, there is often a succession of different populations that rise and fall as different nutrients become available or are exhausted. Some microbes, such as lactic acid bacteria and molds, secrete compounds that inhibit competitors. Spoilage microbes are often common inhabitants of soil, water, or the intestinal tracts of animals and may be dispersed through the air and water and by the activities of small animals, particularly insects (Rawat, 2015).

Spore-forming bacteria are usually associated with spoilage of heat-treated foods because their spores can survive high processing temperatures. These Gram-positive bacteria may be strict anaerobes or facultative (capable of growth with or without oxygen). Some spore-formers are thermophilic, preferring growth at high temperatures (as high as 55ºC). Some anaerobic thermophiles produce hydrogen sulphide (*Desulfotomaculum*) and others produce hydrogen and carbon dioxide (*Thermoanaerobacterium*) during growth on canned/ hermetically sealed foods kept at high temperatures (Gram *et al.*, 2002). Other thermophiles such as *Bacillus* and *Geobacillus* spp. causes a flat sour spoilage of high or low pH canned foods with little or no gas production, and one species causes ropiness in bread held at high ambient temperatures (Rawat, 2015). Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, including species of *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus*, some of which are useful in producing fermented foods such as yogurt and pickles. However, under low oxygen, low temperature, and acidic conditions, these bacteria become the predominant spoilage organisms on a variety of foods. Undesirable changes caused by LAB include greening of meat and gas formation in cheeses (blowing), pickles (bloater damage), and canned or packaged meat and vegetables (Gram *et al.*, 2002).

Enterobacteriaceae are gram-negative, facultatively anaerobic bacteria that include a number of human pathogens (*Salmonella, E. coli, Citrobacter, Shigella, Yersinia*) and also a large number

of spoilage organisms (Rasch *et al.,* 2005). These bacteria are widespread in nature in soil, on plant surfaces and in digestive tracts of animals and are therefore present in many foods. *Erwinia carotovora* is one of the most important bacteria causing soft rot of vegetables in the field or stored at ambient temperatures. *Pseudomonas* and related genera are aerobic, gram-negative soil bacteria, some of which can degrade a wide variety of unusual compounds. Some species grow at refrigeration temperatures (psychrophilic) while other are adapted for growth at warmer, ambient temperatures (Rasch *et al.,* 2005). Many Gram-negative bacteria, including pseudomonads and enterobacteriaceae, secrete acyl homoserine lactones (AHLs) to regulate the expression of certain genes, such as virulence factors, as a function of cell density. These AHL quorum-sensing signals may regulate proteolytic enzyme production and iron chelation during spoilage of some foods although the role of these signals in other spoilage systems is not clear (Gram *et al.,* 2002).

Yeasts are a subset of a large group of organisms called fungi that also includes molds and mushrooms. They can grow with or without oxygen (facultative) and are well known for their beneficial fermentations that produce bread and alcoholic drinks. They often colonize foods with a high sugar or salt content and contribute to spoilage of maple syrup, pickles, and sauerkraut. Fruits and juices with a low pH are another target, and there are some yeast that grows on the surfaces of meat and cheese (Salas *et al.,* 2017). Yeast are best known for their role in production of bread and wine but some strains also spoil wines and other alcoholic beverages by producing gassiness, turbidity and off flavors associated with hydrogen sulfide and acetic acid. Some species grow on fruits, including yogurt containing fruit, and some are resistant to heat processing (Salas *et al.,* 2017).

Molds are filamentous fungi that do not produce large fruiting bodies like mushrooms. Molds are very important for recycling dead plant and animal remains in nature but also attack a wide variety of foods and other materials useful to humans. They are well adapted for growth on and through solid substrates, generally produce airborne spores, and require oxygen for their metabolic processes (Salas *et al.,* 2017). They can tolerate harsh environmental conditions but most are sensitive to heat treatment. Different mold species have different optimal growth temperatures, with some able to grow in refrigerators. They have a diverse secondary

metabolism producing a number of toxic and carcinogenic mycotoxins. Some spoilage molds are toxigenic while others are not (Rawat, 2015).

2.4 Sensory Evaluation

Evaluation of the sensory characteristics of dairy products has been, and will continue to be, the ultimate method for evaluating product quality. Sensory quality is a parameter that can be evaluated only by humans and consists of a series of tests or tools that can be applied objectively or subjectively within the constructs of carefully selected testing procedures and parameters (Schiano *et al.,* 2017). Sensory analysis is considered to be a multidisciplinary science that uses human panelists sensory perception related to thresholds of determination of attributes, the variance in individual sensory response experimental design to measure the sensory characteristics and the acceptability of food products, as well as many other materials. Since there is no one instrument that can replicate or replace the human psychological and emotional response, the sensory evaluation component of any food study is essential and the importance of good experimental design cannot be over emphasized in sensory experiments (Singh-Ackbarali and Maharaj, 2014).

Sensory evaluation can be define as a scientific discipline used to evoke, measure, analyse and interpret those responses to products as perceived through the senses of sight, smell, touch, taste and hearing (Singh-Ackbarali and Maharaj, 2014). Traditionally consumer testing is done by measure preference on different scales. The 9-point hedonic scale was designed already in 1947 (Cardello & Jeager, 2010) and assumes that consumer preferences do exist, likewise that this preference can be categorized based on like-dislike judgment. The word hedonic refers to pleasure and the rating on such a scale is actually more of a measurement of acceptance because the like-dislike judging can be made without the presence of other products (Lawless & Heymann, 2010). The 9-point hedonic scale is commonly used in consumer testing although the outcome not necessary has to reflect on whether the product will be successful or not on the market.

Table 2.1: Definitions of the descriptors (sensory attributes) used for evaluation of the yogurts

Attribute	Description
Appearance	Assess the color (white, whitish, yellowish, yellow) of yogurt
Texture	Evaluate the viscosity when stirring the product with the spoon
Aroma	Detect any aroma defects (e.g., unclean, masked, unnatural, cooked, lacks freshness) by smelling and oral perception of samples
Taste	Evaluate the taste of samples considering several attributes associated with taste (e.g., unclean, unnatural, whey, and refreshing perception) and aftertaste (e.g., sourness, astringency, sweetness, bitterness and saltiness)
Acceptability	Rate the overall score of the sample considering the appearance, taste, texture and aroma

(Soukoulis *et al.*,2007)

2.5 Natural antimicrobials in food applications

A number of preservation techniques are being employed to satisfy consumers demand with regard to nutritional composition and sensory aspects of foods. Generally, foods are subjected to temperatures varying from 60 to 100^{o}C for the duration of a few seconds to a minute in order to destroy vegetative microorganisms. Large amount of energy is transferred to the food during this period. However, this energy can trigger unwanted reactions, leading to undesirable organoleptic changes and nutritional effects (Tiwari *et al.*, 2009). Ensuring the safety of food and at the same time meeting such demands for retention of nutrition and quality attributes has resulted in increased interest in alternative preservation techniques for inactivating spoilage microorganisms and enzymes in foods. Some of the quality attributes of importance include flavor, odor, appearance, texture, and general acceptability. This increasing demand has opened new dimensions for the use of natural preservatives derived from plants and animals (Tiwari *et al.*,2009). In biopreservation, storage life is extended, and/or safety of food products is enhanced by using natural or controlled microflora, mainly lactic acid bacteria (LAB) and/or their antibacterial products such as lactic acid, bacteriocins, and others.

Inactivation, growth delay, or growth prevention of spoilage and pathogenic microorganisms are main objectives of food preservation (Zhang *et al.*, 2015). The use of chemical agents exhibiting antimicrobial activity (by inhibiting and/or reducing microbial growth or even by inactivating undesirable microorganisms) is one of the oldest and most traditional food preservation techniques. Antimicrobial agents are chemical compounds added to, or present in, foods that retard microbial growth or cause microbial death (Salas *et al.*, 2017).

Concerns about the use of antimicrobial agents in food products have been discussed for decades. Both the increasing demand for reduced-additive (including antimicrobial agents) and more "natural" foods and the increasing demand for greater convenience have promoted the search for alternative antimicrobial agents or combinations to be used by the food industry. The application of chemical antimicrobial agents in food preservation is regulated in the United States by the Food and Drug Administration (FDA) and in other countries by appropriate corresponding authorities (Sofos and Busta, 1992). The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations, testing and recommending usage and safety of chemicals in foods and average daily intakes (ADI), regulate chemical antimicrobials internationally. In the United States, chemicals in foods are examined according to the Food Additives Amendment of the Food, Drug and Cosmetic Act, specifying the procedures and conditions required for a chemical food additive to be approved. These obstacles have originated the search of emerging preservatives by examining compounds already used in the food industry, perhaps with other purposes, but with potential as antimicrobials, approved and not toxic in the used levels; many of them are classified as generally recognized as safe (GRAS) (Sofos and Busta, 1992).

2.5.1 Plant based antimicrobials

Plant oils and extracts have been used for a wide variety of purposes for many thousands of years (Swamy *et al.*, 2016). These purposes range from perfume industry to flavouring and application in preservation of stored food crops. In particular, the antimicrobial activity of plant oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. Since ancient times, herbs and their essential oils have been known for their varying degrees of antimicrobial activity.

More recently, medicinal plant extracts were developed and proposed for use in food as natural antimicrobials (Vergis *et al.,* 2013).

2.5.1.1 Lemongrass *(Cymbopogon citratus)*

The scientific name of lemongrass is *Cymbopogon citratus*. The *Cymbopogon* word derives from the Greek words “kymbe” (boat) and “pogon” (beard), referring to the arrangement of the spike of the flower. The word *citratus* derives from the old Latin, meaning lemon-scented leaves (Shah *et al*., 2011). The Cymbopogon genus is comprised of more than 55 species in different countries all over the world possessing various material and chemical properties (Abdulazeez *et al*., 2016). Cymbopogon citra*tus* of the Poaceae family is a tall, monocotyledonous aromatic perennial plant with slender sharp-edged green leaves, pointed apex that is native to tropical Asia. *C. citratus* is known as Guatemala in West Indian, or Madagascar lemongrass.

C. citratus is cultivated in Africa, the West Indies, Central and South America, and tropical regions. The linear leaves can grow up to 90 cm in height and 5 mm in width (Ewansiha *et al.,* 2012). It is grown around the world and has a century long record of extensive therapeutic applications in traditional and Ayurvedic medicine in a number of countries (Ekpenyong *et al.,* 2014). However, *Cymbopogon flexuosus* and *Cymbopogon citratus* (also known as West Indian and East Indian lemongrass, respectively) represent the two major species vastly cultivated in different regions of the world for the high citral content (70–80%) in their essential oils (Haque *et al.,* 2018). In addition to its therapeutic uses, *C. citratus* is also consumed as a tea, added to non-alcoholic beverages and baked food, and used as a flavoring and preservative in confections and cuisines. In cosmetics, its essential oils are used as fragrance in the manufacture of perfumes, soaps, detergents, and creams (Abdulazeez *et al*., 2016).

2.6 Essential oils: overview

An essential oil is a concentrated, hydrophobic liquid containing volatile aroma compounds from plants (Burt, 2004). Essential oils are also known as volatile, ethereal oils or aetherolea. Oil is "essential" in the sense that it carries a distinctive scent, or essence, of the plant. Essential oils are frequently referred to as the “life force” of plants (Dhifi *et al.,* 2016). They have been

associated with different plant parts including leaves, stems, flowers, roots or rhizomes. All parts of aromatic plants may contain essential oils as follows:

- Leaves, most often, including: lemon grass, eucalyptus, mint, thyme, bay leaf, savory, sage, pine needles, and tree underground organs, e.g., roots (vetiver),
- Flowers, of course, including: orange, pink, lavender, and the (clove) flower bud or (ylang-ylang) bracts,
- Fruits, including: fennel, anise, Citrus epicarps,
- Rhizomes (ginger, sweet flag),
- Seeds (carvi, coriander),
- Wood and bark, including: cinnamon, sandalwood, rosewood.

(Dhifi *et al.*, 2016).

Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour (Hyldgaard *et al.,* 2012). The amount of essential oils found in these plants can be anywhere from 0.01% to 10% of the total. These oils have potent antimicrobial factors, having wide range of therapeutic constituents. These oils are often used for their flavor and their therapeutic or odoriferous properties, in a wide selection of products such as foods, medicine, and cosmetics. Although the food industry primarily uses essential oils as flavorings, they represent an interesting source of natural antimicrobials for food preservation (Burt, 2004).

2.6.1 Lemongrass Essential Oil

Lemongrass is a perennial grass plant widely distributed worldwide and most especially in tropical and subtropical countries (Olorunnisola *et al.,* 2014). Several reports have linked its origin to Asia (Indochina, Indonesia and Malaysia), Africa and the Americas. The plant could grow up to 6 inch high and its bulblike stems consist of terete and glabrous linearly venated sheathed leaves with narrow base and acute apex. The leaf height is about 100 cm in length and 2 cm in width. When squeezed, the leaves usually produce yellow or amber colored, aromatic, essential oil (Olayemi *et al.*, 2017). Its aqueous extract is commonly used as an aromatic drink while the whole plant is well incorporated into traditional food for its lemon flavour. It also enjoyed wide application in folk medicine (Vazirian *et al.,* 2012). Traditionally, tea made from lemongrass leaves is popular among countries of South America, Asia and West Africa having

been widely utilized as antiseptic, antifever, antidyspeptic, carminative and anti-inflammatory effects. Others are febrifuge, analgesic, spasmolytic, antipyretic, diuretic, tranquilizer and stomachic agent (Olorunnisola *et al*., 2014).

