EFFECT OF SOME PRESERVATIVE TECHNIQUES (CARBONATION AND PASTEURIZATION) ON THE QUALITY AND STORAGE STABILITY OF ZOBO DRINK

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CERTIFICATION

This is to certify that this project work was carried out by Oshinleye Shola James, Matric number 12/48/0007, Food Technology Department, Moshood Abiola Polytechnic, Abeokuta.

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DEDICATION

This project work is dedicated to Almighty God and to my family with love.

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ABSTRACT

Zobo drink deteriorates in quality within 48 hours of storage at ambient temperature, this therefore makes the beverage very difficult to store for a long time. Therefore, this research work studies the effect of some preservative techniques (carbonation and pasteurization) on the quality (Physiochemical; pH, Total titratable acidity, Total soluble solids, Specific gravity) and storage stability (Microbiological analysis; viable count of bacteria, Isolation of microorganisms, Identification of Isolated microorganisms) of Zobo drink. Zobo drink was extracted from the calyces of H. sabdariffa using water at 100^{o}C. Extracted drinks were given the following

treatments (a) pasteurization at 80ºC for 20 minutes (b) carbonation and pasteurization at 80ºC for 20 minutes. Extracted drinks were packaged in plastic bottles and sealed at ambient temperature (27ºC-29ºC). The following parameters were determined on stored samples at weekly intervals pH, specific gravity, titratable acidity, total soluble sugars and microbial load. Sensory evaluation(Taste, Colour, Flavour, Appearance) of the fresh Zobo drink carried out. Freshly extracted Zobo drink had a total soluble solid content of 15.57^0 Brix, a titratable acidity of 0.45, a pH of 2.49 and a total microbial count 3.6 x 10^2 (cfu/ml). Titratable acidity values increased sharply for samples C and CoP (0.45 to 0.69 and 0.28 to 0.71 respectively at the end of the storage period) while increase was lower for sample P (0.38 to 0.68). Microbial load of untreated Zobo drink increased from $3.6x10^2$ (Aerobic count) to $4.82x10^5$, from $4.6x10^2$ (Yeast and Mould count) to $5.18x10^5$. There were no significant differences ($p>0.05$) in the appearance, colour and flavor of the drinks given different treatments but there was significant difference ($p<0.05$) in the taste of the drinks. Fresh Zobo drink and the pasteurized sample were more acceptable to panelists than the carbonated and pasteurized sample. Pasteurization and carbonation extended the shelf life of the Zobo drink to 3-4 weeks after the day of production.

CHAPTER ONE

1.0 INTRODUCTION

In recent times, there has been an upsurge in the consumption of ready-to-eat food. This is probably due to their convenience. Ready-to-eat foods occur in varieties of food including fruits, fruit juices, nutritional drinks, snacks etc. Generally, they are food items consumed on purchase from vendor, hawkers and consumed immediately without any further preparation. Most ready-to-eat foods are found in public places including markets, motor parks, and streets,

outside schools, hospitals and even express way (Izah *et al*., 2015). Most snacks are purchased to quench hunger and or thirst. They are sometimes used as an appetizer. Some ready-to-eat foods such as fruit juice are mostly consumed due to their refreshing attributes, nutritive values and health benefits. Fruits are potential source of nutrients, micronutrients, vitamins and fiber for humans (Oranusi and Olorunfemi, 2011). Most of these fruits include watermelon. Paw-paw, and pineapples (Izah *et al*., 2015). Some of these fruits are processed into juice wine such as pineapple juice (Nwachukwu and Ezejiaku, 2014), watermelon and paw-paw blend (Adedeji and Oluwalana, 2013), orange, apple, pineapple (Odu and Adeniji, 2013). Other essential nutritional drinks consumed by a large number of the populace in Nigeria are Kunu and Zobo (Izah *et al*., 2015).

In recent years, several developments in society have contributed to changes in the global beverage market. Consumers are increasingly aware of the impact of diet on their health and well-being. Beverages are not only consumed to provide refreshment and hydration but also to increase well-being and to help in preventing nutrition-related disorders (Tenge and Geiger, 2001). Moreover, an increasing number of consumers favour minimally processed products from natural ingredients for reducing intake of chemical additives from food and for obtaining products with improved nutritive and sensory characteristics. For example, studies showing the possible presence of carcinogenic benzene in soft drinks due to the reaction of benzoates (Chemical additive) with ascorbic acid and the possible allergenic effects of sulphites and benzoates have naturally contributed to this consumer trend (Ashurst and Hargitt, 2009).

Zobo is produced from the dried calyces of Hibiscus Sabdariffa. Hibiscus sabdariffa which belongs to malvaceae family is an annual erect bushy branched herb found in tropical and

semi-tropical regions of the world mainly West Africa and the East Indies (Nwachukwu *et al*., 2007). Hibiscus sabdariffa grows to about 3.5metres in height (Padmaja *et al*., 2014). As such can be classified as microphanerophytes (thus trees and shrubs whose height range from 2-8metres) (Izah *et al*., 2015). Hibiscus sabdariffa has cylindrical and dark green to red colored stem, tap root system and the leaves are green to red in colour (Padmaja *et al*., 2014). The flower is pale yellow with fleshy red calyces (Padmaja *et al*., 2014). Hibiscus sabdariffa thrives well in loamy well drained soil with annual rainfall of about 1500-2000mm^3 and can tolerate floods, heavy winds and stagnant waters (Umeh *et al*., 2015). Hibiscus sabdariffa is used to produce diverse food products and beverages in different countries of the world. In Nigeria two botanical varieties of Hibiscus Sabdariffa two botanical varieties exist including red/brown and green (Adelanwo and Ajibade, 2006 and Ilondu and Iloh, 2007). The brown/red type is widespread in the northern guinea and Sudan savanna while the green type is common in southern Guinea savanna (ILondu and Iloh, 2007). The calyces of the green variety of Hibiscus Sabdariffa are used to cook soup, stew and sauces while the calyces of the red variety are used for the production of Zobo drink and soup (Adanlawo and Ajibade, 2006). Zobo is a major nutritional drink consumed by several socio-economic classes of people in Nigeria and some other West African countries (Nwafor, 2012). Zobo is prepared with the addition of flavor such as orange and pineapple etc and preservatives such as garlic, ginger, mixture of garlic and ginger. This is because of its short shelf life of about 24hours after production without refrigeration (Nwachukwu *et al*., 2007; Umeh *et al*., 2015 and Onuoha and Fatokun, 2014). Due to the ease of production and availability of raw material especially Hibiscus sabdariffa, they are source of livelihood to several families in both Northern and Southern Nigeria especially in the rural areas.

Poor sanitary condition at the point of production and contaminated water would put consumers of these products at risk. The quality of Zobo drink depends mainly on the physicochemical constituents of the raw materials, water used in their production and the hygienic condition of the processors. Water is a major resource used in the production of this drink from their raw materials. Poor quality with regard to both physicochemical (colour, pH, turbidity, total suspended solids, total hardness, total alkalinity salinity, electrical conductivity), heavy metals (lead, cadmium, chromium, iron, zinc, copper, nickel, arsenic) and microbial (total heterotrophic bacteria, total fungi, total coliform and fecal coliform) could also impact on the overall quality of the drink (Izah *et al*., 2015). The environment which the drinks are processed could also influence the quality especially in the microbial perspectives. Zobo drink is a nutritional drink consumed by people in Nigeria. However, the consumption of local beverages could be a potential source of transfer of zoonotic and foodborne pathogens including staphylococcosis, salmonellosis, brucellosis, tuberculosis, shigellosis, listeriosis, E.coli, infections etc (Izah *et al.,* 2015). This study is however not intended to put out local producers of this drink by revealing their unhygienic practices, but to help them with knowledge of their products and ultimately lead to improvement in their processing and preservation methods.

1.1 Problem Statement

The major problem of Zobo drink is that of a short shelf life. According to Seiyaboh *et al*., (2013), Zobo drink spoil after 2 days (48hours) of storage at ambient temperature, this

therefore makes the beverage very difficult to store for a long time. Studies on Zobo drink has shown that pasteurization and application of sodium benzoate preserved the product the most (beyond 40 days) followed by pasteurization (for 14 days), control or untreated samples could store for a maximum of three days before spoilage (Egbere *et al*., 2007). However, vitamin C content of the beverage 1,105.88 mg 100 mL in the control samples decreased by 53.4 and 61.46% due to pasteurization and sodium-benzoate plus pasteurization respectively. Apart from the fact that most chemical preservatives may have adverse effect on humans, they are expensive and usually not affordable by the local people that produce this drink. A common problem, however, is the combination of sodium benzoate and ascorbic acid (vitamin C), when they get together, they form benzene, a cancer-causing chemical associated with leukemia and other blood cancers (naturallysavvy.com), and therefore, there is need for an alternative method of preservation. The consumption of Zobo drink when compared to that of the various soft drinks is very low and this can be attributed to the low quality of the drink. If a suitable and health-friendly preservative method is developed it will encourage the large production of the beverage for commercial purpose.

There is little or no information on the preservation of Zobo drink with carbon dioxide (CO_2). Carbonated drinks not only taste nice, they also stay fresh for a long time as CO_2 inhibits the growth of microorganisms. It displaces Oxygen (O_2) and also a small proportion of the Carbon dioxide (CO_2) combines with the water to form Carbonic acid, microbes do not like this environment with the result that carbonated drinks stay fresh for a long time.

The microorganisms associated with the spoilage of Zobo drink include Bacillus sp., Lactobacillus sp., Saccharomyces cerevisiae, Mucor sp., Aspergillus niger, Escherichia coli, and

Staphylococcus aureus Most of these microbes have invaded the products from the dried *H. sabdariffa* bought in the market (Omemu *et al*., 2006). *Bacillus* sp is an opportunistic bacterium of humans, found in soil, leaf surface and wrapping materials (Odom *et al*., 2012). Also the presence of *S. faecalis* is an indication of recent fecal matters (Risiquat, 2013). Coliforms such as *E.coli* are known to cause varying degree of gastroenteritis depending on the strain. The occurrence of *Micrococcus* sp, which is a saprophytic bacterium, may be from the skin of human and animals (seiyaboh *et al*., 2013). *Staphylococcus* and *Pseudomonas* sp may occur in the drink due to handling and other utensils used after processing (Amusa *et al*., 2005). The presence of *S. aureus* could be due to handling. This is because humans are the primary reservoirs of *S. aureus* and is found in the nasal region, hand and skin (Chukwu *et al*., 2010). Also *Streptococci* sp are normal flora of the throat and buccal cavity (Akhigbemidu *et al*., 2015). They could enter the zobo drink during zobo preparations via talking. The consumption of local beverages could be a potential source of transfer of zoonotic and foodborne pathogens including staphylococcosis, Salmonellosis, Brucellosis, Tuberculosis, Shigellosis, Listeriosis, E. coli, infections etc (Umaru *et al*., 2014).

1.2 Justification

The greatest limitation for large-scale production of Zobo drinks is the rapid deterioration of the drink. Its shelf-life is approximately 2 days (48hours) following production, if not refrigerated (Seiyaboh *et al*., 2013). Microorganisms associated with the dried calyces, the production processes and other factors may contribute to its spoilage. Apart from the fact that most chemical preservatives may have adverse effect on humans, they are expensive and usually

not affordable by the local people that produce this drink. Moreover, refrigeration may not be available. There is the need for alternative source of preservative that is natural and human-friendly, affordable and readily available (Jay, 1996) and having seen the effect of sodium benzoate (preservative) on the shelf life of Zobo, and the carcinogenic effect when it combines with vitamin C naturally present in the beverage this research is therefore aimed at substituting sodium benzoate with carbon dioxide (CO_2) as this can serve as an alternative method of preservation which have no effect on the nutrient in the beverage likewise no harmful effect on the consumers. Hence the aim of this research was to do a comparative study of the microbiological, storage quality and physicochemical qualities of locally produced Zobo and laboratory prepared Zobo drink (following the addition of CO_2 and Pasteurization).

1.3 Objectives

The primary objective of this research work is to study the effects of some preservative techniques (Carbonation and Pasteurization) on the quality and storage stability of Zobo drink. The specific objectives of this research are to;

1. To determine the physicochemical qualities of the Zobo drinks.
2. To determine the microbiological and storage qualities of the Zobo drinks.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Zobo

Zobo is a nutritional drink consumed by different class of people irrespective socio economic status (Izah *et al*., 2015; Nwafor, 2012), sex and age in Nigeria especially in the Northern region and other neighboring African countries (Izah *et al*., 2015). The most active ingredient used in the production of zobo drink is *Hibiscus sabdariffa,* which belongs to the Malvaceae family. *H. sabdariffa* are mainly cultivated as vegetables for soup preparations (Fasoyiro *et al*., 2005), folk medicine and tea preparations (Adesokan *et al*., 2013). *H. sabdariffa* is a tropical annual herb. *H. sabdariffa* is native to Asia (India and Malaysia) (Bola and Aboaba, 2004 cited 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 (Alo *et al*., 2012; Ilondu and Iloh, 2007). As at today, *H. sabdariffa* is found in several tropical and subtropical countries of the World (Fasoyiro *et al*., 2005) especially in India and Africa (Nwachukwu *et al*., 2007). In Nigeria, *H. sabdariffa* is mainly found in the Middle belt North eastern region (Joseph and Adogbo, 2015; Obadina and Oyewole, 2007 cited Bolade *et al*., 2009). However, two varieties of *H. sabdariffa* are found in Nigeria including red/brown and green (Ilondu and Iloh, 2007; Adanlawo and Ajibade, 2006). The green type is found in Southern guinea savanna while

the brown type is prevalent in the Northern Guinea and Sudan savanna (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 (Egbere *et al*., 2007) or Zoborodo (in Hausa), Iseipa (in Yoruba) and Sorrel in English (Adebayo-Tayo and Samuel, 2009), Jamaica flower, and karkade (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 (Umaru *et al*., 2014) in different parts of Nigeria. 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; Onuoha and Fatokun, 2014; Nwachukwu *et al*., 2007). Most producers depend heavily on the use of preservatives. Electricity supply in which Zobo processors depend mainly on for refrigeration is grossly inadequate and often characterized by frequent power outage. On the overall, when electricity supply is low Zobo could easily be invaded by spoilage microorganisms. 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). At 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 (Table 1) (Egbere *et al*., 2007; Ezearigo *et al*., 2014; Ezeigbo *et al*., 2015b; Omemu *et al*., 2006; Seiyaboh *et al*., 2013; Braide *et al*., 2012). 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). This paper therefore focused on the preservatives used in enhancing the shelf-life of the Zobo drink.

