NUTRITIONAL & PHYTOCHEMICAL COMPOSITION, FUNCTIONAL PROPERTIES OF ROSELLE (*Hibiscus sabdariffa*) AND SENSORY EVALUATION OF SOME BEVERAGES MADE FROM ROSELLE CALYCES

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Nutritional & Phytochemical Composition, Functional Properties of Roselle (*Hibiscus Sabdariffa*) and Sensory Evaluation of Some Beverages made from Roselle Calyces

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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JKUAT, Kenya.

DEDICATION

This thesis is dedicated to my family with love.

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ABBREVIATIONS AND ACRONYMS

AA Antioxidant activity

AAS Atomic absorption spectroscopy

ANOVA Analysis of variance

AOAC Association of Official Analytical Chemists

CAC Codex Alimentarius Commission

CE Catechin Equivalence

CFU Colony forming units

CHOs Carbohydrates

Ch* Chroma

CIE LAB Commission Internationale Laboratoire

Conc. Concentration

CRD Complete Randomized Design

DMB Dry matter basis

DWB Dry weight basis

DPPH Diphenyl Picryl Hydrazyl Radical

EC_{50} Efficient coefficient

FAO Food and Agricultural Organization

FOSHU Foods for Specified Health Use

FST Food Science and Technology

GAE Gallic Acid Equivalent

GC Gas Chromatography

GC MS Gas Chromatography Mass Spectrometry

GOK	Government of Kenya
HPLC	High Performance Liquid Chromatography
h*	Hue angel
IUFOST	International Union of Food Science and Technology;
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEBS	Kenya Bureau of Standards
KIRDI	Kenya Industrial Research and Development Institute
LSD	Least Significant Difference
NFF	Novel Functional Food
NLEA	Nutritional labeling and education act
NPD	New Product Development
RAD	Roselle apple drink
RD	Roselle drink
RMD	Roselle melon drink
ROD	Roselle orange drink
TPC	Total polyphenol content
USA	United States of America
USDA	United States Department of Agriculture
UV-Vis	Ultra violet visible spectrophotomètre
WHO	World Health Organization
WSV	Water soluble vitamins

ABSTRACT

There have been claims of medicinal benefits obtained from *Hibiscus sabdariffa* L. The main objective of this study was to determine the nutritional composition, functional properties of roselle and evaluate the quality of its novel beverages.

Proximate composition was determined according to established AOAC methods. Antioxidant activity (AA) was determined using 1-1 diphenyl picryl hydrazyl radical (DPPH) and color degradation index (CDI) was done using the Hunter CIE LAB Color Meter. Water soluble vitamins (WSV) and major organic acids were analyzed using HPLC methods. Total polyphenols, tannins, antioxidant activity and flavonoids were analyzed using UV-visible spectrophotometer. Mineral composition was determined using Atomic Absorption Spectroscopy. Roselle product formulations were done at ratios of (roselle extract: fruit juice) 1:1, 3:1and 3:2 for all the product categories of Roselle Apple Drink (RAD), Roselle Orange Drink (ROD) and Roselle Mellon Drink (RMD). Sensory evaluation and consumer acceptability tests were determined based on a 9-point hedonic. Total plate count (TPC), yeast and mold counts were determined after every 14 days to establish the shelf life of the novel beverages.

Results showed that proximate composition of roselle was constituted mainly by soluble carbohydrates at 66.3% (DBM). The physic-chemical properties of roselle extract before and after pasteurization included pH of 3.9 ± 0.00 and 3.4 ± 0.1, total acidity of 2.2 ± 0.0 and 2.2 ± 0.3 %, total polyphenols of 6.1 ± 0.2 and 5.8 ± 0.1 mg/g roselle extract, respectively. The antioxidant activities before and after pasteurization were 230.0 ± 2.4 and 235.3 ± 0.8 μg/ml, respectively. Iron and

calcium contents were 8.6 ± 0.3 and 14.8 ± 0.6 mg/100g respectively. Shelf life studies showed good stability of products where the microbial load was low and within the acceptable limits (10^3g/Kg) for total plate count, yeasts and moulds by Kenya Bureu of Standards and Codex Alimentarius Commission (CAC) up to 90 days.

The RAD and ROD blended with 50 %, 60%, 75% and 100% roselle were highly acceptable in all the sensorial properties of taste, appearance, flavor, consistency and overall acceptability. However, RMD with 75% roselle was the only acceptable with an average score above 5 on a 9-point Hedonic scale. Those with a score lower than 5 were deemed unacceptable. The most preferred pair of beverages for ROD and roselle drink were generally acceptable to consumers. This was an indication for a clear market success scenario in Kenya.

CHAPTER ONE: INTRODUCTION

1.1 Background

The tenet "Let food be thy medicine and medicine be thy food," espoused by Hippocrates nearly 2,500 years ago, is receiving renewed interest. In particular, there has been an explosion of consumer interest in the health enhancing role of specific foods or physiologically-active food components, so-called functional foods (Hasler, 1998). Clearly, all foods are functional, as they provide taste, aroma, or nutritive value. Within the last decade, however, the term functional as it applies to food has adopted a different connotation -- that of providing an additional physiological benefit beyond that of meeting basic nutritional needs. Although a plethora of biologically active compounds have been identified in this regard (Kuhn, 1998), this research focused on novel functional foods, and specific phytochemicals isolated from *Hibiscus sabdariffa* L.

Hibiscus sabdariffa L. Family Malvaceae is believed to be native of tropical Africa. It is known by different synonyms and vernacular names such as roselle (Carmden, and Jordan, 2002) Karkade (Abu-Tarbuoush, 1997) and Mesta (Rao, 1996).

The different parts of roselle include seeds, stems, leaves and calyces. (Purseglove, 1977; Seck, 1997). The roselle calyces (the outer whorl of the flower) groups are red, dark red or green (Schippers, 2000). The fleshy calyces of roselle have been used in various countries in Africa and the Caribbean as food or food ingredient in jellies, syrups, beverages, puddings, cakes, and wines, (Mahadevan *et al.*, 2009; Clydescale, 1979).

In addition to their use in food, various parts of the roselle plant have been used in traditional medicine for the prevention of disease such as cardiovascular diseases and hypertension (CTA, 2001). Among other uses, strong fibre is obtained from the stem (called roselle hemp) which is used for various household purposes including making sackcloth, twine and cord (Mungole and Charvedi, 2011). The calyces have been found to be rich in Vitamin C and antioxidant (Clydescale, 1979; Wong et al., 2002) and also minerals (Babalola, 2001). Roselle plant is highly regarded from the stand point of nutritional and medicinal values as well as economic value. In terms of nutritional values, it has been found to be a good source of essential nutrients (Mohammed and Idris, 1991; Omemu, 2000; Mukhtar, 2007; Wahid, 2008).

This study entailed determination of the nutritional & phytochemicals composition, functional potential and sensory evaluation of novel beverages from roselle blended with selected fruits: apples, oranges and water melons.

1.2 Problem statement

Hibiscus sabdariffa L. is an important flowering plant in the Malvacea family (FAO). But surprisingly very little fundamental research, in terms of .cultivation, agronomy, productivity and various applications, has been carried out. In many developing countries including Kenya, roselle has not been fully exploited despite the existing potential for a wider application in the food processing and other technological applications. There are health and nutritional claims that it has health benefits such as soothing colds, opening blocked nose, clearing up mucous, promoting proper kidney function, helping digestion and helping reduce fever. However, there are limited studies to substantiate these health/nutritional claims. There is need for scientific information to substantiate the claims and validate its applicability in functional food processing. There are no studies on quality changes in the physico-chemical, phytochemicals and nutritional composition at various processing temperature and time regimes as well as storage at various conditions. The problem of this study was to avail the fundamental scientific information as well as proposition of beverage blends with optimized nutrients and functional properties due to beneficial phytochemicals.