2.6.2 Extraction of Essential Oils

Essential oils can be extracted from several plants with different parts by various extraction methods. The method used for extraction of essential oils normally depends on the type of botanical material used, the state and form of the material. Extraction method is one of prime factors that determine the quality of essential oil. It is proved through a number of studies that the quality of essential oil mainly depends on its constituents which is primarily influenced by their extraction procedures (Suryawanshi *et al.* 2016). The basic procedures included steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples (Sasidharan *et al*., 2011). Inappropriate extraction procedure can lead to the damage or alter action of chemical signature of essential oil. This results in the loss in bioactivity and natural characteristics. For severe case, discoloration, off-odor/flavor as well as physical change such as the increased viscosity can occur (Tongnuanchan and Benjakul, 2014).

The following are the methods of extraction and their drawbacks

2.6.2.1 Steam Distillation

Steam distillation is the most widely used method for plant essential oil extraction (Tongnuanchan and Benjakul, 2014). The principle of steam distillation is that it enables a compound or mixture of compounds to be distilled at a temperature substantially below that of the boiling points of the individual constituents (Tongnuanchan and Benjakul, 2014). Basically, fresh or sometimes dried, botanical material is placed in the plant chamber of the still and steam is allowed to pass through the herb material under pressure which softens the cells and allows the Essential Oil to escape in vapor form. The heat applied is the main cause of burst and break down of cell structure of plant material. As a consequence, the aromatic compounds or essential oils from plant material are released. The temperature of heating must be enough to break down

the plant material and release aromatic compound or essential oil. The advantage of Steam Distillation is that it is a relatively cheap process to operate at a basic level, and the properties of oils produced by this method are not altered. As steam reduces the boiling point of a particular component of the oil, it never decomposes in this method. This method apart from being economical, it is also relatively faster than other methods (Tongnuanchan and Benjakul, 2014).

2.6.2.2 Solvent extraction

Solvent extraction has been implemented for fragile or delicate flower materials, which are not tolerant to the heat of steam distillation (Suryawanshi *et al.,* 2016). Different solvents including acetone, hexane, petroleum ether, methanol, or ethanol can be used for extraction. For general practice, the solvent is mixed with the plant material and then heated to extract the essential oil, followed by filtration. Subsequently, the filtrate is concentrated by solvent evaporation. From the concentrate, it is then mixed with pure alcohol to extract the oil and distilled at low temperatures. The alcohol absorbs the fragrance and when the alcohol is evaporated, the aromatic absolute oil is remained. However, this method is a relatively time-consuming process, thus making the oils more expensive than other methods. This is not considered the best method for extraction as the solvents can leave a small amount of residue behind which could cause allergies and effect the immune system (Suryawanshi *et al.,* 2016).

2.6.3 Essential Oils as Food Additives

A wide range of preservatives are used to extend the shelf-life of a product by inhibiting microbial growth. However, an increasingly negative consumer perception of synthetic food additives has spurred an interest in finding natural alternatives to the traditional solutions (Hyldgaard *et al*., 2012). Essential oils from plants have been known to act as natural additives, for example, antimicrobial agents, antioxidant, and so on. Their activities vary with source of plants, chemical composition, extraction methods, and so on. Due to the unique smell associated with the volatiles, this may limit the use of essential oil in some foods since it may alter the typical smell/flavor of foods (Vergis *et al.,* 2013).

2.6.3.1 Antimicrobial Activity

At present, many antibiotics are available for treating various bacterial pathogens. However, increased multidrug resistance has led to the increased severity of diseases caused by bacterial pathogens. In addition, low immunity in host cells and the ability of bacteria to develop biofilm-associated drug resistance have further increased the number of life threatening bacterial infections in humans (Swamy *et al.,* 2016). Thus,. bacterial infections remain a major causative agent of human death, even today. In addition, the use of several antibacterial agents at higher doses may cause toxicity in humans. This has prompted researchers to explore alternative new key molecules against bacterial strains (Swamy *et al.,* 2016). In this regard, plant essential oils and their major chemical constituents are potential candidates as antibacterial agents

Several types of essential oils and their major chemical constituents from various medicinal and aromatic plants have been reported to possess a wide range of bacterial inhibitory potentials (Tongnuanchan and Benjakul, 2014). Anti-bacterial activity in extracts of plant materials has been reported from various sources in recent times with promising results. This characteristic has also been investigated in the volatile oil portion of the aqueous extract of lemon grass. Among the major bioactive compounds identified in the oil were α-citral (geranial) and β-citral (neral) components (Olorunnisola *et al.,* 2014). They have a broad spectrum of antimicrobial activity against most gram-positive and gram-negative bacteria. For gram-positive bacteria, it is able to interact with the membranes of bacteria and alter the permeability for cations like H+ and K+. Lipophilic ends of lipoteichoic acids in cell membrane of gram positive bacteria may facilitate the penetration of hydrophobic compounds of essential oils. On the other hand, the resistance of gram-negative bacteria to essential oils is associated with the protecting role of extrinsic membrane proteins or cell wall lipopolysaccharides, which limits the diffusion rate of hydrophobic compounds through the lipopolysaccharide layer (Burt, 2004). The effect of antibacterial activity of essential oils may inhibit the growth of bacteria (bacteriostatic) or destroy bacterial cells (bactericidal). Nevertheless, it is difficult to distinguish these actions. In relation to this, antibacterial activity is more frequently measured as the minimum bactericidal concentration (MBC) or the minimum inhibitory concentration (MIC) (Swamy *et al.,* 2016).

2.6.3.2 Antioxidant Activity

Essential oils have several modes of actions as antioxidant, such as prevention of chain initiation, free radical scavengers, reducing agents, termination of peroxides, prevention of continued hydrogen abstraction as well as quenchers of singlet oxygen formation and binding of transition metal ion catalysts. With those functions, essential oils can serve as the potential natural antioxidants, which can be used to prevent lipid oxidation in food systems (Tongnuanchan and Benjakul, 2014). Phenolics are organic compounds consisting of hydroxyl group (-OH) attached directly to a carbon atom that is a part of aromatic ring. The hydrogen atom of hydroxyl group can be donated to free radicals, thereby preventing other compounds to be oxidized. The antioxidant capability of phenolic compounds is mainly due to their redox properties, which permit them to act as hydrogen donors, reducing agents, singlet oxygen quenchers as well as metal chelators (Tongnuanchan and Benjakul, 2014).

2.6.4 Identification and Characterization

Due to the fact that plant extracts usually occur as a combination of different types of bioactive compounds with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds (Sasidharan *et al*., 2011). It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as column chromatography, TLC, flash chromatography, Sephadex chromatography and HPLC, should be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity. Besides that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds (Sasidharan *et al*., 2011).

2.6.5 Composition of EO

Essential oils are produced by various differentiated structures, especially the number and characteristics of which are highly variable. Essential oils are localized in the cytoplasm of certain plant cell secretions, which lies in one or more organs of the plant; namely, the secretory hairs or trichomes, epidermal cells, internal secretory cells, and the secretory pockets (Dhifi *et*

al., 2016; Burt, 2004). Essential oil constituents are a diverse family of low molecular weight organic compounds with large differences in antimicrobial activity. Numerous publications have presented data on the composition of the various EOs. Detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the EO or its headspace (Burt, 2004). Most essential oils are composed of terpenes, terpenoids, and other aromatic and aliphatic constituents with low molecular weights (Swamy *et al.*, 2016).

Terpenes or terpenoids are synthesized within the cytoplasm of the cell through the mevalonic acid pathway. Terpenes are composed of isoprene units and are generally represented by the chemical formula $(C_5H_8)n$. Terpenes can be acyclic, monocyclic, bicyclic, or tricyclic. Owing to the diversity in their chemical structures, terpenes are classified into several groups such as monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), and triterpenes ($C_{30}H_{40}$) (Swamy *et al.,* 2016).

The major component (~90%) of bioactive essential oils is constituted of monoterpenes. Some of the major compounds include monoterpene hydrocarbons (*p*-cymene, limonene, α-pinene, and α-terpinene), oxygenated monoterpenes (camphor, carvacrol, eugenol, and thymol), diterpenes (cembrene C, kaurene, and camphorene), sesquiterpene hydrocarbons (β-caryophyllene, germacrene D, and humulene), oxygenated sesquiterpenes (spathulenol, caryophyllene oxide), monoterpene alcohols (geraniol, linalool, and nerol), sesquiterpene alcohol (patchoulol), aldehydes (citral, cuminal), acids (geranic acid, benzoic acid), ketones (acetophenone, benzophenone), lactones (bergapten), phenols (eugenol, thymol, carvacrol, and catechol), esters (bornyl acetate, ethyl acetate), and coumarins (fumarin, benzofuran) (Swamy *et al.*, 2016; Dhifi *et al.*, 2016).

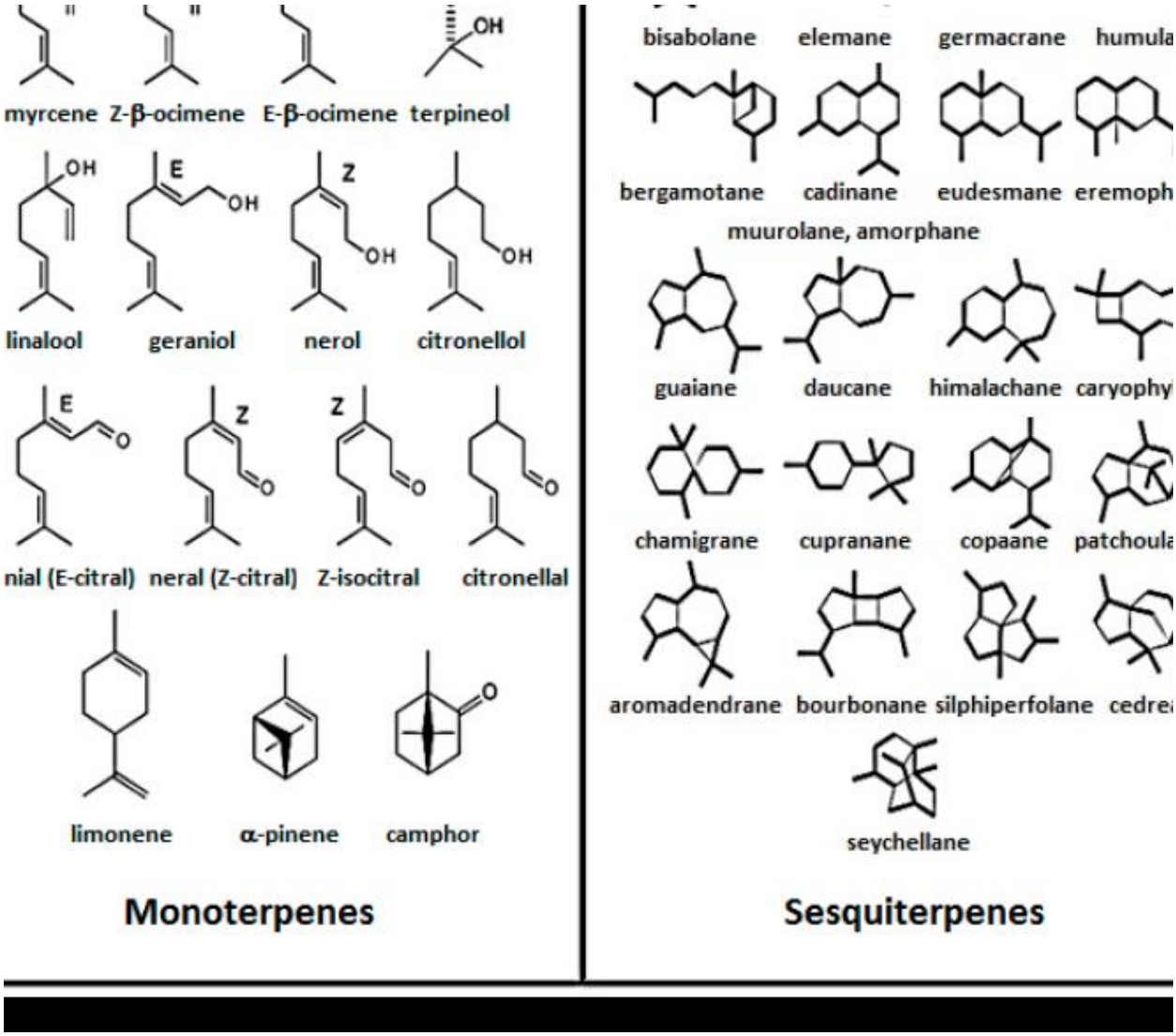


Fig 2.1: Structures of some terpenes (Dhifi *et al.,* 2016).