2.1.1 Zobo drink preparations

Zobo drink is a non-alcoholic nutritive drink prepared from the dried calyces of Roselle (*H. sabdariffa*) (Umaru *et al.,* 2014; Seiyaboh *et al*., 2013; Adebayo-tayo and Samuel, 2009; Fasoyin *et al*., 2005; Adesokan *et al*., 2013; Ezearigo *et al*., 2014; Egbere *et al*., 2007; Braide *et*

al. 2012; Amusa *et al*., 2005; Risiquat, 2013; Ayo *et al*., 2004), thus the flowers of *H. sabdariffa* (Nwafor and Ikenebomeh, 2009; Olawale, 2011). 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 (Nwachukwu *et al*., 2007; Ezeigbo *et al*., 2015a; Onuoha and Fatokun, 2014). 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 (Nwachukwu *et al*., 2007; Umaru *et al*., 2014).

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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 (Ezearigo *et al*., 2014; Nwachukwu *et al*., 2007; Ezeigbo *et al*., 2015a; Onuoha and Fatokun, 2014; Osueke and Ehirim, 2004). 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 and ginger (Adesokan *et al*., 2013; Braide *et al*., 2012), lime (Nwachukwu *et al*., 2007; Ezeigbo *et al*., 2015b; Braide *et al*., 2012), clove, cinnamon and nutmeg (Ezearigo *et al*., 2014), pepper fruit (Ihemeje *et al*., 2013).

2.1.2 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. 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).

Dried calyces of H. sabdariffa

↓

(Cleaning) Removal of dirt, stones and other debris

↓

*(Washing) Addition of water to further aid in the removal of sand debris

↓

**(Extraction) Boiling of washed calyces of H. sabdariffa for 10-15 minutes

↓

Cooling

↓

(Filtration/Seiving) Seive/Muslin cloth is used

↓

(Flavouring) Addition of sugar and other desired flavor such as orange and pineapple

↓

***Stirring of the mixture and boiling for 5 minutes

↓

****Cooling and addition of spices/preservatives of choice

↓

Packaging

Figure 1: Zobo drink production pathway

(*some processors skip this route; ** some processors add natural spices/preservatives during initial boiling at stage 4 and skip step 8*** and 9****)

2.1.2.1 Physical and chemical methods

Zobo drink is usually treated with chemical preservatives such as sodium benzoates at varying concentrations. 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. Electricity supply in Nigeria is of low quality and is characterized by unplanned outage. 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.

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. 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.

The role of acetic acid is 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 (Table 2) 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 1017 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. A laboratory study carried out has showed that bacteria density of zobo drink without any treatment reduced by 90.83% after 3 days of storage. But the pasteurized sample decreased by 99.18% at 14 days of storage and samples pasteurized

followed by the addition of 0.1% sodium benzoates decreased by 99.78% after over >40 days of storage (Egbere *et al*., 2007).

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 0 day 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). This reason for this fluctuation remains unknown.

2.2 Hibiscus sabdariffa-Botanical description

Hibiscus sabdariffa L. or locally known as asam kumbang, asam susur, and asam paya is belonging to the large family of Malvaceae (Osman *et al.,* 2011). It is also commonly known as roselle (English), l'Oiselle (French), Spanish (Jamaica), karkade (Arabic), and Krachiap daeng (Thailand) (Maganha *et al.,* 2010).

2.2.1 Botanical Classification:

Kingdom : Plantae (Plants)

Subkingdom : Tracheobionta (Vascular plants)

Superdivision : Spermatophyta (Seed plants)

Division : Magnoliophyta (Flowering plants)

Class : Magnoliopsida (Dicotyledons)

Subclass : Dilleniidae

Order : Malvales

Family : Mavaceae (Mallow family)

Genus : Hibiscus L. (Rosemallow)

Species : *Hibiscus sabdariffa L*

2.2.2 Description of roselle

Genus Hibiscus which belongs to Malvaceae has more than 300 known species which are used as ornamental plants. Hibiscus sabdariffa is an annual, erect, bushy, herbaceous sub-shrub

that may grow to 8ft (2.4m) tall, with lobed leaves sometimes used for greens, with smooth or nearly smooth, cylindrical, typically red stems. The leaves are alternate, 3 to 5 in (7.5-12.5cm) long, green with reddish veins and long or short petioles. The capsule turns brown and splits open when mature and dry. The narrow leaves and stems are reddish green in color. The main edible part is the fleshy sepal, called a calyx, surrounding the seed boll in the flower. The size of the calyx varies with each variety, but ranges from ½ to 1 ½ inches in diameter (James, 1994). The calyx stems and leaves are acidic and closely resemble the cranberry (Vaccinium spp.) in flavor. It is extensively cultivated in tropical Africa, Asia, Australia, and Central America (Schippers, 2000).

Many parts of *H. sabdariffa* including seeds, leaves, fruits and roots are used in various foods. Among them, the fleshy red calyces are the most popular. The fleshy calyces of *H. sabdariffa* have been used in various countries as food or a food ingredient such as jellies, syrups, beverages, puddings, cakes, and wines (Christian *et al.,* 2006). The red persistent calyx of its flowers is the major component which has a sour taste and is commonly used in the preparation of cold and hot beverages and as a food colorant.

Plate 1: Mature green roselle plant

Table 1: Different names of Hibiscus sabdariffa L.

Region	Vernacular names	Source
English speaking	Roselle, sorrel, jelly okra	Morton 1987
French speaking	Oseille rouge	Morton 1987
Africa	Sorrel	Omobuwajo *et al*., 2000
North Africa	Karkade	Morton 1987; Abu-Tarboush 1997
Malaysia	Assam papaya	Mat Isa *et al*., 1985
Australia	Rosella	Wikipedia [R] 2008
Nigeria	Zobo	Wikipedia [R] 2008

Source: Wikipedia [R] 2008 Sorrel (plant).

2.2.3 Varieties of sorrel seed (*Hibiscus Sabdariffa L*)

Hibiscus Sabdarriffa Var; is an annual erect, bushy, herbaceous subshrub that grows up to 8ft (2.4m) tall, with smooth or nearly smooth cylindrical, typically red stem. The leaves are alternate, 3 to 5 in (7.5 to 12.5cm) long, green with reddish vein and long or short petioles. The leaves of young seedling and upper leaves of older plant are simple, lower leaves are deeply 3 to 5 or even 7 lobed, the margin are toothed flowers, borne singly in the leaf axils are up to 5 in (12.5cm) wide yellow or buff with a rose or maroon eye and turn pink as they wither at the end of the day. At this time the typically red calyx consisting of 5 large sepal with a colar (epicalyx) of 8 to 12 slim or bracteole around the base, begin to enlarge becomes fleshly, crisp and juicy 11/4 to 21/4 in 3.2 to 5.7cm long and fully encloses the3 velvety capsule ½ to ¾ in (1.25 to 2cm) long which is green when immature, 5 valued, with each value containing 3 to 4 kidney shaped, light brown seed 1/8 to 3/16 in (3 to 5mm) long and minutely downy. The capsule turns brown and split open when mature and dry. The calyx stem and leaves are acid and closely resemble the cranberry (Vaccinium Spp) in flavor. It is grown as a fibre crop. The fibre is obtained from the stem of the plant and used for textiles, either alone or mixed with jute. It is used for bags, twines, carpet yarn, and pulp. The fruits are inedible growing period annual, it may be ready for harvest 90-240 days from transplanting and is usually harvested before flowering.

2.2.4 Origin and distribution of roselle

Roselle is native from India to Malaysia, where it is commonly cultivated, and must have been carried at an early date to Africa. It has been widely distributed in the Tropics and Subtropics of both hemispheres, and in many areas of the West Indies and Central America, it has become naturalized (Brouk, 1975). China and Thailand are the largest producers and control much of the world supply. Thailand invested heavily in roselle production and their product is of superior quality, whereas China's product, with less stringent quality control practices is less reliable and reputable. The world's best roselle comes from the sudan, but the quantity is low and processing hampers quality. Mexico, Egypt, Senegal, Tanzania, Mali and Jamaica are also important suppliers but production is mostly used domestically. In kenya, Roselle is cultivated by small scale farmers in Kisimu under the umbrella of REAP (Rural Extension with Africa's Poor)-a registered trust that focuses on teaching about growing and using natural medicines. In Kenya, however, it is not widely grown. It was first introduced to West Indies and cultivated mainly as an ornamental plant. *H. sabdariffa* is relatively a new crop in Malaysia. It was introduced into Malaysia in early 1990s. Its commercial planting was first promoted by the Department of Agriculture in Terengganu in 1993 and has now spread to other states. Presently, the planted area is quite small approximately 150 hectares. Terengganu state used to be the first and the largest producer but now the production has spread more to other states. Despite the dwindling hectarage over the past decade or so, roselle is becoming increasingly known to the general population as an important pro-health drink in the country. In the Indian subcontinent (especially in the Ganges Delta region), roselle is cultivated for vegetable fibres. Roselle is called mesta (or meshta, the *ś* indicating an *sh* sound) in the region. Most of its fibres are locally

consumed. However, the fibre (as well as cuttings or butts) from the roselle plant has great demand in various natural fibre utilizing industries.

2.2.5 Agronomic practices and production of roselle

2.2.5.1 Environmental conditions for roselle growth

Roselle is quite hardy and grows well in most soils that are well drained. It tolerates poor soil, and is often grown as a supplemental rather than a primary crop. It requires 4-8 months with nighttime temperature not below 21^0C. In addition, it requires 13 hours of sunlight during the first 4-5 months of growth to prevent premature flowering. Roselle requires a monthly rainfall ranging from 5-10 in the first 3-4 months of growth. Dry periods can be withstood and are desirable in the last months of growth. Rain or high humidity during the harvest time and drying can downgrade the quality of the calyces and reduce the yield.

2.2.5.2 Planting of roselle

Hibiscus sabdariffa is very sensitive to changes in the length of day as reported by Hacket and Carolene (1982). This photoperiodic quality of blooming, when the days become shorter, requires the planting time to be set according to the day length and not according to the rainfall requirements. Hibiscus, is a deep-rooted crop so deep plowing is recommended in preparing the seedbed. To produce large calyx, 453-906 kilograms of manure are added per acre seeds are planted at a rate of 2.7-3.6 kilograms or less per acre approximately an inch deep. Seeds are best planted at the beginning of the rainy season, 2-3 feet between rows and 18-24 inches within the rows. The reduced planting rate produces larger calyx (Ahmed and Salaheldeen, 2010).

Planting can be done with a modern grain drill and then later requires thinning by hand; planting by seed can also be done by hand. A good alternative tool would be a corn planter small enough to accommodate the hibiscus seeds (Ahmed and Salaheldeen, 2010).

There are over 100 cultivars or seed varieties of Roselle. The major commercial varieties are those grown in China, Thailand, Mexico and Africa, principally Sudan, Senegal and Mali (Mahaderan, 2009).

2.2.5.3 Natural enemies of roselle

Major diseases of Roselle are mostly stem and root rot. Prevention techniques can include monitoring water in an irrigated field as well as avoiding the planting of crops that are also prone to these diseases. Damage done to Roselle by insects is minor but it does exist. In the order Coleoptera is the stem borer and flea beetles, Podgorica spp. In the order hepoptera, the abutilon moth, the cotton bollworm, and the cutworm. The order Hemoptera is a minor problem, the mealy bugs and the leafhopper, and finally in the order Hemiptera the cotton strainer (Elawad, 2001).

Plant enemies usually do not compete in a cultivated field. Weeding can increase yield and Calyx size, but may also reduce profit for the farmer because of differences in available land and labor prices, Chinese Roselle fields are-generally weeded and even hand watered if necessary, for maximum yield, while those in Thailand are given minimal attention.

Plate 2: Flea beetles (natural enemies of roselle)
Source: Kerharo, J. (1971)

2.2.5.4 Growth characteristics of roselle

Flowering of the hibiscus is induced as the days become shorter and light intensity reduces. Flowering begins in September or later depending on the country in question, and continues through October or later when the entire field is in bloom. Flowers begin to drop at the end of October or later. The seed pods begin ripening towards the bottom and proceed to the top. In Sudan, growers will sometimes allow the seed to completely ripen and the leaves to drop prior to harvest (Ahmed and Salaheldeen, 2010).

2.2.5.5 Harvesting of roselle

The harvest is timed according to the ripeness of the seed. The wet red fleshy calyces are harvested after the flower has dropped but before the seed pod has dried and opened. The more time the capsule remains on the plant after the seeds begin to ripen, the more susceptible the

calyx is to sores, sun cracking, and general deterioration in quality. All harvesting is done by hand. Special care must be taken during harvesting operation to avoid contamination by extraneous material. At no time should the calyx come in contact with the ground or other dirt surfaces. Clean bags or containers should be used to transport the harvest from the field to the drying location (Elawad, 2001). In addition to avoiding contamination, the time between harvest and drying should always be kept at a minimum.

Different harvesting methods are in use today. In Mexico the entire plant is cut down and taken to a nearby location to be stripped of the calyces. In China only ripe calyces are harvested with clippers leaving the stalks and immature calyces to ripen in the field. The field is harvested approximately every ten days until the end of the growing season. The calyx is separated from the seed pod by hand, or by pushing a sharp edged metal tool through the fleshy tissue of the calyx separating it from the seed pod (Ahmed and Salaheldeen, 2010).

2.2.5.6 Drying of roselle

Vanvalkenburg *et al*, (2002) highlighted that drying of roselle can be accomplished by different methods. These include drying with adequate ventilation, using woven nylon mats for example, prevent sun baking which can reduce quality. A clean sheet of plastic placed on the ground can also be used with the hibiscus spread thinly on top. This method is still prone to insect infestation and mold. Spreading the calyces on screens or frames would improve ventilation further and reduce drying time such frames cold also be stacked or living in a well ventilated building. Drying the calcyces in forced air dryers would be costly and is rarely done. It heated drying methods are used care must be taken so that the temperature does not exceed 43◦C

so that the phytochemicals are not degraded (Elawad, 2001). Roselle calyx is usually harvested at high moisture content (85%, w.b.). Therefore, drying is an important post-harvest treatment prior to reduce the moisture content and to increase the shelf life. Drying is a process comprising simultaneous heat and mass transfer (Suherman *et al*., 2012). In order to preserve and extend their life, Roselle calyxes are sold in dry form. Their main form of consumption is after leaching them with water (Daniel *et al*., 2012). Saeed *et al*. (2008) found that at different temperatures (35, 45, 55 and 65ºC) and relative humidity (30%, 35%, 40% 45% and 50% RH), the drying kinetics of fresh Roselle calyxes were controlled by internal diffusive mechanisms (falling rate period) and a second model of exponential decay behaviour; the drying time was reduced with increased temperature and prolonged with greater relative humidity. Saeed (2010) used a solar dryer to study the effects of drying conditions on the drying constant (k) and coefficient values (a and c) in order to validate a logarithmic model.