1.3 Justification

This study presented an opportunity to provide scientific information to validate the nutritional and some of the health claims of roselle. The study also provided some scientific information on nutritional, Phytochemicals and functional properties of roselle which can serve as an impetus to those who wish to promote the roselle fruit and beverage industry. The information on the health and nutritional benefits is important for the consumers in general. The gathered and documented information will provide an incentive for commercial utilization of roselle in Kenya. The eventual output is the contribution to the organized body of scientific Knowledge for benefit of other researchers & innovators.

According to market statistics, the global functional food and nutraceutical market is growing at a rate that is outpacing the traditional processed food market. Thus exploring the area of functional foods was interestingly beneficial to a number of actors including the producers diversifying crop farming with roselle as a supplemental crop. Consumers are increasingly becoming interested in healthy life-styles. Therefore, they not only look at the basic nutritional composition of the food, but also the health benefits of the foods in disease prevention and health enhancement. This is combined with a more widespread understanding of how diet affects disease, health-care costs and an ageing population. This trend has produced a skyrocketing demand for functional foods and natural health product. Studies on roselle could partly address the problem.

1.4 Objectives

1.4.1 Main objective

The main objective of this study was to determine the nutritional & phytochemical composition, functional properties of roselle and evaluate its applications in production of beverages.

1.4.2 Specific objectives

The specific objectives were to:

a) Determine the nutritional composition of roselle
b) Determine phytochemicals present in the roselle calyces
c) Formulate beverages using roselle blended with selected fruit juices
d) Determine the changes in the microbial quality and bioactive properties of roselle extract after processing and during storage.
e) Determine the consumer acceptability of the formulated roselle beverages

1.5 Hypotheses

The hypotheses tested were:

1) Phytochemicals that can offer antioxidant activities are present in roselle.
2) Nutritional value of roselle is high
3) Consumer acceptable beverages can be formulated from roselle calyces
4) There are no changes in quality and bioactive properties of roselle extract after processing and during processing.

1.6 Research questions

The research questions were:

i. What is the nutritional composition of roselle grown in Kenya?
ii. What phytochemicals are present in roselle?
iii. Are there any changes in microbial quality and phytochemicals as a result of processing and storage conditions of roselle extract?
iv. Can acceptable beverages be developed from roselle calyces?

CHAPTER TWO: LITERATURE REVIEW

2.1 Description of roselle

Hibiscus sabdariffa L. is an annual, erect, bushy, herbaceous sub-shrub that may grow to 8 ft (2.4 m) tall, with smooth or nearly smooth, cylindrical, typically red stems. The leaves are alternate, 3 to 5 in (7.5-12.5 cm) long, green with reddish veins and long or short petioles. The capsule turns brown and splits open when mature and dry. 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)

Figure 1: Mature green roselle plant.

2.2 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). In Kenya, however, it is not widely grown.

2.3 Agronomic practices and production of roselle

2.3.1 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 temperatures not below 21^{o}C. 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 inches 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.

2.3.2 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).

Figure 2: Roselle plantation at JKUAT Farm

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 (Mahadevan, 2009).

2.3.3 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, Podagrica spp. In the order Lepoptera, 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).

Figure 3: Some natural enemies that attack roselle calyces in the field

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.

2.3.4 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).

2.3.5 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).

2.3.6 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, prevents 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 could also be stacked or hung in a well ventilated building. Drying the calyces in forced air dryers would be costly and is rarely done. If heated drying methods are used, care must be taken so that the temperature does not exceed 43^{o}C.so that the phytochemicals are not degraded (Elawad, 2001).

2.3.7 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 10:1.1. That is, for every 46 kilograms of fresh calyx, 5 kilograms of dry calyx is produced. Increased levels 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, inadequate weed control and harvest problems. High productive potentials has been reported for Roselle grown under rainfed, through various agronomic practices such as weeding and spacing (Babatunde and Zechariah, 2001); nitrogenous fertilizer (Babatunde *et al*., 2002); 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 populations 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.

2.4 Utilization and economic importance of roselle

2.4.1 Calyces

The fruit is about 2.5 cm in length with fleshy 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 parts of Nigeria (Arowogeo, 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 beverages and as coloring reagent for jelly, jam, beverages and foods (Gibbon *et al.*, 1995, Rao, 1996 and Wahid, 2008). Its alleged medicinal values include; prevention and cure of Hypertension and inflammation of the bladder (Qi *et al.*, 2005).

Figure 4: Freshly harvested and dried roselle calyces.

2.4.2 Leaves and roots

Tender young leaves are eaten as vegetables especially with soup, or salads and as a seasoning in curries. They have an acidic, rhubarb like flavor (Fasoyiro, 2005 and Mungole, 2011) Roselle has a tap root system. The roots are deficient of most nutrients as reported by (Ojokoh, 2000).They are claimed to be aphrodisiac (Mungole, 2006).

2.4.3 Stem

The stem is utilized in fibre extraction. Rao (1996) reported that the plant is grown in some regions for fibre and pulp obtained from its stem. Total yield of the dry retted fibre components from one hectare, 30 Tonnes of Green Roselle plants is 1,410 kg. 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 high quality Whereas China's product, with less stringent quality control practices, is less reliable and reputable.

2.4.4 Seeds

The seeds are used as feed meal for fish and domestic animals (Backeit *et al*., 1994; Mukhtar, 2007). There have been attempts to make condiments. Mohamodou, *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 antioxidants and probiotics. Mbuja production and consumption could therefore contribute to the consumer's health

2.5 Functional properties and Phytochemicals

2.5.1 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.

2.5.2 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 cholesterol (Wald and Law, 1995). 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; Cheung, *et al.*, 2002).

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.

2.5.3 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 (Liu & Hutchkis, 1995). 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; 1993). 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.

2.5.3.1 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 (Harborne, 1992). 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 (Grubesic,2007).

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 (Harnely *et al.*, 2007).

Quercetin Quercetin-3-rutinoside Quercetin-3-rhamnose (Quercitrin)

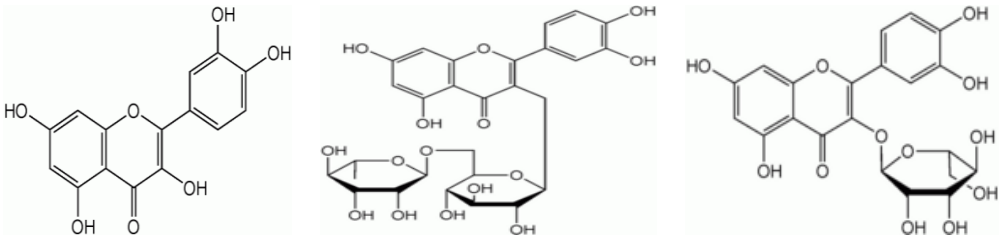

Figure 5: The free aglycone and the glycosylated form of quercetin

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 (Harnely *et al.*, 2007). Literature survey reveals the presence of two classes of flavonoids in the extracts of *Hibiscus sabdariffa*: flavonols (gossypetin), and the anthocyanins (Bisset, 1994; Thomson, 2004).

2.5.3.2 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.

2.5.3.3 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.*, 1996). 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.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Project site

The research study was based at JKUAT in Food Science & Technology department.

3.2 Study design

The research study design entailed:

i. **Field component**: where an identified variety of roselle was planted on JKUAT farm and somewhere in Kitale.

ii. **Laboratory Experimental Analyses:** Postharvest operations, Nutrition & Phytochemical compositional analysis of roselle

iii. **New Product development:** Whereby various product formulations, monitoring quality changes after processing, Shelf life stability, sensory evaluation, and consumer acceptability tests

3.3. Materials Acquisition

Roselle calyces were harvested from the experimental plot in JKUAT farm. Some more calyces of similar variety for NPD were obtained from Kitale, Kenya.

3.4. Analytical Methods

All chemical reagents were of analytical grade (AR) and all determination done in triplicate and results reported on dry weight basis (dwb).