The chemical constituents of plant essential oils differ between species. Some factors that can affect these constituents include the geographical location, environment, and stage of maturity. Furthermore, the stereochemical properties of essential oils can vary and depend upon the method of extraction (Swamy *et al.,* 2016). However, extraction products may also vary qualitatively and quantitatively in their composition. Although essential oils can be recovered using fermentation, extraction, or effleurage processes, commercial production is preferably achieved by the steam distillation process (Burt, 2004). Likewise, the antimicrobial efficiency of essential oils depends on the type of microbes to be inhibited as well as the evaluation methods,

including bioautography, diffusion, and dilution. Methods to evaluate the essential oil chemistry, their biological activities, and various factors that affect bioactivity are detailed in the literature

2.6.6 Mode of action

The hydrophobicity of essential oils enables them to partition the lipid layer of bacterial cell membrane and mitochondrion, making the structures more permeable. This leads to leakage of ions and other cell contents, which when exceeds a limit lead to lysis and death. The mechanism affects disturbance to cytoplasmic membrane disrupting Proton Motive Force (PMF), electron flow, active transport and coagulation of cell contents (Burt, 2004). Two possible mechanisms exist whereby cyclic hydrocarbons act on lipid molecules on cytoplasmic membrane. The lipophilic nature of essential oil forces them to accumulate in lipid bilayer and distort lipid-protein interaction (Vergis *et al*., 2013). Generally, the EOs possessing the strongest antibacterial properties against food borne pathogens contains a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials, chemicals, reagents, equipments and apparatus used

All materials, chemicals, reagents, equipments and apparatus used in this study were of analytical grade and were obtained from laboratory chemicals Department of Biochemistry and Microbiology, Bayero University Kano with detailed list presented in appendix I.

3.2 Method of samples Collection

Raw milk was collected from L and Z Nig LTD Farms. The sample was transported to the lab in an ice pack. Lemongrass sample was purchased from Rimi market and transported to the laboratory. The leaves of *Cymbopogon Citratus* (Lemon grass) were identified at the Department of Plant Biology, Bayero University Kano and were given an accession number BUKHAN 234.

3.3 Essential oil Extraction

The method of EO extraction by (Arifuddin *et al*., 2016; Kamaliroosta *et al*., 2013 and Aziza and Okiy, 2018) was adopted. Lemongrass was washed to remove sand and other foreign particles. It was further reduce by cutting into small sizes and then shade dried to reduce moisture content. The sample was grinded, homogenized and made into a fine powder. Sample (500g) was placed in the Clevenger apparatus. Distilled water (1L) was added to the flask and heated to boiling point. To obtain maximum yield, temperature and time was controlled. The temperature was set to 100^0C.The extraction process was set for about 30-40mins. A beaker was used to collect the extract which was the distillate. Then the extract was further distilled to remove excess water, and get a more concentration of the oil extract (Suryawanshi *et al.,* 2016). The percentage yield of the EO was calculated using:

Yield of essential oil = Amount of essential oil (g) obtained / amount of raw materials (g) used ×100

3.4 Phytochemical Screening of Lemongrass EO

The method of Ewansiha et al. (2012) and Rao et al. (2016) for Phytochemical analysis was performed to screen Lemongrass EO for the presence of the following active principles using standard procedures:

3.4.1 Test for Tannins

Braymers Test: Lemongrass Essential oil (0.5ml) was added to 10ml of distilled water and mixed with few drops of 5% Ferric Chloride (FeCl3) solution. The formation of a green precipitate was an indication of the presence of tannins.

3.4.2 Test for Flavonoids

Alkaline reagent Test: A few drops of dilute sodium hydroxide (NaOH) solution were added to 1ml of LGEO. The appearance of yellow solution which disappeared on the addition of HCL indicates the presence of flavonoids

3.4.3 Test for Saponins

Froth Test: Lemongrass EO was diluted with 20ml of distilled water and was agitated in a graduated cylinder for 15 minutes. The formation of stable foam was taken as an indication for the presence of saponins

3.4.4 Test for terpenoids

Salkowski Test: In a test tube containing 2 mL of chloroform, 0.5 mL of extract was added. This is then followed by the addition of 3 mL conc. H_2SO_4 which forms a layer. Reddish brown coloration of the interface indicates terpenoids.

3.5 Fractionation of compounds

Fractions of the Essential Oil were obtained by simple column chromatography using Silica Gel as describe by Karami-Osboo et al. (2010) and Ewansiha et al. (2012). Four solvents (ethylacetate: toluene and Hexane: Methanol in different proportions to elute fractions according to their affinity to mobile phase) were used for better mobile phase. Mixtures of solvents having

suitable adsorbtion strength were preferred. Admixture was prepared by mixing 5ml of EO with 5g of Silica Gel to get uniform mixing. Also, 30g of silica gel was taken and packed very carefully in a suitable column without air bubble. The admixture was added at the top of the stationary phase and started separation of compounds by eluting with various solvent mixtures at a flow rate of 2ml /min. All column fractions were collected separately and fractions containing similar Rf value were pooled and concentrated under reduced pressure

3.6 Yorghurt preparation

Yogurt sample was prepared at Sensory Evaluation Lab of the Department of Biochemistry (BUK). Cow's milk (12 L) was heated up to 85^0C for 20 min and then cooled to 44 ± 1^0C for the inoculation of starters (*L. bulgaricus* and *S. thermophilus*) added in the milk (Manufacturers Standard) (Soukoulis *et al.,* 2007).

3.7 Bio-preservative study of milk (with EO) and milk (without EO)

The Shelf life extension of milk was determined based on colony count method, Determination of physico chemical properties and sensory evaluation of the yoghurt sample every 48 h after first preservation of yoghurt sample with Lemongrass essential oil and at the expiration of shelf life of the yoghurt. The yoghurt samples was inoculated with Lemongrass EO at 0.5µl/ml, 1µl/, 2µl/ml and artificial preservative which serve as positive control. The packaged yoghurt samples were stored at room temperature for 7 days. Samples were monitored for microbial quality, physico chemical properties and sensory characteristics.

3.7.1 Colony count

The total colony count was carried out using pour plate method as described by De et al. (2014). Yoghurt sample (1ml) was aseptically introduced into 9 ml of peptone water solution. Peptone water was used as the diluents to perform serial dilutions. Serial dilutions for each sample were made up to 10^{-6}. One ml of diluted yogurt was inoculated in each empty Petri dish followed by adding 15 ml melted agar. Microbial counts were taken before biopreservation of products, every

48 h after first preservation of dairy products with Lemongrass essential oil and at the expiration of shelf life of the yoghurt. Discrete colonies that appeared on the plates after appropriate inoculation and incubation were counted using digital colony counter. The total viable count (TVC), Lactobacillus count (LBC), and fungal count (FC) were obtained on Nutrient Agar, Mann Rogosa Sharpe (MRS) Agar and Sabouraud Dextrose Agar, respectively (Dafur *et al.*, 2018). The numbers of colonies counted were multiplied by the reciprocal of the dilution factor plated, and divided by the volume of inoculums used to obtain the colony forming unit per milliliter (cfu/ml) of each sample. This is expressed as:

cfu/ml = (Number of colony counted X Reciprocal of dilution factor)/ Volume inoculated (De *et al.*, 2014)

3.7.2 Physico chemical Analysis

Physico-chemical analyses were carried out according to the method of the Association of Official Analytical Chemists (AOAC, 2005).

3.7.2.1 Measurement of pH

The pH of yoghurt samples was measured using labtech pH meter with a glass electrode

3.7.2.2 Titratable acidity: The titratable acidity was measured by titrating 15 ml of the yoghurt with 0.1 M sodium hydroxide until the substance reached a pH value 8.2, corresponding to the end point of the phenolphthalein.

Readings were done with pH meter (JENWAY 3505). When this value was reached, the spent NaOH volume was recorded and the acid percentage of the substance was calculated using the formula:

Titratable acidity Titre value x M x 90 x 100/ Volume of sample x 1000 (Igbabul *et al.*, 2014).

Where, M = Molar concentration of NaOH

3.7.2.3 Ash content determination: The ash content was determined by direct heating method as described by Igbabul et al. (2014). 2g of the yoghurt samples was weighed in dried glass

crucibles separately. The samples were then incinerated to ash in a muffle furnace for 3 hours at 550℃. The crucibles were then removed, cooled in desiccator and the weight of the ash was determined. The percentage ash content was calculated by the following formula.

% ash = (Z-X/Y-X) ×100 (Igbabul *et al.,* 2014).

where; X=weight of empty crucible; Y=weight of crucible + sample; Z=weight of crucible + ash

3.7.2.4 Moisture content determination: The percentage of moisture content was determined by oven method as described by Igbabul et al. (2014). 2g of yoghurt sample was dried in the oven for 24 hours at 100℃. The percentage moisture content was calculated by the following formula.

% moisture= $W1 - (W2 \times 100/W1)$

where, W1=initial weight of sample; W2=weight of the dried sample (Igbabul *et al.,* 2014).

3.7.2.5 Total solids: The weight of the residue obtained from moisture content analysis was expressed as percentage total solids using the formula below:

Total solids (%) = (100 - %moisture)

3.7.2.6 Viscosity: Viscosity was measured using a viscometer model DV– E viscometer using a glass tube and a normalized ball equipped with a chronometer at 20oC. Viscosity was as expressed as centipoise.

3.7.3 Sensory Evaluation

The sensory evaluation analysis was carried out using the questionnaire adopted by Soukoulis et al. (2007). A group of 8 panelists was chosen from students and teaching staff of Biochemistry Department of Bayero University Kano to evaluate the yoghurt samples. The panel was consumers who were familiar with yoghurt quality. Selection was based on interest and availability. The biopreserved yoghurt was then served randomly in coded plates plus a control sample. Panelists were given the samples in a plastic jar (100 mL) to score (1 the lowest to 9 the highest) and evaluated for colour, aroma, texture, taste, and general acceptability of the biopreserved yoghurt on the 1st, 3rd, 5th, and 7th days of storage by a 9-point hedonic scale which was adapted from Yangilar and Yildiz (2017). All the samples were provided the same

test conditions and panelists were allowed to clean their plates between the samples using water. The organoleptic scores generated for each attribute will be analyzed statistically using Analysis of variance (ANOVA).

Samples were coded using a 3-digit random number and served successively to the panelists in individually partitioned booths (Soukoulis *et al.,* 2007). Samples were served in 100ml plastic jars. The assessment procedure of the sensory attributes of yogurts was divided into 3 major stages. First, flavor was assessed by removing the lid of the cup and rating the intensity of the volatile flavor substances. Second, appearance by visual observation and textural properties by breaking down the yogurt gel and agitating the product were evaluated. Finally, the taste and aroma (aroma sensory attribute was defined as the combined perception of the detected aroma defects) of yogurts was assessed by swallowing (a teaspoon portion) of sample. Overall acceptance was rated at the end of the sensory evaluation of each sample. Panelists were motivated to note any criticisms on the score sheets used for the sensory evaluation (Soukoulis *et al.,* 2007). The sensory score card used is presented in Appendix XIV.

3.8 Isolation of Spoilage Bacteria

Isolation of yoghurt spoilage bacteria was carried out according to the method employed by (Dafur *et al.,* 2018; Lamye *et al.,* 2017; Zakir *et al.,* 2015; De *et al.,* 2014). After the serial dilution of the spoiled yoghurt, 1 mL each from dilutions 10^{-6} was aseptically taken and plated on MacConkey and Nutrient agar using pour plate method. After 24 hours incubation, plates that showed growth of organisms with distinct colonies on the agar were sub-cultured and incubated at 45^{0}C for 24 hours.

3.8.1 Identification and Characterization

The isolated bacteria was identified phenotypically by gram staining and molecular identification to confirm the identity of the isolates

3.8.1.1 Gram Staining

A smear was prepared on a glass slide from cultures incubated, the slide was flooded with crystal violet and allowed to stay for a minute, it was then washed off by dipping the slide into distilled water in a 250ml beaker for 5s, then the slide was flooded with fresh iodine solution for 1min, it was washed for 5s in water. The slide was then decolourized by running 95% ethanol over it for 5s, and then washed with water, then counterstain (safranin) was used to flood the slide for 1minute and it was washed with water for 5s. The slide was allowed to air-dry. The preparation was examined with the oil immersion objective of the compound light (electric) microscope.

3.8.1.2 16S rRNA gene Sequencing and Multiple Alignments

3.8.1.2.1 Genomic Extraction

The DNA of the bacterial isolate was extracted according to the instructions manual using DNA extraction kits (QIAGEN) and stored frozen at -4°C until PCR reaction was carried out.