2.2.5.7 Yield of roselle

Total yield of roselle calyces is approximately 228kg for each acre under cultivation, or about one metric ton per hectare. The drying ratio is 10:1, that is, for every 46 kilogram of fresh calyx, 5 kilograms of dry calyx is produced. Increased level of weeding increases the number of calyces per plant (Ahmed and salaheldeen, 2010). The study showed that weeding three times resulted in high crop vigor score in number of calyces per plant Vanvalkenburg *et al*, (2002) stated that, weeding increased calyx size in roselle crop. The main problems limiting production of roselle as pointed out by Elawad (2001) are; scarcity and reliability of rainfall, limited research and agricultural extension services poor cultural practices, in adequate weed control and harvest problem. High productive potentials have been reported for rosselle grown under rainfed

though various agronomic practices such as weeding and spacing, intercropping, sowing dates, intra row spacing and nitrogen fertilizer (Babatunde , 2003). However Hinrichsen *et al* (1997) highlighted that rainfed agriculture alone is inadequate to feed the bludgeoning population in many parts of the world. It is therefore incumbent upon mankind to resort to both rainfed and irrigation farming for the widest array of food crops possible. This would augment world food production and put the human race a step forward towards attaining food security.

2.2.6 Utilization and economic importance of roselle

2.2.6.1 Calyces

The fruit is about 2.5cm in length with fleshing calyces containing dark brown seed (Kalyane, 1986, Rice *et al*., 1990). The various uses, to which roselle has been put, show that it has been contributing to the livelihood of people in part, show that it has been contributing to the livelihood of people in parts of Nigeria (Arowosoge, 2008). However, its production into non-alcoholic drink (ZOBO-NIGERIA) is at cottage level in Nigeria and Sudan with a very short shelf life (omemu, *et al.*, 2006). The red calyces surrounding the fruit can be used to brew non-alcoholic beverage and as coloring reagent for jelly, jam, beverages, and foods (Gibbon *et al*, 1995, Rao, 1990 and wahid, 2008). Its alleged medicinal values includes; prevention and cure of Hypertension and inflammation of the bladder (QI *et al.*, 2005).

Plate 3: Freshly harvested roselle calyces
Source: Kerharo, J. (1971)

Plate 4: Dried roselle calyces.
Source: Kerharo, J. (1971)

2.2.6.2 Leaves and roots

Tender young leaves are eaten as vegetable especially with soup, or salad and as a seasoning in curies. They have an acidic, rhubarb like flavor (Fasoyiro, 2005 and mungole, 2011). Roselle has a tap root system. The roots are deficient of most nutrient as reported by (Ojokoh, 2006). They are claimed to be aphrodisiac (mungole, 2011).

2.2.6.3 Stem

The stem is utilized in fibre extraction, Rao (1990) reported that the plant is grown is some regions for fibre and pulp obtained from its stem. Total yield of the dry retted fibre component from one hectare, 30 Tonnes of Green Roselle plant is 1,410kg. China and Thailand are the largest producer and control much of the world supply, Thailand invested heavily in Roselle production and their product is of high quality whereas china product, with less stringent quality control practices, is less reliable and reputable.

2.2.6.4 Seeds

The seed are used as feed meal for fish and domestic animal (Backeit *et al*., 1994; Mohamaodu, *et al*., (2007) reported a study that determined the functional potential of Mbuja, a traditional condiment produced by fermentation of Hibiscus Sabdariffa seeds. The study suggested that Mbuja was a cheap functional food that provides both anti-oxidants and probiotic-Mbuja production and consumption could therefore contribute to the consumption to the consumer's health.

2.2.6.5 Medicinal value

Hibiscus Sabdariffa is used in many folk medicines. It is claimed as Thai traditional medicine for kidney stories and urinary bladder stones (Hirunpanich *et al*., 2006). Hibiscus Sabdariffa also is said to have diuretic effect, used effectively in folk medicines for treatment of inflammatory diseases, and cancer (Chewonarin *et al*., 1999). The positive effect of Hibiscus Sabdariffa extract consumption to decrease blood pressure has been proved in study on both man

and rats. More recently, the anti-hypertensive action of hibiscus sabdariffa has been confirmed with experimental hypertension (Odigie *et al*., 2003). In addition studies on human also proves the anti-inflammatory effect of Hibiscus sabdariffa consumption.

H. sabdariffa extract is also reported used as an antibacterial, antifungal, diuretic, uricosuric, and mild laxative substance. In addition, the components of *H. sabdari*ffa extract exhibit anti-tumor characteristics, immune-modulating and anti-leukemic effects. Oil extracted from seeds of *H. sabdariffa* has been shown to have an in vitro inhibitory effect on *Bacillus anthracis* and *Staphylococcus albus*. An ethanol extract of the dried leaves of the plant also has been shown enable to reduce aflatoxin formation, and to have an in vitro inhibitory effect against some fungi that include *Aspergillus fumigatus*, *Rhizopus nigricans* and *Trichophyton mentagrophytes.*

2.2.7 Antioxidant activity and anticholesterol effects of roselle

The calyxes' part of *H. sabdariffa* have repeatedly been studied and shown to have positive health effects especially as a source of antioxidants. The calyx providing antioxidants even higher levels than traditional sources such as raspberries and blueberries. Anthocyanins and protocatechuic acid are among chemical constituents in *H. sabdariffa* that shown to have strong antioxidant and antitumor effects (Lin *et al.,* 2007).

The previous study found that the extract of dried *H. sabdariffa* calyx was shown to be active protect rat hepatocytes from tert-butyl hydroperoxide-induced cytotoxicity and genotoxicity by different mechanisms (Lin *et al.,* 2007). Works done by Lin *et al.,* (2007) also found that protocatechuic acid from *H. sabdariffa* calyx was demonstrated to inhibit lipopolysaccharide-induced rat hepatic damage. Hibiscus protocatechuic acid has also been shown to inhibit the carcinogenic action of various chemicals in different tissues of the rat,

including diethylnitrosamine in the liver. It was proposed that one of the mechanisms of this protective effect was associated with the scavenging of free radicals by antioxidant compounds exist in *H. sabdariffa* calyx. Administering the dried calyx extracts of *H. sabdariffa* was found significantly decreased serum cholesterol, triglycerides and LDL levels. Calyx of *H. sabdariffa* possesses both antioxidant effects against LDL oxidation and hypolipidemic effects in vivo. However, its mechanisms of action remain to be elucidated (Hirunpanich *et al.,* 2006).

2.2.8 Phytochemical constituents

The calyx of *H. sabdariffa* has been known to contain many chemical constituents such as delphinidin-3-glucoxyloside, also known as hibiscin, the major anthocyanin in *H. sabdariffa* calyx. Others flavonoids constituents that exist in *H. sabdariffa* calyx are includes anthocyanins as cyanidin-3-rutinoside, delphinidin, delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, cyanidin-3-sambubioside, cyanidin-3, 5-diglucoside; the flavonol glycosides hibiscetin-3-monoglucoside, gossypetin-3-glucoside, gossypetin-7-glucoside, gossypetin-8-glucoside and sabdaritrin. Alkaloids, ascorbic acid, b-carotene, anisaldehyde, arachidic acid, citric acid, malic acid, tartaric acid, glycinebetaine, trigonelline,; quercetin, protocatechuic acid, pectin, polysaccharides, mucopolysaccharides, stearic acid and wax also reported to be exist in *H. sabdariffa* calyx (Hirunpanich *et al*., 2006).

The calyx yielded 65% (dry weight) of mucilage, which on hydrolysis gave galactose, galacturonic acid and rhamnose. These molecules are bioactive in several biological models and responsible by the pharmacological effects presented by the extracts of this species. Various antioxidant constituents are found in the calyx and flower petals of roselle, such as anthocyanins, quercetin, ascorbic acid, ß-sitosteroid glycoside and protocatechuic acid. Furthermore, *H.*

sabdariffa calyx contained polyphenolic acids (1.7% dry weight), flavonoids (1.43% dry weight) and anthocyanins (2.5% dry weight).

2.2.9 Functional properties and Phytochemicals

2.2.9.1 Functional foods

The term "functional food" first appeared in *Nature* in 1993 in an article titled "Japan Explores the Boundary between Food and Medicine" (Swinbanks and O'Brien, 1993).

Functional food is defined as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains." Health-conscious baby boomers have made functional foods the leading trend in the U.S. food industry (Meyer, 1998). Estimates, however, of the magnitude of this market vary significantly, as there is no consensus on what constitutes a functional food. More significant, perhaps, is the potential of functional foods to mitigate disease, promote health, and reduce health care costs.

2.2.9.2 Functional foods from plant sources

Overwhelming evidence from epidemiological, in vivo, in vitro, and clinical trial data indicates that a plant-based diet can reduce the risk of chronic diseases, particularly cancer. In 1992, a review of 200 epidemiological studies showed that cancer risk in people consuming diets high in fruits and vegetables was only one-half that in those consuming diets low in these items (Block *et al*., 1992). It is now clear that there are components in a plant-based diet other than traditional nutrients that can reduce cancer risk. Steinmetz and Potter (1991) identified more than a dozen classes of these biologically active plant chemicals, now known as "phytochemicals."

Health professionals are gradually recognizing the role of phytochemicals in health enhancement (ADA, 1995; and Kritcheveky, 1997). In USA an act has been enacted, Nutrition Labeling and Education Act of 1990 (NLEA) that requires nutrition labeling for most foods and allow disease- or health-related messages on food labels.

Nowadays in most of the developed and developing countries, hyperlipidemia and thereby atherosclerosis are the leading cause of cardiovascular morbidity and mortality. A major risk factor for the development of cardiovascular diseases is the elevated levels of plasma. It is crucial to maintain the normal body functions by reducing the elevated serum to adequate levels. Since the technology of functional food emerged, more and more functional foods are being developed from plants and regarded as the adjuvant treatment to some diseases (Demigne *et al*., 1998).

Recently there has been an increased interest in research on food components such as anthocyanins and other phenolic compounds because of their possible linkage to health benefits including reduction in heart disease and cancer, partly based on their antioxidant activity (Seeram, 2002). With the global functional food and beverage market estimated at $109 billion by 2010 (Watkins, 2008), diverse sources of phytochemicals are being explored. Polyphenols in beverages are common because of their beneficial physiological effects on health (Bravo, 1998; Ina *et al*., 2002).

Additional research is necessary to substantiate the potential health benefits of those foods for which the diet-health relationships are not sufficiently scientifically validated.

2.2.9.3 Phytochemicals

Phytochemicals—the bioactive non-nutrient plant compounds in fruit, vegetables, grains, and other plant foods have been linked to reductions in the risk of major chronic diseases. A large number of Phytochemicals and bioactive components are reported to be present in foods of plant origins and have become the focus of study in functional foods. Shahidi (2004;2008) reported that their synergistic effects are rendered by a combination of phytochemicals present in source materials, and complementary nature of phytochemicals from different sources are important factors to consider in the formulation of functional foods and in the choice of a healthy diet.

It is estimated that more than 5000 Phytochemicals have been identified, but a large percentage still remain unknown (Shahidi, 1995) and need to be identified before their health benefits are fully understood. However, more and more convincing evidence suggests that the benefits of Phytochemicals in fruit and vegetables may be even greater than is currently understood because oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic diseases (Ames, 1991).

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents may be present in air, food, and water, or they may be produced by metabolic activities within cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiologic conditions in the body. Overproduction of oxidants can cause an imbalance, leading to oxidative stress, especially in

chronic bacterial, viral, and parasitic infections. Oxidative stress can cause oxidative damage to large biomolecules such as proteins, DNA, and lipids, resulting in an increased risk for cancer and cardiovascular disease (Ames, 1991). To prevent or slow down the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed. Fruit and vegetables contain a wide variety of antioxidant compounds (Phytochemicals) such as phenolics and carotenoids that may help protect cellular systems from oxidative damage and lower the risk of chronic diseases.

2.2.9.4 Flavonoids

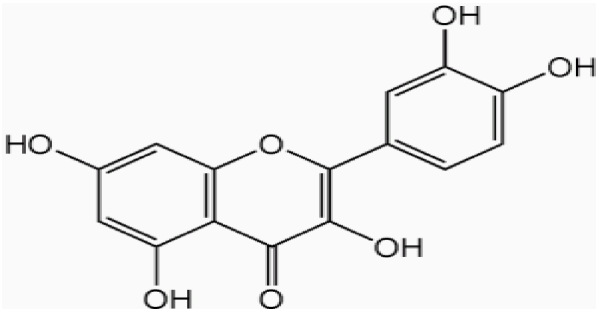
There has been considerable interest in the flavonoid content of foods and plants since the early 1980s when the studies of Steinmetz and Potter (1991) demonstrated a relationship between a diet rich in fruits and vegetables and a reduced risk for chronic diseases. Because reduced risk did not correlate with traditional nutrients, attention has focused on many non-nutrient, potentially bioactive compounds, of which the flavonoids constitute one family (Steinmetz and Potter, 1991).

Flavonoids are naturally-occurring polyphenolic compounds with a C6-C3-C6 backbone. This group of plant pigments which are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea, and can be chemically subdivided into six structural categories: flavones, flavonols, flavanones, flavanonols, flavan-3-ols (catechins), and anthocyanidins. These compounds (aglycones) are commonly glycosylated (at one or more sites with a variety of sugars) and may also be alkoxylated or esterified. As a result, over 5000 different flavonoids have been identified in plant materials. The methods that have been reported for the determination of flavonoids are

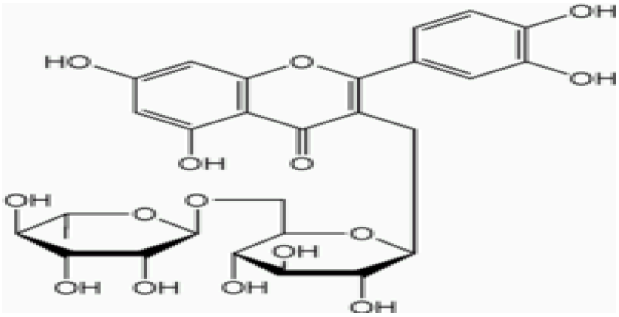
based on the aluminium chloride complex formation, which is one of the most commonly used analytical procedures applied to the flavonoid content determination in various plants.

