STUDY ON SHELF-LIFE OF *ZOBO* DRINK AND MOLECULAR CHARACTERIZATION OF ITS BACTERIAL FLORA

BY

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CERTIFICATION

This is to certify that this Research work was carried out by **Shakirat Afodun ABDULMUMINI** of Matric Number 16/27/MMI002 under my supervision and it is a fair reflection of the student input.

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DEDICATION

This work is dedicated to Almighty Allah.

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I offer my humble thanks to Almighty ALLAH, for His love and all the blessings. He has bestowed on me. I give deep gratitude to my dearest Prophet Mohammad (peace be upon him) who taught us how to remain stead fast in our height of happiness and never to despair in our depth of desolation.

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ABSTRACT

The potential of three preservation techniques: the use of chemical preservative (sodium benzoate), pasteurization and lime for the preservation of *zobo* drink was evaluated. The treated samples were stored for six days at room temperature (28±2 ºC). The proximate composition of the *zobo* drink was determined. Physiochemical parameters (pH and titratable acidity) were assayed. Microbiological analyses were also conducted at one day interval for the duration of storage period. Isolates obtained were subjected to molecular characterization. The proximate composition of *zobo* drink showed that it contains 92.52% moisture content, 0.56% ash content, 1.05% fat and oil, 2.16% protein, 0.17% fibre and 3.54% carbohydrate. The results of the physiochemical analysis carried out on storage showed that the pH values of all samples ranged from 2.8 to 3.4, while the titratable acidity ranged from 0.2586 to 0.2816 %. There was a general reduction in pH of *zobo* samples, with a general increase in the titratable acidity. Bacterial counts ranged from 3.0×10^2 to 3.15×10^3 $cfuml^{-1}$ while fungal count ranged from 3.5×10^2 to 2.35×10^3 $cfuml^{-1}$. Result obtained indicate the presence of six organisms comprising of four bacteria and two fungi in the *zobo* drink samples. The two fungi were identified to be *Aspergillus niger* and *Penicillium citrinum.* Molecular characterization of the four bacterial isolates identified them to be *Bacillus cereus* ZB1 (MH566234), *Lactobacillus brevis* ZB2 (MH566235), *Staphylococcus aureus* ZB3 (MH559826) and *Micrococcus luteus* ZB4 (MH566236). It was deduced from the results obtained that pasteurized samples containing preservatives were most effective followed by lime and sodium benzoate in reduction of bacterial load. This study revealed that the synergistic effect of pasteurization and lime, pasteurization and sodium benzoate introduction into *zobo* sample can be used to minimize bacterial load to acceptable limit for at least six days after production.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Zobo is a nutritional drink consumed by different classes of people irrespective of socio-economic status, sex and age in Nigeria especially in the Northern region and other neighbouring African countries (Nwafor, 2012; Izah *et al.*, 2015). It is produced from the dried calyces of the roselle plant *Hibiscus sabdariffa* by boiling and filteration. The demand for *zobo* drink is due to its low prices, nutritional and medicinal properties (Ilondu and Iloh, 2007). *Zobo* drink has been shown to be a good source of natural carbohydrate, protein and vitamins C which constitutes the major reason for consuming soft drink and fruit juice (Ogiehor and Nwafor, 2004). It is served indoors or at special occasion to people of various tribes and tradition, in Nigeria. Inspite of the increasing popularity of *zobo* drink, one of its limitations for large scale production is that it deteriorates rapidly. The drink supports growth of microorganisms which can cause food spoilage (Omemu *et al.,* 2006). Several groups of microorganisms (*Bacillus, Streptococcus, Staphylococcus, Leuconostoc, Lactobacillus, Aspergillus, Penicillium, Geotrichum, Fusarium* and *Alternaria*) have been associated with spoilage of *zobo* drink during storage (Ogiehor and Nwafor, 2004). Some health implications of these spoilage microorganisms include: food poisoning, food intoxication, and sometimes death in severe cases (Omemu *et al.,* 2006). The proliferation of the associated microorganisms potentiates spoilage and the short shelf life associated with this relish beverage (Ogiehor and Nwafor, 2004). Considering the increasing acceptance, socio-economic potentials and ready sources of vitamin C, the need to enhance and extend the shelf life by an effective means becomes imperative.

It is possible to improve the shelf life of *zobo* drink by slowing down the fermentation process, thus inhibiting discolouration of *zobo* drink and possibly permitting large-scale production and preservation for a longer period with maximum retention of nutritive values. In addition, less than 50% of the about 170 million Nigeria are connected to the national grid (Ohimain, 2014). As such, most people rely heavily on gasoline and diesel to power generators. Hence the use of refrigeration as the sole source of preservation could be challenging since most *Zobo* processors are small-scale producers and this could result in increased cost of production.

1.2 Statement of Research Problem

Since refrigeration is the only dependable means of preserving *zobo* drink after production, the inadequate supply of electricity as experienced in our nation could lead to microbial contamination of the unrefrigerated *zobo* drink. Running of generator for refrigeration could also lead to increase in the cost of production.

1.3 Justification of the Study

Using different preservation methods, at low cost to prevent and reduce the spoilage of *zobo* drink by microbial activities in order to increase its shelf life.

1.4 Aim

The main aim of this research is to determine the effectiveness of different preservation methods on the shelf-life of *zobo* drink.

1.5 Specific Objectives

i. To determine the nutritional, chemical and microbiological quality of *zobo* drink
ii. Identify fungi and bacterial isolates present in *zobo* drink
iii. Carry out molecular characterization of bacterial isolates present in *zobo* drink.

CHAPTER TWO

LITERATURE REVIEW

2.1 Description of *H. sabdariffa*

Hibiscus has more than three hundred species distributed in tropical and subtropical regions around the world and are used as ornamental plants (Yadong *et al.*, 2005). Research have shown that some species of *Hibiscus* possess certain medicinal properties of which *H. sabdariffa* is one (Yadong *et al.*, 2005). *H. sabdariffa* is commonly named as "red sorrel" or "roselle". Even though permeable soil is the best, Roselle can adapt to a variety of soil in a warmer and more humid climate (Robert, 2005).

H. sabdariffa, a member of Malvaceae family, is a known medicinal plant with a worldwide fame. The plant can be found in almost all warm countries such as India, Saudi Arabia, Malaysia, Indonesia, Thailand, Philippines, Vietnam, Sudan, Egypt and Mexico (Abbas *et al.*, 2011). Roselle is mainly cultivated to be consumed as vegetables for soup preparation (Fasoyiro *et al.*, 2005). It is used in folk medicine and tea preparations (Adesokan *et al.*, 2013). The main producers of Roselle blossoms are Egypt, Sudan, Mexico, Thailand and China. Other hibiscus varieties are planted for their fibers (Naturland, 2002).

2.2 Origin of *H. sabdariffa*

There is a big argument about the origin of Roselle among different scholars. *H. sabdariffa* is native to Asia (India and Malaysia) (Bola and Aboaba, 2004; Egbere *et al.*, 2007; Ezearigo *et al.*, 2014). Specifically, *H. sabdariffa* is native to India from where

it was introduced to other part of the World including Central America, West India and Africa (Fasoyiro *et al.*, 2005). *H. sabdariffa* was introduced to Central America and West India when the plant became popular in Jamaica in early 18th century (Ilondu and Iloh, 2007; Alo *et al.*, 2012). As at today, *H. sabdariffa* is found in several tropical and subtropical countries of the World especially in India and Africa (Nwachukwu *et al.*, 2007). In Nigeria, *H. sabdariffa* is mainly found in the Middle belt North eastern region (Obadina and Oyewole, 2007; Bolade *et al.*, 2009; Joseph and Adogbo, 2015).

2.3 Species of *H. sabdariffa*

Among numerous varieties of Hibiscus, *Hibiscus altissima* and *H. sabdariffa* are the most common and better introduced. *Hibiscus altissima* is branchless plant with yellow flowers and red or green colored calyxes. Though this species is not used for food, this plant is more economically important than *H. sabdariffa* because of its high fiber content. The other distinct type, *H. sabdariffa* or "Roselle" (plate 1) grows many branches. The flowers of Roselle are axillaries or in terminal racemes, the petals are white with reddish center at the base of the stamina column and this species is widely used as food (Mady *et al.*, 2009).

2.4 Composition of *H. sabdariffa*

Roselle is mainly cultivated for its calyx, which is of three types: green, red and dark red. The red calyxes are the most used and are characterized by their concentration of anthocyanin (Plate 2). Delphinidin 3-Sambubioside and Cyanidin3-Sambubioside are the major anthocyanin. Roselle is also rich in organic acids, minerals, amino acids, carotene, vitamin C and total sugar in its calyx, leaves and seeds at variable levels

depending on the variety and geographical area (Arvind and Alka, 2011). According to Manita–Mishra (1999), a number of compounds have also been isolated and characterized from Roselle including flavonoids, anthocyanidins, triterpernoids, steroids and alkaloids. Nutrient contents of different part of *H. sabdariffa* per 100 gram are shown in Table 1.

Plate 1: *H. sabdariffa* plant

Source: (Mady *et al.*, 2009)

Plate 2: Dried calyces of *H. sabdariffa*

Source: (Arvind and Alka, 2011)

Table 1: Nutrient contents of different part of *Hibiscus Sabdariff* per 100g

Nutrients	**Calyxes**	**Seeds**	**Leaves**
Protein [g]	2	28.9	3.5
Carbohydrates[g]	10.2	25.5	8.7
Fat [g]	0.1	21.4	0.3
Vitamin A [I.E.]	-	-	1000
Thiamine [mg]	0.05	0.1	0.2
Riboflavin [mg]	0.07	0.15	0.4
Niacin [mg]	0.06	1.5	1.4
Vitamin C [mg]	17	9	2.3
Calcium [mg]	150	350	240
Iron [mg]	3	9	5

Source: (Naturland, 2002)

2.5 Uses of *H. sabdariffa*

The plant is primarily cultivated for the production of bast fibre from the stem. The fibre may be used as a substitute for jute in making burlap (Pau *et al.,* 2002). Hibiscus, specifically roselle, has been used in folk medicine as a diuretic and mild laxative (Mohamad *et al.*, 2002).

The red calyces of the plant are increasingly exported to the United States and Europe, particularly Germany, where they are used as food colourings (Chau *et al.,* 2000). It can be found in markets (as flowers or syrup) in places, such as France, where there are Senegalese immigrant communities (Pau *et al.,* 2002). The green leaves are used like a spicy version of spinach. They give flavour to the Senegalese fish and rice dish *thieboudienne*. Proper records are not kept, but the Senegalese government estimates national production and consumption at 700 t (770 short tons) per year (Braid *et al.*, 2012). In Burma their green leaves are the main ingredient in chin baungkyaw curry (Bolade *et al.,* 2009). Brazilians attribute stomachic, emollient, and resolutive properties to the bitter roots (Pau *et al.*, 2002).

2.5.1 Vegetable

In Maharashtra, roselle is called Ambadi (Mohamad *et al.,* 2002). The Ambadi leaves are mixed with green chillies, salt, some garlic to prepare a chutney which is served with Jowar or bajra made bhakri. This is eaten by Farmers as breakfast to start their day. A dry vegetable or SukhiSabzi made of Ambadi leaves tastes good with Bhakri.

In Andhra cuisine, roselle is called gongura and is extensively used. The leaves are steamed with lentils and cooked with dal. Another unique dish is prepared by mixing fried leaves with spices and made into a gongurapacchadi, the most famous dish of Andhra cuisine that is often described as king of all Andhra foods (Bukar *et al.,* 2010).

In Burmese cuisine, called *chin baungywet* (lit. sour leaf), the roselle is widely used and considered affordable. It is perhaps the most widely eaten and popular vegetable in Burma (Pau *et al.,* 2002). The leaves are fried with garlic, dried or fresh prawns and green chili or cooked with fish. A light soup made from roselle leaves and dried prawn stock is also a popular dish. Among the Paites tribe of the Manipur *H. sabdariffa* and *Hibiscus cannabinus* locally known as 'anthuk' are cooked along with chicken, fish, crab or pork or any meat, and cooked as a soup as one of their traditional cuisines (Chau *et al.,* 2000). In the Khasi Hills of Meghalaya, the plant is locally known as Jajew, and the leaves are used in local cuisine, cooked with both dried and fresh fish.

In the Philippines, the leaves and flowers are used to add sourness to chicken dish "Tinola" (chicken stew) (Gupta *et al.,* 2013). In Vietnam, the young leaves, stems and fruits are used for cooking soups with fish or eel (Gupta *et al.,* 2013).

In Mali, the dried and ground leaves, also called Djissima, are commonly used in Songhaï cuisine, in the regions of Timbuktu, Gao and their surroundings (Braide *et al.,* 2012). It is the main ingredient in at least two dishes, one called Djissima-Gounday, where rice is slowly cooked in a broth containing the leaves and lamb, and the other dish is called Djissima-Mafé, where the leaves are cooked in a tomato sauce, also including lamb. Note that Djissima-Gounday is also considered an affordable dish (Chau *et al.,* 2000).

2.5.2 Beverage

In the Caribbean, sorrel drink is made from sepals of the roselle. It is prepared by boiling dried sepals and calyces of the sorrel/flower of the plant in water for 8 to 10 minutes (or until the water turns red), then adding sugar (Ilondu *et al.,* 2003). It is often served chilled. This is done in Trinidad and Tobago, Guyana, Antigua, Barbados, St. Lucia, Dominica, Grenada, and Jamaica where it is called 'sorrel' (Ilondu *et al.,* 2007). The drink is one of several inexpensive beverages (aguasfrescas) commonly consumed in Mexico and Central America; they are typically made from fresh fruits, juices or extracts. It is very popular in Trinidad and Tobago especially as a seasonal drink during christmas where cinnamon, cloves and bay leaves are preferred to ginger (Bernard *et al.,* 2014). It is also popular in Jamaica usually flavored with rum (Braide *et al.,* 2012). In Mali, Senegal, The Gambia, Burkina Faso and Benin calyces are used to prepare cold, sweet drinks popular in social events, often mixed with mint leaves, dissolved menthol candy, and/or fruit flavors (Bolade *et al.,* 2009). The Middle Eastern and Sudanese "Karkade" is a cold drink made by soaking the dried Karkade calyces in cold water overnight in a refrigerator with sugar and some lemon or lime juice added. It is then consumed with or without ice cubes after the flowers have been strained. In Lebanon, toasted pine nuts are sometimes added (Ilondu *et al.,* 2007). Roselle is used in Nigeria to make a refreshing drink known as *Zobo* and natural fruit juices of pineapple and watermelon are added. Ginger is also sometimes added to the refreshing drink (Ayo *et al.,* 2004).