3.4.1. Proximate Composition and determination of dry matter

3.4.1.1. Moisture content

Moisture was determined according to AOAC methods specification 950.46 Method 925. 10-32.1.03 (AOAC, 1995). Results were reported on dry weight basis. About 5g of well-ground sample was accurately weighed into a moisture dish and transferred to an air-oven previously heated to a temperature of 130°C and drying done for 1 hour to constant weight. The final weight of the sample was taken after the drying period and cooling in a desiccator. The residue was calculated as percent total solids and loss in weight as percent moisture by the formula:

Calculations;

$$\% \text{ Moisture} = \frac{\text{Wt of sample before drying} - \text{Wt of sample after drying} \times 100}{\text{Wt of sample before drying}}$$

3.4.1.2 Determination of crude protein

Crude Protein was determined using the Semi-MicroKjeldal method. AOAC Specification 950.46, Method 20.87-32.1.22. (AOAC, 1995).

About 1g of sample was weighed exactly into a digestion flask together with a catalyst composed of 5g of K_2SO_4 and 0.5 g $CUSO_4$ and 15ml of concentrated H_2SO_4. The mixture was heated in a fume hood till the digest colour turned blue. This signified the end of the digestion process. The digest was cooled, transferred to a 100 ml volumetric flask and topped up to the mark with distilled water. A blank digestion with the catalysts and acid was also made. Ten (10) ml of diluted digest was transferred into the distilling flask and washed with about 2 ml distilled water. 15 ml of 40% NaOH was added and this was also washed with about 2 ml distilled water. Distillation was done to a volume of about 60 ml distillate. The distillate was titrated using 0.02N-HCl to an orange colour of the mixed indicator which signified the end point.

Calculations were done using the formula below;

Nitrogen % = $(V_1 - V_2) \times N \times f \times 0.014 \times 100/V \times 100/S$

Where: V_1 = Titer for sample (ml); V_2 = Titer for blank (ml)

N = Normality of standard HCl solution (0.02)

f = Factor of standard HCl solution

V = Volume of diluted digest taken for distillation (10ml)

S = Weight of sample taken (g)

Protein % = Nitrogen × protein factor.

3.4.1.3 Fat content

Fat content was determined using the Soxhlet according to AOAC Method 920.85-32.1.13 (AOAC, 1995). About 5 g of samples was accurately weighed into extraction thimbles and the initial weights of the extraction flasks taken. Fat extraction was done using petroleum spirit in soxhlet extraction apparatus for 8 hours. The extraction solvents were evaporated and the extracted fat dried in an air-oven at 100^0C for about 15 minutes cooled in a dessicator and weighed.

Calculations were done using the formula:

$$\text{Crude lipid \%} = \frac{\text{Weight of residue} \times 100}{\text{Weight of sample}}$$

3.4.1.4 Determination of crude ash

Ash content was determined by incinerating in a muffle furnace Method 923.03-32.1.05 (AOAC, 1995). Sample weights of between 2-5 g were measured in pre-conditioned crucibles. The samples were first charred by flame to eliminate smoking before being incinerated at 550°C in a muffle furnace until a grey-white ash was obtained. The residues were cooled in desiccators and the weights taken

Calculations were done follows:

$$\text{\% Crude ash} = \frac{\text{Weight crucible + ash-weight of crucible} \times 100}{\text{Weight of sample}}$$

3.4.1.5 Crude fibre

Crude fibre was determined according to, AOAC Method 920.86-32.1.15 (AOAC, 1995). Approximately 2g (W) of sample were weighed accurately into a 500 ml conical flask. About 200 ml of boiling 1.25% H_2SO_4 was added and boiling done for 30 minutes under reflux condenser. Filtration was done under slight vacuum with Pyrex glass filter (crucible type) and the residue washed to completely remove the acid with boiling water. Approximately 200 ml of boiling 1.25% NaOH was added to the washed residue and boiling done under reflux for another 30 minutes. Filtration was done using glass wool filter previously used with the acid. The residue was rinsed with boiling water followed by 1% HCl and again washed with boiling water to rinse the acid from the residue. The residue was washed twice with alcohol and thrice with ether. It was then dried in a Hot-air oven at 105ºC in a porcelain dish to a constant weight (W_1). Incineration was done in a muffle furnace at 550ºC for 3 hours, the dish was then cooled in a dessicator and the final weight (W_2) taken crude fibre was determined as:.

$$\% \text{ Crude fibre} = \frac{W_1. - W_2}{.W} \times 100$$

Where W_1= weight of crucible +dry residue, W_2= weight of crucible + ash and W= weight of sample

3.4.1.6 Mineral determination

The ash was cooled after ash determination, 15 ml of 6N HCl was added to each of them in the crucibles before transferring to 100 ml volumetric flasks. Distilled water was used to top up to the mark before mineral analysis (AOAC, 1995). Atomic Absorption Spectroscopy (AAS) was used for Magnesium, Manganase, Copper, Calcium, Iron and Zinc. Potassium and Sodium where determined using Flame Emission spectrophotometer was used (Model A A-6200, Shimadzu, Corp., Kyoto, Japan).

3.4.2 Determination of changes in physico-chemical attributes of roselle beverages

3.4.2.1 Colour

Tri-stimulus colorimeter was used to take colour measurements (Simple Spectrophotometer NF 333-Model 99061, Nippon Nenshoku Ind., Tokyo, Japan). The instrument expresses colour measurement in the CIELAB (L*, a*, b*) form. The instrument was first calibrated using standard black and white plates (with transparent papers placed on the standard plates). After calibration colour measurements were randomly taken in triplicates. The hue angle (h*) which describes the visual sensation according to an area which appears to be similar to one or proportions of two of the perceived colours, red yellow, green, and blue was calculated according to the formula given below.

Hue angle (h*) = $\tan^{-1}$(‘b*’ / ‘a*’)

Where ‘L*’, ‘a*’, and ‘b*’ are values. Appendix 1 shows the Tristimulus colour match solid and code table used in colour analysis.

3.4.2.2 pH

This was done by the method of Ofori and Hahn (1994). The pH meter was standardized using buffer solutions of acidic and basic values of 4.01 and 9.08 at 25°C (TOA pH Meter HM–7B, Tokyo, Japan). Calibration was done by dipping the electrode in the acidic buffer solution, adjusting the pH, cleaning the electrode, dipping it into the basic buffer solution and again adjusting the meter. The electrode was rinsed with distilled water before taking measurements. The beverages were homogenized by stirring before measurements of pH were taken to achieve uniformity. The pH readings were made by dipping the electrode in drink system and letting it display stability. Measurements were taken from the display screen when the readings were stable.

3.4.2.3 Total titratable acidity

Total titratable acidity analysis was done using AOAC, 1995 method. Approximately 10 ml of sample was pipetted into a conical flask and 2 drops of phenolphthalein indicator added. Titration was done using 0.1N NaOH to a faint pink colour which persisted for at least one minute compared against a white background. The titre volume was noted and used for calculations of TTA which was expressed as percentage Malic acid. Calculations of TTA was done as follows;

$$\% \text{ Malic acid} = A \times 0.009 \times 100/V$$

Where: A = ml of 0.1 NaOH required for the titration; and V = ml of sample taken for the test and 0.009 is a Constant.

3.4.2.3 Major organic acids of roselle

Organic acids were analyzed using HPLC system based on the method of Chen *et al*., (2006). Two grams of sample were mashed with 10 ml of 1M HCl. The volume was made up to 20 ml with 1M HCl. The samples were flushed with nitrogen and centrifuged at 2000 rpm for 15 minutes. The samples were placed in a water bath at 90 degrees for 30 minutes, and then allowed to cool to room temperature. The supernatant was collected and filtered through a 0.45µm membrane filter. The injection volume was 20 µl and column temperature maintained at 40 degrees C. An isocratic phase of 0.5 % ammonium phosphate, pH adjusted to 2.8 with phosphoric acid, was used at a flow rate of 0.5ml/min. Detection was done at 214 nm. Means were done based on triplicate determinations. The major organic acids determined by this method included: Malic acid, Citric acid, Tartaric acid, Oxalic acid and Acetic acid.