3.8.1.2.2 Polymerase Chain Reaction (PCR)

The PCR reaction was carried out using Kapa Taq DNA polymerase. The PCR was performed in BIOER GENE TOUCH THERMAL CYCLER. The Total reaction volume was 15µL. Reaction mix comprise of 1µL each of the genomic DNA, 1.5µL of 10 TaqA Buffer, ~0.4M(0.5µL) each of the forward and reverse primers, 1.25mM (0.75µL) of $MgCl_2$, 0.25mM (0.15µL) of dNTP mixes and 0.12µL of Taq DNA polymerase, in dH_2O. Amplification was carried out using the following conditions: initial denaturation of 5min at 95°C, followed by 35cycles each for 30s at 94°C (denaturation), 1min at 57°C (primer annealing) and 1min at 72°C (extension). This was followed with 10min final extension at 72°C. The PCR product was run on a 1.5% agarose gel stained with ethidium bromide with side marker to determine the size of the DNA.

S/N	Primer Name	Sequence (5′-3′)	Base Pair	Annealing temperature (°C)

1	Bact1442-F	AGAGTTGATCCTGGCTCAG	1,200	60
	Bact1492-R	GGTTACCTTGTTACGACTT		

3.8.1.2.3 Purification of Amplified PCR Products and Sequence Analysis

The amplified PCR product was sent to Inqaba Biotech Lab, South Africa for purification and sequencing.

3.8.1.2.3.1 Phylogenetic Analysis

BLAST search program at the NCBI website was used to identify the sequences obtained. The sequences were aligned using BIOEDIT version 7.2.5, then blasted on the NCBI website and all closely related species were downloaded for further analysis. Mega version 7 software was used to align sequences and for phylogenetic trees construction using neighbour-joining methods to determine the relationship of isolates in this study with those on the NCBI GenBank database. Sequences were then submitted to NCBI to obtain the accession number.

3.9 Antibacterial activity

Identified cultures isolated from spoiled yoghurt were sub cultured from nutrient agar slant into nutrient broth and will be incubated at 37^0C for 24 hours. The microorganisms were grown overnight at 37°C in 10 mL of nutrient broth. The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 0.5 standard (1.0×10^7 cfu/mL).

The Broth dilution method was employed as described by Clinical and Laboratory Standards Institute (CLSI) guidelines to determine the MIC and MBC of the oil fractions (Delaquis *et al.,* 2002). Firstly, the stock of EO fractions was dissolved in DMSO and added in the 10 ml sterile NB medium to achieve a concentration of 100 mg/ml. Then a serial two fold diluted to obtain final concentrations ranging from 50 to 1.5625 mg/ml. Finally, 50 µl of bacterial suspensions (adjusted to 1×10^7 cfu/ml) were added in each test tube. After 24 hours incubation at 37°C, the broth dilution tubes were tested for the absence or presence of visible growth in comparison with that of the growth in essential oil-free control well. The MBC will be determined by inoculating the mixture from the tube showing no microbial growth onto the surface of Nutrient agar plate. Plates were incubated at 37°C for 24-48 hours. The MIC is defined as the lowest concentration

of the oil fraction with no visible (no turbidity) bacterial growth and the MBC is define as the lowest concentration of oil fraction with initial inoculum bacteria killed (Moussaoui and Alaoui, 2016; Zhang *et al.*, 2015). Values are described as mean ± SD of experiments performed in triplicate

3.10 Analysis of Lemongrass EO fractions

The most active fraction was characterized by FTIR and GCMS according to Shigwenya et al. (2012) and Sa-Nguanpuag et al. (2011). The IR spectrum of the most active fraction was recorded in Bayero University, Department of Biochemistry, using a computerized Tensor 27 FTIR spectrometer (Bruker Co., Germany) in the range of 4000–650 cm−1 and structure was determined with the help of correlation charts. The samples were examined neat by placing them in between potassium bromide cells. The solvent spectrum was also obtained to aid in analyte identification.

GC-MS analysis was carried out using an Agilent 6890 GC system with a HP-5MS capillary column (30m × 0.25mm film thickness 0.25 μm) fitted with an Agilent HP-5973 mass selective detector. Helium as a carrier gas was used at a flow rate of 1ml min^{-1}(split ratio 1:10). Essential oils (1 µL) will be injected manually. The column temperature will be held at 45^0C for 8 min and then increased to 250^0C at a rate of 28C/min and held at 250^0C for 16 min. The compounds of the EO will be identified by comparing their retention indices (RI).

CHAPTER FOUR
RESULTS

4.1 Essential Oil Extractions

Table 4.1 shows the mean volume of EO yield from lemongrass. From 481.25±25.88g of plant material used a total of 3.16±0.17ml (0.66%) of EO was obtained. The obtained oil was found to be pale yellow, strong, with pungent lemon scent and cooling taste. The extraction yield was calculated considering the volume of the obtained EOs and the mass (g) of dried material processed (Appendix II).

Table 4.1 Mean volume and yield of Lemongrasss Essential oil obtained

Mean weight of plant material (g)	Mean volume of Essential oil (ml)	Yield of Essential oil (%)
481.25±25.88	3.16±0.17	0.66

4.2 Qualitative phytochemical Screening of Lemongrass EO

Table 4.2 shows the result of phytochemicals screened from EO. The result shows the presence of tannins, flavonoids, Saponins and terpenoids.

Table 4.2 Qualitative phytochemical screening of oil obtained from leaves of lemongrass.

Phytochemical test	Result
Tannins	+
Flavonoids	+
Saponins	+
Terpenoids	+

+ ----- Presence

4.3 Fractionation by Column Chromatography

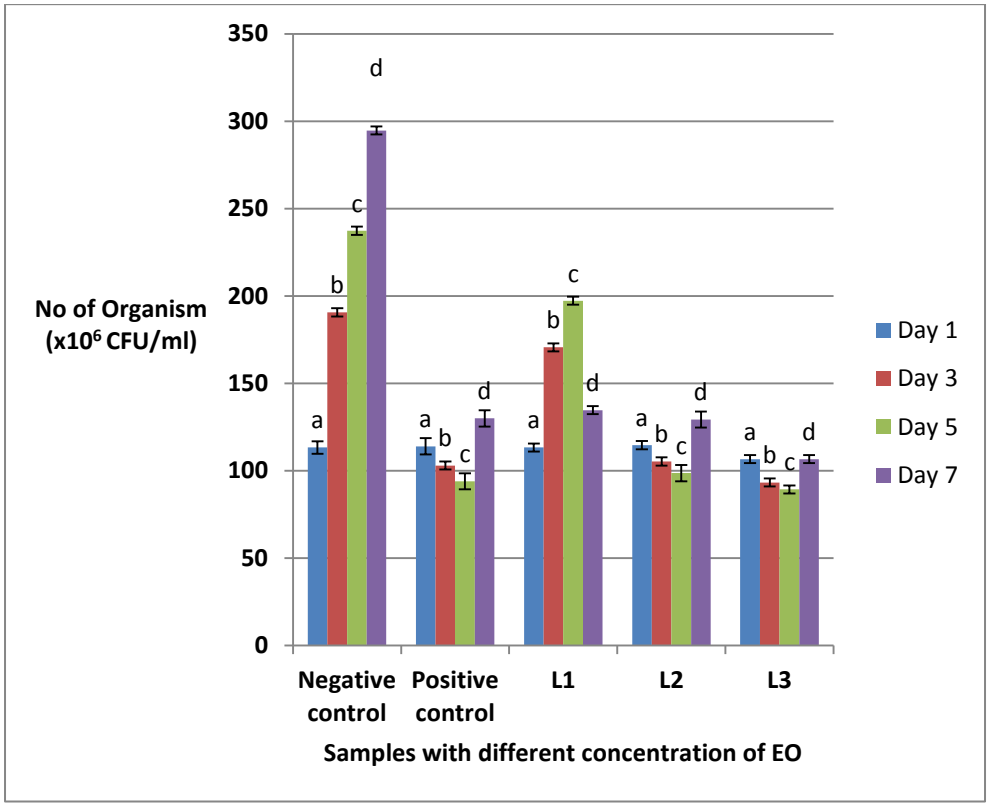
The column chromatography was carried out using different solvent combination for mobile phase and 45 fractions were collected. Fractions collected containing similar Rf values were pooled together which result in 4 fractions named A, B, C and D. Using 70% Hexane: 30% Methanol and 40% Toluene: 60% Ethyl acetate one fraction each was pooled. However, two fractions were pooled using 70% Toluene: 30% Ethyl acetate.

Table 4.3 Fractions obtained from column chromatography

Mobile phase	**Number of fractions**	**Pooled fractions**
Hexane	5	-
70% Hexane: 30% Methanol	10	1
40% Hexane: 60% Methanol	10	-
70% Toluene: 30% Ethylacetate	10	2
40% Toluene: 60% Ethylacetate	10	1

4.4 Colony Count

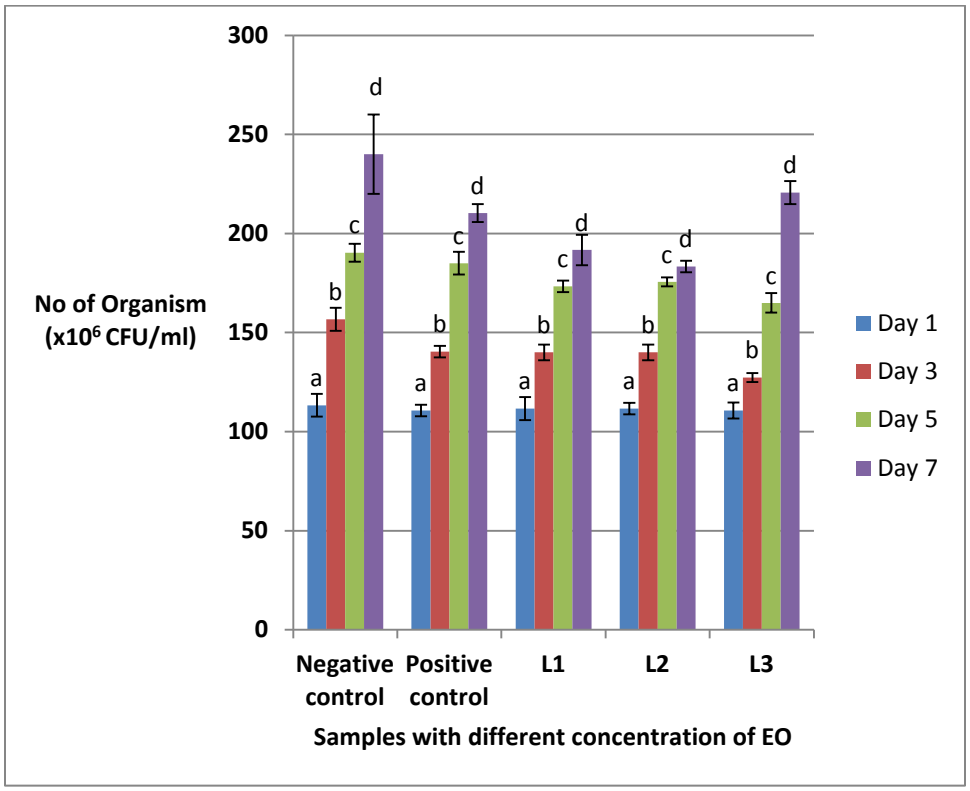
Fig 4.1 depicts the result of total bacterial count on produced yoghurt sample treated with different concentration of EO extracted from lemongrass. The TBC tends to decrease with increase in concentration of EO. The count was calculated as $\times 10^6$cfu/ml.



Negative control= without preservative, positive control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml.

Figure 4.1: Total Bacterial Count (TBC) of yoghurt sample treated with different concentration of essential oils

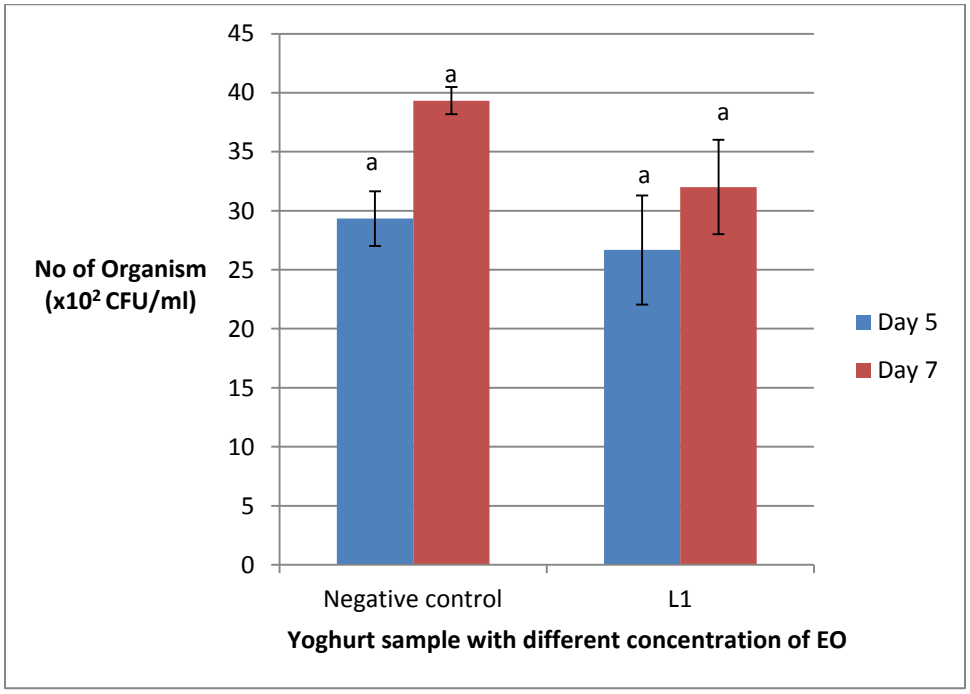
Fig 4.2 represent the result of total lactic acid bacterial count on produced yoghurt sample treated with different concentration of EO extracted from lemongrass. The count was calculated as $\times 10^6$cfu/ml.