With the advent in the U.S. of interest in south-of-the-border cuisine, the calyces are sold in bags usually labeled "Flor de Jamaica" and have long been available in health food stores in the U.S. for making tea (Bernard *et al.,* 2014). In addition to being a popular

homemade drink, Jarritos, a popular brand of Mexican soft drinks, makes a Flor de Jamaica flavored carbonated beverage. Imported Jarritos can be readily found in the U.S. (Bernard *et al.*, 2014). In the UK, the dried calyces and ready-made sorrel syrup are widely and cheaply available in Caribbean and Asian grocers (Bhomilk *et al.*, 2012). The fresh calyces are imported mainly during December and January to make Christmas and New Year infusions, which are often made into cocktails with rum. They are very perishable, rapidly developing fungal rot, and need to be used soon after purchase — unlike the dried product, which has a long shelf-life.

In Africa, especially the Sahel, roselle is commonly used to make a sugary herbal tea that is sold on the street. The dried flowers can be found in every market. Roselle tea is quite common in Italy where it spread during the first decades of the 20th century as a typical product of the Italian colonies (Kumari *et al.*, 2014). The Carib Brewery Trinidad Limited, a Trinidad and Tobago brewery, produces a 'Shandy Sorrel' in which the tea is combined with beer.

In Thailand, roselle is generally drunk as a cool drink, and it can be made into a wine (Braide *et al.*, 2012). Hibiscus flowers are commonly found in commercial herbal teas, especially teas advertised as berry-flavoured, as they give a bright red colouring to the drink. Rosella flowers are sold as Wild Hibiscus flowers in syrup in Australia as a gourmet product. Recipes include filling them with goats cheese; serving them on baguette slices baked with brie; and placing one plus a little syrup in a champagne flute before adding the champagne — the bubbles cause the flower to open (Kanoma *et al.*, 2014).

2.5.3 Jam and preserves

In Nigeria, rosella jam has been made since colonial times and is still sold regularly at community fetes and charity stalls (Bankole *et al.,* 2013). It is similar in flavour to plum jam, although more acidic. It differs from other jams in that the pectin is obtained from boiling the interior buds of the rosella flowers. It is thus possible to make rosella jam with nothing but rosella buds and sugar.

In Burma, the buds of the roselle are made into 'preserved fruits' or jams. Depending on the method and the preference, the seeds are removed or included. The jams, made from roselle buds and sugar, are red and tangy (Gupta *et al.,* 2013). In India, Roselle is commonly made into a type of pickle (Pau *et al.,* 2000). "Sorrel jelly" is manufactured in Trinidad (Braide *et al.,* 2012). Rosella jam is made in Queensland, Australia as a home-made or speciality product sold at fetes and other community events (Bhowmilk *et al.,* 2012).

2.5.4 Herbal medicine applications

Roselle is used in many folk medicines. It is valued for its mild laxative effect, ability to increase urination, relief during hot weather and treatment of cracks in the feet, bilious, sores and wounds (Yadong *et al.,* 2005). Traditionally in Sudan, Roselle has been used for relief of sour throat and healing wounds (Aziz *et al.,* 2007). In African folk medicine, Roselle leaves are used for their, antimicrobial, emollient, antipyretic, diuretic, anti-helmentic, sedative properties and as a soothing cough remedy, whereas in India, leaves are poultice on abscesses (Duke *et al.,* 2003).

2.5.4.1 Hypo-lipidemic effects: According to a study conducted among hyper-cholesterolemic patients, two capsules of Roselle extract (1 g), given three times a day (for a total of 3 g/day), significantly lowered serum cholesterol (Lin Tzu-Li *et al.,* 2007). Another scientific study also confirmed that ethanolic extract from the leaves of Roselle significantly exhibit hypo-lipidemic effect (Sandeep *et al.,* 2010). Roselle extract was also studied among subjects, some with and some without metabolic syndrome. Subjects with metabolic syndrome receiving ethanolic extract of Roselle had significantly reduced glucose, total cholesterol and low density lipoprotein, with increasing high density lipoprotein (Hassan *et al.,* 2009).

2.5.4.2 Blood pressure lowering effect: The effectiveness of an aqueous extract of Roselle on mild to moderate hypertension was investigated in many researches. Aqueous extract of roselle was as effective as captopril in treating mild to moderate hypertension and there is no adverse effect with the treatment, confirming the effectiveness and safety of the extract (Herrera-Arellano *et al.,* 2007). Even though the possible mechanism(s) of action of Roselle extract is not investigated, daily consumptionof an aqueous Roselle extract resulted in decrease in systolic and diastolic blood pressure (Herrera-Arellano *et al.,* 2004).

2.5.4.3 Anti diabetic activity: Penq *et al.* (2011) extracted the polyphenolic components of roselle and studied their effect in a type II diabetic rat model (high fat diet model). Studied revealed anti-insulin resistance properties of extract at a dose level of 200 mg/kg, and reduction in hyper glycaemia and hyper insulinemia. The extract was found effective in lowering serum cholesterol, triacylglycerol, the ratio of low density lipoprotein/high-density protein (LDL/ HDL), and also Advanced Gycated End product

(AGE) formation and lipid per oxidation. Intestinal α-glycosidase and pancreatic α-amylase help in digestion of complex carbohydrates present in the food into bioavailable monosaccharide and plays an important role in postprandial hyperglycaemia; therefore inhibition of these enzymes has been reported as an effective mechanism for the control of postprandial hyperglycaemia. Hibiscus acid (hibiscus- type (2S,3R)-hydroxycitric acid lactone) have been shown as a potent inhibitor of pancreatic α-amylase and intestinal α-glucosidase and pancreatic α-amylase activity (Yamada *et al.,* 2007). In another study, Adisakwattana *et al.* (2012), conducted an *in vitro* study and reported roselle extracts as an effective inhibitor of pancreatic α-amylase.

2.5.5 Anti helmentic and anti microbial effects

Roselle is known for its antibacterial, antifungal and anti-parasitic actions. Oil extracted from seeds of Roselle has been shown to have an *in vitro* inhibitory effect on *Bacillus anthracis* and *Staphylococcus albus (*Hansawasdi *et al.,* 2000). Aqueous and ethanol extracts were also found to be effective against *Schistosoma mansoni* and other microorganisms (Yamada *et al.,* 2007). Afolabi *et al.* (2008) demonstrated the antibacterial effect of hibiscus extract on *Streptococcus mutans,* a bacterium from oral cavity. In a similar study, antibacterial potential of hibiscus was also observed on *Campylobacter* species (Yin and Chao, 2008). An ethanol extract of the dried leaves of Roselle reduce aflatoxin formation and have *in vitro* inhibitory effect against some fungi (Okasha *et al.,* 2008).

2.5.5.1 Anti-oxidant effect: Protective property of a compound to inhibit the oxidative mechanisms by scavenging reactive oxygen and free radicals is known as antioxidative activity. It protects lining organelles from premature cell damage and reduces ageing. A large number of *in vitro* and *in vivo* studies have shown that Roselle calyxes contain potent antioxidant. According to Augustine (2011), both the whole aqueous and anthocyanin-rich extracts of Roselle are effective antioxidant. Studies have also highlighted that poly-phenolic acid, flavonoids and anthocyanins which are found in Roselle are potent antioxidants (Bako *et al.,* 2014).

2.5.5.2 Other Pharmacological effects: Roselle has been reported to possess a lactogenic activity. Okasha *et al.* (2008), observed enhancement in the serum prolactin level of lactating female Albino Rats on administration of seed extract of Roselle. Bako *et al.* (2014), studied the lactogenic effect of ethyl acetate fraction of *H. sabdariffa,* from 3-17 days of lactation. The results showed an increase in serum prolactin level and milk production in lactating female albino rats, which confirms the lactogenic property of *H. sabdariffa.* Studies have shown that Roselle tea contains an enzyme inhibitor which blocks production of amylase and it is possible that drinking a cup of *hibiscus* tea after meals can reduce the absorption of dietary carbohydrates and assist in weight loss (Da-costa-Rocha, 2014). It was also reported that Roselle is considered as a possible anti-obesity agent. Extracts from Roselle are also known to have effect on inflammatory disease (Dafallah and Al-Mustafa, 1996). It also have effect on cancer (Chewonarin *et al.,* 1999).

2.6. *Zobo* drink

Zobo is a nutritional drink consumed by different class of people irrespective socio economic status, sex and age in Nigeria especially in the Northern region and other neighboring African countries (Nwafor, 2012; Izah *et al.,* 2015). The most active ingredient used in the production of *zobo* drink is *H. sabdariffa.* Two varieties of *H. sabdariffa* are found in Nigeria including red/brown and green (Adanlawo and Ajibade, 2006; Ilondu and Iloh, 2007). The green type is found in Southern guinea savanna while the brown type is prevalent in the Northern (Ilondu and Iloh, 2007). The calyces of the red variety are used for the production of *zobo* drink and soup, while the calyces of the green variety are used to cook soup, stew and sauces (Adanlawo and Ajibade, 2006).

This popular drink is called *zobo* or Yakwua or *zobo*rodo (in Hausa), *Iseipa* (in Yoruba) and Sorrel in English, Jamaica flower, and Karkade (Egbere *et al.*, 2007; Adebayo-Tayo and Samuel, 2009; Cid-Ortega and Guerrero-Beltrán, 2014). Some times *zobo* could be spelled as Sobo. However, based on the name of this useful drink in different languages, the popular name it's known in Nigeria is derived from its Hausa name (Ezeigbo *et al.,* 2015a, b). *Zobo* has gained prominence in several parts of the country and are sold in public places. *Zobo* is one of the nutritional drinks that are served during festivals and in a number of other ceremonies in different parts of Nigeria (Umaru *et al.*, 2014). The increased consumption of *zobo* is due to the nutritional, medicinal properties and low cost (Oboh and Elusiyan, 2004). At present, *zobo* drink is consumed by several millions of people cutting across different socio-economic classes in West African (Ogiehor and Nwafor, 2004).

Zobo drink is characterized by short shelf-life span of about 24 hours after production without refrigeration (Omemu *et al.*, 2006; Nwachukwu *et al.*, 2007; Onuoha and Fatokun, 2014). Food safety is an essential concern of both the consumers and the producers (Witkowska *et al.*, 2013). Microorganism in food is not always detrimental, because their growth may result in pleasant taste and texture (Bukar *et al.*, 2010). But microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Vibro cholera, Salmonella, Bacillus, Clostridium* species etc. could contaminate food and transfer a wide range of disease conditions in food (Bukar *et al.*, 2010). Also listed are *Pseudomonas, Saccharomyces, Rhizopus, Streptococcus, Bacillus, Erwinia, Aspergillus, Chromobacteria, Penicillium, Fusarium, Flavobacterium, Xanthomonas, Enterobacter* species as microbes that could biodegrade food materials such as fruits and vegetables (Seiyaboh *et al.*, 2013). Specifically, *Bacillus, Streptococcus, Staphylococcus, Leuconostoc, Lactobacillus, Aspergillus, Penicillium, Geotrichum, Fusarium* and *Alternaria* species are potential microbes that could deteriorate *zobo* drink (Bankole *et al.*, 2013). Laboratory studies have shown that *Bacillus, Aeromonas, Corynebacterium, Veilonella, Micrococcus, Pseudomonas, Streptococcus, Staphylococcus, Lactobacillus, Enterococccus, Escherichia, Proteus* (bacteria), *Aspergillus, Penicillium, Saccharomyces* (Fungi/yeasts) are the genera of microbes that cause spoilage of *Zobo* drink (Omemu *et al.*, 2006; Egbere *et al.*, 2007; Braide *et al.*, 2012; Seiyaboh *et al.*, 2013; Ezearigo *et al.*, 2014; Ezeigbo *et al.*, 2015b). Generally, contaminated ready–to-eat foods and drinks are the potential source of various food borne disease conditions especially the ones associated with gastroenteritis in human (Bello *et al.*, 2014).

2.6.1 *Zobo* drink preparations

Zobo drink is a non-alcoholic nutritive drink prepared from the dried calyces of roselle (Nwafor and Ikenebomeh, 2009; Adebayo-Tayo and Samuel, 2009; Risiquat, 2013; Adesokan *et al.*, 2013; Ezearigo *et al.*, 2014; Umaru *et al.*, 2014). *Zobo* is a red liquid drink with fruit punch taste (Ezeigbo *et al.*, 2015a). During *zobo* preparation, the dried calyces are hurled in water for 1- 2 hours and then allowed to cool prior to sieving (Umaru *et al.*, 2014). A stricter soaking period of 10 – 15 minutes has been severally reported by authors (Onuoha and Fatokun, 2014; Ezeigbo *et al.*, 2015a). The resultant filtrate is then consumed as hot tea or taken as a refreshing drink when chilled (Ezeigbo *et al.*, 2015b). Thereafter preservatives/spices such as ginger (*Zingiber officinalis)* and sugar are added before cooling in the refrigerator (Umaru *et al.*, 2014). The choice of preservatives and flavor depend on the processors. After refrigerating, the nutritive drink is ready for consumption. Figure 1 presents the preparation processes of *zobo* drink in Nigeria.

More recently, other plant materials are added as a blend in the preparation of *zobo* drink so as to improve the flavor and their shelf life. The sharp sour taste derived from the raw extract of *H. sabdariffa* is usually sweetened with sugar cane or granulated sugar, pineapple, orange or other fruits depending on choice (Onuoha and Fatokun, 2014; Ezearigo *et al.*, 2014; Ezeigbo *et al.*, 2015a). Straw berry is another additives/ flavor used in *zobo* drink preparation (Egbere *et al.*, 2007). The sweetness of *zobo* does not last probably due to the activities of spoilage microorganism (Nwachukwu *et al.*, 2007). Some of these preservatives include garlic, ginger, lime, clove, cinnamon, nutmeg and pepper fruit (Ihemeje *et al.*, 2013).