In general, aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. The analytical methods were used either to determine the glycosylated or the nonglycosylated flavonoids shown in figure 5.

Quercetin (Quercitrin)



Quercetin-3-rutinoside



Quercetin-3-rhamnose

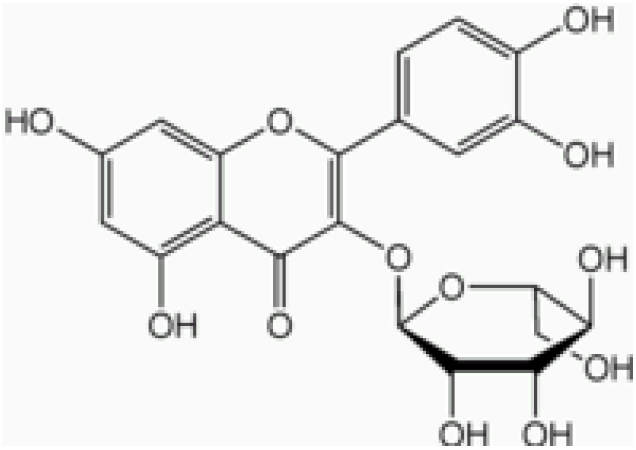


Figure 2: The free aglycone and the glycosylated form of quercetin

Source: Bisset, 1994.

The hydrolysis is performed in order to produce aglycones, to serve multiple purposes: to reduce the number of compounds and make chromatographic separation easier to achieve; Permit quantification of flavonoids because standards for a large number of the glycosylated flavonoids are not available; and to provide consistent data with the earlier view that flavonoids are absorbed only in the intestine as aglycones. Unfortunately, hydrolysis also leads to degradation of the aglycones, thus giving more importance to those methods based on the glycosylated flavonoid. Literature survey reveals the presence of two classes of flavonoids in the extracts of Hibiscus sabdariffa: flavonols (gossypetin), and the anthocyanins (Bisset, 1994).

2.2.9.5 Tannins

Tannins are secondary metabolites of plants. They are non-nitrogenous and phenolic in nature. They have the ability to tan animal skin to convert it to leather or hide. Conversion

imparts resistance to water, heat, and abrasives. They can be extracted using water-acetone/alcohol mixture. They also have the ability to precipitate gelatin & heavy metals. Complex tannins are macro molecules with many sugar molecules. The three major classes of tannins are the: hydrolysable tannins, non-hydrolysable tannins (condensed tannins) and the pseudo tannins. Hydrolysable tannins on heating with hydrochloric or Sulphuric acids yield Gallic or Ellagic acids. Examples of hydrolysable tannins include: - myrobalon, bahera, which hazel. Non-hydrolysable tannins on heating with hydrochloric acid yield phlobaphenes like phloroglucinol.

2.2.9.6 Anthocyanins

Anthocyanins are another group of pigments in plants. The structure of these anthocyanins differs in the types of anthocyanidins, sugar molecule and numbers, and types of acylation groups. Due to their bright color and high water solubility, anthocyanins are considered a potential natural pigment to replace artificial food colorants (Mazza & Miniati, 1993). Besides the coloring functions, anthocyanins in foods also possess potent antioxidant capacity and health promoting properties. For instance, anthocyanins in foods are believed to be able to reduce the risk of cardiovascular diseases for people who consume wine, berry, Roselle and grape. (IUFOST, 2009)

The mechanism postulated is that anthocyanins act as antioxidants by donating hydrogen atoms to highly reactive free radicals, breaking the free radical chain reaction (Rice, *et al*., 1990). The field of functional foods, however, is in its infancy. Claims about health benefits of functional foods must be based on sound scientific criteria (Clydescale, 1997). A number of

factors complicate the establishment of a strong scientific foundation, however. These factors include the complexity of the food substance, effects on the food, compensatory metabolic changes that may occur with dietary changes, and, lack of surrogate markers of disease development.

2.2.9.7 Encapsulation of anthocyanin

Encapsulation facilitates light and heat-labile molecules to maintain their stability and improve their shelf lives and activity. It is a rapidly expanding technology, highly specialized, with affordable costs. Among the diverse encapsulation techniques available, only few were evaluated for anthocyanin encapsulation, these include: spray drying using different coating materials, gelation using polymers as sodium alginate, pectin, curdlan and glucan and liophilization (Santos *et al*., 2013). Spray drying technique has been widely used in the microencapsulation of food ingredients susceptible to deterioration by external agents and consists of entrapping an active agent (solid particles, liquid droplets or gaseous compounds) in a polymeric matrix, in order to protect it from adverse conditions. The immediate drying of the mixture leads to the formation of a matrix system in which the polymer forms a tridimensional network which contains the encapsulated material (Tonon *et al*., 2010). Microencapsulation has been used by the food industry in order to protect sensitive food ingredients during storage, to mask or preserve aromas and flavours, to protect food against nutritional losses or even to add

nutritive materials to food after processing (Re, 1998). The spray dry technique minimizes the loss of nutrients' content during processing and storage, the powder obtained is soluble and convenient to carry anywhere, it requires less storage space and it is one of the most commonly used methods to transform a wide range of liquid food products into powder. Some of the most used materials include pectin, whey protein, carrageenan, carboxymethyl cellulose (CMC), gelatin and xanthan gum amongst others, trying to protect the core of the particles against adverse environmental conditions, providing at the same time, an easy handling and release rate control (Diaz *et al*., 2015).

2.2.10 Phytochemistry

Roselle is rich in anthocyanins and protocatechuic acid. The dried calyces contain the flavonoids gossypetine, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscine has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), chrysanthenin (cyanidin 3-monoglucoside) and delphinidin are also present. Roselle seeds are a good source of lipid soluble antioxidants, particularly ■-tocopherol (Mohamed *et al*., 2012). The anthocyanin content of H. sabdariffa in five strains of the plant reportedly reached 1.7% to 2.5% of the dry weight during calyx growth (Khafaga and Koch, 1980b). H. sabdariffa calyces contain high amounts of organic acids, namely: citric acid, malic acid, tartaric acid and hibiscus protocatechuic acid (Kerharo, 1971; Khafaga and Koch, 1980a). The acid content of the calyces increases during growth but decreases when it reaches maturity

or ripens. The aqueous extract of H. sabdariffa calyces has a very rich red pigmentation due to the presence of anthocyanins and the colour properties has been the subject of intense scientific investigations (Ali *et al*., 2005; Aishah *et al*., 2013). Hibiscus sabdariffa calyces were found to contain a higher amount of iron content (164.78 mg/kg) (Maregesi *et al*., 2013). The plant is also found to be rich in minerals especially potassium and magnesium. Vitamins (ascorbic acid, niacin and pyridoxine) were also present in appreciable amounts (Puro *et al*., 2014).

2.2.11 Value addition of Roselle

Roselle is cultivated for its leaves, seeds and calyces. Nutritionally young leaves of Roselle contain nutrients such as phosphorus, calcium, magnesium and potassium Roselle as a Source of Natural Colour 517 (Atta *et al*., 2010). The leaves are consumed as a green vegetable and prepared like spinach (Delgado and Parcedes, 2003). However, the seed of Roselle is a valuable food resource on account of its protein, calorie and also substantial amount of fiber and valuable micronutrients (Akanbi *et al*., 2009). Indeed, young leaves and stems are eaten raw or cooked in salads, and as a seasoning in curries. Different food products, fermented foods and beverages of Hibiscus sabdariffa are widely used in different countries. From the fresh flowers of Hibiscus sabariffa cold and hot beverages are produced. The juice from the calyces of Sorrel (Roselle) is called “zoborodo” (soborodo), a non-alcoholic drink in Nigeria, which involves the production process of solid-liquid extraction leaving the calyx pulp as raffinate, which is a heavy organic material that could be converted as glucose (Ajayi *et al*., 2012). In Mexico, this beverage is called “flor de jamaica”. In Senegal (a state of West Africa) this Hibiscus sabariffa known as bissap and drink is prepared through aqueous extraction from a solid-to solvent ratio. This drink consumption is widespread in Asia and Africa. In Senegal, it is more popular and more

consumption is observed during the month of Ramadan (Padmaja *et al*., 2014). In Egypt, this beverage is called as “drink of the Pharaohs”. In Sudan, it is called as “tea Karkade”. In Mali (Africa) it is called as “da Bilenni”. Tea from the Hibiscus sabdariffa also called Sudan tea, sour tea which is prepared from ground dried calyces also have medicinal properties and is considered as herbal tea and Roselle is also used as main ingredient in many other tisanes (herbal tea) (Diane *et al*., 2009). ‘Bikalaga’ is a fermented food produced from the seeds of Hibiscus sabdariffa in African countries, including Burkina Faso, Mali Niger, Nigeria, Cameroon and Sudan among others (Parkouda *et al*., 2008). Fresh calyx (the outer whorl of the flower) is eaten raw in salads, or cooked and used as a flavouring in cakes and is also used in making jellies, soups, sauces, pickles, puddings etc. The calyx is rich in citric acid and pectin and is useful for making jams, jellies (Pacome *et al*., 2014). The seeds contain 17 to 20% edible fixed oil, which is similar in its properties to cotton seed oil. On the other hand, the colour extract from the dry calyces is rich in anthocyanin, amino acids, organic compounds, mineral salts and source of vitamin C (Al-Ansary *et al*., 2016). Calyces extract is also a potential source of natural colourant to replace red synthetic colouring agents for carbonated soft drinks, jams, juices, jellies, sauces, chutneys, wines, preserves and other acidic foods (Delgado and Parcedes, 2003). In fact, some Roselle varieties/cultivars are identified according to calyxes anthocyanin content. For example, the Sudani Roselle variety has dark red calyces, while the calyx has light red colour in Masri variety (Al-Ansary *et al*., 2016). Roselle, also known as sorrel, Jamaica flower and karkade, has been used by people for preparing soft drinks and in traditional medicine. It has been observed that its components, such as vitamins (C and E), polyphenols acids and flavonoids, mainly anthocyanins, have functional properties. The Roselle calyces production, in developing

countries, is of great importance since its production represents a very important income for people from rural communities. Today, several studies have shown that compounds found in aqueous and ethanol Roselle calyces extracts may have antioxidant properties. These compounds could work in several ways in humans; for instance, they could have anticancer characteristics. They may also reduce chronic diseases such as diabetes mellitus, dyslipidemias, hypertension and cardiovascular diseases. Some of these compounds (flavonoids and anthocyanins) are natural which have no toxic or mutagenic effects (Cid and Guerrero, 2014). The whole plant can be used as beverage, or the dried calyces can be soaked in water to prepare a colourful cold drink, or may be boiled in water and taken as a hot drink. It also has some medicinal properties. The seeds contain 17.8–21% non-edible oil and 20% protein, and are sometimes used for animal feed (Mohamed *et al*., 2012). The calyx of Hibiscus sabdariffa is widely used by humans, as food, jams, jellies, juice drinks, wine and as medicinal syrups (Akanya *et al*., 1997). It is used effectively in folk medicines for treatment of hypertension, inflammatory diseases and cancer (Lin *et al*., 2007). The calyces are used to decrease blood viscosity and reduce hypertension (Christian *et al*., 2006). Hibiscus pigments reduce the incidence of liver lesions including inflammation, leucocyte infiltration and necrosis (Kong *et al*., 2003). Anthocyanins are approved as food colourants in the USA under the category of fruit (21 CFR 73.250) or vegetable (21 CFR 73.260) juice colour. The EU classifies anthocyanins as ‘natural colourants’ under classification number E163. The use of anthocaynins may show benefits over that of synthetic colours. A potential use for Roselle extract may include production of fruit juice, drinks and jam (Duangmal *et al*., 2008).

2.2.12 Extraction of colour

Colour is one of the most important quality attributes affecting the consumer's acceptance of food since it gives the first impression of food quality. Many convenience foods such as confectionery products, gelatin desserts, snacks, cake, pudding, ice cream and beverages would be colourless, and would thus appear undesirable without the inclusion of colourants (Hirunpanich *et al*., 2006). Due to perceived safety and physiological advantage of the natural colourants over synthetic ones, interest are being geared into search of new natural colourants and the verification of the safety of existing ones. Roselle is a tropical plant of considerable economic potential. Its calyces have been suggested as food colourants for food industries; emulsifier for carbonated drinks, jam manufacture, juices and natural food colourants (Duangmal *et al*., 2008). The calyces are rich in anthocyanin, ascorbic acid and hibiscus acid. It is water soluble with brilliant and attractive red colour and with sour and agreeable acidic taste, which aids in digestion. In this respect, Roselle calyces appear to be good and promising sources of water soluble red colourants that could be utilized as natural food colourants (Abou *et al*., 2011).

Anthocyanins are one of the most important groups of water soluble pigments visible to the human eye. They are responsible for many of the attractive colours, from scarlet to blue, of flowers, fruits, leaves and storage organs. Hibiscus anthocyanins were identified as having Delphinidin-3-sambubioside (Dp-3-sam) (70% of the anthocyanins) and Cyanidin-3-sambubioside (Cyn-3-sam) as the major pigments, with Delphinidin-3-glucoside (Dp- 3-glu) and Cyanidin-3-glucoside (Cyn-3-glu) as the minor ones (Amor and Allaf, 2009). Cahlikova *et al*. (2015) explained that the active constituents of the extracts of Roselle, which widely used in folk medicine to combat many illnesses and have been shown on several occasions to be anthocyanins and they ascertained through UHPLC-ESI MS/MS that

delphinidin-3-sambubioside is the major one but the predominant one is cyaniding-3-sambubioside. Also Roselle extracts are reported to have an antimicrobial effect on different pathogenic and food spoilage micro-organisms due to its metabolities of phenolic compounds such as anthocyanins. They are associated with the prevention of illnesses generated by oxidative stress (Heba *et al*., 2014). H. sabdariffa calyx extracts has been studied extensively as a food colourant (Duangmal *et al*., 2008; Alobo and Offonry, 2009) and found to be a suitable replacement for various artificial colourants.