Negative control= without preservative, positive control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml.

Figure 4.2: Total Lactic Acid Bacteria (LBC) Count of yoghurt sample treated with different concentration of essential oils

Fig 4.3 shows the result of total fungal count (TFC) on produced yoghurt sample treated with different concentration of EO extracted from lemongrass. The count was calculated as $\times 10^2$ cfu/ml.



Negative control= without preservative and L1=0.5µl/ml,

Figure 4.3: Total Fungal Count (TFC) of yoghurt sample treated with different concentration of essential oils

4.5 Physico chemical Analysis

Table 4.3 showing the pH of Yoghurt sample containing different concentrations of Lemongrass essential oil which were analyzed after 1, 3, 5 and 7 days of storage. The pH reduced from 4.64 to 3.65 in yoghurt samples at normal room temperature.

Table 4.4 pH value of yoghurt samples treated with Lemongrass EO at room temperature for 7 days

Days	-ve Control	+ve Control	L1	L2	L3
1	4.64 ± 0.01^{a}	4.64 ± 0.01^{a}	4.64 ± 0.01^{a}	4.64 ± 0.01^{a}	4.64 ± 0.01^{a}
3	4.25 ± 0.02^{a}	4.45 ± 0.00^{c}	4.25 ± 0.0^{a}	4.33 ± 0.02^{b}	4.48 ± 0.02^{c}
5	3.98 ± 0.02^{a}	4.06 ± 0.01^{a}	4.06 ± 0.03^{a}	3.97 ± 0.01^{a}	4.15 ± 0.02^{b}
7	3.65 ± 0.01^{a}	3.91 ± 0.02^{c}	3.71 ± 0.0^{b}	3.85 ± 0.02^{a}	4.07 ± 0.01^{d}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml. mean values in the same row with different superscript indicates significant difference ($p<0.005$)

Titrable acidity of yoghurt sample of yoghurt treated with essential oil (1.0 µl/ml, 2.0 µl/ml) and artificial preservative significantly increased from 68.8 to 86.4 over the storage period.

Table 4.5 Titrable Acidity of yoghurt sample treated with Lemongrass EO at room temperature for 7 days.

Days	-ve Control	+ve Control	L1	L2	L3
1	68.8 ± 1.39^{a}	67.2 ± 2.4^{b}	68.8 ± 1.39^{a}	68 ± 1.39^{a}	65.6 ± 3.67^{c}
3	76.8 ± 2.4^{a}	68.8 ± 1.39^{c}	73.6 ± 1.39^{b}	70.6 ± 1.21^{b}	68.8 ± 1.39^{c}
5	81.6 ± 2.4^{a}	71.3 ± 1.21^{c}	76.8 ± 2.4^{b}	72.8 ± 1.39^{c}	70.6 ± 1.21^{d}
7	86.4 ± 2.4^{a}	72.8 ± 1.39^{c}	81.6 ± 2.4^{b}	73.6 ± 1.39^{c}	70.9 ± 1.21^{d}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml. mean values in the same row with different superscript indicates significant difference ($p<0.005$)

The ash contents of yoghurt treated with high concentration of essential oil (2.0 µl/ml) stored at room temperature slightly decreased from 0.79 to 0.73 over the storage period. While ash content decreased in yoghurts sample with low concentration of EO and the sample without preservative.

Table 4.6 Ash content of yoghurt sample treated with Lemongrass EO at room temperature for 7 days.

Days	-ve Control	+ve Control	L1	L2	L3
1	0.73 ± 0.01^{a}	0.76 ± 0.01^{b}	0.74 ± 0.01^{a}	0.74 ± 0.01^{a}	0.79 ± 0.01^{c}
3	0.69 ± 0.01^{a}	0.74 ± 0.01^{b}	0.70 ± 0.01^{a}	0.71 ± 0.01^{a}	0.76 ± 0.01^{c}
5	0.64 ± 0.01^{a}	0.70 ± 0.01^{c}	0.68 ± 0.01^{b}	0.71 ± 0.01^{c}	0.73 ± 0.01^{d}
7	0.61 ± 0.01^{a}	0.70 ± 0.01^{b}	0.64 ± 0.01^{a}	0.68 ± 0.01^{b}	0.73 ± 0.01^{c}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml. mean values in the same row with different superscript indicates significant difference ($p<0.005$)

The moisture content of the yoghurt samples ranges from 87.67 to 89.73 during storage at room temperature. Moisture content remains stable with the addition of high concentration of EO.

Table 4.7 Moisture content of yoghurt sample treated with Lemongrass EO at room temperature for 7 days.

Days	-ve Control	+ve Control	L1	L2	L3
1	87.67 ± 0.02^{a}	87.64 ± 0.02^{a}	87.66 ± 0.01^{a}	87.65 ± 0.01^{a}	87.63 ± 0.01^{a}
3	88.02 ± 0.01^{a}	87.65 ± 0.01^{b}	88.06 ± 0.03^{a}	87.7 ± 0.03^{b}	87.62 ± 0.01^{b}
5	89.34 ± 0.02^{a}	87.7 ± 0.01^{c}	88.23 ± 0.02^{b}	88.1 ± 0.02^{b}	87.87 ± 0.16^{c}
7	89.73 ± 0.03^{a}	87.96 ± 0.02^{c}	88.59 ± 0.09^{b}	88.35 ± 0.02^{b}	87.87 ± 0.01^{c}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml mean values in the same row with different superscript indicates significant difference ($p<0.005$)

The total solid contents of yoghurt treated with essential oil (2.0 µl/ml) and artificial preservative ranged from 12.36 to 12.04 over the storage period. While total solid content decreased in yoghurts samples without preservatives and samples with low concentration of EO.

Table 4.8 Total Solid content of yoghurt sample treated with Lemongrass EO at room temperature for 7 days.

Days	-ve Control	+ve Control	L1	L2	L3
1	12.33 ± 0.02^{a}	12.36 ± 0.01^{a}	12.34 ± 0.01^{a}	12.35 ± 0.01^{a}	12.37 ± 0.01^{a}
3	11.98 ± 0.01^{a}	12.35 ± 0.01^{b}	11.94 ± 0.03^{a}	12.3 ± 0.03^{b}	12.38 ± 0.01^{b}
5	10.66 ± 0.02^{a}	12.3 ± 0.01^{c}	11.77 ± 0.02^{b}	11.9 ± 0.02^{b}	12.13 ± 0.16^{c}
7	10.27 ± 0.03^{a}	12.04 ± 0.02^{c}	11.41 ± 0.09^{b}	11.65 ± 0.02^{b}	12.13 ± 0.01^{c}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml. mean values in the same row with different superscript indicates significant difference ($p<0.005$)

The viscosity of yoghurt treated with high concentration of essential oil (2.0 µl/ml) stored at room temperature remains stable over the storage period.

Table 4.9 viscosity of yoghurt sample treated with Lemongrass EO at room temperature for 7 days.

Days	-ve Control	+ve Control	L1	L2	L3
1	61.23 ± 0.87^{a}	62.17 ± 0.29^{b}	61.23 ± 0.87^{a}	61.57 ± 0.51^{a}	62.83 ± 0.76^{b}
3	57.8 ± 0.76^{a}	61 ± 0.87^{b}	58.5 ± 0.29^{a}	59.93 ± 0.5^{b}	61.33 ± 0.28^{c}
5	55.17 ± 0.36^{a}	61 ± 0.66^{c}	55.93 ± 0.62^{a}	58.27 ± 0.28^{b}	61.33 ± 0.28^{c}
7	53.73 ± 0.75^{a}	59.93 ± 0.75^{c}	54.17 ± 0.87^{a}	55.33 ± 0.87^{b}	61 ± 0.36^{c}

-ve control= without preservative, +ve control= with artificial preservative, L1=0.5µl/ml, L2= 1.0 µl/ml and L3= 2.0 µl/ml. mean values in the same row with different superscript indicates significant difference ($p<0.005$)

4.6 Sensory Result

Table 4.9 shows the results of total mean score of sensory characteristics for control and yoghurt treated with different concentration (0.5, 1.0 and 2.0 µl/ml) of lemongrass essential oil for day 1, 3, 5 and 7.

Table 4.10: Sensory scores of yoghurt samples treated with Lemongrass essential oil at room temperature for 7 days.

		EO Dosage (µl/ml)			
		Control	0.5	1.0	2.0
Day 1	Appearance	8.63±0.52^{a}	8.63±0.52^{a}	8.75±0.46^{a}	8.75±0.46^{a}
	Taste	7.63±0.52^{a}	7.63±0.52^{a}	7.75±0.46^{a}	7.88±0.35^{a}
	Texture	7.88±0.35^{a}	8.13±0.35^{a}	8.25±0.46^{a}	8.38±0.52^{a}
	Aroma	7.75±0.46^{a}	7.88±0.35^{a}	8.13±0.35^{a}	8.38±0.52^{b}
	Acceptability	8.38±0.52^{a}	8.5±0.53^{a}	8.75±0.46^{a}	8.75±0.46^{a}
Day 3	Appearance	4.50±0.53^{a}	4.63±0.52^{ac}	5.25±0.46^{bc}	5.75±0.46^{bd}
	Taste	2.88±0.64^{a}	3.00±0.53^{ac}	3.38±0.52^{a}	3.88±0.35^{bd}
	Texture	3.50±0.53^{a}	3.63±0.52^{ac}	4.38±0.92^{a}	4.88±0.83^{ad}
	Aroma	3.78±0.52^{a}	3.50±0.53^{ac}	4.13±0.35^{bc}	4.75±0.46^{bd}
	Acceptability	3.13±0.64^{a}	3.38±0.52^{a}	3.88±0.64^{a}	4.13±0.64^{b}
Day 5	Appearance	3.50±0.53^{a}	3.63±0.52^{ac}	4.13±0.35^{a}	4.75±0.46^{bd}
	Taste	2.25±0.71^{a}	2.38±0.52^{ac}	3.13±0.64^{b}	3.63±0.52^{bd}
	Texture	3.13±0.83^{a}	3.38±0.74^{ac}	4.13±0.64^{a}	4.50±0.76^{bd}
	Aroma	2.63±0.51^{a}	2.75±0.46^{ac}	3.38±0.52^{b}	3.75±0.46^{bd}
	Acceptability	2.50±0.53^{a}	2.88±0.83^{ac}	3.38±0.52^{a}	3.88±0.83^{bd}
Day 7	Appearance	3.00±0.76^{a}	3.13±0.64^{ac}	3.75±0.71^{a}	4.25±0.46^{bd}
	Taste	1.88±0.64^{a}	2.00±0.53^{ac}	2.88±0.35^{bd}	3.25±0.46^{bd}
	Texture	2.50±0.53^{a}	2.88±0.35^{ac}	3.5±0.53^{bc}	4.00±0.76^{bd}

Aroma	2.13±0.64[a]	2.25±0.71[a]	3.00±0.76[a]	3.63±0.52[b]
Acceptability	1.63±0.52[a]	1.75±0.46[ac]	2.38±0.52[bc]	2.63±0.53[bd]

*Results are mean score of 8 judges.

*Values are described as mean ± SD

*Mean values followed by different letters in the same row are significantly different ($P<0.05$)

4.7 Isolation and Identification of Bacterial Isolates

The cultural and morphological characteristics of isolates obtained were labeled Unknown X and Unknown Y. The results of the 16S rRNA sequencing indicated that Unknown X belongs to Citrobacter genus with sequence similarity of 96% and Unknown Y belongs to Acinetobacter genus with sequence similarity of 98%. The phylogenetic relationships of the isolates are shown in Fig 4.4 and Fig 4.5.