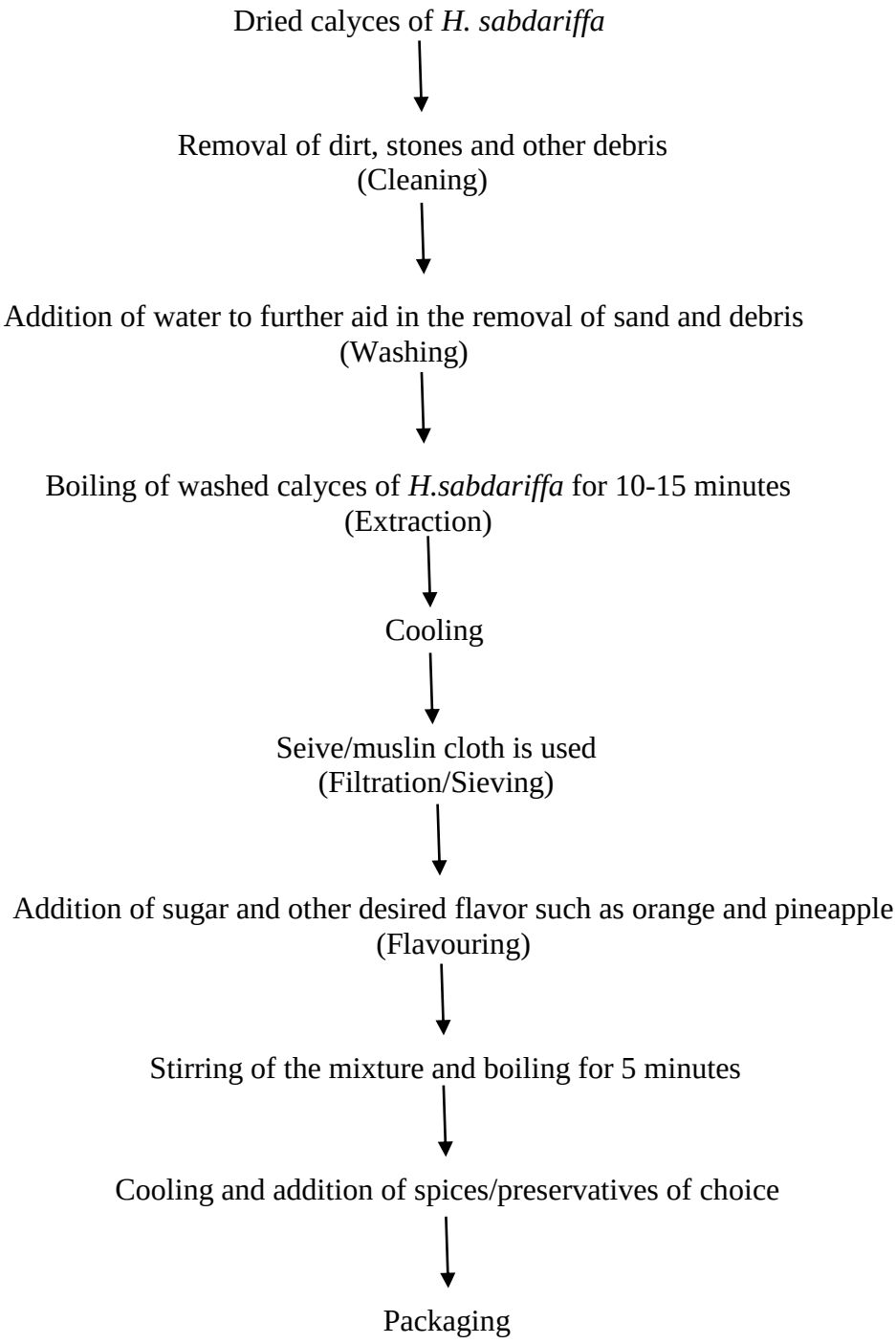

Figure 1: *Zobo* drink production pathway

Source: (Izah *et al.,* 2016)

2.6.2 Preservatives used in the preparation of *Zobo* drink

Preservatives are chemical additives that are utilized to improve the shelf-life of food products so as to prevent or retard the growth of microorganisms such as bacteria and mould and prevent changes in the chemicals quality of the food such as color, taste, aroma and texture (Bankole *et al.,* 2013). Spices are flavored or aromatic substances obtained from plants and are mostly used as food condiments (Singh *et al.,* 2012). However, most spices are also used as preservatives and vice versa. Typically, preservatives are products or substances added to food products to sustain the physicochemical and microbial quality of the food (Ayo *et al.,* 2004). Several studies have been carried out on the suitability and sustainability of potential preservatives for used in *zobo* drink. These include physical treatment (freezing, refrigeration and pasteurization), chemical treatment (e.g. acetic acid and sodium benzoate) and biological treatment (several plant extracts) (Izah *et al.,* 2016).

2.6.2.1 Physical and chemical methods

Zobo drink is usually treated with chemical preservatives such as sodium benzoates at varying concentrations (Izah *et al.,* 2016). These are basically aimed at reducing the spoilage microorganisms and to enhance the shelf life of the products. Pasteurization is one of the physical methods employed in the preservation of nutritive drink such as *zobo*. Pasteurization of *zobo* drink by small holder processors is by immersion of the bottled *zobo* drink in sterile water and heated to ≤100ºC for few minutes. Other physical methods include freezing and refrigeration.

Chemical methods used in the preservation of *zobo* drink are mainly sodium benzoates and acetic acids. The use of acetic acid from synthetic sources for food preservative has received worldwide restriction (Izah *et al.,* 2016). Other potential chemical that could be used include sorbate and propionate. However, the use of chemical in the control of spoilage microorganisms could have negative effects on the nutritional composition of the drink especially in the loss of vitamins. Acetic acid produced from biomass via microbial methods such as oxidative fermentation and its derivatives has been recommended as best preservatives for food (Olawale, 2011).

The role of acetic acid in preventing spoilage of *Zobo* drink is the elimination of spoilage organisms. Acetic acid have antimicrobial properties against *B. subtilis, B. megaterium, B. sphaericus, B. polymyxa, S. aureus E. coli, P. oxalicum, A. flavus, A. luchuensis, Rhizopus stolonifer, Scopulariopsis* and *Mucor* species (Pundir *et al.,* 2010). Studies have shown that pasteurization, acetic acid and sodium benzoates act at different concentrations could reduce the microbial load of *zobo* drink and enhance the shelf life as reported by an author in *zobo* drink (Braide *et al.*, 2012). Acetic acid appears to have a superior effect on bacteria than sodium benzoates. This is because no growth was observed at 24 hours of preservation of *zobo* drink using 2w/v of acetic acid (Braide *et al.*, 2012). The same authors reported that sodium benzoate appears to have a superior effect on the fungi than acetic acid. This is because at 48 hours of preservation the fungi counts was too numerous to count for acetic acid and is in order of 10^{17} using sodium benzoates. Generally, the microbial load of *zobo* drink increases at the beginning of storage and when the nutrient has been exhausted, the growth begins to decline. This typically occurs between few hours to 4 days of storage depending on the concentration of preservatives and spices added during processing.

Typically, storage period influences the microbial load of the *zobo* drink. Also preservation of *zobo* drink reduces the nutritional content such as vitamin C. When *zobo* drink is pasteurized for 0 days the content decreased by 53.41 % and by 14 days it further reduced by 60.37 % (Egbere *et al.*, 2007). Also when *zobo* is pasteurized and 0.1 % sodium benzoates are added at day 0 the vitamin C content reduced by 60.46 % and after 40 days it further declined by 70.34 % (Egbere *et al.*, 2007). On the microbial diversity, the type of preservation used is selective on the microbes. For instance, preserved *zobo* drink treated with different preservatives including sodium benzoates and pasteurization contain *M. lutens, M. roseus, B. subtilis, S. aureus, E. faecalis* (found in pasteurized samples), while *M. lutens* and *B. subtilis* were only found in samples treated with acetic acid, and *M. lutens, B. subtilis* and *S. aureus* were found in samples treated with sodium benzoates (Braide *et al.*, 2012). Furthermore, fungi including *R. stoloifer, A. flavus, F. poae, Mucor* sp, *Saccharomyces cerevisiae, S. ellipsoideus, P. caseicolum* were found in pasteurized samples, and acetic acid treated samples lack only *F. poae* among the fungi diversity and *Mucor* sp, *Saccharomyces cerevisiae, S. ellipsoideus* were found in samples treated with sodium benzoates while the pasteurized samples contained all the type of fungi isolates found in the samples (Braide *et al.*, 2012). However, it appears that sodium benzoates is the best chemical method for preserving *zobo* drink against fungi and yeasts while acetic acid is best against bacteria. Pasteurization only reduces the microbial load slightly. The efficacy of the pasteurization method could be due to short period of denaturation and low temperature i.e. 60 - 70ºC. It was noted that bacteria counts of sodium benzoates treated *zobo* drink preserved at ambient temperature and refrigerated at 4ºC fluctuates between the storage periods (Ezearigo *et al.*, 2014). The reason for this fluctuation remains unknown.

2.6.2.2 Biological methods

Biological preservation of food is the best method of avoiding microbial food spoilage and contamination. Studies have shown that the use of synthetic chemical for food preservation could have adverse human health. Moreover they are expensive when compared to the financial/economic status of the average local processors (Nwachukwu *et al.,* 2007). Some synthetic chemicals contain toxic, mutagenic, clastogenic and genotoxic compounds (Omoruyi and Emefo, 2012). Due to these problems, research has focused on the use of natural products as potential preservatives to mitigate food borne pathogens. To these effects, natural spices have been widely used as food additives or flavor enhancer (Omoruyi and Emefo, 2012). Some of these plant extracts that are used as preservatives are also spices including kola nut or *Cola* species, lime, nutmeg, clove, ginger, garlic, cinnamon, pepper fruit etc.

2.6.2.2.1 Lime

Lime (*Citrus aurantifolia*) which belongs to the *Rutaceae* family is widely distributed in Nigeria and several other tropical countries. Typically, the sub-genus *Citrus* (Swingle) comprises of *C. aurantium, C. sinensis, C. reticulata* (*Tangerine*), *C. limon, C. aurantifolia, C. grandis, C. paradisi, C. indica* and *C. tachibans* etc (Piccinelli *et al.,* 2008; Nwauzoma *et al.,* 2013). *C. aurantifolia* have several medicinal properties. For instance, in traditional setting especially in rural area in Nigeria, *C. aurantifolia* is used for the treatment of several types of skin diseases. Other uses include antioxidants, throat and mouth wash. Typically, *C. aurantifolia* is rich in phytonutrients with health benefits. *C. aurantifolia* contain bioactive metabolites including alkaloids, flavonoids, phenols, saponins and tannins; vitamins including ascorbic acid, thiamine, riboflavin; and nutrients including potassium, phosphorus, magnesium, sodium and calcium (Okwu, 2008).

The effects of lime on the preservation of *zobo* drink have been demonstrated (Egbere *et al.,* 2007). For instance, lime as low as 0.1 concentrations could reduce the population of coliforms and total viable counts in *zobo* drinks (Nwachukwu *et al.*, 2007; Onuoha and Fatokun, 2014). The ability of the lime to reduce the microbial load of the *zobo* drink could be due to the acidic nature of the lime. Most microorganisms cannot survive an acidic condition as such their survival in the *zobo* could be inhibited (Nwachukwu *et al.*, 2007; Onuoha and Fatokun, 2014). Lime has lesser microbial load among other organic spices/preservatives such as garlic, ginger and clove (Braide *et al.*, 2012). The authors also reported a lesser microbial population compared to sodium benzoate at the same concentration of preservatives added at 24 hours of preservation. But thereafter, the bacterial density was similar to other preservatives from 24 hours to 336 hours. On effect of lime on fungal spoilage organisms on *zobo* drink, higher fungi density have been reported among other organic preservatives from 24 hours to 48 hours and from 12 hours to 336 hours the growth was too numerous to count (Braide *et al.*, 2012). The reduction in the population of microbial counts in *zobo* drink that lime was added to completely control *S. aureus* and *F. poae* from the *Zobo* samples but other microbes including *M. lutens, M. roseus, B. subtilis, Enterobacter fecalis* (bacteria) *R. stoloifer, A. flavus, Mucor sp, Sacchraomyces cerevisiae, S. ellipsoideus, P. caseicolum* (fungi) still persist in the *zobo* drink though the population is lower than the sample without lime (Braide *et al.*, 2012**)**.

2.6.2.2.2 Nutmeg (*Myristica fragrans* Houtt.)

Nutmeg (*Myristica fragrans* Houtt) belongs to the *Myristicaceae* family. *M. fragrans* is the most common commercial evergreen tree native to Banda Islands in eastern Indonesia (Moluccas) and is found in Banda Island (Saxena and Patil, 2012; Pooja *et al.*, 2012). Typically, nutmeg tree which grows slowly and could reach about 20 m in height and are mainly cultivated in India, Ceylon, Malaysia and Granada, Caribbean, South India, Sri Lanka, Sumatra, and Brazil (Sanghai-Vaijwade *et al.*, 2011; Pooja *et al.*, 2012). When the fruit of nutmeg tree matures, it splits open and the stony endocarp or seed surrounded by a red, slightly fleshy aril are laid bare (Sanghai-Vaijwade *et al.*, 2011). The resultant nut is then dried and called nutmeg. The dried kernel seed of nutmeg is broadly utilized as spice and active ingredient by the alternative medicine practitioners. The *M. fragrans* produced essential oil which is useful in the pharmaceutical sector and some perfume production industry (Saxena and Patil, 2012). The oil of *M. fragrans* is colourless or light yellow and is used in the oleochemical industry and natural food flavorings including baked foods, syrups, beverages and sweets (Saxena and Patil, 2012). Nutmeg has antioxidant and antimicrobial properties and contains active phytochemicals such as vitamins, carotenoids, terpenoids, alkaloids, saponins, flavonoids, lignans, terpernoids, phlobatanins, tannins, quionines and phenolics etc (Gupta *et al.*, 2013; Kumari *et al.*, 2014). Also *M. fragrans* essential oil contains secondary metabolites including steroids, tannins, alkaloids, flavonoids, phenolics and glycosides and lacks saponins (Saxena and Patil, 2012). Nutmeg has strong antioxidant potentials and a good preservative agent (Tan *et al.*, 2013). Nutmeg contain active molecules including myristicin (main psychoactive constituent), macelignan (Lignans are a class of phytoestrogens having several potential pharmacological activities such as anticancer, anti-

inflammatory, antimicrobial, antioxidative, and immunosuppressive activities), eugenol (4-allyl-2-methoxyphenol) which is mainly used as antiseptic, analgesic and antibacterial agent (Demeco, 2013).