3.4.2.4 Determination of the seven water soluble vitamins of roselle

A reversed-phase HPLC method by Ekinci and Kadakal (2005), modified from Cho *et al.,* (2000) was used. The sample treatment consisted of SPE with Sep-Pak C_{18} (500 mg) cartridges that enabled separation of water-soluble vitamins and removed most of the interfering components. Twenty grams of water were added to 5 g of the sample. The mixture was homogenized using a homogenizer at medium speed

for 1min. The homogenized samples were centrifuged for 10 minutes at 14×10^3g (Centrifuge Model H–2000C Shimadzu Corp., Kyoto, Japan). The stationary phase preparation involved flushing with 10 ml methanol and 10 ml water (pH 4.2) to activate it. The homogenized and centrifuged samples were then loaded. The sample was eluted with 5ml acidified water (pH 4.2) then 10 ml methanol at a flow rate of 1 ml min^{-1}. The eluent was collected in a bottle and evaporated to dryness. The residue was dissolved in mobile phase and then filtered through 0.45 μm pore size filters. Approximately 20 μl of samples was injected into the HPLC column. The column elute was monitored with a photodiode-array detector at 234 nm for thiamine, 265 for vitamin C, 266 for riboflavin, 324 nm for pyridoxine, 282 nm for folic acid, 204 for panthothenic acid and 261 nm for niacin. Riboflavin was analyzed separately since the HPLC conditions are different; hence the method is outlined differently.

The vitamins were analyzed in a HPLC (Model SCL-10A, Shimadzu Corp., Kyoto, Japan) using a column of inertsil ODS 5μm 4.6 × 250 mm 5LI0101Z with 0.1 mol /L KH_2PO_4 (pH 7.0)–methanol, 90:10 mobile phase (filtered through 0.45μm membrane and degassed by sonication), flow rate of 0.5 ml/min, a photodiode-array detector (Model Waters 2996, Waters Corp., Mailford, USA), oven temperature of 25ºC, and a sample volume of 20 μl.

Identification of compounds was achieved by comparing their retention times and UV spectra with those of standards stored in a data bank. Five different concentrations of each standard were used to prepare calibration plots for each vitamin. This was done by plotting concentration (μg/ml) against peak area (mAU).

Their correlation coefficients were greater than 0.997 (Appendix 2). Concentrations of the water-soluble vitamins were calculated from integrated areas of the sample and the corresponding standards.

Vitamin content (mg/g) = (y/b) × (dilution factor / weight of sample (g) × 1000)

Where y=is the y intercept of obtained from the standard curve of the vitamin in question, and b is the peak area of the injected sample

Riboflavin

Riboflavin was determined using HPLC (AOAC, 1995). Approximately 1 g of ground sample was mixed with 10 ml of distilled water and homogenized. The pH was adjusted to 3.0 using acetic acid glacial (1:1 v/v). The sample was vortexed before centrifugation at 10,000 rpm for 20 minutes and the resulting supernatant transferred into a 25 ml volumetric flask (Centrifuge Model H–2000C, Shimadzu Corp., Kyoto, Japan). The sediment was washed with 5 ml of 2% acetic acid solution, the washings combined and centrifuged. The second supernatant was added to the first and volume made to the mark using 2% acetic acid solution. Filtration was done and the sample injected into the HPLC.

The HPLC (Model C–R7A plus chromatopac, Shimadzu Corp., Kyoto, Japan). Operating conditions included: Column; Inertsil, ODS (C_{18}) 5μm 250 × 4.6 mm, Mobile phase; Methanol: Water: acetic acid (40:59.5:0.5), Detector; UV-VIS Model MDS510M-24-F2 Shimadzu Corp., Kyoto, Japan. The Analysis wavelength was 270 nm at 0.02 sensitivity, Flow rate; 0.5-1.0 ml/min, Injection volume: 10 μl. The

centrifuge micro-filters size was 0.45 μm. Riboflavin standard stock solution of 1000 ppm in 2% aqueous acetic acid was prepared and serial dilutions of 100-600 ppm were made. The standards were injected into the HPLC and corresponding peak areas obtained and used to plot a standard curve (Appendix III) whose equation was used to calculate the quantity of riboflavin as follows;

Calculations;

Vitamin content (mg/g) = (y/b) × (dilution factor / weight of sample (g) × 1000)

Where y=is the y intercept of obtained from the standard curve of the riboflavin, and b is the peak area of the injected sample.

3.4.3 Phytochemicals in roselle

All analytical methods for quantifying the biologically active compounds present in roselle calyces involved extraction, separation and analysis. (Gordana *et al*., 2008). A procedure by Suhad and Viorica (2008) that was simple, rapid and economical was used in the quantitative analysis of the phytochemicals.

3.4.3.1 Tannins

This was done according to the Vanillin-Hydrochloric Acid method (Burns, 1963; Price *et al*., 1978). Approximately 0.25 g of ground samples were weighed into Erlenmeyer flasks. Ten (10) ml of 4% HCl in methanol was pipetted into each of the flasks and closed with parafilm. The flasks were gently shaken for 20 minutes in a shaker (Model KS 250 basic, Germany) and the resulting extracts centrifuged for 10 minutes at 4500 rpm (Model H–2000C, Kokusan Corp., Tokyo, Japan). The

supernatant aliquots were transferred to 25 ml volumetric flasks. Second extractions were done by adding 5 ml of 1% HCl in methanol to the residue from the first extraction and repeating the extraction process. The aliquots of the first and second extracts were combined and made up to 25 ml in a volumetric flask. Approximately 1 ml of each extract was pipetted to a corresponding labeled test tube. A set of catechin standard solutions was prepared ranging from 100 to 1000 ppm using methanol as the solvent. Approximately 1 ml of each respective standard and sample extract were pipetted into test tubes and 5 ml of freshly prepared vanillin-HCl reagent added to each. Sample blanks were prepared by adding 5 ml of 4% HCl in methanol to 1ml of the aliquots of the extracts pipetted into the test tubes. The absorbance of the standard solutions, sample extracts and blanks were read in a UV-VIS spectrophotometer at 500 nm 20 minutes after adding Vanillin-HCl reagent to the samples and standards (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan).

A standard curve was prepared from the readings of the catechin standard solutions (Appendix ii). The blank absorbance were subtracted from the samples absorbance and the corrected absorbance substituted into the regression equation ($y = 0.0004x$, $R=0.9972$) in order to calculate the concentration of the sample extracts.

The concentration in µg per ml was converted in to mg catechin per ml. The percent catechin equivalents (% CE) were calculated as follows:

$$\% CE = (CC \times VM) / (VE \times Wt) \times 100$$

Where: CC = catechin concentration (mg/ml); VM = volume made up (25 ml); VE = volume of extract (1 ml); and Wt

3.4.3.2 Total polyphenol content

The total polyphenol content (TPC) was determined using the Folin-Ciocalteu method. Solvent extraction was carried out using 50% methanol and ethanol solutions at 25^{o}C. A measure of 0.4 g of the ground sample was put in a conical flask and 40 ml of the solvent was added. The mixture was covered with aluminium foil and allowed to settle without shaking for 2 hours. The extract was then be filtered using a 0.45 um microfilter. The filtrate was directly used for analysis using UV-Vis spectrometer at 760 nm after color development.

3.4.3.3 Flavonoids

The flavonoids were determined as described by Chang *et al*., (2002).

Sample extracts were evaporated to dryness and re-dissolved in 80 % ethanol to be ready for the analytical test. 1 mL of a sample (ethanolic solutions or rselle extract) was mixed with 3 mL 95 % ethanol *(V/V*), 0.2 mL 10 % aluminum chloride *(m/V*), 0.2 mL of 1 mol L^{-1} potassium acetate and 5.6 mL water. A volume of 10 % (*m/V*) aluminum chloride was substituted by the same volume of distilled water and used as a blank.