Table 4.11: Morphological and cultural characteristics

Bacterial isolate	Grams reaction	Characteristic on culture media
Unknown X	Gram negative rods (single and in pairs)	Mucoid grayish with shiny surface
Unknown Y	Gram negative rods	Mucoid and milky in appearance

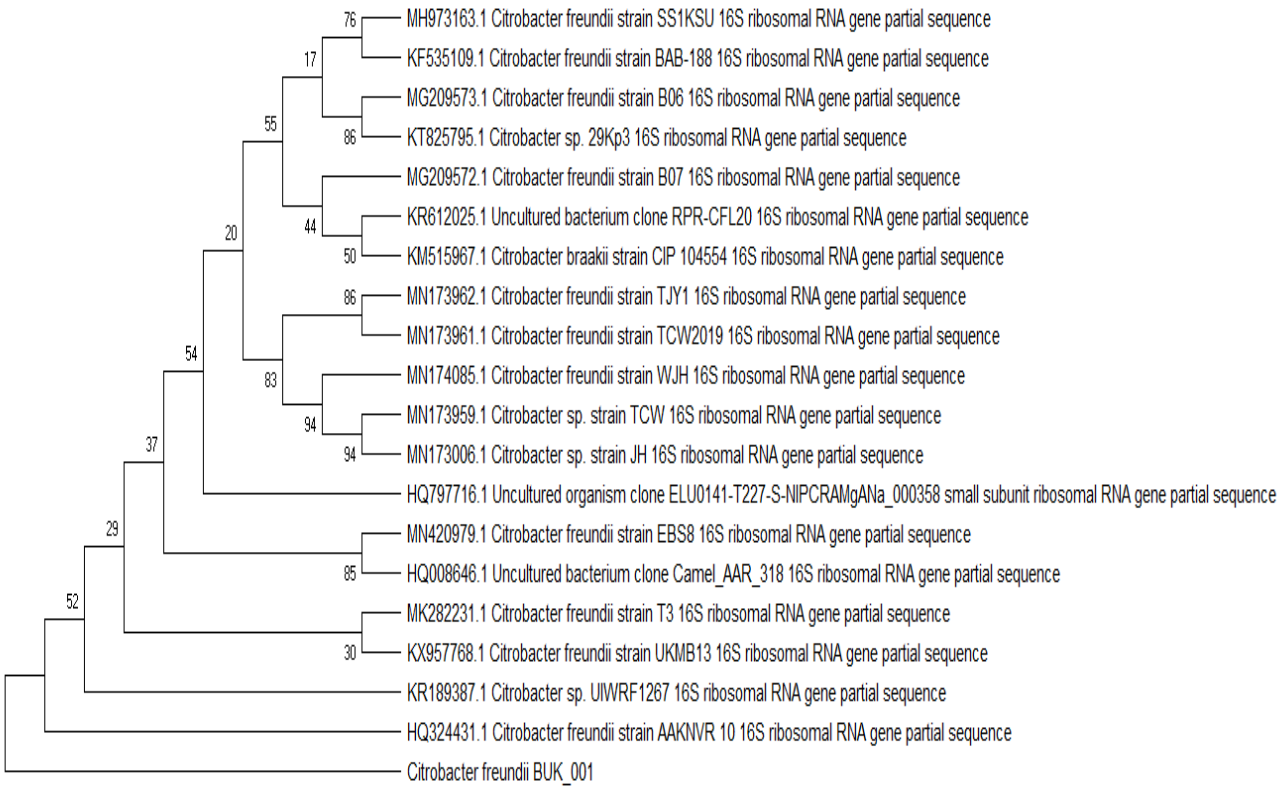


Figure 4.4: Phylogram (neighbor-joining method) indicating the genetic relationship between *Citrobacter freundii BUK_001* and referenced related microorganisms based on 16S rRNA gene sequence analysis.

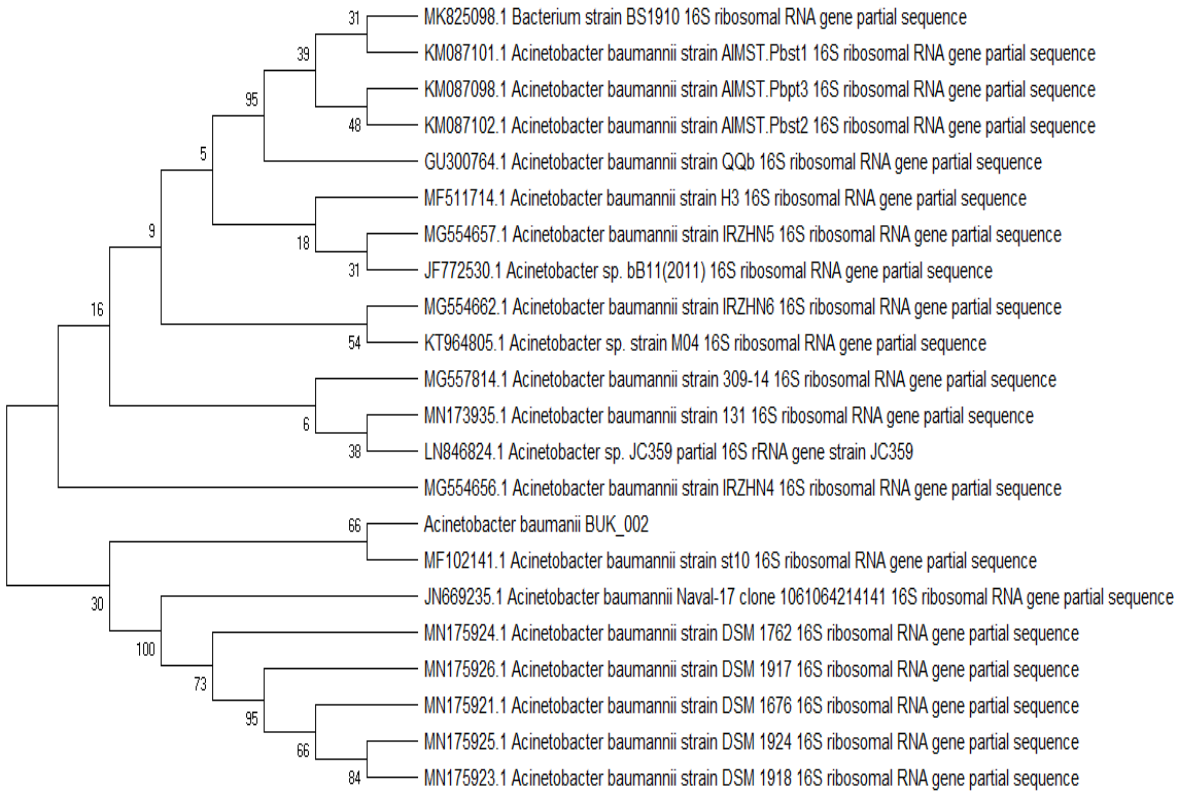


Figure 4.5: Phylogram (neighbor-joining method) indicating the genetic relationship between *Acinetobacter baumanii BUK_002* and referenced related microorganisms based on 16S rRNA gene sequence analysis.

4.8 Antibacterial Potential

The antibacterial activities of Lemongrass essential oil fractions were assayed in vitro by a broth macro-dilution method against two gram negative bacteria. Table 4.12 summarizes the microbial growth inhibition by each fraction.

4.9 Characterization of the effective fraction by FTIR and GCMS

Table 4.13 shows the infrared spectra and the characteristic bands observed in fraction B of the Lemongrass essential oil in the range of 4000-650 cm. The examination of the spectra reveals the presence of some functional groups found. The FT-IR spectrum reveals its peak at 2854cm-^{1} which is attributed to CH_2 stretching. While peak at 1748cm^{-1} was attributed to the presence of carbonyl group (C=0).

A total of 36 components, with different retention time, were eluted from the GC column as indicated by the chromatogram (Appendix XI), representing 100% of the total oil. The main composition of fraction B reveals citral (17.13%), 9-Octadecenoic acid, 1,2,3-propanetriyl ester (10.73%), Hexadecanoic acid, 2-hydroxy-1-(hydroxyme (9.72%) and Geranial (7.57%) as the major components (Table 4.14).

Table 4.13 Funtional Groups Identified in the effective fraction by FTIR

Absorption wave number (cm-1)	**Functional groups**	**Remark/inference**
2854	CH_2	Alkanes
1748	C=0	Aldehyde, ketone, ester, carboxylic acid
1164	C-O	Alcohol, ether, ester, carboxylic acid

Table 4.14: Major components of the effective fraction identified by GCMS

S/N	Compound Name	Retention Time	Area %
1	Citral	6.958	17.13
2	9-Octadecenoic acid, 1,2,3 propanetriyl ester	32.959	10.73
3	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl	30.847	9.72
4	Geraniol	6.749	7.57

4.11 Discussion

Plant essential oils and extracts have been used for many thousands of years, especially in food preservation, pharmaceuticals, alternative medicine and natural therapies (Al-bayati, 2008). It has long been acknowledged that some plant essential oils exhibit antimicrobial properties and it is necessary to investigate those plants scientifically, which have been used, in traditional medicine to improve the quality of healthcare. Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens.

Essential oils are aromatic and volatile oily liquids obtained from plant material through steam distillation and named after the plant from which they are derived. The antibacterial properties of essential oils have long been recognized, the recent interest in alternative naturally derived preservative/antimicrobials has led to a renewed scientific attention in these substances. Extraction is the crucial first step in obtaining Essential oil. The basic operations include steps such as pre-washing, drying of plant materials and grinding to obtain a homogenous sample. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. Steam distillation is the most commonly used method for producing EOs on a commercial basis (Burt, 2004).

Factors such as geographical location, harvest time, plant parts used and method of isolation all affect the chemical composition of the crude material separated from the plant. The quality and quantity of essential oil mainly depends on the extraction procedures. Each essential oil is characterized by a special odor attributed to the presence of some low molecular weight compounds like alcohols, esters, phenols and oxygenated compounds which have the future of

being highly volatile at room temperature. Because it is highly volatile, it was stored in a well air-tight container protected from light in cool place. The essential oil was insoluble in water, miscible in alcohol and in oil. The percentage yield (0.66%) obtained was similar to what Nadjib *et al.* (2014) while Suryawanshi *et al.* (2016) report percentage yield at 0.70%. The odor of the essential oils is regarded as one of the important diagnostic futures, because every essential oil has its own special odor and hence regarded as a diagnostic tool for the plant that contain the oil (Al-Jumaily and Al-Amiry, 2012)

Phytochemical screening of *Cymbopogon citratus* revealed the presence of tannins, flavonoids and terpenoids. This result is in agreement with Ewansiha et al. 2012 and Mishra et al. 2010. Extraction and Phytochemical screening of bioactive agents from medicinal plants permits the demonstration of their physiological activities (Ewansiha *et al.*, 2012). Tannins have been reported to have antidiarrheal, homeostatic and antihemorrhagal activity. Accumulated evidence has shown that the phytochemicals present in *C. citratus* are responsible for its wide range of pharmacologic and physiologic actions (Ekpenyong *et al.,* 2014).

Essential oils are very complex mixtures that can be composed of 20-80 constituents at significantly different concentrations which are very useful. These volatile compounds belong to various chemical classes: alcohols, ethers or oxides, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and mainly the terpenes (Burt, 2004). Identifying the most active compounds of essential oils is cumbersome because essential oils are complex mixtures of up to different constituents. Furthermore, they can vary greatly depending upon the geographical region, the variety, the age of plant, the method of drying and the method of extraction of the oil.

Although complex essential oils are mainly evaluated for various activities, little is known about constituents of essential oils. Hence, instead of taking whole essential oil for bio-preservative and antimicrobial activities, some components of the oil are fractionated and studied for their potential activity in preservation and control for microbial contamination (Hyldgaard *et al.*, 2012).

Solvent selection for achieving a good separation was very critical to our work. The choice of the column mobile phase is achieved after TLC study in different solvent systems. Four solvents were chosen as better mobile. Fractionation of essential oil was done using silica gel column chromatography technique with a mobile phase ethylacetate: toluene and Hexane: Methanol in different proportions to elute fractions according to their affinity to mobile phase. This process depends on the difference in degree of polarity among compounds, which play a key role in the degree of dissolving these structures in two differed solvents in its polarity.

Yoghurt sample treated with different concentration of lemongrass essential oil was subjected to microbiological analyses. Total microbial count of different yoghurt treatments with 0.5, 1.0, and 2µl/ml^{-1} of lemongrass EO, artificial preservative and of untreated yoghurt (Control) were followed up through 7 days at room temperature. Result shows mean values of total bacterial count (TBC) of yoghurt sample treated with essential oil in higher concentration decreased the presence of bacterial count compared with the control sample. The mean total bacterial counts ranged from 110.33±3.61 in day 1 to 294.67±2.31 in day 7 for yoghurt sample without treatment. The obtained results suggest that the bacterial populations were not inhibited by low concentrations of the different essential oils. However, increases in the oil concentrations lead to

decreases in bacterial counts significantly ($P<0.05$). It has previously been reported that addition of some essential oils to yoghurt and labneh cheese during its manufacture had a stimulatory effect on LAB by enhancing their growth and acid production (Mutlag and Hassan, 2008).

Count of lactic acid bacteria (LBC) increases with increase in essential oil. It has been reported that addition of some aromatic and essential oils to yoghurt and labneh during its manufacture had a stimulatory effect on lactic acid bacteria by enhancing their growth and acid production (Thabet *et al*., 2014). LAB enumerating indicates the levels of added starter culture and its development during the storage and shelf-life (Rodrigues *et al.,* 2010).