Nutmeg have antimicrobial properties against *B. subtilis, S. aureus, P. putida, P. aeruginosa, A. fumigates, A. niger* and *A. flavus, P. fluorescens, S. aureus* ATCC 26923, *E. fecalis* ATCC 2912, *Candida albicans* and *A. niger* (Pooja *et al.*, 2012; Gupta *et al.*, 2013; Witkowska *et al.*, 2013; Lawal *et al.*, 2014). Also, Omoruyi and Emefo (2012) reported that *M. fragrans* have antibacterial activity to both gram positive and gram negative microbes including *S. aureus, S. epidermidis, Klebsiellia pneumoniae, B. cereus, E. coli, Salmonella typhi* and *P. aeruginosa.* Nutmeg have been tried as a possible preservatives of *zobo* drink but the bacteria growth at 0 days of preparation at ambient storage temperature were higher than that of other organic preservative such as ginger, garlic, cinnamon and even samples without preservatives (Ezearigo *et al.*, 2014).

2.6.2.2.3 Garlic (*Allium sativum* L.)

Garlic (*Allium sativum*) which belongs to the family *Alliaceae,* is a common spice used to add flavor in food. Garlic is an erect bulbous herb that reaches 30–60 cm tall, and possess strong smell when crushed (Enyi-Idoh *et al.*, 2011). *A. sativum* is aromatic sulphur based compounds, which contribute to its odour and taste properties (Pundir *et al.*, 2010). *A. sativum* contain bioactive constituents including terpernoids, glycosides, flavonoids, saponin, tannins and hydrocynaides (Enyi-Idoh *et al.*, 2011; Youssef *et al.*, 2013). As such, *A. sativum* has antimycotic properties against *A. flavus, A. niger, A. ostianus, Alternaria alternate, Fusarium solani* and *C. albicans* (Youssef *et al.*, 2013), *B. subtilis, B.*

megaterium, B. sphaericus, B. polymyxa, S. aureus, E. coli, P. oxalicum, A. flavus, A. luchuensis, Rhizopus stolonifer, Scopulariopsis and *Mucor* species (Pundir *et al.*, 2010). The antimicrobial properties of *A. sativum* to its key component allicin, which is a volatile molecule gives it odour (Pundir *et al.*, 2010). Garlic is effective against heart disease, stroke and hypertension (Padhye *et al.*, 2014).

Garlic has been severally used as a spice for the preparation of *zobo* drink. A lower microbial counts for ginger treated *zobo* drink was noted as compared to the sample without any treatment (Braide *et al.*, 2012; Adesokan *et al.*, 2013). Specifically, garlic reduce the population of *M. lutens, M. roseus, S. aureus, B. subtilis, Enterobacter faecalis,* completely eliminate *R. stoloifer, A. flavus, P. caseicolum*, *F. poae* and reduces the population of *Mucor* species. However, do not have effect on the population of *S. cerevisiae* and *S. ellipsoideus* (Braide *et al.*, 2012).

2.6.2.2.4 Ginger (*Zingiber officinale* Rosc.)

Ginger (*Zingiber officinale* Rosc), is a tropical and subtropical plant native to South East Asia from where it was introduced to other parts of the world (Lawal *et al.*, 2014). Presently, it is extensively cultivated in Jamaica, Nigeria, China, India, Fiji, Sierra Leone and Australia (Bhargava *et al.*, 2012). Ginger which belongs to the *Zingiberaceae* family, is one of the most widely utilized herbs for food flavouring and it possesses medicinal properties (Gaurav *et al.*, 2013; Lawal *et al.*, 2014).

Ginger aids in stimulation of heart and circulatory system and has ability to reduce inflammation (Padhye *et al.*, 2014). In addition, garlic have hepatoprotective, nephroprotective, antioxidant, larvicidal, anti-diabetic, anti-diarrhea, anti-inflammatory,

antifungal, antibacterial, anti-helminthes, cytotoxic and analgesic effects (Gaurav *et al.*, 2013). Ginger has been widely employed for broad range of ailment including arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis (Ali *et al.*, 2008). *Z. officinale* have variously been reported to have antimicrobial properties against *S. aureus* 25923 MRSA, *P. aeruginoa, E. coli, S. aureus, E. faecalis, K. pneumoniae, B. cereus, E. aerogenes and P. mirabilis* (Bello and Adeleke, 2012; Bhargava *et al.*, 2012; Lawal *et al.*, 2014).

Ginger is a major spice used in the production of *Zobo* drink. Typically, ginger reduces the microbial density of *zobo* drink (Braide *et al.*, 2012; Adesokan *et al.*, 2013). Like garlic, ginger reduced the population of *M. lutens, M. roseus, S. aureus, B. subtilis, Enterobacter faecalis, R. stoloifer, A. flavus, F. poae* and *P. caseicolum*, but do not have effect on the population of *S. cerevisiae, S. ellipsoideus* (Braide *et al.*, 2012). The ability of ginger to have effects on the microbial quality of *zobo* could be due to the presence of secondary metabolites found in them. Blended ginger and garlic has been reported to have superior effect on the bacterial density of *zobo* when compared to separate blends (Adesokan *et al.*, 2013).

2.6.2.2.5 Clove (*Syzygium aromaticum* L.)

Clove (*Syzigium aromaticum*) belongs to the family of *Myrtaceae*. Cloves are an evergreen aromatic herb that could reach 30 – 40 feet high with leathery textured leaves covered with many depressions (Bhowmik *et al.*, 2012). Cloves thrive in tropical climates such as the Islands of Indonesia (Bhowmik *et al.*, 2012). *S. aromaticum* is native to India,

Indonesia, Zanzibar and Mauritious (Reji and Rajasekaran, 2015). The flower buds of the plant produced oil known as clove oil (Ayoola *et al.*, 2008; Bhowmik *et al.*, 2012). Clove oil contains ß-caryophyllene, representing 14-21% of its compounds, 10-13% of tannins as well as sesquiterpenes and phenols (Rodríguez *et al.*, 2014). Phenylpropene is the most important part of clove oil, apart from eugenol, which is responsible for the scent properties of the plant and its main component (Rodríguez *et al.*, 2014).

Different parts of Clove plants (oils, dried flower buds, leaves, and stems) have several medicinal properties including expectorant, enhancing blood circulation and increasing body temperature slightly. The aromatic oil have stimulating and irritant effects, enhancing the flow of saliva and gastric juices, relieving chronic rheumatism, toothache, lumbago stomach pain, muscle cramps and some nerve conditions, nausea and vomiting, mouth and throat inflammation, diarrhoea, hernia, and bad breath (Bhowmik *et al.*, 2012). Pundir *et al.* (2010) also reported that *S. aromaticum* have antimicrobial properties against *B. subtilis, B. megaterium, B. sphaericus, B. polymyxa, S. aureus E. coli, P. oxalicum, A. flavus, A. luchuensis, Rhizopus stolonifer, Scopulariopsis* and *Mucor* spp. Reji and Rajasekaran (2015) also reported that *S. aromaticum* can be used against *E. coli*, *P. mirabilis* and *K. pneumoniae*. *S. aromaticum* contain several metabolites including terpernoids, glycosides, phylobatannin, tannins, saponins, sugars, steroids, flavonoids and coumarins (Youssef *et al.*, 2013).

Clove has been studied as a preservative for *zobo* drink. Braide *et al.* (2012) showed that at 24 hours of preservation, the bacterial density were higher than the control, but reduced from 48 – 72 hours before increasing again at 96 hours. The authors further reported that no fungal growth was observed at 24 hours and the population was lesser than the

control throughout the period 24 – 336 hours. It appears that clove has superior effects on the fungal spoilage organisms of *zobo* as compared to bacteria. Clove reduces the density of *M. lutens, B. subtilis, R. stoloifer, A. flavus, S. cerevisiae* and *S. ellipsoideus* moderately and scanty growth of *M. roseus, S. aureus, Enterobacter faecalis, Mucor sp* and *P. caseicolum*, and completely eliminate the growth of *F. poae* (Braide *et al.*, 2012).

2.6.2.2.6 Cinnamon (*Cinnamomum zeylanicum* L.)

Cinnamon (*Cinnamomum zeylanicum* L.) which belongs to the *Lauraceae* family is an ever green plant that could reach 10-15 m tall. It originated from Sri Lanka and South India (Reji and Rajasekaran, 2015). The flower of *C. zeylanicum* is green in color with distinct odour and arranged in panicles, while the berry fruit contain a single seed (Reji and Rajasekaran, 2015). Like most spices, *C. zeylanicum* can be used to treat diarrhea, and other digestive system upset. The plant also has antioxidant activity and the oil is effective against microbes, which help in some food preservation (Reji and Rajasekaran, 2015). Bioactive agents found in *C. zeylanicum* include alkaloids, flavonoids, terpernoids, glycosides, coumarins, tannins (Youssef *et al.*, 2013). The seed, leaf, root-bark and stem-bark of *C. zeylanicum* contain phytosterols, phenols, tannins, saponins and terpernoids (Bernard *et al.*, 2014). Reji and Rajasekaran (2015), also reported that *C. zeylanicum* contain alkaloids, killer killani, quinine, steroids, tannins, terpernoids, carbohydrate and saponin as bioactive constituents. The authors further reported that the plant can be used against *E. coli, P. mirabilis* and *K. pneumoniae.* Youssef *et al.* (2013) reported that *C. zeylanicum* has antimycotic properties against *A. flavus, A. niger, A. ostianus, Alternaria alternata* and *Fusarium solani* and *Candida albicans*. Shareef, (2011) also reported that cinnamon has antibacterial activity against *E. coli, S. aureus, P. aeruginosa, K. pneumoniae, Brucella* and *Proteus* sp.

The potentials of cinnamon as a preservative for *zobo* drink has been studied. Ezearigo *et al.* (2014) have demonstrated that the cinnamon has effects on the reduction in the microbial counts of *zobo* which is similar to other preservatives such as ginger, garlic, nutmeg after 24 hours of preparation. The role of cinnamon in the reduction of microbial counts in *zobo* drink could be due to the presence of phytochemical ingredients found in the plant.

2.6.2.2.7 Kola nut

Kola nut has wide range of traditional uses in Nigeria especially during ceremonies. Kola nut has antioxidant properties (Muhammad and Fatima, 2014). Typically Kola nut belongs to the *Sterculiaceae* family. Kola nut trees could reach 20 - 40 feet tall. The seed of kola nut are used as a condiment by Western and Central tropical African natives. As such, it constitute essential commodity for trade in West Africa possibly due to their caffeine content (Bankole *et al.*, 2013). Several species of Kola nut exist. In Nigeria about three species are edible including *Cola acuminate*, *Cola nitida* and *Cola verticilata*. The *C. nitida* and *C. acuminate* often referred to red and white kola respectively (Muhammad and Fatima, 2014). Typically, Kola nuts constituents include caffeine (with tinge of theobromine), kolanin (combination of kola red and caffeine), glocuside, starch, sugar, fatty matter, tannins, oils, some anti-oxidants like phenolics, anthrocyanin and protein (Theimcgroup, Undated). Odebunmi *et al.* (2009) reported the nutritional and proximate composition of *C. nitida* as 66.0 %, 33.60 %, 5.71 %, 2.63 %, 1.50 %, 7.13 %, 28.56 %, 3480.67 mg/kg, 124.40 mg/kg, 392.00 mg/kg, 16.43 mg/kg, 5.24 mg/kg, 411.43 mg/kg for moisture, dry matter, crude fat, crude protein, ash, crude fibre, total carbohydrate, potassium, calcium, magnesium, iron, zinc and phosphorus respectively.

Kola nut products are energy boosters because of the caffeine content, but excess consumption could be deleterious to humans and produces the following side effects, stomach upset**,** insomnia**,** high blood pressure (Theimcgroup, Undated). Despite the adverse effects, it also have some benefits including helps in digestion**,** decrease high blood pressure**,** helps in weight loss, acts as pain reliever (Theimcgroup, Undated). Muhammad and Fatima (2014) reported that *C. nitida* possesses chemotherapeutic and antibacterial activities that could be useful against odontopathogens. Sonibare *et al.* (2009) reported that *C. acuminate* is effective against *S. albus, K. pneumoniae, A. niger* and C. *albicans; C. ntida* effective against *C. albicans, A. niger and S. albus.* The Secondary metabolites found in fresh leaves *Cola acuminate*, *Cola nitida*, include alkaloids, saponins, tannins and cardenolides. Muhammad and Fatima, (2014) reported that alkaloid, saponin, and glycosides, steroids, flavonoids, tannins, volatile oil and balsam as the bioactive constituents of *C. nitida*. Kanoma *et al.* (2014) also reported secondary metabolites found in *C. nitida* and *C. acuminate* such as alkaloid, tannins, glycoside, steroids and saponins glycoside.

Bankole *et al.* (2013) reported that *Kola acuminate* and *Kola nitida* could be used as preservatives in improving the shelf life of *zobo* drink. The author further reported that extract could reduce the *E. coli* count in *zobo* drink from 2.0 x 10^4 cfu/ml (0 days) to 1.5 x 10^3 cfu/ml (2 days) and to 1.5 x 10^2 cfu/ml (4 days) (*Kola nitida*) and 2.0 x 10^4 cfu/ml (0 days) to 1.4 x 10^4 cfu/ml (2 days) and to 1.0 x 10^3 cfu/ml (4 days) (*Kola acuminate*). The trends in this studies showed that Kola species can be used as preservatives. However, *C. nitida* appear to have superior results than *C. acuminate* in the control of *E. coli* in *zobo* drink during storage (Bankole *et al.*, 2013).

2.6.2.2.8 Pepper fruit (*Dennettia tripetala*)

Dennettia tripetala is commonly known as pepper fruit. The plant belongs to the *Annonaceae* family. It is found in West Africa and the fruit is edible. The plant is widely distributed in southern and to a lesser extent in the savanna regions of Nigeria. Typically, the fruit of *D. tripetala* is red and green when ripe and unripe respectively. Most parts of the plants such as leaves, fruit, bark and root possess strong pepperish and pungent spicy taste with aroma and fragrance properties (Ihemeje *et al.*, 2013). *D. tripetala* is chewed in different perspectives including fresh (green), ripe (red), dried (black). *Dennettia tripetala* fruits and leaves are used as seasonings in food preparation such as meat, soup, fish etc (Ihemeje *et al.*, 2013).