After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm.

Each extract was hydrolyzed with 4 N HCl (1:1) for 30minutes, then the flavonoids were extracted with three quantities of ethyl acetate (each of 15 mL), after which the ethyl acetate layer was evaporated to dryness under reduced pressure, then the residue was re-dissolved using 80 % ethanol to a volume of 25 mL. This solution was used for the analytical procedure. Flavonoids in roselle extracts were expressed as quercetin equivalent. Quercetin (Sigma, Germany) was used to perform the calibration curve (standard solutions of 6.25, 12.5, 25.0, 50.0, 80.0 and 100.0 µg mL^{-1} in 80 % ethanol *(V/V)*.

3.4.4 Antioxidant activity

The radical-scavenging activity was determined using diphenyl picryl hydrazyl radical (DPPH) according to Ayoola *et al.* (2006). The following concentrations of the extracts were prepared, 0.05, 0.1, 0.5, 1.0, 2.0 and 5 mg/ml in methanol in cuvette placed in the spectrophotometer (Analar grade). Ascorbic acid was used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5 and 0.75 mg/ml. One ml of the extract was placed in a test tube, and 3 ml of methanol added followed by 0.5 ml of 1 mM DPPH in methanol. The mixture was shaken vigorously and left to stand for 5 minutes. A blank solution was prepared containing the same amount of methanol and DPPH. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. The radical scavenging activity was calculated using the following formula:

% inhibition = {[Ab-Aa]/Ab} x 100

Where Ab is the absorption of the blank sample and Aa is the absorption of the extract. All tests were run in triplicate, and analyses of all samples run in duplicate and averaged.

3.5 New product development from roselle

The roselle beverages was extracted 1:40 of calyces to purified water. Natural preservative as lemon dash (juice already pasteurized), this also imparted flavour. The beverages with the characteristic colour and flavour of roselle were sweetened with natural sugar at 10% as minimum sugar (KEBS, 1996). The beverages were sieved with size 14 sieves and strained with a piece of white cloth until they were crystal clear (isotonic) (Fellows, 1997).

3.5.1 Processing of roselle drink

Roselle calyces were sorted, washed and soaked for 2 hours in water. They were pasteurized at 100^0C, 80^0C and 60^0C for 5, 15 and 30 minutes, respectively in order to ascertain temperature/ time regime for optimal retention of both the nutrients and functional properties due to Phytochemicals present. The extract was then filtered while still hot and packaged at 45^0C to avoid collapsing of the packaging bottles and ensure good manufacturing practices GMP are followed. Packaging while still hot minimized the possible chances of microbial growth Figure 6 is a summary of the processing of the 100% Roselle drink.

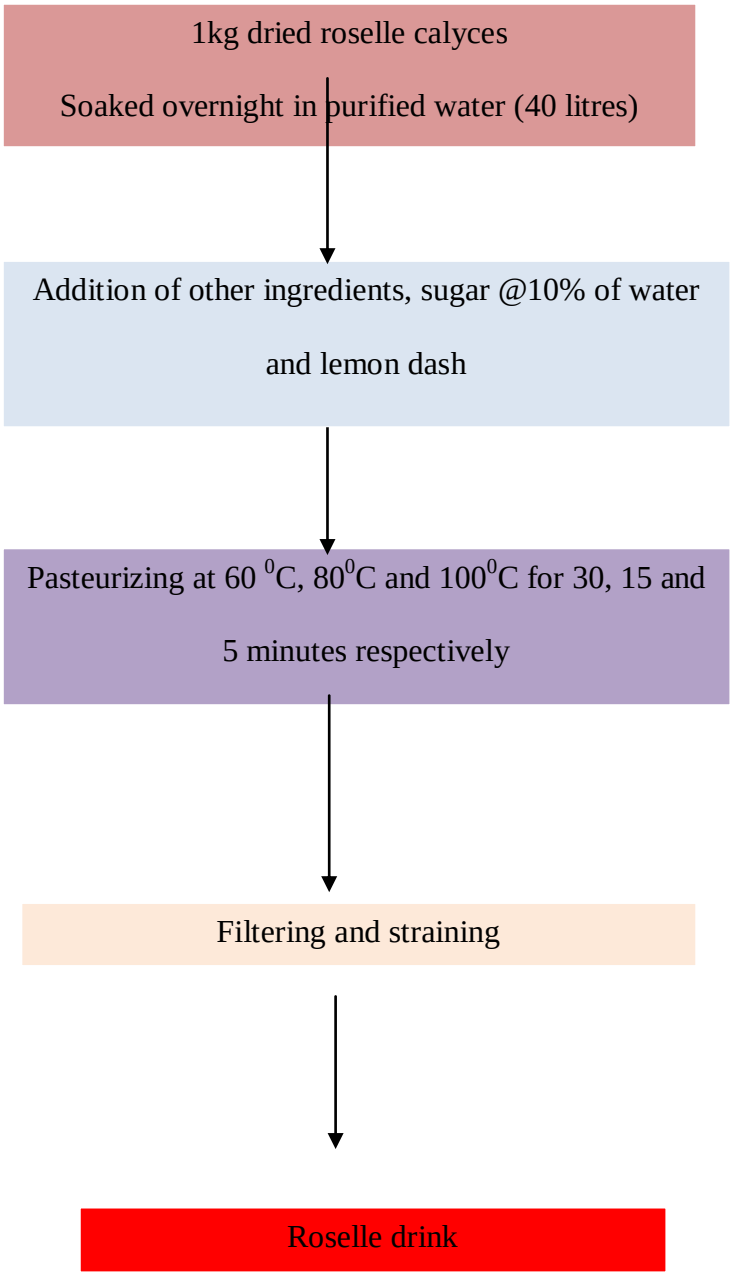

Figure 6: Production of pure roselle drink

3.5.2 Processing of roselle beverages blended with selected juices.

Roselle beverages incorporating roselle calyces extract with either apples, oranges or watermelons formulated. Figure 7 is a summarized production flow chart.

Roselle Apple Drink (RAD):

Forty apples of about 500g were washed with tap water, sliced and using a fruit blender the juice was squeezed, the residue was mixed with the juice and 10% water.

Roselle Orange Drink (ROD):

Forty kilograms of oranges were washed with tap water, cut into halves and using a squeezer juice was extracted by pressing. The juice extract was filtered, measured.

Roselle Watermelon Drink (RMD):

The water melons were subsequently washed in tap water with food grade soap rinsed and sliced into sizeable pieces. The pulp was extracted and using a fruit juicer, juice was extracted. The juice was filtered, measured and then mixed with (50%), (60%) and (75%) of roselle extract respectively. All other ingredients including Lemon dash (extracted and pasteurized lemon juice) and 10% sugar (weight of dispersing medium in this case water) (KEBS, 1996) were added to each formulation.

Pasteurizing of the blended mix was done at 60 ^{0}C, 80^{0}C and 100^{0}C for 30,15 15 and 5 minutes respectively in order to establish the optimal processing temperature/ time regime for better phytochemical extraction as well as nutrition and functional property retention.