Plant materials generally contain a small amount of high added value active solute. Extraction and purification of bioactive compounds from natural sources have become very important for the utilization of phytochemicals in the preparation of dietary supplements or neutraceuticals, functional food ingredients, food additives, pharmaceutical and cosmetic products. The polar character of anthocyanins makes them soluble in several types of polar solvents such as methanol, ethanol, acetone, and water. Solvent extraction of anthocyanins is the initial step in the determination of total and individual anthocyanins prior to quantification, purification, separation, and characterization (Rivas, 2003). The extraction of anthocyanins is commonly carried out under cold conditions using methanol or ethanol containing a small amount of acid in order to obtain the flavylium cation form, which is red and stable in a highly acid medium (Du and Francis, 1973; Amor and Allaf, 2009). Extraction of anthocyanins is commonly carried out under cold conditions with methanol or ethanol containing a small amount of acid with the objective of obtaining the flavylium cation form, which is red and stable in a highly acid medium. However, acid may cause partial hydrolysis of the acyl moieties in acylated anthocyanins, especially in anthocyanins acylated with dicarboxylic acids such as malonic acid

(Bronnum and Flink, 1985). Abou *et al*. (2011) used ethanol acidified with 1.5 N/L HCl (85:15), ethanol acidified with 1% citric acid, 2% citric acid solution and distilled water in extracting the pigments from Roselle calyces. They concluded that the addition of acids to water or ethanol increased the efficiency of anthocyanins extraction compared with distilled water alone. In general, HCl was more effective than citric acid. Ethanol acidified with HCl, showed the strongest influence on the amount of anthocyanins extracted, followed by 2% citric acid solution and ethanol acidified with 1% citric acid. Frimpong *et al*. (2014) investigated the potential of aqueous extract of Hibiscus sabdariffa calyces as colouring agent in three pediatric oral pharmaceutical formulations and they found that Aqueous extract of H. sabdariffa calyces at a concentration of 33% w/v solution was successfully used to colour three pediatric oral formulations. Formulations coloured with the extract were susceptible to deterioration on exposure to light, pH and high temperatures. It can be concluded that the extract of H. sabdariffa calyces can be a good substitute for amaranth as a colouring agent for pediatric oral pharmaceutical formulations when buffered at pH 5.0 and protected against high temperatures and light.

2.2.12.1 Stability of colour

Anthocyanins are highly unstable molecules in food matrix. The colour stability of Anthocyanins is strongly affected by pH, solvents, temperature, anthocyanin concentration and structure, oxygen, light, enzymes, and other accompanying substances (Arueya and Akomolafe, 2014). Colour stability of anthocyanins depends on a combination of various factors including: structure of anthocyanins, pH, temperature, oxygen, light and water activity. Enzymatic degradation and interactions with food components such as ascorbic acid, sugars, metal ions,

sulfur dioxide and co-pigments are no less important (Abou *et al*., 2011). Among these factors, pH is one of the major factors significantly influenced the pigment colour variations and stability. In general, anthocyanins are more stable in acidic media at low pH values than in alkaline solutions. Anthocyanin is relatively unstable and because of their high reactivity it may be easily degraded and form colourless or undesirable brown – coloured compounds during extraction processing and storage (Durst and Wrolstad, 2001) Indeed, temperature, pH, light oxygen, metals, organic acids, sugars, ascorbic acid, enzymes, sulfer dioxide, co-pigmentation and interactions with food components may affect both the structure and stability of anthocyanins (Zuhaili *et al*., 2012). Bronnum and Flink (1985) reported that the efficiency of extracting solvent increased with increasing the concentration of citric acid and concluded that pH of extracting medium was the determining factor for anthocyanins extractability. Anthocyanins are easily susceptible to pH changes due to the ionic nature of anthocyanin. Anthocyanins exist as four equilibrium forms, namely the quinonoidal base, the flavylium cation, the carbinol (pseudobase) and the chalcone. Under low pH, the anthocyanin exists primarily in the the form of flavylium cation in red. As the pH is raised (>5), a rapid loss of proton occured to form quinoidal base that tend to become blue or violet. In addition, the increasing of pH causes the hydration of the flavylium cation to form a carbinol (pseudobase) or chalcone which are colourless (Amelia *et al*., 2013). Light is another factor, which affects the stability of anthocyanin. Palamidis and Markakis (1975) have studied the role of light on the stability of anthocyanin in grape juice and showed that exposure of the pigments to light accelerates their destruction. Their experiments showed that after placing the juice samples containing anthocyanin in dark for 135 days at 20°C, almost 30% of the pigments were destroyed, but

placing the same samples in the same temperature and same period of time in the presence of light destroyed more than 50% of total pigments. Palamidis and Markakis (1975) have studied the effect of temperature on the stability of anthocyanin in soft drinks and have shown that increase in the storage temperature greatly accelerate the destruction of pigments in soft drinks. Maccarone *et al*. (1985) reported the anthocyanin in red orange juice in 15°C, 25°C and 35°C during a 15 day period and found that the increase in temperature accelerates the destruction of anthocyanins.

2.2.13 Potential Health Benefit

Maccarone *et al*., (1985) compared the effects of diet based on soya bean protein (Plant protein) and casein (animal protein) supplemented or not with 0.1 % cholesterol and plasmalipoprotein lipid amount and their fatty acid composition lecithin; cholesterol acyl transferase activity, and lipid per oxidation. Their results suggested that the origin of dietary protein affects lipid peroxidation and polyunsaturated fatty acid bio synthesis and distribution among liver and different lipoprotein lipid classes, but plays only a minor role in the regulation of plasma and lipoprotein cholesterol concentration. Maccarone *et al*., (1985) compared of soya protein with animal protein or even the casein of cow milk and established that soya protein helps prevent atherosclerosis', where as animal protein from beef and milk contribute to atherosclerosis.

Prospective epidemiological evidence is conflicting regarding the role of animal protein vs plant protein in bone loss. Maccarone *et al*., (1985) found cross cultural relationship between hip fracture rates and dietary protein was positively related to animal protein intake and inversely related to vegetable protein intake. They explained that increases in dietary animal

protein intake. They explained that increase in dietary animal protein are associated with increase in urinary Ca excretion; an increase in osteoporotic fractures has frequently been attributed to an increase in dietary animal protein. On the other hand, a report by massey (2003) summarized that urinary Ca excretion is strongly related to net renal acid excretion. The catabolism of dietary protein generates ammonium ion and sulfates from sulfur containing amino acids. Bone citrate and carbonate are mobilized to neutralize these acids, so urinary Ca increase when dietary protein increases. However vegetable protein are known to have poorer nutritional quality than animal protein for human because they are inbalanced in the ratio of cystein to methionine needed to meet requirement, not because they are all lower in sulfur per gram of protein as eggs, milk and muscle from meat, poultry and fish. Therefore increasing intake of purified protein from either animal or plant sources similarly increases urinary Ca, plus, the effects of a protein on urinary Ca and bone metabolism are modified by other nutrient found in that protein food source. This concluded that animal and plant foods may have different effect on bone health, although these effects are mainly attributable to other constituent of the food and diet not protein.

Studies revealed that fibre rich foods such as wheat, bran, beans, oat bran, grain and legumes have important effect on serum cholesterol (Maccarone *et al*., 1985). Hainida *et al.,* (2008) indicated that the inclusion of 50 g/kg and 150 g/kg of dried sorrel seeds exhibited positive hypocholesterolemic effects but did not hold true when added at less than 50g/kg in hypercholesterolemic diets. Hainda and co workers observed significant substantial negative correlstion between high density lipoprotein (HDL-C) with total cholesterol (TC) and low density lipoprotein (HDL-C) with total cholesterol (TC) and low density lipoprotein (LDL-C) respectively. A positive correlation was observed between LDL-C and TC, since HDL-C is

negatively correlated to coronary heart diseases, consumption of sorrel seeds might be beneficial for cardio protective effect.

Numerous studies have been conducted to evaluate the possible effects of ingesting various sources of dietary fiber, Maccarone *et al*., (1985) found that serum triglycerides responses were lowered in the presence of oat bran, wheat fibre, or wheat germ and that chylomicron triglycerides were reduced by wheat fibre. Moreover, cholesterolemia decreased postprandially for 6 h, and was further lowered in the presence of oat bran. This was in agreement with Hainida *et al.,* (2008) who found that diet rich in soluble fibre had significantly lowered cholesterol concentration in the plasma and liver. Subsequently sufficient consumption of dietary fibre could reduce the risk of civilization diseases such as cardiovascular diseases, colon cancer and obesity. Some dietary fibres have also been reported to decrease the digestion and absorption of carbohydrate and postprandial serum glucose level.

Sorrel seeds had a considerable good ratio of soluble to insoluble fraction (Hainda *et al.,* 2008) compared to other common source of Dietary fibre such as wheat bran , oat bran and Rice bran (Hainida *et al.,* 2008). Kritchevsky *et al*., (1997) reported that the ratio of soluble to insoluble fraction in dietary fibers must be within the range of 1.0 to 2.3 in order to be able to exert a physiological effect. Dietary fibers may lower serum cholesterol by modifying the bile acid absorption and metabolism, producing short chain fatty acid from fiber fermentation in colon and alternating the concentration of the insulin and hormones (Kritchevsky *et al*., 1997). Previous research demonstrated that bile acid binds strongly to vegetable fiber (Hainida *et al.,* 2008). The hypocholesterolemic effect may be due to binding of dietary fibre from sorrel seed with bile acids which in turn increases its feacal excretion (Hainida *et al.,* 2008). A subsequent

increase in demand for cholesterol by the bile acid synthesis pathway, may divert cholesterol by the bile acid synthesis pathway may divert cholesterol away from the lipoprotein synthethic pathway, thereby reducing the serum cholesterol. Nevertheless, since other dietary components in the seeds could also influence the total cholesterol and distribution of cholesterol and distribution of cholesterol in different lipoprotein classes, it is not possible to definitvely assume that dietary fibersnalone linked to cholesterol lowering effects.

Despite dietary fiber, a high content of protein in the seeds might also be the reason for the hypochelsterolemic effect. Hainida *et al.,* (2008) reported that the consumption of vegetable protein may lead to change in the level of hormones that modulate plasma cholesterol. However, Kritchevsky *et al*., (1997) did not indicate any clear relationship between hormones levels and serum lipids. Hainida *et al.,* (2008) reported that methionine in the diet plays a central role in cholesterol lowering the effects through the regulation of Apo A-1 secretion from liver into the blood circulation. Sorrel seed powder contain a higher methionine content (Rao 1990; Hainda *et al.,* 2008) than other established cholesterol lowering foods such as soybean and grain. Hence it is reasonable to assume that amount of methionine in seed might play an important role in cholesterol lowering effets.

Kritchevsky *et al*., (1997) showed that the ratio of lysine to arginine might be an important determinant of atherogenicity. A higher ratio of lysine to arginine is associated with enhanced atheroscelerosis (Kritchevsky *et al*., 1997) Rao (1990). Abu Tarboush *et al.,* (1997) and Hainda *et al*., (2008) found that the lysine to arginine ratio was low in sorrel seed (0.4-0.5). Thus, despite dietary fibre, protein and methionine content, this amino acid ratio could also be one of the indicators of cholesterol lowering effects of sorrel seed.

Anti-oxidant are also considered to be important in the prevention of disease like cardiovascular disease. However Hainda *et al*., (2008) indicated that the anti-oxidant capacity of sorrel seeds was low and might not be an important determinant bin lowering the total cholesterol level. Abu Tarboush *et al.,* (1997) reported that some grain such as oat had significantly reduced LDL., myocardial infarction (MI) risk drops by 2% and for every 1% increase in HDL., MI risk drops 3-4%. This suggest that even a slight increase in HDL and decreased in LDL helps to reduce the risks of cardiovascular disorders. Therefore, the inclusion of sorrel seeds in the diet might potentially prevent the risk of atherosclerosis and coronary heart diseases.

2.2.14 Toxicology Effect

An experiment was concluded to study the effect of sorrel seeds on broiler performance, functions and bistopathology in liver. Histopathology studies demonstrated no damage in the liver due to sorrel seed in the diet. An in vitro study indicated that sorrel seeds can be included in levels up to 30% without adverse effect on weight gain on liver function.

Sorrel seeds, like other seeds and legumes may have anti nutritional factors that have been associated with a reduction in food digestibility, a decrease in nutrient bio availability and flatulence production. Abu-Tarboush (1997) reported that deffated flour and protein isolate of sorrel seeds contained protease inhibitor, phytic acid and gossypol but theywould not pose a problem if the seed are properly processed. Moreover, a study done by Schippers (2005) found that the replacement of soybean meal protein by 60% of sorrel seed meal into a catfish diet did not affect weight gain, growth response, feed conversion, protein utilization or carcass composition and histological abnormalities in the liver , it was observed by earlier worker that

different cooking methods improve the nutritional quality of food legumes to various extents(Kritchevsky *et al*., 1997).

2.2.15 Development of Atherosclerosis

Hardening of the arteries wall or also called atherosclerosis is a condition when fat, cholesterol, and other substances accumulate in the walls of arteries and form hard structures called plaques. Over the time, these plaques can block the arteries and cause problems throughout the body. Atherosclerosis is assumed one of the major causes for cardiovascular mortality. Plasma lipoprotein profiles in blood serum play an important role in the development of atherosclerosis. Proper maintenance of the high density lipoprotein (HDL) level and reduction of the low density lipoprotein (LDL) level has been a major goal in treating cardiovascular diseases.

LDL and HDL have opposite roles in body cholesterol regulation. LDL is always labeled as 'bad' lipoprotein due to its relationship in development of atherosclerosis. There is general agreement that the presence of small dense LDL is associated with an increased risk for coronary heart disease. Small dense LDL may play a role in the development of coronary heart disease due to its physiological properties, e.g. its high susceptibility to oxidation. Oxidation of low-density lipoprotein (LDL) is believed to be an important step in the atherogenic process. Lots of research on atherosclerosis reported elevated levels of oxidized LDL (Ox-LDL) in plasma of patients with atherosclerosis cardiovascular disease (Kritchevsky *et al*., 1997). Other risk factors which contribute to coronary heart disease (CHD) are age, blood pressure, cigarette smoking, diabetes, lack of physical activity, obesity and atherogenic diets.

As oppose to LDL, HDL is called 'good' lipoprotein. A number of reports have indicated that raising high density lipoprotein (HDL), can result in significant cardiovascular benefit (Kritchevsky *et al*., 1997). The protective effect of HDL toward atherosclerosis has been attributed primarily to its role in reverse cholesterol transport, the process whereby cholesterol is brought from peripheral cells to the liver for excretion in the bile. Recently, attention is being given to the possible anti-inflammatory activity of HDL, which may be mediated in part by enzymes and apolipoproteins that can inactivate LDL-derived oxidized phospholipids or prevent their formation.