According to Okwu and Morah (2004), *D. tripetala* fruits possess essential nutritive properties such as vitamins (ascorbic acid, thiamine, riboflavin and niacin), minerals (calcium, phosphorus, potassium, magnesium), trace element (iron, copper, zinc etc) and others such as total carbohydrate, moisture, crude protein, lipid and fibre. *D. tripetala* is a spicy indigenous medicinal plant (Elekwa *et al.*, 2011). Elekwa *et al.* (2011) studied the nutritional properties of *D. tripetala* and reported the percentage of protein, moisture content, fat, ash, fiber, carbohydrate, calcium, magnesium, iron, phosphorus, potassium, sodium, ascorbic acid, niacin, thiamine, riboflavin and vitamin A contents on wet basis as 6.59 %, 15.26 %, 5.52 %, 4.13 %, 17.05 %, 51.45 %, 181.69 mg/g, 229.78 mg/g, 0.2 mg/g, 285.8 mg/g, 360.8 mg/g, 6.12 m/mg, 85.65 mg/g, 0.40 mg/g, 0.10 mg/g, 0.05 mg/g and 65. 58 mg/g respectively (unripe) and 4.67 %, 18.73 %, 5.78 %, 3.18 %, 14.32 %, 53.32 %, 138.94 mg/g, 173.68 mg/g, 0.23 mg/g, 243.8 mg/g, 324.27 mg/g, 5.47 mg/g, 115.57 mg/g, 0.37 mg/g, 0.08 mg/g, 0.05 mg/g and 388.10 mg/g respectively (ripe). Also, *D. tripetala*

seed is also rich in phytochemicals including saponins, flavonoids, tannins, cyanogenic glycosides, alkaloids, steroids, terpernoids and phenol (Elekwa *et al.*, 2011; Ihemeje *et al.*, 2013). Ihemeje *et al.* (2013) studied the potentials of *D. tripetala* seed as possible replacement of ginger in *zobo* drink. It is reported that there was no significant difference at 95 % confidence interval in color, aroma, taste, mouth-fee and general acceptability of both category of the *zobo* drink blend. But information about the microbial quality of *zobo* drink blended with *D. tripetala* is scanty in literature.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Source of Sample

The dried calyces of *H. sabdariffa* were purchased from Oja Oba market in Ilorin and was authenticated at department of Plant Biology, University of Ilorin and a voucher number UILH/601/1218 was assigned.

3.2 Production of *zobo* drink

The dried calyces of *H. sabdariffa* were sorted, after which 25 grams was weighed and rinsed. It was then boiled in 1 litre of sterile water for 40 minutes to enable extraction. The mixture was then filtered with a clean muslin cloth and was allowed to cool before dispensing into various containers plastic bottles used for the analysis.

3.3 Proximate analysis of *zobo* drink

The proximate composition of the prepared *zobo* without preservative (moisture content, ash content, fat and oil content, crude protein content, fibre content and carbohydrate content) were determined at department of Chemistry, University of Ilorin following the methods described by the Association of Official Analytical Chemists (AOAC, 2000).

3.3.1 Determination of moisture content

Procedure: A clean crucible was first dry in an oven for about 30 minutes and was then cooled in a dessicator. The cooled crucible was weighed as W_1, after which 2g of sample was then introduced into the crucible and weighed as W_2 before drying. This was put in an oven set at 105ºC for an interval of two hours and cooled in a dessicator and weighed as W_3, this was done until constant weight was obtained. Then the water/moisture content which is represented by losses in weight was calculated as follows:

Weight of clean crucible = W_1

Weight of clean crucible + sample = W_2

Weight of clean crucible + dried sample = W_3

Total loss in weight = $W_2 - W_3$

Weight of sample = $W_2 - W_1$

% moisture content = $\frac{W^2 - W^3}{W^2 - W^1} \times 100$

3.3.2 Determination of Ash content

Procedure: A clean crucible was first weighted W_1. About 2g of the sample was added into the clean crucible and weight as W_2. The crucible and its content was then transferred into the murfle furnaces set at 600ºC for about 4 hours. The grey colour observed shows that it was fully ashed. The crucible and its contents was removed from the murfle furnaces and placed inside dessicator to cool. After cooling it was then weighted as W_3.

The ash content of the sample was calculated as follows:

Weight of clean crucible = W_1

Weight of clean crucible + sample = W_2

Weight of clean crucible + sample after ashing = W_3

Weight of sample = $(W_2 – W_1)$g

Weight of ash = $(W_3 – W_1)$g

Weight of organic = $(W_2 – W_3)$g

% ash = $\frac{W_3 - W_1}{W_2 - W_1} \times 100$

3.3.3 Determination of fat and oil contents

Procedure: Lipid contents were determined using soxhlet extractor and was done continuously using suitable solvent such as petroleum spirit (40^0-60°C). Exactly 2g of each sample was weighed into a clean filter paper and was then tied up properly using a thread. This was then weighed again. The tied up sample in the filter paper was then transferred into the extraction column of the soxhlet extractors for extraction. Petroleum ether was introduced into the round bottom flask below the extraction column until it was half filled. Then more was added to it to make it up to about 300cm^3. The soxhlet extractor was then connected to a water tape source which run round to cool the extractor. It was then immersed into a heating mantle to heat the petroleum ether in the round bottom flask below the extractor. The petroleum ether boils between 40°C to 60°C of which it evaporated into the extracting column where the sample was. It was allowed to siphon. This was allowed to continue for about six (6) hours. This is to ensure complete extraction of the fat from the sample. After 6 hours of extraction, the set up was disconnected and the defatted sample

(sample in the extracting column) was then remove and transferred into the oven and dried to constant weight.

Weight difference was taken and percentage lipids were calculated as follows:

% lipids were calculated as follows:

$$\% \text{ lipids} = \frac{weight\ loss}{weight\ of\ sample} \times 100$$

3.3.4 Determination of Crude Protein using the Micro Kjedahl Method Principle

This method does not include nitrogen from nitrites and nitrates, but includes nitrogen form protein, alkaloids, nucleic acids. The organic matter is oxidized by concentrated sulphuric acid (H_2SO_4) in the presence of catalyst and the nitrogen converted to ammonium sulphate. This was then made alkaline and the liberated ammonia is distilled and estimated. The crude protein is estimated by multiplying the percentage of nitrogen by a appropriate factor.

This determination is based on the fact that most nitrogen-containing food materials are protein, and protein on the average is approximately 16% nitrogen. Actually individual protein range from about 15% to 18% nitrogen.

Procedure: Exactly 2g of the sample was carefully weighed into a kjeldahl flask. Thereafter 0.5g of $CuSO_4$, $7H_2O$ and 10g of $NaSO_4$ were all added into digesting flask containing 2g of the sample. Following this, 25cm^3 of concentrated sulphuric acid was added to the mixture in digesting flask. The flask and it content were clamped in an incline position and then heated gently for about 15 minutes and then vigorously for further 45 minutes to digest in a

fume cupboard after the mixture was clear or oxidation is complete and a green colour is obtain (colour changed from black to a clear brilliant green colour) and this indicated complete digestion.

Digestion flask was removed from the burner and allowed to cool. The resulting product was then transferred into 250 mls volumetric flask and diluted with distilled water and made up to the graduation mark. This method depends on the fact that when organic compound containing nitrogen are heated at 80^{o}C with concentrated sulphuric acid in the presence of a catalyst. The organic nitrogen is converted to ammonium sulphate $(NH_4)_2\ SO_4$ (See step 1 in equation of reaction below):

Digestion: The distillating apparatus was then connected and 5 mls of the digested sample was pipetted into micro-Kjeldahl distillation unit (Malchan distillating apparatus) through the filling part of the apparatus in the inner chamber. Five milimetres of 40% NaOH was pipetted and added into it to make the solution strongly alkaline.

(See step 2 in the equation of the reaction below):

Distillation: To force out the ammonia form the ammonium salt, NH_3 was given off and collected into *5* mls of 2% boric acid indicator which was pipette into a clean conical flask.

The ammonia was distilled into 5mls of 2% boric acid indicator in 100cm^3 conical flask. About 45cm^3 of the distillate was collected. The blue indicator mixture turned green after the absorption.

(See step 3 in the equation of the reaction below):

Absorption: The borate was determined by titration.

The borate was determined by titration with or against 0.01m HCI (freshly prepared) to regenerate the blue botic acid indicator and produced ammonium chloride. End point of the titration is the pathway between green and purple.

The titration was continued until the green colour was no longer visible.

(See step 4 in the equation of the reaction below):

EQUATION OF REACTION

STEP 1: DIGESTION

$$R\text{-}NH_2 + H_2SO_4 \xrightarrow[\text{Selenium}]{\text{Catalyst}} (NH_4)_2SO_4 - CO_2 + H_2O$$

Clear brilliant Green colour

STEP 2: DISTILLATION

$$(NH_4)_2SO_4 + 2N_aOH \longrightarrow Na_2SO_4 + 2NH_3 + 2H_2O$$

STEP 3: ABSORPTION

$$3NH_3 + H_3BO_3 \longrightarrow (NH_4)_3BO_3$$

STEP 4: TITRATION

$$(NH_4)_3BO_3 + 3HCl \longrightarrow 3NH_4Cl + NH_3BO_3$$

CALCULATIONS FROM THE EQUATION

3 moles of HCl = 3 moles of NH_4Cl

1 mole of HCl = 1 mole of N

1 mole of HCl = 14g of N

0.01 of HCl was used:

i.e. 1000cm^3 of HCl contains 0.01M of N

1cm^3 of HCl contain $\frac{0.01}{1000}$ - 1 x 10^{-5}N

Therefore 1 x 10^{-5} mole of HCl = 1.4 x 10^{-4} moles of N

5cm^3 of the digest used will contain (1.4 x 10^{-4} x 5)g of N

250cm^3 of the digest will contain (1.4 x 10^{-4} x $\frac{250}{5}$)g of N

If the titre value is Ycm^3

Then Ycm^3 of HCl will be (1.4 x 10^{-4} x Y)g of N

Then 250cm^3 of digest will contain (1.4 x 10^{-4} x $\frac{250}{5}$ x Y)g of N

2g of the sample was used.

Therefore 2g of the sample will contain (1.4 x 10^{-4} x $\frac{250}{5}$ x Y x $\frac{1}{2}$)g of N

100g of sample will therefore contain (1.4 x 10^{-4} x $\frac{250}{5}$ x Y x $\frac{1}{2}$ x 100)g N

This gives the percentage of nitrogen in the sample.

It was then multiplied by 6.25 to obtain the percentages of crude protein (since protein is 16% nitrogen: 6.25 times the amount of nitrogen in the sample would equal the amount of 100% of protein in the sample).

Therefore % protein = 1.4 x 10^{-4} x $\frac{250}{5}$ x 4 x $\frac{1}{2}$ x 100 x 6.25

PREPARATION OF REAGENTS

0.01M HCl, 0.83cm^3 of concentrated HC1 was measured into 1000cm^3 standard flask and distilled water was added to it and made up to the graduation mask: 40% NaOH, 40g of NaOH pellets was weighed into a beaker and dissolved with distilled water in to 100cm^3 standard flask, the beaker was then rinsed continually with distilled water until everything goes to the standard flask and was then make up to the mark with distilled water.

BORIC ACID INDICATOR

0.lg of methelene red was weighed and transferred into a 100cm^3 standard flask. Absolute ethanol was then added to it to dissolve and then more was added to make it up to the graduation mark. This was then kept aside for further procedure. 0.1g of rnethelene blue was also weighed into an empty 100cm^3 standard flask. Following this, distilled water was added to it a little and then shook to enable it to dissolve. More distilled water was then added to make up to graduation mark. Five grams of boric acid (solids) was weighed and transferred into an empty 1000cm^3 standard flask. About 250cm^3 distilled water was added and shook vigorously until the solids were completely dissolved. Thereafter 16cm^3 of methelene blue from the already standardized solution were added and shook to ensure proper mixing up. More distilled water was then added to make it up to the graduation mark.

3.3.5 Determination of fibre content

The crude fibre determination was done by the method of A.O.A.C. (2000). The crude fibre is the bulk of roughage in foods.

Procedure

Crude fibre determination is done in accordance with the A.O.A.C. (2000) method. A known weight of the sample (2g) was taken and defatted with petroleum ether for 2 hours: It was boiled under reflux for exactly 30 minutes with 200 cm^3 of 1.25% H_2SO_4. It was filtered and washed with boiling water until the washings no longer acidic. The residue was boiled in a round bottom flask with 200 cm^3 of 1.25% NaOH for anther 30 minutes.

This was filtered into a previously weighed crucible and the crucible with sample (residue) was dried in the oven at 100^0C. It was left in a desicator to cool and then weighed (X_2). This was then incinerated in a muffle furnace at about 600^0C for 3 hours, left to cool in a desicator and weighed as X_3.

% fibre = $\frac{weight\ of\ fibre}{weight\ of\ original\ sample} \times 100$

= $\frac{x_2 - x_3}{weight\ of\ original\ sample} \times 100$

x_2 = weight of filtered sample before incineration in a muffle furnace

x_3 = weight of filtered sample after incineration into a muffle furnace

3.3.6 Determination of Carbohydrate content

The total carbohydrate content of the sample was derived by difference.

% Total carbohydrate = 100 – (% water = % protein + % lipids + % ash + % crude fibre)

3.4 Physicochemical analysis of *zobo* drink

3.4.1 pH determination

The pH of each group of containers with different treatment was determined for the period of storage starting from the day of production (day zero) using a pH meter (HI 96107). The pH of each of the treated samples and the control was measured twice and recorded.

3.4.2 Titrable acidity

Ten (10)ml of *zobo* drink was measured into a 250 ml conical flask, and four drops of phenolphthalein indicator was added. This was titrated with 0.1N NaoH to golden brown point. The titer for the total acidity was expressed as percentage of citric acid (Umme *et al.*, 2001).

3.5 Treatment of *zobo* drink with chemical preservative

Six containers with 100ml of *zobo* drink served as the control. Then 2ml (0.02%) of sodium benzoate was aseptically added to six containers containing 98ml of *zobo* drink. Another group of six containers containing 98ml of *zobo* drink were pasteurized and 2ml (0.02%) of sodium benzoate was aseptically added and stored at room temperature for the period of the experiment.

3.6 Pasteurization

Six containers of *zobo* drink were transferred into a hot water bath and pasteurized at 60^{o}C for 1 hour (Maji *et al.*, 2011). After pasteurization the containers was then removed, cooled and stored at room temperature for the period of the experiment.