There was no fungal growth observed across each concentration of EO used from day 1 to day 3. However, fungal growth was observed in day 5 and day 7 at control and 0.5µl/ml with TFC ranged from 30.67±2.31 to 38.67±2.31 in control and 22.67±2.31 to 30.67±2.31 CFU/ml using 0.5 µl/ml of EO respectively. The quality and the shelf life of yoghurt was evaluated with fungal counts, fungi was not detected in yoghurt sample containing high concentration of lemongrass EO (1 and 2 $\mu l/ml^{-1}$) throughout the storage period, while in yoghurt sample containing lower concentration and sample without treatment fungi were detected at day 5 and day 7 of storage. These results are in agreement with both Abd-El Fattah *et al.,* 2010 who reported that 0.1% of the EO extract of lemongrass was effective in inhibition of both mold growth and mycotoxin production for 30 days at 5^0C and (Mutlag and Hassan) in 2008 reported that essential oil from thyme, sage and marjoram had antifungal and antimicrobial activities.

High bacteria count was expected because of the presence of starter cultures, which are mainly lactic acid bacteria. The standard aerobic bacterial count is 10^6 to 10^7 cfu/ml^{-1} (Rodrigues *et al.,* 2010). Very high count however is used as an indication of post pasteurization contamination, due to inadequate hygienic measures during production. In most foods, the total bacterial count is often an indication for the sanitary quality, safety, and utility of foods. It may reflect the conditions under which the product is manufactured such as contamination of raw materials and ingredients, the effectiveness of processing, and the sanitary conditions of equipment and utensils at the processing plants (Lamye *et al.,* 2017).

pH plays an important role in the shelf life of milk during fermentation. The pH value for control yogurt was approximately the same as pH of yoghurt treated with low concentration of essential oil. pH values for all yoghurts were reduced ($P<0.05$) from the initial values of approximately 4.65±0.01 to between 3.91±0.15 by day 7 of storage. The pH values were stable in the samples treated with higher concentration of lemongrass essential oil throughout the storage period. This finding is in agreement with Ghalem and Zouaoui (2013) who reported pH to be stable in the yogurt samples fortified with essential oil during the storage period while that of the control sample decreased significantly. Ghalem and Zouaoui, 2013 reported pH ranged from 4.08 to 4.66 for yogurt sample fortified with Chamaemelum spp. extract and from 4.52 to 4.61 for the sample enriched with Lavandula spp. oils. In the first three days there wasn't significant change in pH value, while between the third and seventh day of storage, pH decreased approximately for 0.11 units.

According to Mutlag and Hassan, 2008 acidity is considered as one of the significant factors affecting shelf life and acceptability of yoghurt. The values of titratable acidity % and pH values

gradually increased and decreased respectively during refrigerated storage of all samples of yoghurt. This may be due to fermentation of lactose, which produces lactic and acetic acid during fermentation and storage period. These results are in agreement with the findings of Falade *et al.,* 2015 and Dzigbordi *et al*., 2013. The decrease in pH of yoghurt samples could be as a result of the breakdown of lactose into lactic acid. The lactic acid produced during fermentation period is known to be responsible for the characteristic flavour and aroma of yoghurt and this helps to maintain the quality of yoghurt during storage and packaging (Akande and Adegoke, 2018).

The increasing oils rate in yogurts increased ash contents, viscosity and total solid during storage. The ash values remained stable in samples treated with high concentration of Lemongrass EO throughout the storage period at 0.79%. Yangilar and Yildiz, 2017 mention about the rearrangement contact of proteins effective in viscosity increase during storage period. High hardness degree was reported in yogurt samples depending on moderate amount of probiotic inoculums. These results may show that EO addition strengthened the structure of gel system.

Sensory attributes are of great importance to measure consumer attitudes and their influence on food choice and acceptability. The quality or integrity of a particular food sample can be determined by evaluating its sensory characteristic (USDA, 2001). The sensory properties were evaluated by a panel group of 8 sensory analysts by ranking test and the 9 point hedonic system ranging from 1 (Extremely poor) to 9 (Extremely good) during storage which are summarized above. The appearance, taste, texture, aroma and acceptability in different concentrations (0.5, 1.0 and 2µ/ml) of essential oil fractions were compared. The samples were presented to sensory analysts in a series, and each sample was ranked according to an appropriate scale.

The change in colour may be referred to chemical changes occurred during storage of yoghurt samples. The lemongrass EO pretreated yoghurt samples received higher sensory scores than yoghurt sample without treatment, but the differences was non-significant ($P<0.05$) for all sensory characteristics in day 1 except for aroma. The addition of lemongrass EO in yoghurt had significant ($P<0.05$) impact on taste and aroma but insignificant ($P<0.05$) on texture and appearance characteristics. Yoghurt sample treated with 2.0µl/ml EO received better score for taste and aroma. On the contrary, lower concentration of essential oil had lower score for all sensory characteristics at all samples especially in taste and aroma characteristics. However, concentration of lemongrass EO had a positive influence on the general acceptability of yoghurt samples. Similar results were reported by (Elsaadany *et al.,* 2017; Thabet *et al.,* 2014; Ghalem and Zouaoui, 2013; Mutlag and Hassan, 2008)

It is obvious that higher concentration of EO gained high scores from the panelists. This could be attributed to the effect of the lemongrass EO that improves the keeping quality and prevents/reduce the growth of spoilage microorganisms till the end of storage time. On the other hand, the control samples had lower scores as a result of especially spoilage microorganisms growing which might impart off-flavour, bitterness and discoloration in some cases.

The results of the 16S rRNA sequencing indicate the presence of *Citrobacter freundii* and *Acinetobacter baumanii* with sequence similarities between 96% and 98% respectively. Lamye et al. 2017 isolate *Citrobacter freundii* a coliform bacterium from locally prepared yoghurt sold in Cameroon. *Acinetobacter baumanii,* a psychrotropic bacteria have been reported to be associated with dairy spoilage (Amorim and Nascimento, 2017). Contamination of milk by

gram-negative microorganisms is usually associated with a humid environment, equipment, and the water used to clean the systems for obtaining and storing milk (S*amaržija et al.,* 2012). The National Agency of Foods and Drugs Administration Control (NAFDAC) stipulated that coliforms generally must not be detectable in 100 ml of yogurt samples products (Mbaeyi-Nwaoha and Egbuche, 2012). The presence of coliforms is of public health concern as these organisms have been implicated as the causal agents of one or more human diseases such as gastrointestinal discomfort or disorders, pneumonia, listerosis and others (Mbaeyi-Nwaoha and Egbuche, 2012).

Psychrotrophic bacteria usually account for more than 90% of the total microbial population in cooled raw milk. They are able to grow at 7°C or less regardless of their optimal growth temperature. They are the most commonly isolated organisms which caused the spoilage of the heat treated milk and dairy products as the result of post-pasteurization contamination of the products (S*amaržija et al.,* 2012). In addition to the ability to grow and multiply at low temperatures, psychrotophic bacteria have the ability to produce heat stable extracellular thermostable proteolytic and lipolytic enzymes which can survive pasteurization thus affecting the shelf life and quality of milk and milk products during storage. Many of these enzymes retain their activity even after the conventional heat treatment of milk (S*amaržija et al.,* 2012). Also, psychrotropic bacteria are said to develop a rapid resistant to a wide range of antimicrobials (Doughari *et al.,* 2011).

To achieve precisely the antimicrobial properties of essential oils for potential application in food preservation, determination of MICs and MBCs were necessarily performed on fraction of

essential oil. Experiments were carried out to determine the lowest concentration that inhibit visible microbial growth (MICs) and also the lowest concentration required to kill the organisms (MBCs). After incubation at 30^0 C for 24 hours, some tubes were turbid while some were not.

The addition of fractions of lemon grass essential oil in broth culture inoculated with *Citrobacter freundii* and *Acinetobacter baumanii* inhibited the growth of these organisms. Results revealed that the fractions possess antibacterial activity with varying magnitude depending on the concentration of the fractions. The strongest antibacterial activity was observed in fraction B against *Citrobacter freundii* with MIC value of 6.25±0.00mg/ml and MBC value of 10.42±3.61mg/ml followed by MIC value of 7.29±4.77 mg/ml for *Acinetobacter baumanii* and MBC value of 8.33±3.61 mg/ml, while lowest antibacterial activity was observed in fraction A and fraction C for both *Citrobacter freundii* and *Acinetobacter baumanii* with MIC value of 16.67±7.22 mg/ml and MBC value of 16.67±7.22 and 20.83±7.22 mg/ml respectively.

The tolerance of Gram-negative bacteria to the inhibitory effect of essential oils has been attributed to the unique composition of their outer membrane due to the presences of high ratio of hydrophilic lipopolysaccharides. The lipopolysaccharides can act as a barrier toward macromolecules and hydrophobic compounds like those found in essential oil extracts (Elsaadany *et al.,* 2017). Concerning the antimicrobial activity reported in this study, *C. freundii* appeared to be more susceptible to essential oil extracts compared to *A. baumanii*. *Acinetobacter species* are of major concern because of their rapid development of resistance to a wide range of antimicrobials and their long persistence in the environment (Doughari *et al.,* 2011).

Although the mechanisms associated with the antimicrobial activities of essential oils are not fully understood numerous modes of action have been proposed involving, for example, degradation of the bacterial cell wall, modification of proteins of the cytoplasmic membrane, alteration of membrane permeability, inactivation of extracellular enzymes, reduction of intracellular ATP, leakage of cellular contents, coagulation of cytoplasm, and interruption of electron flow and active transport (Delaquis *et al.,* 2002)**.** It has been reported that EOs containing mainly aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol, or thymol were characterized by the highest antibacterial activity, followed by EOs containing terpene alcohols (Dhifi *et al.,* 2016). Similarly, Delaquis et al. (2002) determined that the essential oil of cilantro was particularly effective against *Listeria monocytogenes,* potentially because of long chain alcohols and aldehydes since the antimicrobial properties of alcohols are known to increase with molecular weight.

IR Spectroscopy is an extremely effective method for determining the presence or absence of a wide variety of functional groups in a molecule (Shigwenya *et al.*, 2012). The functional groups present were determined by comparing the vibration frequencies in wave numbers of the sample spectrograph obtained from an FT-IR spectrophotometer with those of an IR correlation chart. The vibration frequencies of the solvent were also obtained to aid in the determination of sample vibration frequencies.

The FTIR spectra revealed the presence of alkanes, aldehydes, ketone, carboxylic acid and esters. These functional groups identified are in agreement with compounds reported in the literature (Olayemi *et al.,* 2017) as well as the ones identified in the GC-MS analysis. For

instance, citral and Geranial are aldehydes, hexadecanoic acid and 9-octadecanoic acids are carboxylic acids. Compounds such as hydrocarbon terpenes, alcohols, ketones, esters and mainly aldehydes have constantly been registered in the literature. Lemon grass contains active ingredients like myrcene, which is an alkene, citral are aldehydes, citronellol and geraniol are alcohols.

It appears that geranial, neral, geraniol, limonene and β-myrcene have been found as major compounds in many other *Cymbopogan* species with the main chemical component of lemongrass oil as citral (Mirgani *et al*., 2012). Both citral and geraniol have immense commercial significance due to their characteristic lemon and rose-like smell in the flavour, fragrance, cosmetics, perfumery and pharmaceutical industries (Olayemi *et al.,* 2017). Citral is used in the manufacture of perfumes, colored soaps, synthesis of vitamin A and β-ionones and other specialty chemicals (Mirgani *et al*., 2012). The biological activity of the essential oil obtained from *C. citratus* has been reported to be due to the presence of citronellal and citral (Shigwenya *et al.,* 2012). Geranial, neral, geraniol, limonene, β-myrcene, citral have been found as major compounds in many other *Cymbopogon citrates* (Olayemi *et al.,* 2017).

CHAPTER FIVE
SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary

Lemongrass (*Cymbopogan citratus*) an aromatic perennial grass was subjected to steam distillation to extract Essential oil. Phytochemical screening showed active ingredients: tannins, flavonoids and terpenoids. The essential oil was fractionated using column chromatography (Silica column) and all fractions were tested against yoghurt spoilage organisms isolated. Lemongrass EO was added to yoghurt at different concentration (0.5, 1.0 and 2 μlml^{-1}) for 7 days. Bio-preservative potential of EO was observed based on microbial quality, physico chemical parameters and sensory characteristics for 7 days at room temperature which shows the impact of EO in improving the shelf-life of yoghurt. Sensory evaluation of the bio-preserved yoghurt and control sample at 48 hours interval for 7days revealed that yoghurt sample inoculated with $2\mu lml^{-1}$ of EO has the highest score. The antibacterial activity was assessed for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the macrodilution method against two gram negative (*Citobacter freundii* and *Acinetobacter baumanii*) bacteria isolated. The results obtained in this study demonstrate that the fractions of Lemongrass essential oil exhibit *in vitro* antimicrobial activities against these organisms. The rate of inhibition was greater on *C. freundii* than what was observed with *A. baumanii*. The GC-MS and FTIR spectra of the effective sample revealed the presence of alkanes, aldehydes (citral), ketone, carboxylic acid (9-Octadecenoic acid and Hexadecanoic acid) and esters. Essential oil from Lemongrass can be good preservative of yoghurt as they reduce pathogens and increase shelf life of yoghurt.