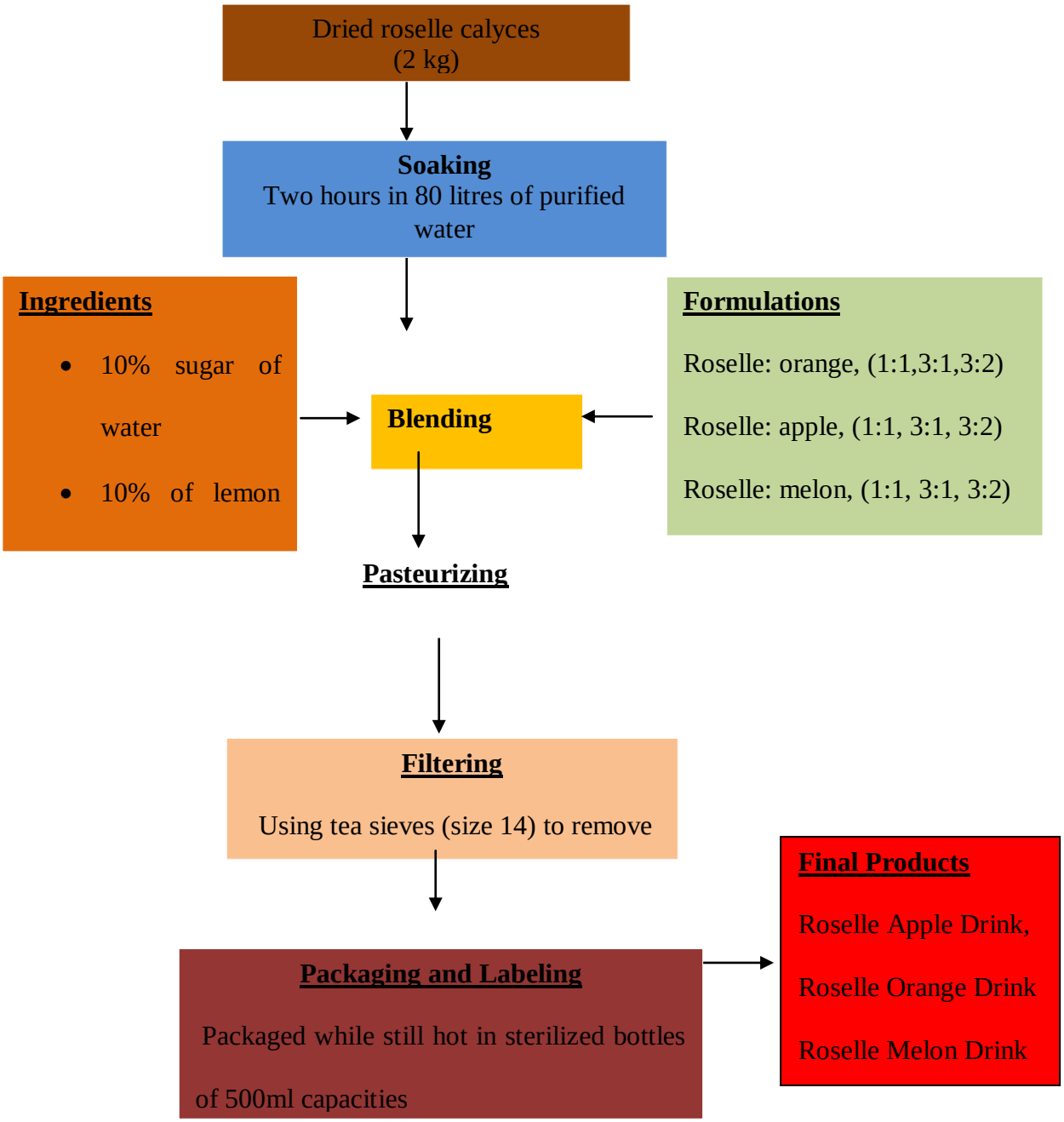

Figure 7: Flow chart for processing of fruit flavored roselle beverages

3.6 Shelf life Stability of roselle beverages

Shelf life stability was analyzed by determining the microbial load of the beverages**.** Also observation was used to monitor the quality changes in the product. Samples of 500 ml of the beverages were packaged in transparent bottles and stored at 25^0C and 80% RH (room temperature conditions of temperature and humidity respectively). A portion of the sample was taken from the same bottle each time during the analysis after which the bottle was carefully sealed and kept at the aforementioned condition. The total plate count (TPC), yeast and mold counts were determined after every 14 days to determine how long the product would store and still be suitable for consumption. The general aesthetic appeal (that is the appearance in terms of colour and luster) of the products was also observed and colour changes monitored over a period of two months, 60 days.

3.6.1 Total plate count

Total plate count was done according to AOAC, (1995) methods**.** Initial product sample homogenates were prepared in sterile diluents in ratios of 1:10. For each homogenate, 1ml was aseptically diluted through a series of tubes containing 9ml sterile diluents. Approximately 1ml of diluents of each tube were spread plated on to Plate Count Agar (PCA) and incubated for 48 hrs at 35ºC. Plates with less than 300 colonies were counted and the number of bacterial colonies expressed as colony forming units per gram (CFU/g) of the sample using the formula from International Dairy Federation method (IDF, 1996) as follows;

$$\text{Log } C = \Sigma\, x/n_1 + (0.1n_2)^{x} d$$

Where: C = Count CFU/g; x = Total number of colonies in all plates; n_1 = number of plate from initial dilution where counts were made; n_2 = number of plates from second dilution from where counting was done; and d = initial dilution of counting.

3.6.2 Yeast and mould count

The mould count was carried out using potato dextrose agar (PDA) AOAC (1995). Initial product sample homogenates were prepared in sterile diluents in ratios of 1:10. 1 ml of each homogenate was then aseptically diluted in series up to a dilution of 10^{-3}. The diluents were then pour plated in duplicates. Incubation of the plates was done at 25ºC for 72 hours. The number of yeast and molds were expressed as colony forming units per gram (CFU/g) using the formula in TPC determination.

3.7 Sensory evaluation of roselle beverages

The products developed were randomly subjected to sensory evaluation to determine the most preferred using a semi structured questionnaire (Appendix iii). This was done by a team of 60 untrained panelists. Each recorded their degrees of likes and dislikes on the taste, color, consistency, flavor and overall acceptability of the formulated products using a 9-point hedonic scale (Ihekoronye and Ngoddy, 1985). Where by a score of 9 implied the panelist liked very much, while a score of 1 implied dislike very much. Conversely, a score less than 5 meant the product was generally not acceptable and therefore reformulation or total disposal of the product development process. Before each sample testing, the panelists rinsed their mouth

with water. The assessment was carried out under natural light at a temperature of 25ºC (room temperature). A copy of the questionnaire used is attached as Appendix III. The data was analyzed using the two way analysis of variance (ANOVA). Significant differences in the means were separated by Duncan's Multiple Range tests (Steel and Torrie, 1980).

3.8 Data management and analysis

Data was verified, analyzed using Analysis of Variance (ANOVA) with Genstat. Mean comparisons for treatments were made using Duncan's Multiple Range Tests (Steel and Torrie, 1980). Significance difference was set at $p \leq 0.05$.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Proximate composition of roselle

Table 1 shows proximate composition of the roselle calyces. Soluble carbohydrates were the most abundant nutrient at 66.3%; this was followed by crude fibre with 14.6%. The calyces had appreciable amounts of crude protein (5%) and crude ash of 12%. These results were converted into dry matter basis (dmb) similarly, the results were found to be in agreement with those found by other researchers like Babalola (2001) and Ojokoh (2003). However, the various were observed in that Ojokoh found higher contents of dry matter in roselle .The high carbohydrate content which is mainly sugars is promising in product development of roselle beverages, Omemu (2006).

Table 1: Proximate composition of roselle calyces (g/100g of sample) dmb

Parameter	Amount (g/100g)
Soluble Carbohydrates	66.3 ± 0.7
Crude Fibre	14.6 ± 0.5
Ash	12.2 ± 0.3
Crude Protein	4.7 ± 0.1
Crude lipids	2.2 ± 0.1

Values are presented as means of triplicate determinations ±SD

4.2 Mineral Content of roselle

Table 2 summarizes the elemental composition of the roselle calyces. Potassium was the most abundant mineral (101.5 mg /100 g), followed by magnesium (100 mg /100 g,). The mineral occurring in the least quantity was zinc (0.2 mg /100 g). Iron and calcium were 14.8 and 8.5 mg/100 g, respectively. Potassium is important not only as the main cation in the intra- cellular fluids but also essential for the nervous systems, maintenance of fluid volume in the body, contractile mechanism of muscles, maintenance of correct rhythm of heart beat and clotting of blood (Shahnaz *et al*., 2003). Iron is an important transition element with several vital functions in the body; as a carrier of oxygen to the tissues from lungs (hemoglobin), storage of oxygen in the muscle tissue (myoglobin). Babalola (2001) states similar importance of micronutrients in body functions.