3.7 Treatment of *zobo* drink with lime

Lime fruits used were first surface-sterilised (70% ethanol) and peeled using a pre-sterilised knife. The fruits were then halved (using a pre-sterilized knife) and was squeezed aseptically (sterile gloves worn during preparation) into a sterile 100ml conical flask. Two millemetres of lime juice was added to six containers containing 98ml of *zobo* drink. Another set of five containers containing 98ml *zobo* drink were pasteurized at 60^{o}C for 1

hour and 2ml of lime juice was aseptically added and stored at room temperature for the period of the experiment.

3.8 Microbiological analysis of samples

One millimeters of each sample was transferred into sterile 9ml of distilled water and diluted serially. Then aliquot portion (0.1ml) of the 2nd dilutions was inoculated onto freshly prepared potato dextrose agar and nutrient agar respectively using the pour plate method. The nutrient agar plates were incubated at 37^{o}C for 24 hours and the PDA plates were incubated at room temperature 28 ± 2^{o}C for 48 hours (Cheesbrough, 2002). This procedure was repeated throughout the period of the experiment.

3.8.1 Enumeration and Characterizaiton of Fungi

Total fungi count was done, in which the total numbers of viable microorganisms (colonies) developed were enumerated and expressed as colony forming unit per millimeters (cfu/ml). Fungi isolates were identified by staining with lactophenol cotton blue and viewed under the microscope.

3.8.2 Enumeration and biochemical characterization of bacterial isolates

Total bacterial count was done in which the total numbers of viable microorganisms (colonies) developed were enumerated and expressed as colony forming units (cfu/ml).

3.8.2.1 Gram staining

A very small innoculum of bacteria was smeared on a clean grease free slide using a sterile innoculting loop. A drop of sterile water was added to make a smear and then air dried. The slide was heat fixed using naked flame. The slide was stained with crystral

violet for 1 min. The slide was stained with lugols iodine for 1 min to fix the dye. The slide was then flooded with 95% ethanol for 30 secs and rinsed with water. The slide was counter stained with safranin for 1 min, rinsed with water and then air-dried. The slide was then viewed using microscope under oil immersion (Cheesbrough, 2006).

3.8.2.2 Catalase test

A thick emulsion of each isolates was prepared on clean slides. Drops of 3% hydrogen peroxide were added to each of the slides. A positive result showed effervescence which is caused by the liberation of oxygen gas as a result of catalase enzyme production by the bacterium (Cheesbrough, 2006).

3.8.2.3 Coagulase text

The coagulase test identifies whether an organism produces the exoenzyme coagulase which cause the fibrin of blood plasma to clot. Emulsion of the isolates was prepared on a clean glass slides, then plasma was added to the side to watch for agglutination if positive (Cheesbrough, 2006).

3.8.2.4 Oxidase test

This was done to determine the production of enzyme oxidase by bacteria. A filter paper was soaked in 1% sodium oxalate solution. A portion of each bacterial isolates was picked and rubbed on the filter paper. A blue colour change within 10 seconds indicates the production of the enzyme oxidase (Cheesbrough, 2006).

3.8.2.5 Citrate test

Following the manufacturer's instruction, citrate agar was prepared. The agar was then inoculated with bacterial isolates using streaking method. The plates were incubated at 37ºC for 24 hours. A colour change from green to blue on the plates indicates citrate utilization by the test organisms (Cheesbrough, 2006).

3.8.2.6 Indole test

One percent tryptone broth was prepared in different test tubes. It was inoculated with each bacterial isolates. The tubes were incubated at 37 ºC for 24 hours. After incubation, 2ml of kovac's reagent was added to the broth culture and gently shaken. The tubes were allowed to stand for 20 mins in order to allow the reagent rise to the top. A red colour at the reagent top layer indicates indole production (Cheesbrough, 2006).

3.8.2.7 Sugar test

Glucose, sucrose and fructose broths were prepared according to manufacturers instruction. Each broth was then dispensed into different strerile bijou bottle. The bottles were then inoculated each with the test bacteria. They were then incubated at 37ºC for 24 hours. A change from reddish orange colour to yellow after incubation indicates positive utilization of the sugar by the test bacteria (Cheesbrough, 2006).

3.8.3 Molecular Characterization

All the five isolates were subjected to molecular characterization as follows:

3.8.3.1 DNA Extraction Protocol and PCR Condition

(Cetyltrimethyl ammonium bromide) (CTAB) DNA Extraction Buffer pH 8.0

Chemical Reagents

- ✓ 2 % CTAB (Cetyltrimethyl ammonium bromide) powder (w/v)
- ✓ 100 mM Tris-HCl
- ✓ 20 mM EDTA
- ✓ 1.4 M NaCl
- ✓ 0.2 % β-mercaptoethanol (v/v and did adjust before use)

Steps involved

Overnight culture of entomotoxic isolates was prepared in nutrient broth. 1000 µL of the broth medium was pipetted and centrifuged at 14,500 rpm for 15 min followed by decanting of the supernatant. This was repeated four times and 500 µL of extraction buffer was added to it in a sterile 1.5 ml microcentrifuge and was thoroughly mixed and vortexed. It was incubated in water bath at 60 ^{0}C for 15 mins, brought to room temperature and added equal volume of phenol, chloroform and iso-amyl alcohol in the ratio of 25:24:1. It was mixed and centrifuged at 13,000 rpm for 10 mins before it was removed and transferred 450 µl of the supernatant into new and sterile 1.5 ml tube.

Four hundred micro litre of ice-cold isopropanol was added for precipitation of the DNA. It was mixed and incubated for 2 hrs-overnight at -20 ^{0}C. It was removed and centrifuged at 14,000 rpm for 15 mins to sediment the DNA. The supernatant was gently decanted and ensured the pellets were not disturbed before 500 µl of 70 % ethanol was added to the pellets and centrifuged at 13,000 rpm for 5 mins to wash the pellet. The ethanol was decanted and the DNA was air-dried at room temperature. The Pellets were suspended in 100 µl of nuclease free water for further use (Abarshi *et al.,* 2010).

3.8.3.2 PCR Amplifications of the Extracted DNA Samples

PCR amplification assay consisted of 2.0 µL 100 ng/u LDNA, 2.5µL of 10 x buffer (Bioline, Taunton, USA), 1.5 µL of 50 mM $MgCl_2$ (Bioline, Taunton, USA), 2.0 µL of 2.5 mM dNTPs (Bioline, Taunton, USA), and 0.2 µL 500 U DNA Taq polymerase (Bioline, Taunton, USA), 1.0 µL of 10 pm each primer per 16S ribosomal gene and was made up to a total volume of 25 µL by adding 15.8 µL of 500 ml DEPC-treated water (Invitrogen Corporation, USA). The PCR cycling profile for the reaction consisted of an initial step at 94 ^{0}C for 5 min, 35 cycles at 94 ^{0}C for 30 secs, then changed to 55 ^{0}C for 1min, then 72 ^{0}C for 1 min, and 8 min final extension at 72 ^{0}C. Five micro litres (5 µL) of the amplicon was electrophoresed in 1.5 % agarose gel containing 0.5 mg/ml ethidium bromide and photographed on UV Transilluminator light (Abarshi *et al.,* 2010).

3.8.3.3 Electrophoresis

Procedure

The solution was heated in a boiling water bath until agarose was completely dissolved. It was then allowed to cool in a water bath set at 50 – 55^{0}C. Casting tray was

prepared by sealing ends of gel chamber with tape or appropriate casting system. Combs were placed in gel tray. 5ul of ethidium bromide was added to cooled gel and pour into gel tray. It was allowed to cool for 15-30 min at room temperature. The comb(s) was removed placed in electrophoresis chamber and cover with buffer (TAE previously). The DNA and standard (Ladder) was loaded onto gel. Electrophorese at a given Voltage for at least 1hour. Visualize DNA bands was visualized using UV lightbox or gel imaging system.

3.8.3.4 Method of Sequencing

The sequencing results was generated from the ABI Prism 3130X1 Genetic automated sequencer (Applied Biosystems) and were carefully edited, filtered and assembled for polymorphism detection using BioEdit software (Abarshi *et al.,* 2010).

3.8.3.5 Sequence alignments

Sequence similarity was estimated by searching the homology in the Gene bank DNA database using BLAST. The sequence information was then imported into the MEGAS software program for assembly and alignment. The 16SrDNA sequences of isolated bacterial strains were compared to sequences from typed strains held in Gene Bank.

3.9 Data analysis

The data was subjected to analysis of variance (ANOVA) and Duncan test was carried out using pre-packaged computer statistical software (SPSS 20.0).

CHAPTER FOUR

RESULTS

4.1 Proximate Composition

The proximate composition of *zobo* drink shows that the sample contains high moisture content (92.52 %), Ash content (0.56 %), fat and oil (1.05 %), crude protein (2.16 %), fibre (0.17 %) and carbohydrate content (3.54 %) (Table 2).

4.2 Physicochemical Parameters

The pH values of the *zobo* samples with different preservative techniques over a period of 120 hours ranged from 2.8 to 3.4 with control (untreated) having pH of 3.4. All samples were acidic irrespective of the type of preservative added as shown in Figure 2. Generally there was decrease in the pH values over the period of examination as all samples remained acidic.

The titratable acidity (TTA) ranged from 0.25856 to 0.2816. Values increased with time irrespective of the preservative added as shown in Figure 3.

Table 2: Proximate composition of *zobo* drink

Nutritional quality	% composition
Moisture content	92.52%
Ash content	0.56%
Fat and oil	1.05%
Crude protein	2.16%
Fibre	0.17%
Carbohydrate content	3.54%