There are increasing evidence to suggest an association between inflammation and the risk for cardiovascular disease. Pathologic studies have found that atherosclerotic lesions contain infiltrates associated with inflammation (Kritchevsky *et al*., 1997). The complex anti-inflammatory effect of HDL is related in part to the activity of HDL-associated enzymes. These include paraoxonase, which can prevent the oxidative modification of LDL (Reddy *et al.,* 2001) and platelet-activating factor (PAF) acetyl hydrolase, which hydrolyzes PAF, a proinflammatory phospholipids produced by activated platelets, leukocytes, and endothelial cells. Levels of both enzymes have been shown to decrease during the acute-phase response in mice.

The positive effect of antioxidant in preventing the formation of atherosclerosis is widely studied. Antioxidant substances are believed enable to suppress the onset and development of atherosclerosis. Compounds such as probucol, flavonoids and phenolic compounds have also been shown to have antioxidative effects and effective in preventing the formation and progression of atherosclerosis (Diaz *et al.,* 1997). These antioxidants were proved to give

positive effect by prevent LDL from oxidative damage in vitro and eventually interrupt the progression of atherosclerosis.

Besides antioxidant, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors (Statins) are drug used to prevent atherosclerosis development. Statins are one class of many drugs used to lower the level of cholesterol in the blood by reducing the biosynthesis of cholesterol by the liver. Statins block the HMG-CoA reductase enzyme in the liver that is responsible for making cholesterol (Kritchevsky *et al*., 1997). HMG-CoA reductase is membrane-bound enzyme found in the liver. It is involve in reduction process of HMG-CoA to mevalonate, the precursor in biosynthesis of cholesterol. HMG-CoA reductase is considered to be the rate-limiting enzyme of cholesterol synthesis and the pharmacological target of all statins. By blocking the HMG-CoA reductase enzyme, the level of cholesterol in blood serum can be reduced and eventually lowering the risk of atherosclerosis development. There are many types of HMG-CoA reductase inhibitors drug available in the market such as Lovastatin, Pravastatin, Simvastatin, Fluvastatin, Atorvastatin and Rosuvastatin.

2.2.17 Nutritional composition of sorrel seed

2.2.17.1 Protein and Amino acid

Protein are formed by amino acid linked by pepetide bonds, all amino acid have the same basic structure containing a central carbon atom surrounded by four groups; a hydrogen atom, an amine group (H=NH2), an acid group (COOH) and a distinctive side group. The side group, which vary from a single hydrogen atom to a complex ringed structure, distinguish the 21 different amino acid, generally dietary protein source are divided into two categories;

Animal protein such as meat, poultry, fish, egg, milk and milk product; 2. Plant protein such as legumes (peanut, peas, beans and lentils), cereal, vegetable and fruit, Animal product and the properties of amino acid in the protein of the animal origin comes closer to meet the human need than in most plant sources. Plant protein is potentially important in undeveloped and developing countries. Several countries in the south Asia such as Banglalesh , Bhiltan, india, Maldives, Nopa, Pakistan and sri lanka, the most staple; wheat legumes and edible oil are main import foods although a variety of animal foods are used by economicallhy priviledge people for a large section of the population animal food don not constitue part of their diet due to economic reason (Seeram,2002). Similarly a substancial part of their food is heightened during times of drought thaleads to poor cereal harvest. Thus the wild plant foods of the western sahel play an essential role in the survival an health of human population who utilizes them. Bean protein are found to be relatively high in, isoleucin, leucin, phenylalanine and valine. But they are different in sulphur containing amino acid. In particular methionine is the first limiting essential amino acid in legumes because the major storage protein globulin is low in the amino acid (Kritchevsky *et al*., 1997). Amino acid can be divided into essential and non essential. The non essential are those that can be synthesized by the body. In adults,12 of the amino acid can be synthesized and nine are essential, namely cystein, arginine. Protein is an essential component of the diet needed for the survival of living being. The basic fun ction of protein in nutrition is to supply adequate amount of amino acid. In developing countries, legumes are cheap and valuable potential source of protein an they are consumed in large quantities . Additionally legumes are consumed as economical supplementary protein with cereal. Today despite many advances in agriculture food and nutrition, there are still large segment of the population in developing and under developed

countries that suffers from protein malnutrition. Protein energy mal nutrition (PEM) remains the common nutritional disorder facing by the majority of the world's poor population. Therefore the most logical way to increase the world protein supply would be to make more plant protein available for human consumption. Hence research effort have be directed towards identification and evaluation of under exploited sources such as alternative protein crop (QI *et al*., 2005). Among plant based protein sources, cereal are the most important sources of dietary protein as well as energy for people in most of the developing countries of the world. However cereal protein are found to be low in concentration and poor in quality. In this regard, the potential of legumes that are still not widely used as dietary sources of protein are increasingly being assessed for the improvement of traditional legumes crop (QI *et al*., 2005).although several researcher showed that soya bean (Glycine Max.L) can supply more potential protein than any other crops (Messina,1999) and be excellent sources of low cost protein, the use of soya bean is currently limited to under developed countries especially in African countries.

Therefore, there is a need to explore other sources of concentrated plant protein (Vose 1980) which ideally should be crops that are widely grown in tropical countries.

Many studies have been carried out to asses the potential of sorrel source as a new source of protein and oil (El-Adawy and Khalil 1994;Rao 1990; Abu tarboush *et al*., 1997).

2.2.16.2 Lipids

The value of sorrel seed fat was considerable even when compared with other seed, palmitic, oleic, linoleic acid (Rao 1990) and stearic acid (El-Adawy and Khalil 1994) were the major fatty acid constituent in sorrel seed. A high content of unsaturated fatty acid was 1.2 (El-Adawy and khalil 1994). In contrast Mohumuddin and Zaidi (1975) found a ratio of saturated

to unsaturated fatty acid to be 1;3, sorrel seed have been reported to have similar properties for cotton seed oil as a substitute for crude castor oil. There are different fatty acid present in sorrel seed namely myrisitic (2.1%), palmitic,(33.2%), palmioleic (2.0%),stearuc (3.4%), oleic (34.0%), linoleic (14.4%).13-epoxy-cis -9 octadecenic (12,13-epoxoliec) (4.5%) stenilic (2.9%) and Malvalic (1.3%) (QI *et al*., 2005). However there is limited published data on sorrel seed at or lipids.

2.2.16.3 Other Components

Carbohydrate supply the greatest percentage of calories and bulk in an average diet, yet they make up less than 1% of total body weight. The main function of dietary carbohydrate is to supply energy. The carbohydrate content of sorrel has been understudied. Nevertheless, El Adawy and Khalil (1994) found that the sorrel seed of different cultivars have a high content total carbohydrate content in the ranging from 36-38%. A study by Hamida *et al*., (2008) however, found a low (-13%) carbohydrate content in the seeds.

Dietary fiber is a collective term for a variety of plant substances that are resistant to digestion process of human gastrointestinal enzymes. Different type of fiber are distinguished by their differing physicological properties and systemic effects, Dietary fiber is grouped into two namely; water soluble and in soluble, depending on their solubility in water. Water soluble fiber will dissolve in hot water, whereas insoluble fiber will not (QI *et al*., 2005). The structural matrix fiber (Lignin, cellulose an some hemicelluloses) are insoluble. The natural gel forming fibers, which are pectin,, gums, mucilages and the remainder of the hemicelluolose are soluble (Bravo,

1998). According to Rao (1990), sorrel seed are rich in dietary fiber (39-42%). This contribute to the strength in the seed if compared to other varieties of common sources of dietary fiber.

2.3 Dry ice

Dry ice, sometimes referred to as "**cardice**" (chiefly by British chemists), is the solid form of carbon dioxide. It is used primarily as a cooling agent. Its advantages include lower temperature than that of water ice and not leaving any residue (other than incidental frost from moisture in the atmosphere). It is useful for preserving frozen foods where mechanical cooling is unavailable. Dry ice sublimates at 194.65 K (−78.5 °C; −109.3 °F), at Earth atmospheric pressures. This extreme cold makes the solid dangerous to handle without protection due to burns caused by freezing (frostbite). While generally not very toxic, the outgassing from it can cause hypercapnia (abnormally elevated carbon dioxide levels in the blood) due to buildup in confined locations.

2.3.1 History of Dry Ice

It is generally accepted that dry ice was first observed in 1835 by French inventor Adrien-Jean-Pierre Thilorier (1790–1844), who published the first account of the substance (Khanna and Kapila, 2008). In his experiments, he noted that when opening the lid of a large cylinder containing liquid carbon dioxide, most of the liquid carbon dioxide quickly evaporated. This left only solid dry ice in the container. In 1924, Thomas B. Slate applied for a US patent to sell dry ice commercially. Subsequently, he became the first to make dry ice successful as an industry. In 1925, this solid form of CO2 was trademarked by the DryIce Corporation of America as "Dry ice", thus leading to its common name (Khanna and Kapila, 2008). That same year the Dry Ice Company sold the substance commercially for the first time; marketing it for

refrigerating purposes. The alternative name "Cardice" is a registered trademark of Air Liquide UK Ltd. It is sometimes written as "card ice" (Treloar, 2003).

2.3.2 Manufacture of Dry Ice

Dry ice is easily manufactured. First, gases with a high concentration of carbon dioxide are produced. Such gases can be a byproduct of another process, such as producing ammonia from nitrogen and natural gas, oil refinery activities or large-scale fermentation (Khanna and Kapila, 2008). Second, the carbon dioxide-rich gas is pressurized and refrigerated until it liquefies. Next, the pressure is reduced. When this occurs some liquid carbon dioxide vaporizes, causing a rapid lowering of temperature of the remaining liquid. As a result, the extreme cold causes the liquid to solidify into a snow-like consistency. Finally, the snow-like solid carbon dioxide is compressed into small pellets or larger blocks of dry ice. A teaching laboratory demonstration for the production of dry ice is to use a 15 lb aluminum carbon dioxide fire extinguisher with a porous fabric-collecting bag over the nozzle. The Joule-Thomson expansion of the gas lowers the temperature enough to produce an approximate 50/50 mixture of CO2 gas and dry ice snow also called dust that is collected in the bag. This is an inefficient process, and unsuitable for even lab-scale production. The expansion is inefficient, fire extinguishers are an expensive way to buy carbon dioxide, especially when a signed and dated fire extinguisher certification is required.

Dry ice is typically produced in three standard forms: large blocks, cylindrical small (5/8 or 1/2 inch diameter) pellets and cylindrical tiny (1/8 inch diameter), high surface to volume pellets that float on oil or water and do not stick to skin because of their high radii of curvature. Tiny dry ice pellets are used primarily for ice blasting, quick freezing, fire fighting, oil solidifying and have

been found to be safe for experimentation by middle school students wearing appropriate Personal Protective Equipment such as gloves and safety glasses. A standard block weighing approximately 30 kg covered in a taped paper wrapping is most common. These are commonly used in shipping, because they sublime relatively slowly due to a low ratio of surface area to volume. Pellets are around 1 cm (0.4 in) in diameter and can be bagged easily. This form is suited to small scale use, for example at grocery stores and laboratories where it is stored in a thickly insulated chest

Plate 5: Picture of Dry Ice
(http://www.dryiceinfo.com/selling.htm, 2015).

2.3.3 Applications of Dry Ice

2.3.3.1 Commercial use of Dry Ice

The most common use of dry ice is to preserve food (Yaws, 2001), using non-cyclic refrigeration. It is frequently used to package items that must remain cold or frozen, such as ice cream or biological samples, without the use of mechanical cooling. Dry ice can be used to flash-freeze food) or laboratory biological samples carbonate beverages make ice cream, solidify oil spills and stop ice sculptures and ice walls from melting.

Dry ice can be used to arrest and prevent insect activity in closed containers of grains and grain products, as it displaces oxygen, but does not alter the taste or quality of foods. For the same reason, it can prevent or retard food oils and fats from becoming rancid. When dry ice is placed in water, sublimation is accelerated, and low-sinking, dense clouds of smoke-like fog are

created. This is used in fog machines, at theaters, haunted house attractions, and nightclubs for dramatic effects. Unlike most artificial fog machines, in which fog rises like smoke, fog from dry ice hovers near the ground. Dry ice is useful in theater productions that require dense fog effects (McCarthy 1992). The fog originates from the bulk water into which the dry ice is placed, and not from atmospheric water vapor (as is commonly assumed) (Kuntzleman *et al*., 2015). It is occasionally used to freeze and remove warts (Lyell, 1966). However, liquid nitrogen performs better in this role, since it is colder so requires less time to act, and less pressure (Goroll and Mulley, 2009). Dry ice has fewer problems with storage, since it can be generated from compressed carbon dioxide gas as needed (Goroll and Mulley, 2009).

2.3.3.2 Scientific use of Dry Ice

In laboratories, a slurry of dry ice in an organic solvent is a useful freezing mixture for cold chemical reactions and for condensing solvents in rotary evaporators (Housecroft, 2001). Dry ice/acetone forms a cold bath of −78 °C, which can be used for instance to prevent thermal runaway in a Swern oxidation. The process of altering cloud precipitation can be done with the use of dry ice (Keyes, 2006). It was widely used in experiments in the US in the 1950s and early 60s before it was replaced by silver iodide (Keyes, 2006). Dry ice has the advantage of being relatively cheap and completely non-toxic (Keyes, 2006). Its main drawback is the need to be delivered directly into the supercooled region of clouds being seeded (Keyes, 2006).

2.3.4 Properties of Dry Ice

Dry ice is the solid form of carbon dioxide (CO2), a molecule consisting of a single carbon atom bonded to two oxygen atoms. Dry ice is colorless, non-flammable, with a sour zesty odor, and can lower the pH of a solution when dissolved in water, forming carbonic acid

(H2CO3) (Yaws, 2001). At pressures below 5.13 atm and temperatures below −56.4 °C (−69.5 °F) (the triple point), CO2 changes from a solid to a gas with no intervening liquid form, through a process called sublimation (Yaws, 2001). The opposite process is called deposition, where CO2 changes from the gas to solid phase (dry ice). At atmospheric pressure, sublimation/deposition occurs at −78.5 °C (−109.3 °F) or 194.65 K. The density of dry ice varies, but usually ranges between about 1.4 and 1.6 g/cm3 (87 and 100 lb/cu ft).(Häring, 2008) The low temperature and direct sublimation to a gas makes dry ice an effective coolant, since it is colder than water ice and leaves no residue as it changes state (Yaws, 2001) Its enthalpy of sublimation is 571 kJ/kg (25.2 kJ/mol).

Dry ice is non-polar, with a dipole moment of zero, so attractive intermolecular van der Waals forces operate (Khanna and Kapila, 2008) The composition results in low thermal and electrical conductivity (Khanna and Kapila, 2008).