5.2 Conclusion

Essential oils could be exploited as effective alternatives or complements to synthetic compounds of the chemical industry, without inducing the same secondary effects. The consumers nowadays have greater concern over on high quality, natural and safe food products in order to maintain a healthy lifestyle. The trend of using EOs as natural antimicrobial agents is, therefore, gradually becoming an attractive approach in the field of food preservation. The regulation and new method of application of natural antimicrobials agents are important factor that was addressed. Optimization of application methods and regulation will enhance the consumer confidence. The application methods for the natural antimicrobial agents to different food products require higher efficiency. Lemongrass EO enhances the flavor and taste of yogurt and recorded as best in Overall Acceptability. It was also concluded from the research that as the amount of Lemongrass EO increases in yogurt; it affects the total bacterial viability, total lactic acid bacteria viability and fungal growth in yogurt. Therefore, addition of Lemongrass EO in the process of yoghurt production is recommended because Lemongrass EO is a natural herbal product with a wide range of beneficial and nutritional properties. It may also serve as alternative to conventional chemical preservatives in the preservation of yoghurt.

5.3 Recommendation

Essential oils classified as Generally Recognized as Safe (GRAS) and considered at low risk for developing resistance to pathogenic microorganisms could be a credible alternative. However, aromatic plants and their extracts should be standardized and properly controlled in their extraction and composition, in order for the study of these plants to yield meaningful data. Based on the outcome of this study the following recommendations are made:

1. For the practical use of this oil, further research is needed on safety issues for human health and acceptability by consumers.
2. Identifying the main components of the essential oil and testing their safety to uncover their full potential.
3. Many studies have investigated the site of action, while few proceed to reveal the mode of action. Future research should thus explore the mode of action of individual essential oil constituents further, while also initiating systematic investigations into the mechanisms of synergy among different constituents.
4. Hazard analysis critical control point system implementation and control measures from farm to post pasteurization milk handling should be undertaken in order to minimize food borne diseases
5. Consequently, more restriction and preventive measures should be taken to improve the quality of milk and dairy products to protect consumers from being infected by the discussed microorganism.

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APPENDICES

APPENDIX I: LIST OF EQUIPMENT, GLASS WARES AND CHEMICALS

MATERIALS

1. Clevenger Apparatus
2. Cooking stove
3. Mortar and pistil
4. Autoclave
5. Incubator
6. Digital Colony Counter
7. Spectrophotometer

REAGENTS/MEDIA

1. Nutrient Agar
2. Sabourand dextrose agar (PDA) medium
3. peptone water
4. Distilled water
5. Mann Rogosa Sharpe (MRS) Agar
6. MacConkey Agar
7. Hexane
8. Methanol
9. Ethyl acetate
10. Toluene
11. Nutrient broth

12. Starter culture (Yogourmet produced by Lyo-San Inc. 500, Aeroparc, C. P. 598 Lachute, Quebec)

GLASS WARES/APPARATUS

1. Conical flask
2. Beaker
3. Glass rod
4. Erlenmeyer
5. Separating funnel
6. Thermometer
7. Stop watch
8. Petri dish
9. Pipette
10. Test tubes
11. Spatula
12. Electric weighing balance
13. Inoculating needle
14. Beaker
15. Measuring cylinder 10ml, 50ml and 100ml
16. Cotton wool
17. Foil paper
18. Spirit lamp.

Appendix II

Extraction of Lemongrass Essential oil using Steam distillation

Appendix III

Volume and yield of Lemongrasss Essential oil obtained

Experiment	Weight of plant material (g)	Volume of Essential oil (ml)	Yield of Essential oil (%)
1	500	3.3	0.67
2	450	3.1	0.69
3	450	3.0	0.67
4	500	3.4	0.68
5	500	3.3	0.66
6	450	2.9	0.64
7	500	3.1	0.62
8	500	3.2	0.64
Total	3850	25.3	0.66

Appendix IV

Total Bacterial Count (TBC) of yoghurt sample treated with different concentration of essential oils

			Dosage of EO (µl/ml)		
Days	-veControl	+veControl	0.5	1.0	2.0
1	110 ± 3.61^{a}	114 ± 4.67^{c}	113.33 ± 2.31^{b}	114.67 ± 2.31^{c}	106.67 ± 2.3^{d}
3	190.67 ± 2.31^{a}	103 ± 2.31	170.67 ± 2.31^{b}	105.33 ± 2.31^{c}	93.33 ± 2.31^{d}
5	237.33 ± 2.31^{a}	94 ± 4.62	197.33 ± 2.31^{b}	98.67 ± 4.62^{c}	89.33 ± 2.31^{d}
7	294.67 ± 2.31^{a}	130 ± 4.62	234.67 ± 2.31^{b}	129.33 ± 4.62^{c}	106.67 ± 2.31^{d}

*Values are described as mean ± SD

*Values are calculated as $\times 10^{4}$ CFU/ml

*Mean values followed by different letters in the same row are significantly different ($P<0.05$)

Appendix V

Total Lactic Acid Bacteria (LBC) of yoghurt sample treated with different concentration of essential oils

			Dosage of EO (µl/ml)		
Days	-ve Control	+veControl	0.5	1.0	2.0
1	113.33±5.77[a]	110.67±2.89	111.67±5.77[a]	111.67±2.88[a]	110.67±4.00[b]
3	156.67±5.77[a]	140.33±2.89	140±4.00[b]	140±4.00[c]	127.33±2.31[d]
5	190.33±4.51[a]	185±5.77	173.33±2.89[b]	175.6±2.31[c]	165±5.00[c]
7	240±20.00[a]	210.33±4.51	191.67±7.64[b]	183.33±2.89[c]	220.67±5.77[d]

*Values are described as mean ± SD

*Values are calculated as $\times 10^4$ CFU/ml

*Mean values followed by different letters in the same row are significantly different ($P<0.05$)

Appendix VI

Total Fungal Count (TFC) of yoghurt sample treated with different concentration of essential oils

			Dosage of EO (µl/ml)		
Days	Control	+ve Control	0.5	1.0	2.0
1	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND
5	30.67±2.31	ND	22.67±2.31	ND	ND
7	38.67±2.31	ND	30.67±2.31	ND	ND

*Values are described as mean ± SD

*Values are calculated as $\times 10^2$ CFU/ml

*ND- Not detected

Appendix XII

Showing panel scoring yoghurt samples

APPENDIX VIII

SENSORY SCORE CARD FOR SENSORY EVALUATION OF BIO-PRESERVED YOGURT

Name: Madani Tijjani
ID Number: SPS/16/MBC/00063 **DATE:**

Kindly evaluate the given samples for attributes like appearance, Taste, Texture, Aroma and general acceptability using the following 9- point hedonic scale and enter the scores in the space provided in the table below.

HEDONIC RATING SCORE

Like extremely	**9**
Like very much	**8**
Like moderately	**7**
Like slightly	**6**
Neither like nor dislike	**5**
Dislike slightly	**4**
Dislike moderately	**3**
Dislike very much	**2**
Dislike extremely	**1**

	Control	L11	L12	L13
Appearance				
Taste				
Texture				
Aroma				
Acceptability				

Remarks (if any):

Appendix IX

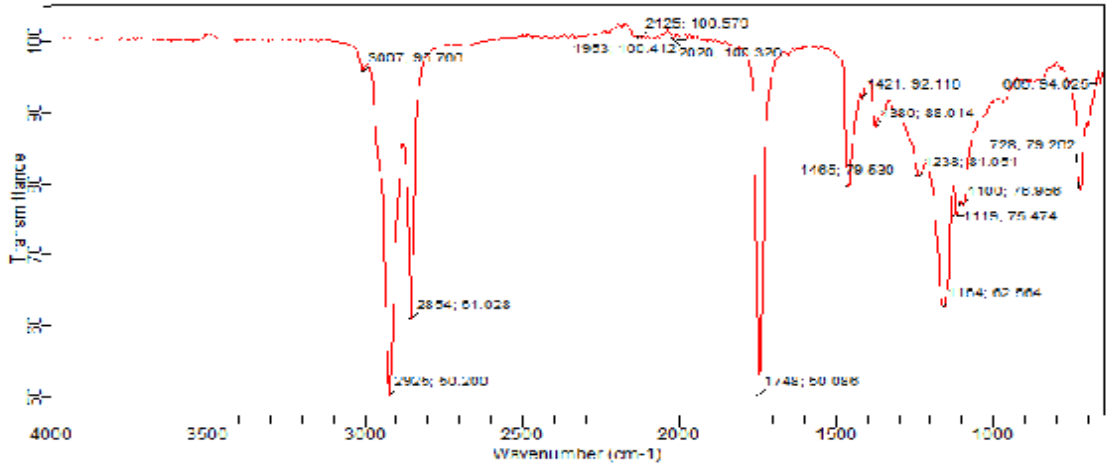
POLYMERASE CHAIN REACTION MIXTURE

REAGENTS	VOLUME (µL)
10 Taq buffer A	1.5
$MgCl_2$	0.75
dNTPs MIX	0.12
Bact 1442-F	0.51
Bact 1492-R	0.51
KappaTaq	0.12
dH_20	10.49
gDNA	1.0
TOTAL	15

Appendix X

Agilent Technologies

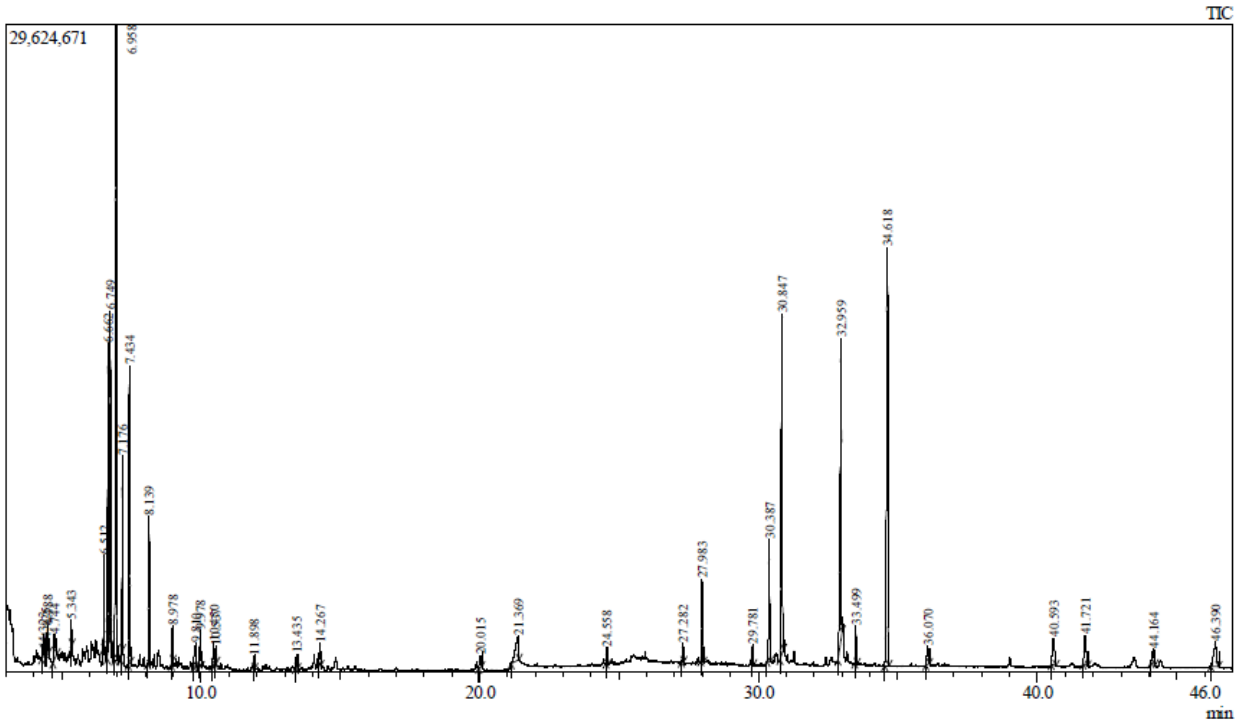
Sample ID:	SAMPLE B (MADANI)	Method Name:	Default
Sample Scans:	32	User:	biochemistry
Background Scans:	32	Date/Time:	3/21/2019 11:31:57AM
Resolution:	8 cm-1	Range:	4,000.00 - 650.00
System Status:	Good	Apodization:	Triangular
File Location:	C:\Program Files\Agilent\MicroLab PC\Results\SAMPLE B (MADANI)_0000.a2r		



03/21/2019 11:33:41 Printed by:biochemistry SAMPLE B (MADANI)_0000.a2r Page 1 of 1

FTIR result of fraction B

APPENDIX XI



Gas chromatogram of fraction B