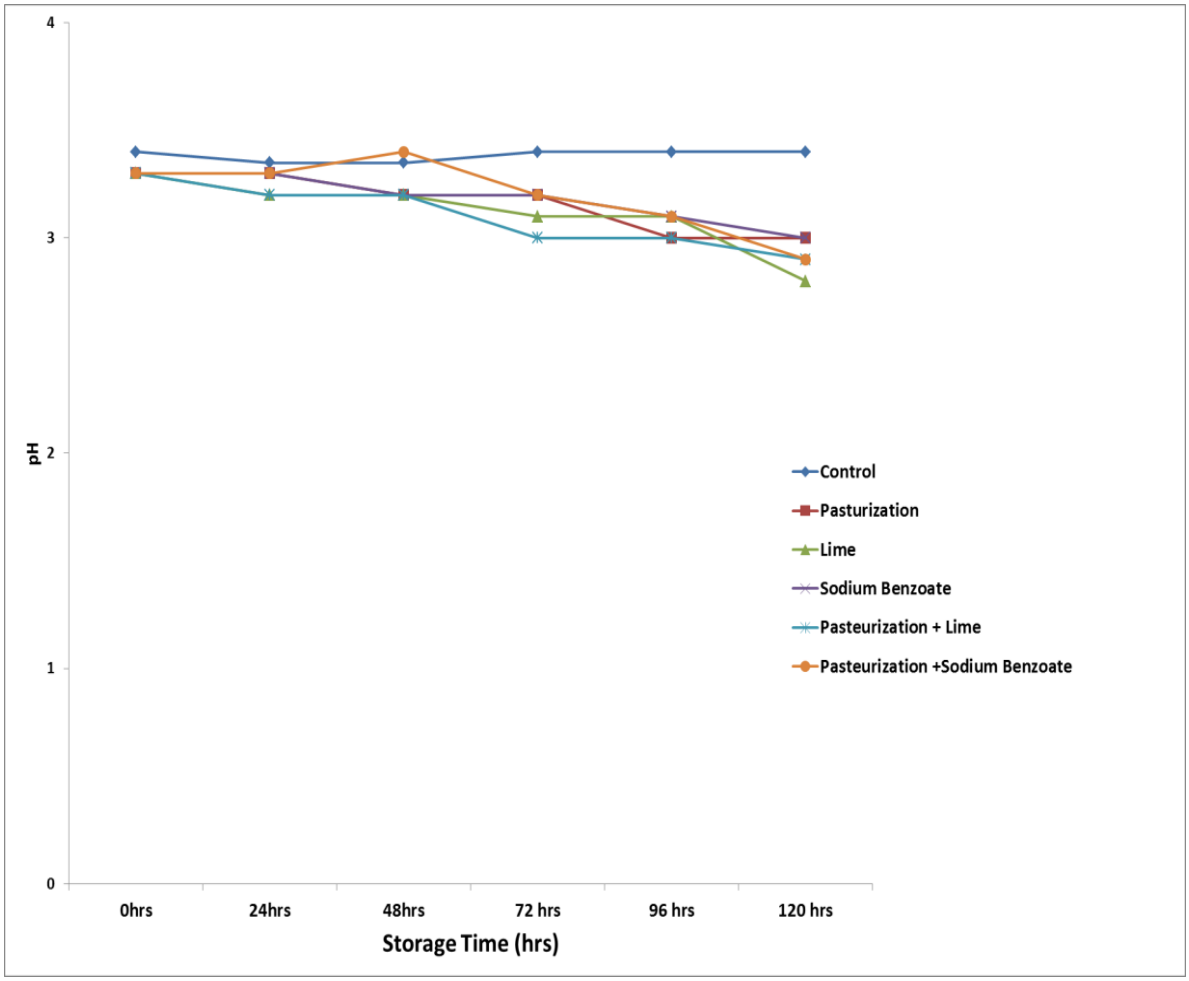

Figure 2: Changes in pH of *zobo* drink during period of storage

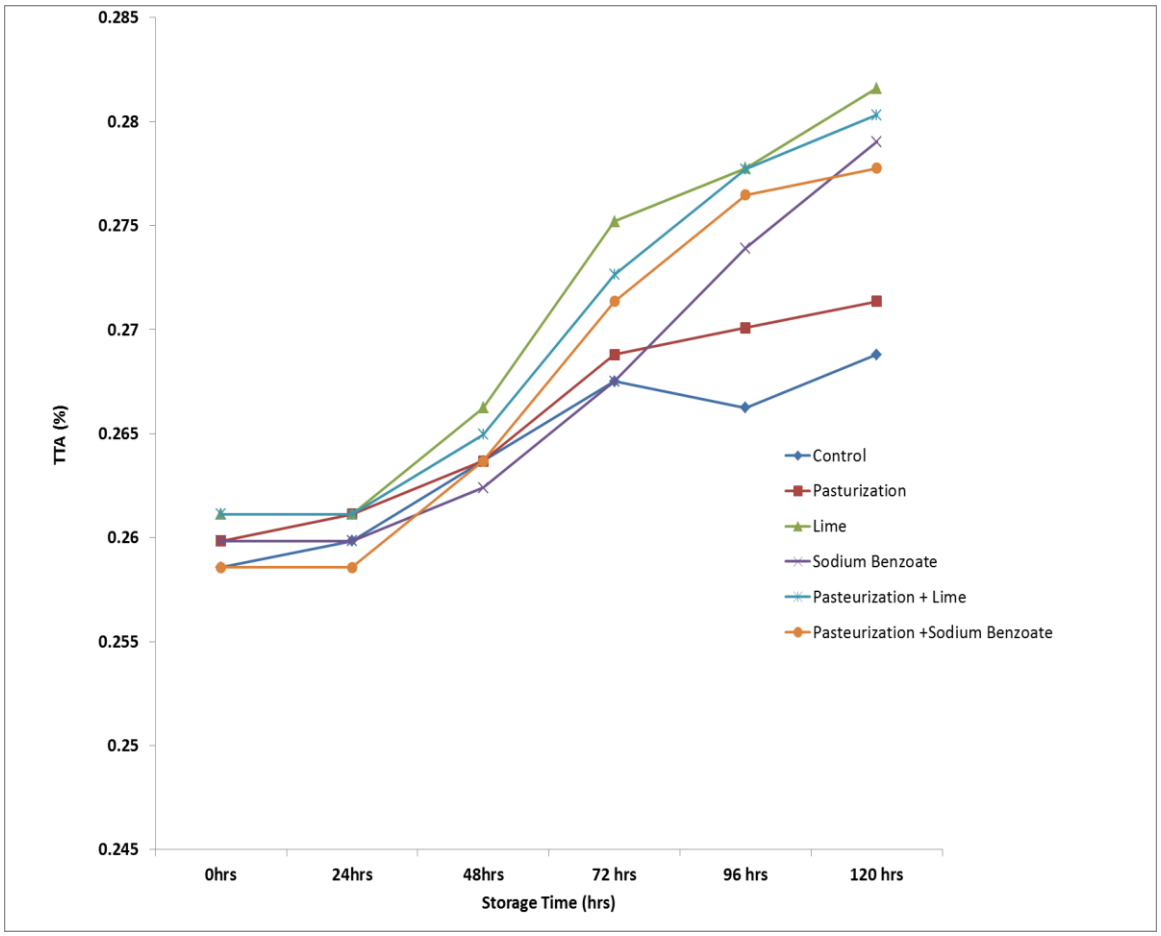

Figure 3: Changes in TTA of *zobo* drink during period of storage

4.3 Microbiological Analysis

The effect of different preservative techniques on bacteria load of the *zobo* drink for a period of 120 hours is shown in Table 3. Bacterial count for control sample increased from 1.6 x 10^3 cfuml^{-1} at 0 hour to 3.15 x 10^3 cfuml^{-1} at 120 hours. For pasteurized sample, visible growth was observed at 24 hours till 120 hours. Bacterial count for *zobo* sample treated with sodium benzoate decreased from 1.85 x 10^3 cfuml^{-1} at 48 hours to 9.5 x 10^2 cfuml^{-1} at 120 hours. Bacterial count for *zobo* sample treated with lime decreased from 1.25 x 10^3 cfuml^{-1} at 0 hour to 3.5 x 10^2 cfuml^{-1} at 120 hours. For pasteurized sample treated with lime, bacterial count ranged from 0 to 7.5 x 10^2 cfuml^{-1}. Bacterial count for pasteurized sample treated with sodium benzoate decreased from 1.1 x 10^3 cfuml^{-1} at 24 hours to 3.5 x 10^2 cfuml^{-1} at 120 hours.

Fungal count for control sample increased from 6.5 x 10^2 cfuml^{-1} at 0 hour to 2.35 x 10^3. Fungal count for pasteurized sample increased from 3.5 x 10^2 cfuml^{-1} at 0 hour to 1.3 x 10^3 cfuml^{-1} at 120 hours. Fungal count for sample treated with lime increased from 6.0 x 10^2 cfuml^{-1} at 0 hour to 2.3 x 10^3 cfuml^{-1} at 120 hours. For pasteurized *zobo* sample treated with lime, fungal count increased from 4.0 x 10^2 cfuml^{-1} at 0 hour to 2.35 x 10^3 cfuml^{-1} at 120 hours. There was no visible growth observed till 48 hours for samples treated with sodium benzoate and pasteurized sample treated with sodium benzoate.

Two different fungal isolates were observed during storage period (Table 5). They were identified to be *Aspergillus niger* and *Penicillium citrinum* (Plate 2 and 3). A total of four (4) bacterial was observed based on their colonial morphology during storage period. The four (4) bacteria observed were coded as ZB1, ZB2, ZB3 and ZB4. Table 6 shows the

distribution of fungi isolates. Table 7 shows the biochemical characteristics of the four bacterial isolates. Table 8 shows the distribution of bacterial isolates.

4.4 Molecular characterization

Figure 4 shows the results of the Agarose gel Electrophoresis of the amplified DNA of the four bacterial isolates.

Table 9 shows the identification of the bacterial isolates after comparing with sequence in the gene bank. ZB1, ZB2, ZB3 and ZB4 were identified to be *Bacillus cereus, Lactobacillus brevis, Staphylococcus aureus and Micrococcus luteus* respectively.

Table 3: Bacterial Count of *zobo* sample during storage period (cfu/ml)

SAMPLES	HOURS					
	0	**24**	**48**	**72**	**96**	**120**
Control	$1.6x10^{3c}$	$1.9x10^{3c}$	$2.2x10^{3c}$	$2.5x10^{3d}$	$2.9x10^{3d}$	$3.15x10^{3d}$
Pasteurization	NG	$8.0x10^{2a}$	$1.15x10^{3b}$	$1.65x10^{3c}$	$2.1x10^{3c}$	$2.6x10^{3c}$
Lime	$1.25x10^{3b}$	$9.0x10^{2a}$	$8.0x10^{2a}$	$7.0x10^{2b}$	$5.0x10^{2a}$	$3.5x10^{2a}$
Sodium benzoate	$1.5x10^{3c}$	$1.65x10^{3c}$	$1.85x10^{3c}$	$1.45x10^{3c}$	$1.1x10^{3b}$	$9.5x10^{2b}$
Pasteurization + Lime	NG	$7.5x10^{2a}$	$6.0x10^{2a}$	$4.0x10^{2a}$	$2.5x10^{2a}$	$2.0x10^{2a}$
Pasteurization + Sodium benzoate	$3.0\ x10^{2a}$	$1.1x10^{3b}$	$6.0x10^{2a}$	$5.0x10^{2a}$	$4.0x10^{2a}$	$3.5x10^{2a}$

Values are means of duplicate determinations. Values within a column with the same superscripts are not significantly different ($p<0.05$).

KEY: NG – No growth

Table 4: Fungal Count of *zobo* sample during storage period (cfu/ml)

SAMPLES	HOURS					
	0	**24**	**48**	**72**	**96**	**120**
Control	$6.5x10^{2c}$	$9.5x10^{2c}$	$1.4x10^{3b}$	$1.75x10^{3c}$	$2.1x10^{3b}$	$2.35x10^{3b}$
Pasteurization	$3.5x10^{2b}$	$5.5x10^{2b}$	$7.5x10^{2b}$	$9.0x10^{2b}$	$1.04x10^{3b}$	$1.3x10^{3a}$
Lime	$6.0x10^{2c}$	$1.05x10^{3c}$	$1.35x10^{3b}$	$1.7x10^{3c}$	$2.1x10^{3b}$	2.3×10^{3b}
Sodium benzoate	NG	NG	NG	$4.5x10^{2a}$	$4.0x10^{2a}$	$4.0x10^{2a}$
Pasteurization + Lime	$4.0x10^{2b}$	$8.5x10^{2c}$	$1.2x10^{3c}$	$1.55x10^{3c}$	$1.9x10^{3c}$	$2.35x10^{3b}$
Pasteurization + Sodium benzoate	NG	NG	NG	$5.5x10^{2a}$	$5.5x10^{2a}$	$6.5x10^{2a}$

Values are means of duplicate determinations. Values within a column with the same superscripts are not significantly different ($p<0.05$).

KEY: NG – No growth

Table 5: Characteristics of Fungal isolated from sample

Colonial characteristics	Microscopic characteristics	Suspected isolates
Dark Green granular powdry colonies	Septate hyphac; smooth walled conidiophores conidia is produced in well-defined chains	*Penicillium citrinum*
Black powdry colonies	Septate hyphae, colourless conidiophores, very dark brown spores	*Aspergillus niger*

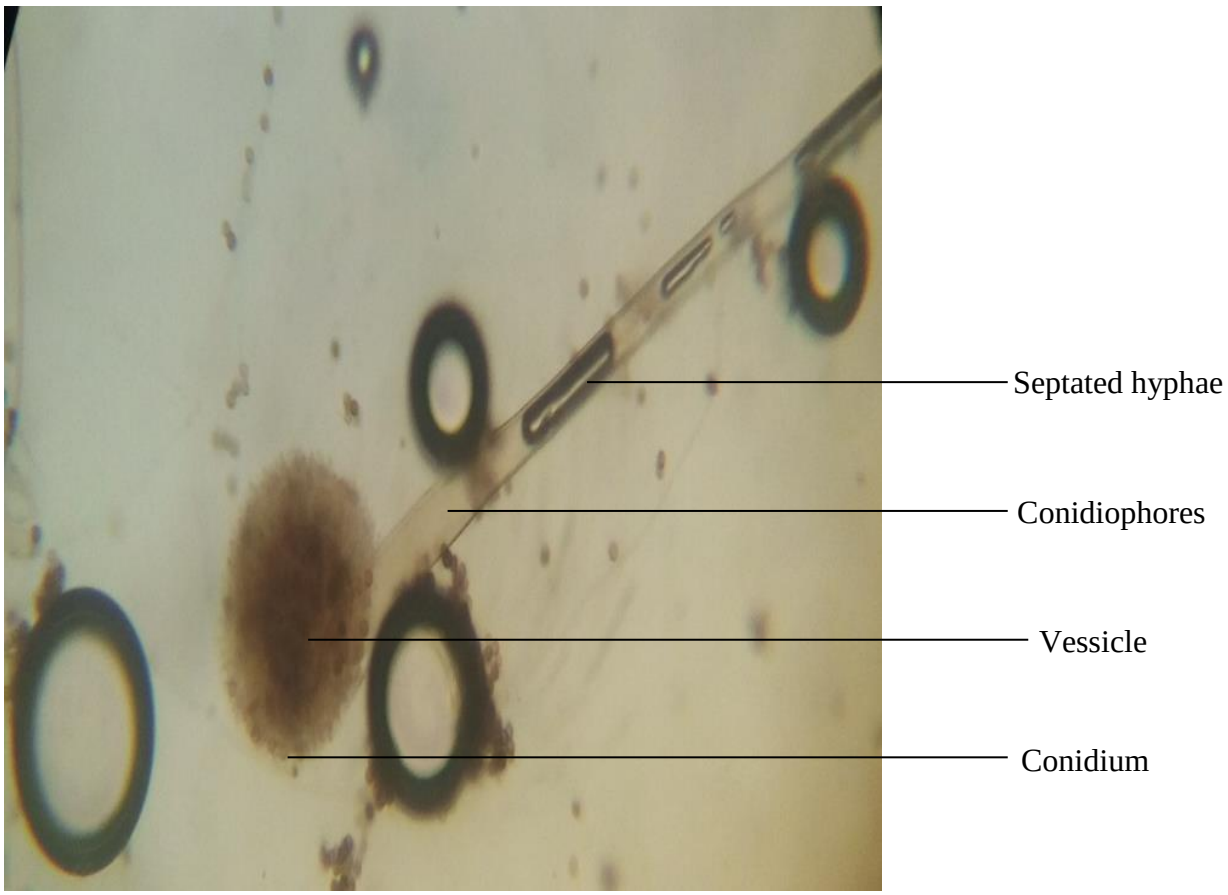

Plate 3: Photomicrograph of *Aspergillus niger* x 400

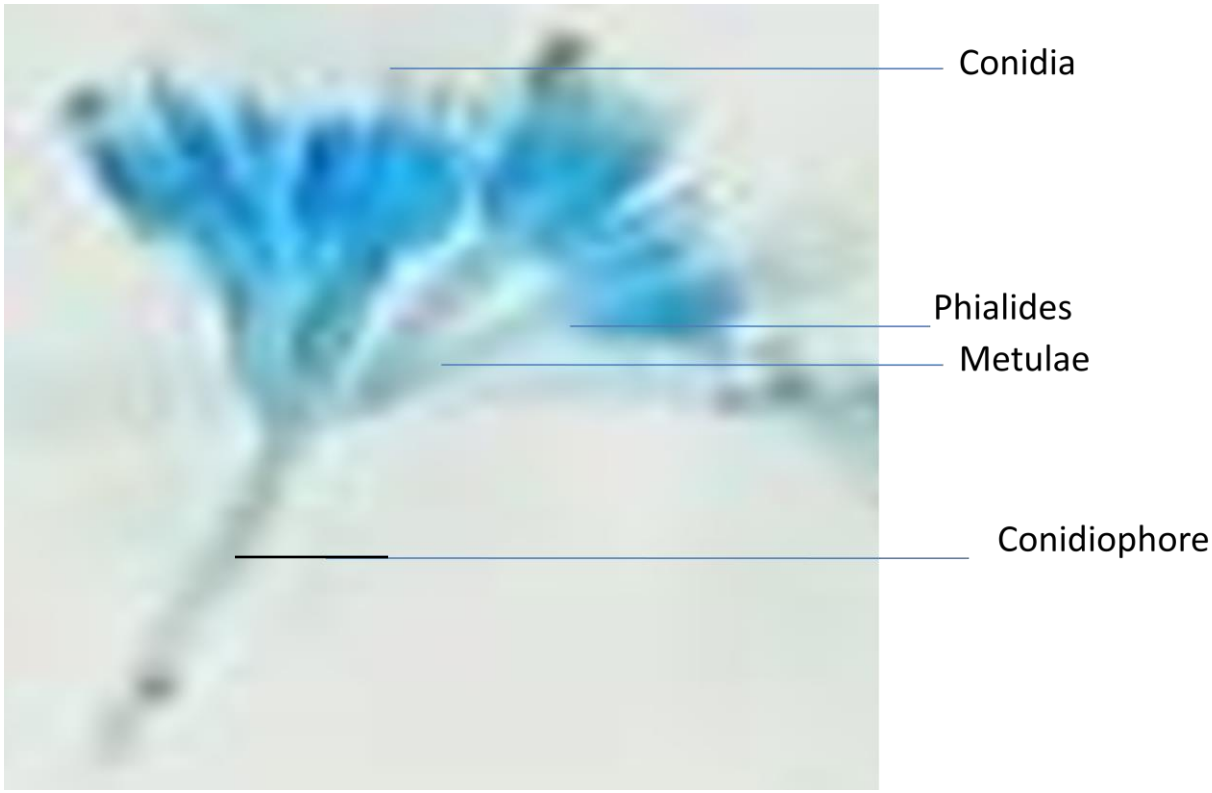

Plate 4: Photomicrograph of *Penicillium citrinum* x 400

Table 6: Distribution of Fungal Isolates

ISOLATES	Control	Pasteurization	Lime	Sodium benzoate	Pasteurization + Lime	Pasteurization + Sodiun benzoate
Asperrgillus niger	+++	+++	+++	++	++	++
Penicillium citrinum	++	++	++	+	+	++