2.3.5 Safety of Dry Ice

Prolonged exposure to dry ice can cause severe skin damage through frostbite, and the fog produced may also hinder attempts to withdraw from contact in a safe manner. Because it sublimes into large quantities of carbon dioxide gas, which could pose a danger of hypercapnia, dry ice should only be exposed to open air in a well-ventilated environment (Horrell, 1961). For this reason, dry ice is assigned the S-phrase S9 in the context of laboratory safety. Industrial dry ice may contain contaminants that make it unsafe for direct contact with foodstuffs (Khanna and Kapila, 2008). Tiny dry ice pellets used in dry ice blast cleaning do not contain oily residues.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Materials

Dried calyces of Hibiscus Sabdariffa, Sugar, Strawberry flavor, Dry Ice, Plastic bottle. The fresh dried calyces of Hibiscus sabdariffa (dark red in colour), granulated sugar and strawberry flavor was purchased from Ifo main market (Nigeria). Dry ice was purchased from Greatsmith Resources Limited, Lagos (Nigeria).

3.1.1 Equipment

Water bath (W-10), Oven (ASTM F1360-17), Incubator (Fisher scientific 630D), pH meter (3015, Jenway®, U. K), Blender (Kenwood blender BL227), Hand refractometer (BS eclipse 43-03), Microscope (Nikon eclipse E200), Colony counter (EM science MAS-100), Aspirator (FTA-1).

Utensils: Sieve, Plastic bowl, Pot, Knife, Stirrer.

3.2 Methodology

3.2.1 Processing of Zobo drink

According to Okolie and Ojimelukwu (2003), four hundred (400) gram of Hibiscus Sabdariffa Calyx was boiled with 12 liters of distilled water for 5 minutes, cooled for 30 minutes and filtered. The extract was sieved with muslin cloth, 1.4kg of granulated sugar was added to enhance quality. Prepared Zobo drink was given the following treatments. A= Bottling (control), B= Bottling followed by pasteurization at 80^0C for 20 minutes, C= Carbonation followed by bottling and then pasteurization at 80^0C for 20 minutes.

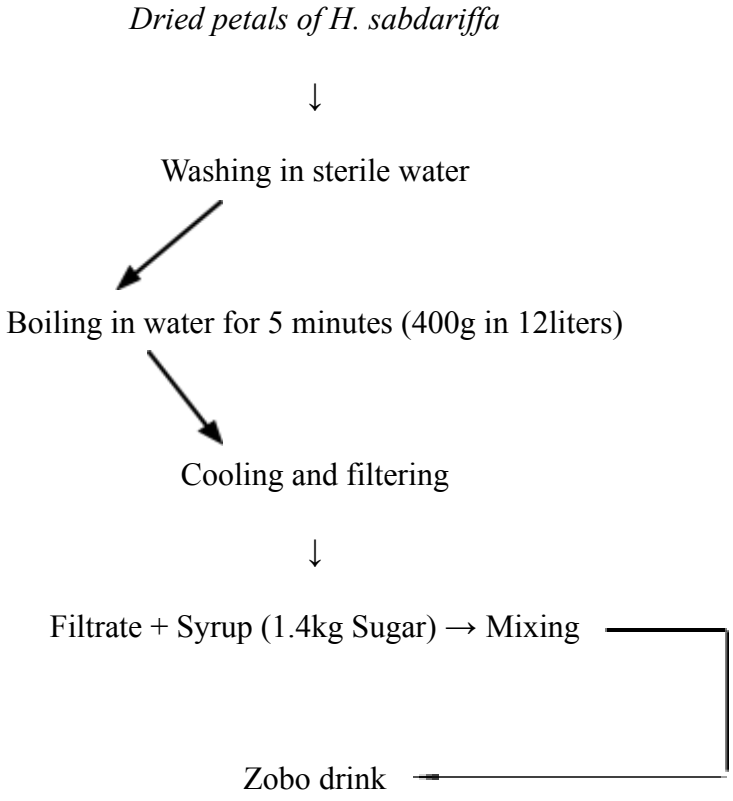


Figure 3 : Steps in the preparation of Zobo drink

(Adapted from: Nwafor and Ikenebomeh, 2009 with little modification)

3.2.2 Carbonation and Pasteurization

2kg of dry ice was added to each gallon of the Zobo drink (www.eagledis.com). The carbon dioxide produced by the dry ice dissolves in the drink producing a carbonated beverage. The dry ice was added to the drink prior to sealing. According to Okolie and Ojimelukwu (2003), the Zobo samples for pasteurization was labelled accordingly and then pasteurized at 80^0C for 20 minutes.

3.2.3 Physicochemical analysis of Zobo

The physicochemical qualities of the samples was determined using the methods described by Ademoranti (1996) and Sadiq and Malami (2009). The parameters determined include; pH, lactic acid content, total suspended solids and turbidity.

3.2.3.1 Determination of pH

This was determined using pH meter 3015, Jenway®, U. K. Ten milliliter of the sample was placed in a beaker. A buffer solution was used to zero and calibrate the pH meter. Then the electrode of the pH meter was inserted into each of the samples and the pH readings were taken.

3.2.3.2 Determination of Specific gravity

The Specific gravity of Zobo drinks given different treatments was determined using a pynconometer according to AOAC method (AOAC, 1970).

Specific gravity was calculated as follows;

$$\text{Specific gravity} = \frac{W_2}{W_1}$$

Where W_1 = weight of equivalent volume of distilled water.

W_2 = weight of sample.

3.2.3.3 Determination of lactic acid

A mass of 10g was weighed out of the beverages and added into a conical flask. About 30 ml of distilled water was added to the sample and mix well by shaking. This was followed by the addition of 5 drops of Phenolpthalein indicator. The burette was filled with 0.1 M Sodium hydroxide solution. The solution was titrated until it retains a very slight pink tinge after mixing. After which the volume of sodium hydroxide used was recorded. The titration for each sample was repeated three times, and the percentage of lactic acid in the sample was calculated using the equation below:

$$\% \text{ acid} = \frac{(\text{mL of NaOH used}) * (\text{conc. Of NaOH}) * 0.09 * 100}{\text{Weight of sample used}}$$

3.2.4 Microbiological analysis

An aliquot of 1mL of the various serially diluted samples was placed each on Nutrient agar (for total bacterial load estimation), Potato Dextrose Agar (PDA) (for fungal isolation) according to Bradshaw (1979). The nutrient agar plates were incubated at 37^0C while the PDA plates were incubated at room temperature 28 ± 2^0C for 3-5 days. Pure cultures of the isolates morphological cultural and biochemical characteristics were established.

3.2.4.1 Viable Count of Bacteria

The method used for the serial dilution of samples was that described by Pelczar, et. al., (1993). The spread-plate method was used in determining the viable count of the various samples of zobo drink. One milliliter of the various dilutions were transferred to sterile nutrient agar in Petri dish that had been oven dried at 37^0C for 30 minutes. A bent sterile glass rod was used in spreading the diluted samples properly on the surface of the nutrient agar plates. The plates were incubated at 37^0C for 28-48 hours. Different bacterial colonies present in the plates were counted and multiplied by the reciprocal of the appropriate dilution factor to obtain the viable count.

3.2.4.2 Isolation of Microorganisms

The streak plate method was used in isolating pure cultures as described by Seiyaboh, *et al.,* (2013). The organisms were isolated from the Zobo drink samples on the first day of storage. Loopful of the samples (a, b, c) were aseptically collected and streaked on agar plates. The plates were incubated at 37^0C for 24 hours.

3.2.4.3 Identification of Isolated Microorganisms

Discrete colonies were picked with a sterile wire loop and transferred aseptically to fresh agar plates. The essence being to obtain pure cultures of organisms that can be used for further analysis. The identification of microorganisms was based on such tests as:- Gram Reaction, Colonial Morphology, Cell Morphology, Biochemical tests and sugar fermentation reactions. Biochemical tests carried out included:- motility citrate utilization, methyl red, vogues posteur, indole, coagulase and catalase tests. The identification sequence for single bacterial culture in the manual for identification of medical bacteria (Cowan and Steel, 1974) was used in identifying the isolates.

3.2.5 Storage Studies

The different samples control, pasteurized (PO) and sample with CO_2 plus pasteurization (COP) will be kept at room temperature (28±2°C) (Egbere *et al*., 2007). They were then monitored periodically for a month on a weekly basis for changes in their microbial and physicochemical characteristics.

3.3 Determination of sensory properties of fresh zobo drinks

According to Okolie and Ojimelukwe (2003) the sensory evaluation of zobo drink samples given different treatments was carried out using a 22-member untrained panelists drawn from the Polytechnic community. The sensory attributes evaluated by the panelists were colour, taste, flavor, and general acceptability. A 9-point hedonic scale was used to evaluate the ratings of the product where 1 = extremely disliked, 9 = extremely liked. Statistical differences between the treatments were determined by the analysis of variance (ANOVA). The means were separated using Least significant difference (LSD) values.

3.4 Statistical Analysis

Data obtained were subjected to SPSS 19.0 of mean, standard deviation and analysis of variance (ANOVA). The significant value was determined using Duncan multiple test (Steel and Torrie, 1980).

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

4.1 RESULT

Table 2 shows the quality characteristics of fresh sweetened Zobo drinks. Freshly extracted Zobo drink had a total soluble solid content of 15.57^0 Brix, a titratable acidity of 0.45, a pH of 2.49 and a total microbial count 3.6×10^2 (cfu/ml).

The change in pH with period of storage is shown in Figure 4. Titratable acidity values increased sharply for samples C and CoP (0.45 to 0.69 and 0.28 to 0.71 respectively at the end of the storage period) while increase was lower for sample P (0.38 to 0.68) (Figure 5). Total soluble solids also decreased with the period of storage (Figure 6). Decrease in total soluble sugar content was rapid and significant higher ($p<0.05$) in the control sample and sample CoP (carbonated and pasteurized) than in sample P (pasteurized). The initial specific gravity values of all the samples were markedly similar. This result is shown in Table 3.

Table 4 shows the microbial counts of freshly prepared Zobo drink prepared under proper hygienic conditions and given different treatment. Table 5 shows the total aerobic count of the Zobo samples from week 1 through to week 4 of storage. The control sample has the highest the aerobic count at the end of the storage period with a total count of 4.82×10^5 followed by carbonated and pasteurize (CoP) sample with a total count of 3.02×10^5 and lastly the pasteurized sample with a total count of 1.46×10^5. Table 6 shows the yeast and mould count of Zobo samples form week 1 to week 4. The Morphological details of the Fungal isolates from the Zobo samples are shown in Table 7. A total of four Fungal isolates were identified which include *Penicillium sp*, *Fusarium*, *Aspergillus flavus*, *Rhizopus sp*. The biochemical characteristics of the bacteria isolates form the Zobo samples are shown in Table 8. A total of six bacterial were identified which include *Micrococcus sp*, *Streptococcus sp*, *Bacillus sp, Proteus, Enterobacter sp, Escherichia coli.*

Effects of the different treatments on the organoleptic quality such as appearance, taste, flavor, and colour on the freshly prepared zobo samples is shown in Table 9. The various quality attributes were different in the different treatment given to zobo samples. Overall acceptability score was in the order: P (pasteurized), C (control), CoP (carbonated and pasteurized).

Table 2: Quality Characteristics of Fresh, Sweetened Zobo Drink

Parameter	**Value**
Titratable acidity (%)	0.45
pH	2.49
Total Soluble Solids (^{0}brix)	15.57
Microbial Count (Cfu/ml)	$3.6x10^2$ (Aerobic count) $4.6x10^2$ (Yeast and Mould count)

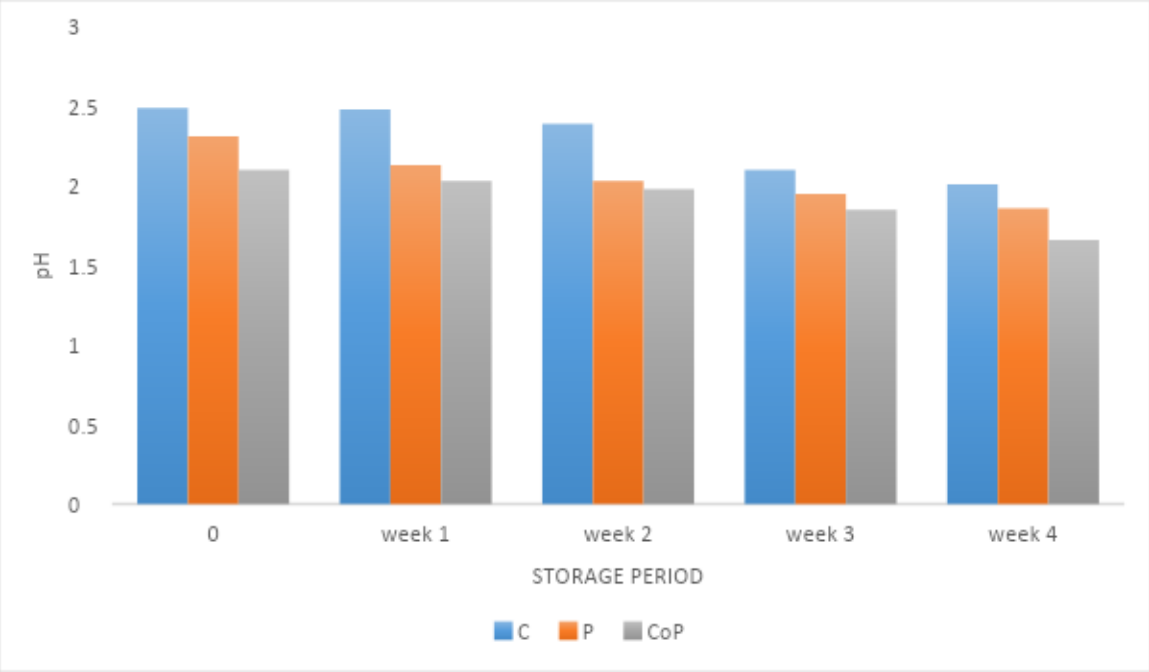

3
2.5
2
1.5
1
0.5
0
pH
0
week 1
week 2
week 3
week 4
STORAGE PERIOD
C
P
CoP

Figure 4: pH of Zobo drink samples at different storage periods.