Table 2: Minerals contents of dried roselle calyces (Mg/100g,) dmb

Metal	Amount (mg/100g)	RDA
Potassium	101.5 ± 0.1	2g
Magnesium	100.7 ± 0.4	240mg
Sodium	72.1 ± 0.1	500mg
Phosphorus	35.3 ± 0.1	700mg
Calcium	14.8 ± 0.6	1g
Manganese	10.8 ± 0.1	2mg
Iron	28.5 ± 0.3	27mg
Copper	3.6 ± 0.1	1mg
Zinc	0.2 ± 0.0	10mg

Values are presented as means of triplicate determinations ±SD

Assuming high bioavailability of the minerals, 100 g of roselle provided adequate amounts of copper, manganese and iron to meet the RDAs of adults.

4.3 Water soluble vitamin in roselle calyces

Table 3 presents the content of water soluble vitamins (WSV). Ascorbic acid was the most abundant vitamin (6.7 mg) followed by niacin (3.8 mg) and pyridoxine (1.5 mg). All the other vitamins were at concentrations less than 1 mg / 100 g. There was 25-30% reduction in vitamins upon drying the roselle calyces. This is because vitamins are labile and degrade upon exposure to light, oxidation and thermal processes. Pyridoxine (B6) was relatively stable in the roselle calyces in comparison to the rest this was inferred based on the level of vitamin loss upon drying. Additionally, roselle was found to provide sufficient amounts of vitamin B6 to meet the RDAs of adults.

Table 3: Water soluble vitamins in fresh and dried roselle calyces

Constituent Vitamins	Calyces(fresh) mg/100g	Calyces (dried) mg/100g	RDA (mg)
Ascorbic acid	6.7± 0.1	4.7 ± 0.1	45.0
Niacin (B3)	3.8 ± 0.0	2.6 ± 0.0	16.0
Pyridoxine (B6)	1.6 ± 0.1	1.1 ± 0.1	1.5
Panthothenic (B5)	0.3 ± 0.1	0.2 ± 0.0	5.0
Riboflavin (B2)	0.3 ± 0.0	0.2 ± 0.0	1.1
Thiamin(B1)	0.2 ± 0.0	0.1 ± 0.0	1.1
Folic acid	0.2 ± 0.0	0.1 ± 0.0	0.4

Values are presented as means of triplicate determinations ±SD

4.4 Levels of the major organic acids in roselle calyces

Table 4 presents some of the major organic acids that were found in roselle extract. As a characteristic of the Malvacea family, high amounts of Malic acid content was found (560 mg/100 g), Oxalic acid, usually found in green leaves, was found in the calyces in considerable amounts (150 mg/100 g). Other organic acids identified in roselle included Tartaric acid, Citric acid, and Acetic acid. Unidentified peaks were also noted and suspected to be some unclassified organic acids like Succnic, Hibiscicic. Organic acid content of a fruit is an important factor for the development of its flavor (Gansch, 2009).

Table 4: Major organic acids in dried roselle calyces.

Organic acid	Amount (mg/100g)
Malic	560 ± 13
Citric acid	70 ± 2.5
Tartaric acid	46 ± 2.6
Oxalic acid	148 ± 7.2
Acetic acid	115 ± 5.5

Values are presented as means of triplicate determinations ±SD

4.5 Levels of phytochemicals in roselle calyces

The quality and bioactive properties of the roselle products before and after pasteurization as well as storage for 90 days are shown in Tables 5 and 6.

4.5.1 Quality Changes in Acidity and Antioxidant Activity

Table 5 shows quality changes in acidity and antioxidant activity. These are changes in pH, TTA and Antioxidant activity (E50 ug/ml). The pH was significantly decreased after pasteurization. Conversely there was no significant change at storing for 90 days.

Total titratable acidity was not significantly affected by neither pasteaurization nor storage (time/temperature) conditions. Pasteurization enhances complexes of chemical reactions leading to by-products that increase acidity, hence the increase in pH. The antioxidant activity expressed as EC_{50} of the product increased significantly with after pasteurization and storage. There's a connection between pH and antioxidant activity.

Table 5: Quality changes in acidity and decreasing capacity of roselle extract

Parameter	Before pasteurization	After pasteurization	Storage for 90 days	
			5^0C	27^0C
pH	3.9 ± 0.0^{bx}	3.4 ± 0.0^{a}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}
TTA[xx]	2.2 ± 0.0^{a}	2.2 ± 0.0^{a}	2.2 ± 0.0^{a}	2.3 ± 0.0^{a}
EC 50 ug/ml	230.0 ± 2.4^{d}	235.3 ± 0.7^{c}	359.9 ± 0.9^{b}	390.6 ± 4.8^{a}

[x] Determination as means ± standard deviation, [xx] as malic acid (mg/g) Rows with the same letters are not significantly different at ($p>0.05$).

According to Azizah *et al.,* 1999, the value of the pH affects antioxidant activity of products.

4.5.2 Quality changes in the phytochemicals with functional properties

Table 6 presents the quality changes in the phytochemicals with the potential of functional properties.

There was a decrease in the level of total polyphenols after pasteurization which was not significant. In continuation of the same, there was significant intermittent decrease in the total polyphenols during storage. Possibly, this decrease is due to gradual degradation of the anthocyanins, residual enzyme action and condensation of the other Phytochemicals.

Table 6: Quality changes in functional properties of roselle extract product

Phytochemicals with functional potential	Before pasteurization	After pasteurization	Storage for 90 days	
			5^0C	27^0C
Total polyphenols[x]	6.06 ± 0.2 [a]	5.82 ± 0.0[a]	3.3 ± 0.4[b]	2.58 ±0.4[c]
Tannins [xx]	2.26 ± 0.0[a]	3.21 ± 0.0 [a]	4.17 ± 0.0[b]	4.07 ± 0.0 [b]
Flavonoids [xxx]	6.3 ± 0.34[a]	5.57 ± 0.05[a]	9.33 ± 0.3[b]	7.33 ±0.3[b]

[xxx] total polyphenol content as gallic acid (mg/g) [xxxx]Tannins as mg catechin/g extract, [xxxxx]Flavonoids as Quercetin (mg/g), Rows with the same letters are not significantly different at ($p>0.05$).

There was significant increase in tannins after pasteurization, subsequently during storage for 90 days. Monomeric compounds tend to polymerize with time forming complexed tannins.

There was decrease in flavonoids measured as Quercetin equivalent; however, this reduction was not significant. This phenomenon was similar upon storage for 90 days. Thermal processing and storage conditions reduces the anthocyanidins, especially pelargodin which are the building blocks of flavonoids. Anthocyanins are constituents of flavonoids and they have an arithmetic decrease upon exposure to both light and heat. Roselle is red in color; the anthocyanins are responsible for this pigment. To enhance the functional properties the thermal conditions should be optimized as well as conditions of storage maintained at low temperatures below 5^0C.

The results shown in Tables 5 % 6 suggested that storage at 5 ^{0}C provides greater retention of phytochemicals with functional properties. Additionally the roselle products should be consumed within a shorter time (<3 months) upon processing to enhance the health benefits from Phytochemicals.

4.5.3 Antioxidant activity and radical scavenging capacity of roselle extract

Figure 9 presents the antioxidant activity of roselle extract at different conditions of processing and storage.