KEY: +++ $\geq$ 4 times

++ $\geq$ 2times,< 4times

\+ 1time

Table 7: Biochemical Characterization of Isolates

Isolates Code	Colonial characteristics	Gram reaction	Catalase	Coagulase	Oxidase	Citrte	Indole	Glucose	Sucrose	Frustose	Suspected organism
ZB1	Large cream colonies	+ rod	+	-	-	+	-	+	-	+	*Bacillus* sp
ZB2	Small circular creamy white colonies	+ rod	-	-	-	-	-	+	+	+	*Lactobacillus* sp
ZB3	Small circular golden yellow colonies	+ cocci	+	+	-	+	-	+	+	+	*Staphylococcus aureus*
ZB4	Small circular bright yellow colonies	+ cocci	+	-	+	-	-	-	-	-	*Micrococcus* sp

Key: gram positive; +v, positive +, negative -

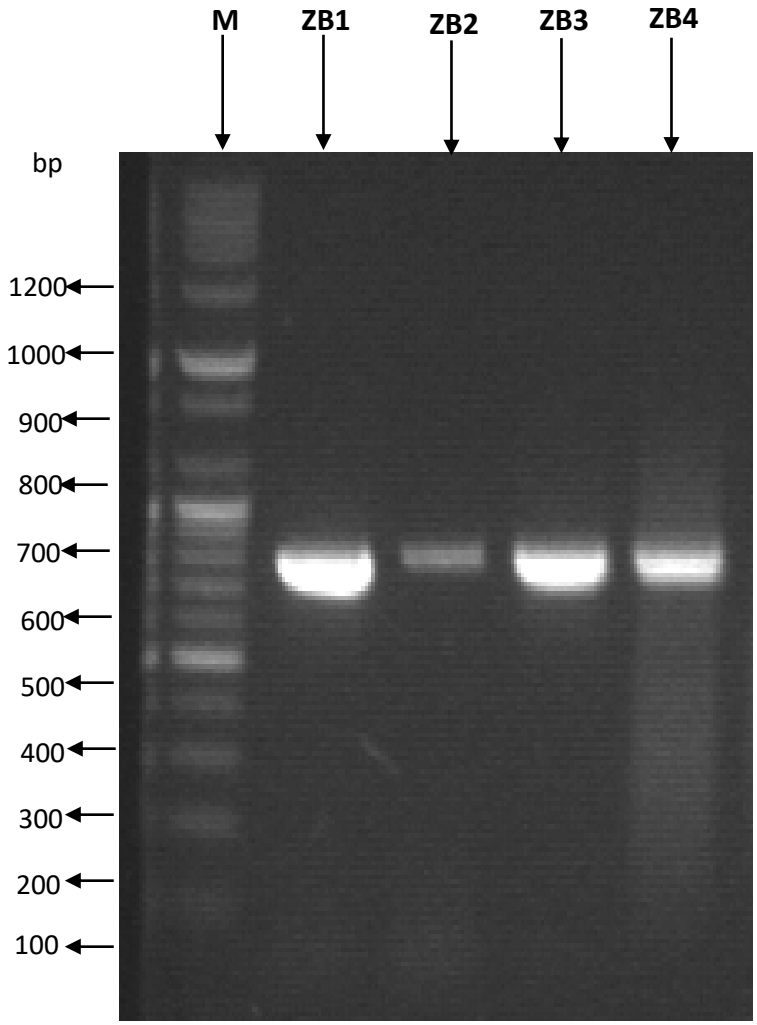

Figure 4: Agarose gel electrophoresis of bacterial DNA.

M: DNA ladder

Key: ZB1: *Bacillus cereus,* ZB2: *Lactobacillus brevis*, ZB3: *Staphylococcus aureus*, ZB4: *Micrococcus luteus*, bp: base pair

Table 8: Distribution of Bacterial Isolates

ISOLATES	Control	Pasteurization	Lime	Sodium benzoate	Pasteurization + Lime	Pasteurization + Sodiun benzoate
ZB1	++	++	++	++	+	+
ZB2	+++	++	+++	++	++	++
ZB3	+	-	+	+	-	-
ZB4	+	-	+	+	-	+

Key: +++ ≥4times

++ ≥2times, < 4times

\+ 1time

_ No appearance

Table 9: Molecular Identification of bacterial Isolates

S/N	Isolate code	Reference from NCBI database	Accession numbers	Percentage % similarity
1	ZB1	*Bacillus cereus*	MH566234	100
2	ZB2	*Lactobacillus brevis*	MH566235	99
3	ZB3	*Staphylococcus aureus*	MH559826	100
4	ZB4	*Micrococcus luteus*	MH566236	100

CHAPTER FIVE

DISCUSSION

The microbial quality of pasteurized and unpasteurized *zobo* drink, *zobo* drink treated with sodium benzoate and *zobo* drink treated with lime was investigated. The effects of the varying treatments were investigated with a view to prolong the shelf-life of *zobo* drink. Biodeterioration of the drink is influenced by factors like temperature, pH, chemical composition and microbial load (Nwachukwu and Ezeigbo, 2013).

It was deduced from the result of the proximate composition that *zobo* drink contains high water content (92.52%) which is helpful in thirst-quenching. The low fat content (0.56%) of *zobo* drink makes it an ideal drink for obese people. The result of the proximate composition obtained is in accordance to result obtained by Egbere *et al*. (2007) who investigated the effects of some preservation techniques on the quality and storage stability of *zobo* drink. They reported that the low protein value of *zobo* drink obtained is of disadvantage to populations with protein malnutrition.

The pH values of *zobo* samples with different preservation techniques over a period of 120 hours ranged from 2.8 to 3.4 which implies that the sample was generally acidic irrespective of the preservation methods. This result is in accordance with Egbere *et al*. (2007) who reported *zobo* sample to be acidic over the period of storage irrespective of the additives added. Fasoyiro *et al.* (2005) also reported similar pH value when they worked on the chemical and storability of fruit-flavoured *H. sabdariffa* drink. According to the authors, the pH of the samples ranged from 2.19 to 3.32. The study also reported that the acidic nature of the samples was due to the acidic nature of the roselle calyx and that the roselle calyx is characterized as a highly acidic fruit rich in organic acids; oxalic, tartaric, malic and

succinic acids. The acidic nature of the drink might also be due to the presence of lactic acid bacteria in the drink. The advantage of the drink being acidic is that it helps to restrict the growth of other bacteria that cannot withstand acidic condition. The titratable acidity (TTA) of *zobo* drink samples obtained ranged from 0.25856% to 0.2816%. Values increased with time irrespective of the preservatives added. Similar increment was observed in the work carried out by Fasoyiro *et al.* (2005), Egbere *et al.* (2007), Gbadegesin and Odunlade, (2016). The increase in acidity accounts for the decrease in microbial population.

During the 120 hours period of storage of *zobo* drink samples, the microbial analysis (Bacteria and fungi) was carried out. Bacterial count for control sample ranged from $1.6x10^3$ $cfuml^{-1}$ at 120 hours. This implies that there was increase in bacterial load with time. This was due to the fact that no preservative was added or preservation treatment was not given thus suitable for continuous bacterial growth and this agreed with Adeoye *et al.* (2018). For pasteurized sample, bacterial count ranged from 0 at 0 hour to $2.6x10^3$ $cfuml^{-1}$ at 120 hours. This is in accordance with Ekeke *et al.* (2015) who worked on optimal conditions for the preservation of *zobo* drink where it was reported that unpasteurized sample had growth from the initial day and visible spoilage was observed after several days of storage. The study also reported that pasteurized sample showed no growth at day 1 (0 hour) but over time (further storage) showed growth. The presence of growth in the pasteurized sample after the initial day may be due to the inactiveness of the bacteria after pasteurization, and this agreed with Braide *et al.* (2012). For sample treated with lime, it was noticed that bacterial load was reduced with time. This result is in accordance with the findings of Onuoha and Fatokun (2014) that reported similar result with the use of *citrus aurantofokia* juice (lime) on the shelf-life of *zobo* drink produced locally in Afikpo, Ebonyi State, Nigeria that Lime reduced

bacteria load in *zobo* drink. They also explained that the reduction in bacteria load is due to the acidic nature of the line juice, also that lime juice unlike other preservatives which are used to store food substances create unfavourable microenvironment for bacteria. They further explained that lime juice may have created hurdles which the organisms were not able to survive and that the organisms may have survived to some extent, the effect of the preservatives but further attempt to overcome the hurdles may cause them to become metabolically exhausted, hence the continuous decrease in population observed (Nwafor 2012, Nwafor and Ikenebomeh, 2009). The *zobo* sample preserved with sodium benzoate, bacteria count started reducing after 48 hours from $1.85x10^3$ $cfuml^{-1}$ to $9.5x10^2$ $cfuml^{-1}$ at 120 hours. In support of this finding was the report from research work carried out by Braide *et al.* (2012) that sodium benzoate had no impact on microbial community of the sample but after days of application, a notable effect was observed most especially after 144 hours of application. Pasteurized sample containing lime had no growth at 0 hour and fewer bacteria count was recorded during further storage. This might be due to synergistic effect of pasteurization at the initial day and presence of preservative during further storage. Pasteurized sample with sodium benzoate had growth at 0 hour, this may have occurred during the addition of sodium benzoate after pasteurization.

There was increase in fungal count with storage time in samples treated with lime, the explanation for this might be that since the efficacy of lime as preservative is based on its acidic nature, fungi are organisms that thrive under acidic condition. That means lime was not effective against them. There was increase in fungal count in pasteurized sample, this may be that the pasteurization temperature was not effective against the spores of the molds isolated. It was observed that there was no growth up till 48 hours of storage for

samples treated with sodium benzoate. Nwafor and Ikenebomeh (2009) had earlier reported the efficacy of sodium benzoate as an antimicrobial agent against the growth and survival of *Aspergillus niger* and *Penicillium citrinum.* They further explained that sodium benzoate may have created hurdles, which the organisms could not overcome which may have led to physiological homeostatic and metabolic distortion, further attempts to overcome this adverse condition led to increased stress which inturn brought about metabolic exhaustion, death and subsequent decrease in population.

The two molds isolated were identified to be *Aspergillus niger* and *Penicillium citrinum* in which *Aspergillus niger* was predominant. *Aspergillus niger* is an asexual, filamentous, saprophytic fungus that can be isolated from many habitats such as plant debris, rotting fruits, indoor air environment, soil and stored grams (Braide *et al.*, 2012). *Penicillium citrinum* is also found in decaying vegetation and in the air.

Molecular characterization of bacterial species showed that ZB1 has 100% similarity with *Bacillus cereus*, ZB2 has 99% similarity with *Lactobacillus brevis*, ZB3 has 100% similarity with *Staphylococcus aureus*, ZB4 has 99% similarity with *Micrococcus luteus* and ZB5 has 100% similarity with *Bacillus subtilis* after searching the homology in Gene bank DNA Database using BLAST.

Results obtained showed that *zobo* drink, raw or preserved supports the growth and proliferation of a wide variety of microorganisms. *Lactobacillus brevis* followed by *Bacillus cereus* were the predominant isolates. This findings is in accordance with Egbere *et al.* (2007). *Lactobacillus brevis* belong to the group of bacteria called Lactic acid bacteria (LAB). *Bacillus cereus* is a spore former that can withstand adverse effects of additives.

Spores are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectant and dessication (Prescott *et al.*, 2002). *Staphylococcus aureus* and *Micrococcus luteus* have lesser occurrence in all samples. *Staphylococcus aureus* is a member of the normal flora of the body, frequently found in the nose, respiratory tract and on the skin. *Micrococcus luteus* is found in soil, dust, water and in human skin flora. It has also been isolated from foods such as milk. It is an atmospheric microorganism commonly found on environmental monitoring plates and it is one of the most common contaminants of lab cultures (Prescott *et al.*, 2002). Braide *et al.* (2012) also reported the presence of *Micrococcus luteus* and *Staphylococcus aureus*.

Conclusion

Result from the study showed that treatment of *zobo* drink with lime and sodium benzoate were effective in reducing the growth of microorganisms. A combination of pasteurization and lime, pasteurization and sodium benzoate are more effective in reducing microbial load of *zobo* drink.

Recommendation

- From the result of the proximate analysis, it is deduced that *zobo* drink can serve as ideal drink for people watching their weight since it contains low fat, it can also help in thirst quenching because of its very high moisture content.
- From the result obtained, lime was effective in reducing the bacteria load, so adding lime juice to *zobo* drink should be encouraged since lime fruits are not hazardous and hence safe for human consumption. Also lime fruits are readily available, and cheap for use by low processors of *zobo* drink.
- Local processors can place the finished product in a boiling water for some minutes (pasteurization) in order to inhibit or reduce microbial load encountered during processing and packaging.
- The microbial load obtained for all the samples is within the limit 10^3 for ready to drink soft drink (FDA, 2013). This might be due to the fact that the drink was produced under precautionary laboratory conditions. This implies that personal hygiene and Good Manufacturing Practice (GMP) should be employed during production, processing and packaging.

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APPENDICES

Penicillium citrinum on PDA Plate

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Aspergillus niger on PDA Plate