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

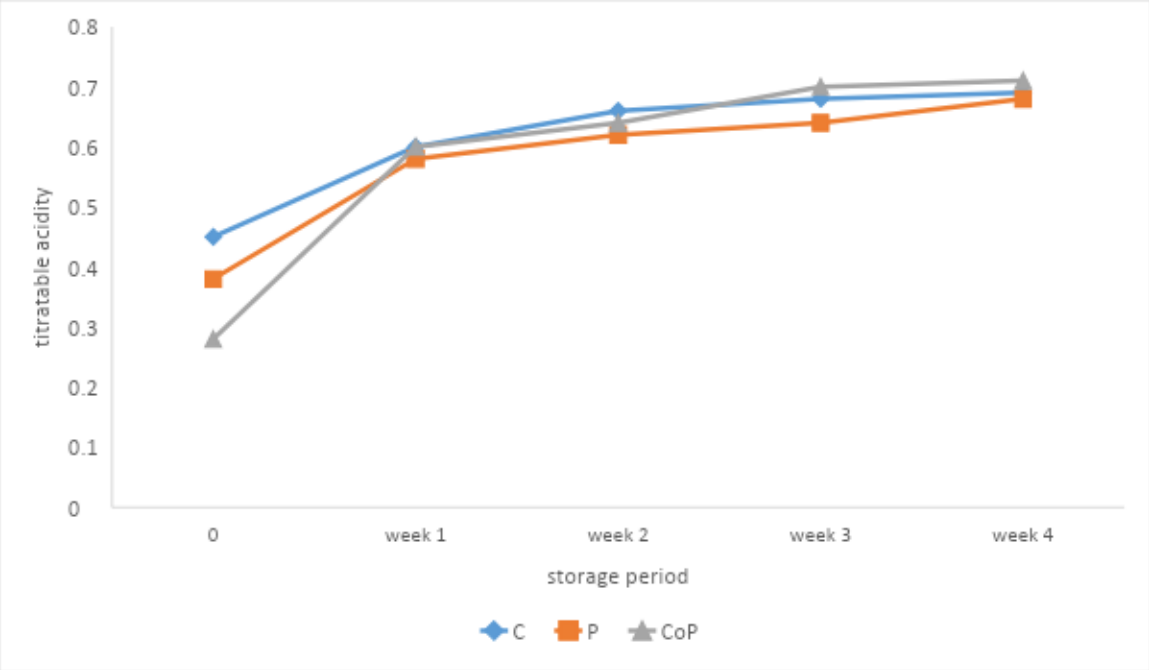


Figure 5: Titratable acidity of Zobo drink samples with different treatments.

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

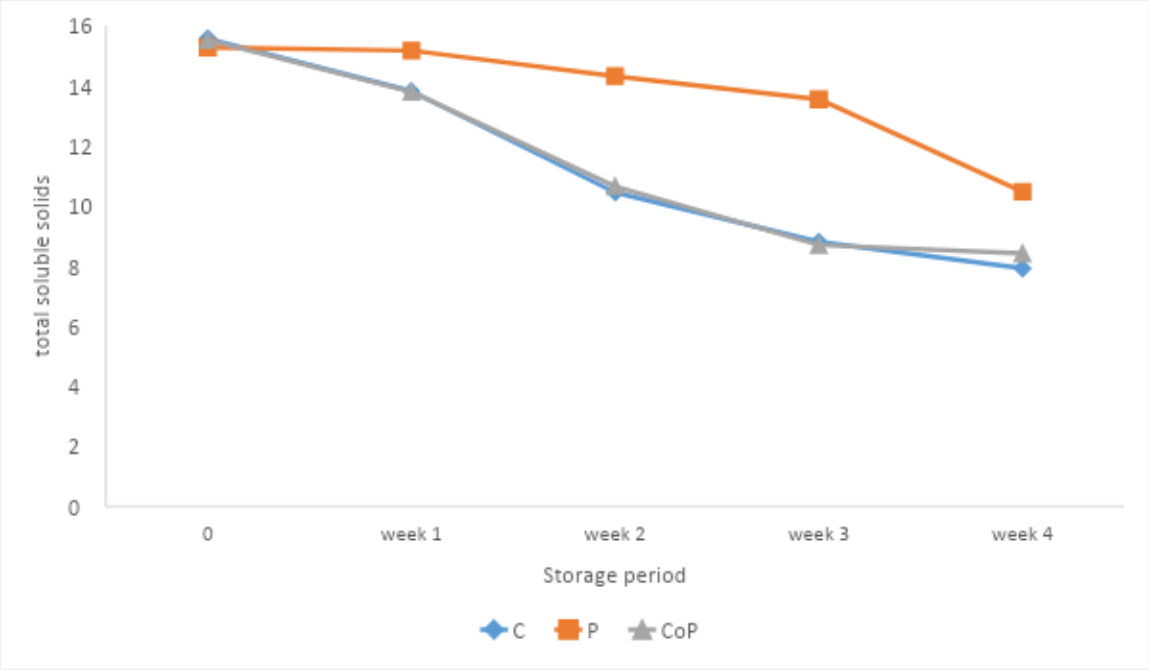


Fig 6: Total soluble solids of Zobo drink samples at different storage periods.

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

Table 3: Effect of Storage period on the Specific Gravity of Zobo drinks given different treatments.

Storage Period	C	P	CoP
0	1.03^{e} (0.00)	1.03^{a} (0.00)	1.02^{e} (0.00)
Week 1	1.03^{d} (0.00)	1.03^{a} (0.00)	1.02^{e} (0.00)
Week 2	1.03^{c} (0.00)	1.03^{a} (0.00)	1.02^{b} (0.00)
Week 3	1.03^{b} (0.00)	1.03^{a} (0.00)	1.02^{a} (0.00)
Week 4	1.02^{a} (0.00)	1.02^{a} (0.00)	1.02^{a} (0.00)

Each value is the mean standard deviation of duplicate determinations

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

Table 4: Microbial counts of freshly prepared Zobo drink

Sample	**Total Aerobic Count**	**Total Fungal Count (Yeast and Mould count)**
C	3.6×10^2	4.6×10^2
P	2.0×10^2	3.9×10^2
CoP	2.9×10^2	2.8×10^2

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

Table 5: Total aerobic count of the Zobo samples

Sample	**C**	**P**	**CoP**
Week 1	2.42×10^3	1.18×10^3	2.42×10^3
Week 2	2.74×10^4	3.48×10^4	2.24×10^4
Week 3	4.22×10^4	1.58×10^4	2.92×10^4
Week 4	4.82×10^5	1.46×10^5	3.02×10^5

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

Table 6: Yeast and mould count of Zobo samples

Sample	**C**	**P**	**CoP**
Week 1	4.52×10^3	8.0×10^2	1.12×10^3
Week 2	3.14×10^4	1.62×10^4	2.08×10^4
Week 3	3.11×10^5	0.93×10^4	1.98×10^5
Week 4	5.18×10^5	2.27×10^5	3.78×10^5

C = Control P = Pasteurization CoP = Carbonation and Pasteurization

Table 7: Morphological details of yeast and moulds isolates of Zobo sample

Isolate	**Colony colour on PDA on front slide**	**Colony colour on PDA on reverse side**	**Microscopic features**
Penicillium spp	Ivy green	Creamy	Short conidiosphores bearing a compactverticil of mutalae, phialides closely packed in clusters bearing catenate conidia arranged in basipetal manner, conidia elliptical, smooth, and hyaline.
Fusarium	Wooly white	Colourless	Conidiophores slender and simple, short or branched irregularly or bearing a whorl of phialides; conidia hyaline, variable, principally of two kinds, macroconia several celled slightly curved or bent at the point ends, microconida 1-celled, ovoid or oblong, borne singly or in chains.
Aspergillus flavus	Yellow green	Colourless	Conidiophores arise separately from foot cell, phialides uniseriate and sometimes biseriate; conidia globose to subglobose.
Rhizopus sp.	Grey	Colourless	Zygomycete, spores, dark upright, and cylindrical shape.

Table 8: Biochemical characteristics of bacteria isolates from Zobo samples

Isolate Code	Gram Reaction	Shape	Motility	Catalase	Coagulase	Oxidase	Citrate	Hydrogen Sulphide	Urease	Indole	Methyl Red	VP	Glucose	Lactose	Mannitol	Sucrose	Probable Identity
Zo1	+	Cocci	-	-	-	-	-	-	-	-	-	+	A	A	-	A	**Micrococcus sp**
Zo2	+	Cocci	-	-	-	-	-	-	-	-	-	+	A	A	-	A	**Streptococcus sp**
Zo3	+	Rods	-	+	-	+	+	-	-	-	-	+	A	-	A	A	**Bacillus sp**
Zo4	-	Rods	+	+	+	+	+	+	-	+	+	+	AG	-	-	-	**Proteus**
Zo5	-	Rods	+	+	+	-	+	-	-	-	-	+	A/G	A/G	-	-	**Enterobacter sp**
Zo6	-	Rods	+	+	+	-	-	-	-	+	+	-	A/G	A/G	-	-	**Escherichia coli**

KEY:	VP	Voges-Poskauer	A Acid production only
	+	Positive	A/G Acid production with gas
	-	Negative	

Table 9: Sensory Characteristics of Zobo drink given different treatment

	Appearance	**Taste**	**Flavour**	**Colour**	**Overall acceptability**
C	7.40[a]	8.00[b]	7.50[a]	7.50[a]	7.80[a]
P	7.35[a]	7.60[ab]	7.35[a]	7.75[a]	7.90[a]
CoP	7.35[a]	7.00[a]	7.60[a]	7.80[a]	7.45[a]

Each value is the mean standard deviation of duplicate determinations

4.2 DISCUSSION

The pH value of the freshly prepared Zobo drink 2.49 (Table 2) imply that it belongs to a class of foods referred to high acid foods (Frazier and westhoff, 1988). An advantage of an acid food is that it does not support survival of many pathogenic organisms but favours the proliferation of acidic organisms (Okolie and Ojimelukwe, 2003). This explains the domination of in occurrence in the beverage (Table 8) and this corroborates the result of Okolie and Ojimelukwe, 2003. The graphical representation of the pH of the beverage with respect to storage time indicated a general decrease in pH and corresponding increase in acidity. The pattern is obviously due to the acid producing activities of spoilage bacteria, Bacillus sp (Egbere *et al*., 2007). Metabolic activities of microorganisms may have caused the release of metabolites such as organic acids resulting in increased titratable acidity and subsequent lower pH values recorded. Increase in titratable acidity is indicative of increased microbial activity. The high titratable acidity and low pH values would be responsible for the sour taste (Okolie and Ojimelukwe, 2003). The high total soluble sugar content is attributable to the sugar added during processing (Okolie and Ojimelukwe, 2003). The high titratable acidity and low pH values would be responsible for the sour taste as reported by Okolie and Ojimelukwe (2003). On the other hand reduction in total soluble solids (TSS) in the packaged sample may be attributed to utilization of TSS by the microorganisms especially fungi (Nwafor and Ikenebomeh, 2009). This is similar to the observed decrease in reducing sugar content of tomatoes inoculated with

Alternaria tenuis (Efiuvwevwere and Eka, 1991). The pattern is obviously due to the acid producing activities of spoilage bacteria, Lactobacillus, and Bacillus sp. (Okolie and Ojomelukwe, 2003). There were no significant differences between the specific gravity values ($P > 0.05$) within the storage period under storage (for samples P) while there is significant difference between the specific gravity values ($p<0.05$) of sample C and CoP. The slight decrease in specific gravity was due to gradual degradation of soluble sugars (Okolie and Ojimelukwe, 2003).

The Zobo juice produced from the calyces of Hibiscus sabdariffa was very acidic with low pH values as was reported by Frazier and Westhoff (1986). The high acidity of the juice could account for the low numbers and few types of organisms isolated from the fresh preparation (Table 7&8). There was gradual increase in the population as the organisms utilize nutrients from the Zobo beverage and some of the preservatives (5&6). Zobo drink when left for two or three days at room temperature turns sour. This may result from fermentation due to microbial action. This fermentation process has been found to lead to loss of taste and nutritional value, increased rate of browning and offensive odour, and perharps presence of cloudy materials at the bottom of the container (Adenipekun, 1998).

Results obtained showed that Zobo drink, raw or preserved suppots the growth and proliferation of a wide variety of microorganisms. Some of the isolates have been found to be associated with food spoilage and intoxication (Stainer *et al*., 1987; Prescott *et al*., 1999) and portends health risk to consumers. Omemu *et al*. (2005) suggested that the presence of microorganisms in Zobo drink produced with a boiling method is indicative of post-production contamination during the addition of sugar and other additive(s). Bacillus sp are spore former and can withstand adverse

effects of preservative(s). Spores are extraordinarily resistant to environmental stress such as heat, ultraviolet radiation, chemical disinfectant and desiccation (Prescott *et al*., 1999; Prescott *et al*., 2002). The microbial load of samples given different treatments, increased with storage period. The control sample was still safe for consumption after two weeks of storage since its microbial load count was still lower than the value reported as indicative of unsafe samples (above 1x 10^5 cfu/ml) (NAFDAC, 2004), the carbonated and pasteurized samples were still safe for consumption after three weeks of storage. At the end of the storage period the drinks were no longer safe for consumption.

Sensory evaluation of the drinks were made on freshly treated samples before they were put into bottles and corked. Essentially, Pasteurization negatively affected the overall acceptability of the Zobo drinks. There was no significant difference ($p>0.05$) in the appearance, colour and flavor of the Zobo samples given different treatments, but there was significant difference ($p<0.05$) in the taste of the Zobo samples. Pasteurization is known to drive off volatiles thereby affecting the flavor of juices. Physical and chemical properties are altered (El-Hashiny *et al*., 1980). Reconstituted pasteurized juices have worse flavor changes (Shaw *et al*., 1993). Pasteurization in food processing is primarily used to destroy pathogenic microrganisms. In the process, enzymes may be inactivated and ascorbic acid and volatiles may be lost.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The conclusion of this research work can be deduced from the following strings;

1. This research found that pasteurization and carbonation were really capable of extending the shelf life of the drink to three to four weeks after the day of production, being capable of either eliminating some bacteria or inhibiting the rapid acid, or alcohol production by the spoilage microbial agents.
2. The study found that pasteurized and the carbonated plus pasteurization samples were more preserved than the control samples.

5.2 Recommendation

From the conclusion above it is obvious that pasteurization and carbonation elongates the shelf life of the drink but it can be further worked upon to extend the shelf life more. Therefore, attention should be focused on the carbonation, pasteurization and addition of natural preservatives such as Kolanut, Lime, Clove.

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APPENDICES

Appendix I: Picture of Control sample of the Zobo drink

Appendix II: Picture of Pasteurized sample of Zobo drink

Appendix III: Picture of Pasteurized and Carbonated sample of Zobo drink

Appendix IV: Picture of Dry Ice