There was no significant change in the antioxidant activity after pasteurization However; there was a significant linear increase in antioxidant activity during storage. There is no clear understanding as to why the anti oxidant activity increases with storage. Possibly the polymerization of the monomeric anthocyanins leading to polymeric antioxidants could be the confounding phenomenon as advanced by the

consistent findings of (Wrolstad, 2005) studies on kinetics of anthocyanins degradation.

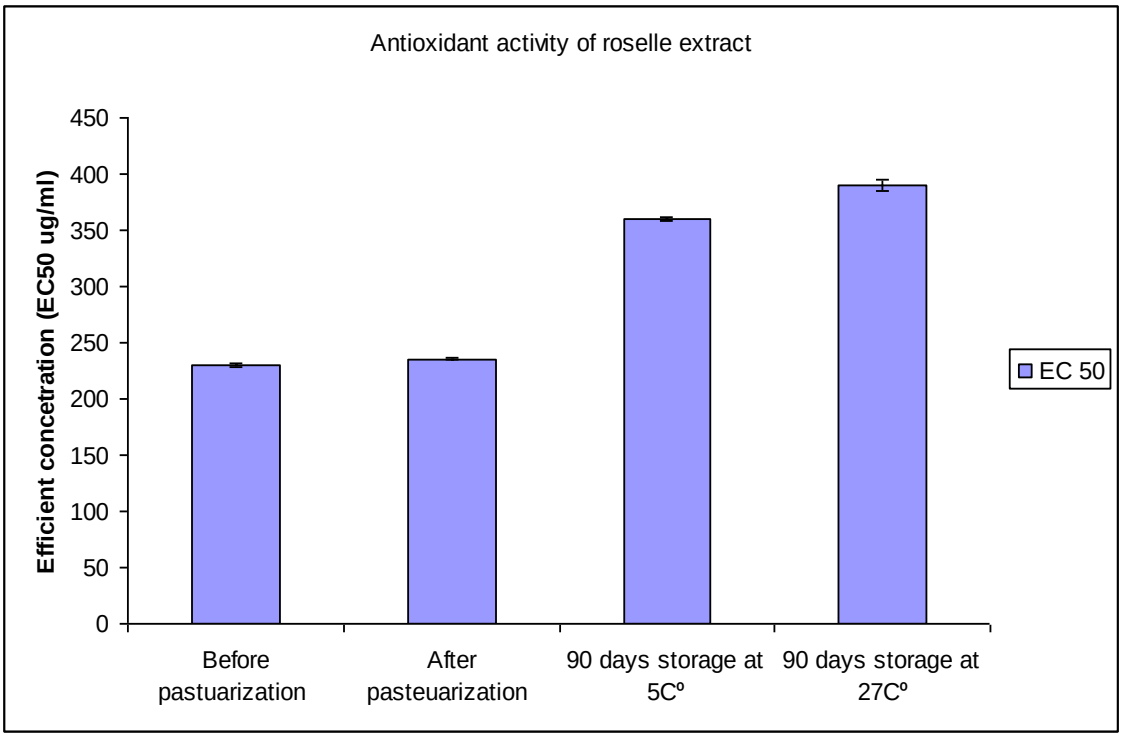

Figure 8: Radical reduction of roselle extract as influenced processing

Figure 9 shows the capacity of roselle extract to scavenge for free radicals using ascorbic acid as the standard and DPPH as the radical.

The ability to inhibit radical oxidation was demonstrated by roselle at various levels of concentrations as presented.

At low concentration, inhibition was almost minimum at 5% increasing gradually to almost 95% at 5mg/ml. This shows a logarithmic capacity to scavenge for free radicals. As compared to the ascorbic acid radical scavenging capacity (RSC), the

results suggested the potential of roselle extract to scavenge for free radicals. This capacity is what constitutes the functional properties important for formulation of Functional food products.

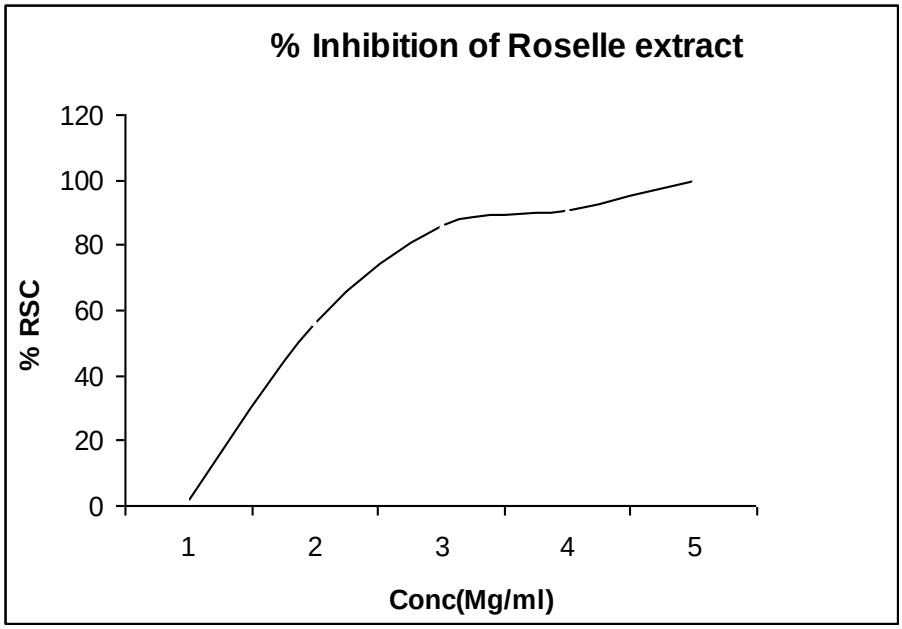

Figure 9: Percentage reduction of roselle extract at different concentrations

Total polyphenol content (TPC) in roselle was measured and related to their DPPH radical scavenging capacity. As expected, they showed a close relationship (0.95). The total polyphenol content and DPPH radical scavenging capacity of roselle was high.

There was direct correlation between Gallic acid concentration and spectrophotometer absorbance at 760 nm with a gradient of 0.7936 and correlation coefficient (R^2) of 0.9857. The TPC generally increased with increase in temperature of 60^0C, 80^0C, 100^0C for 51.7mg/g, 62.5mg/g and 73.58mg/g respectively. Gansch (2009) tested TPC in Raspberry cultivars and it ranged from 342.0 to 875.3 mg of GAE/100g of fresh weights.

The relatively lower levels of total polyphenol in *Hibiscus sabdariffa* could be attributed to the type of food matrix; roselle is a flower while raspberry is a fruit. Fruits are more concentrated in total polyphenols (Ganch,2009)

And in view of the abundant total phenolcs, flavonoids, pleasing aesthetic appeal and other phytochemicals, roselle can be considered as a functional food/ ingredient with potential to offer functional properties.

4.6 Indices for polymeric color changes of roselle beverages.

Fig. 11 shows how the color of roselle drink changed in a period of two months as depicted by the calculated hue (h*) angle. The intensity of the pigment degradation was depicted by plotting the chroma (c*) calculated as $(a^{*2}+b^{*2})^{1/2}$ values against time as shown in Figure 11

The color changed gradually darkened. This was anticipated because of the decrease in pH, co-pigmentation, oxidation and certainly the thermal processing of the products. Several studies have reported a logarithmic course of anthocyanin destruction with an arithmetic increase in temperature (Drdak and Dancik, 1990).

Color degradation in roselle may have been as a result of anthocyanin degradation**.** According to Wrolstad (2005) a coloured sample of different dilution strengths will have the same color (hue angle) but different chroma (saturation or intensity) values.

He adds that a confounding phenomena regarding chroma, is that it will increase with pigment concentration to a maximum, and then decrease as colour darkens. Thus a pink and a dark red colour can have identical values for chroma.

For commercial production of roselle drink, processing time, temperature regimes will be critical to maintain color stability, quality and Phytochemicals with functional potential like flavonoids, tpc and tannins as well as capacity to scavenge for radicals.

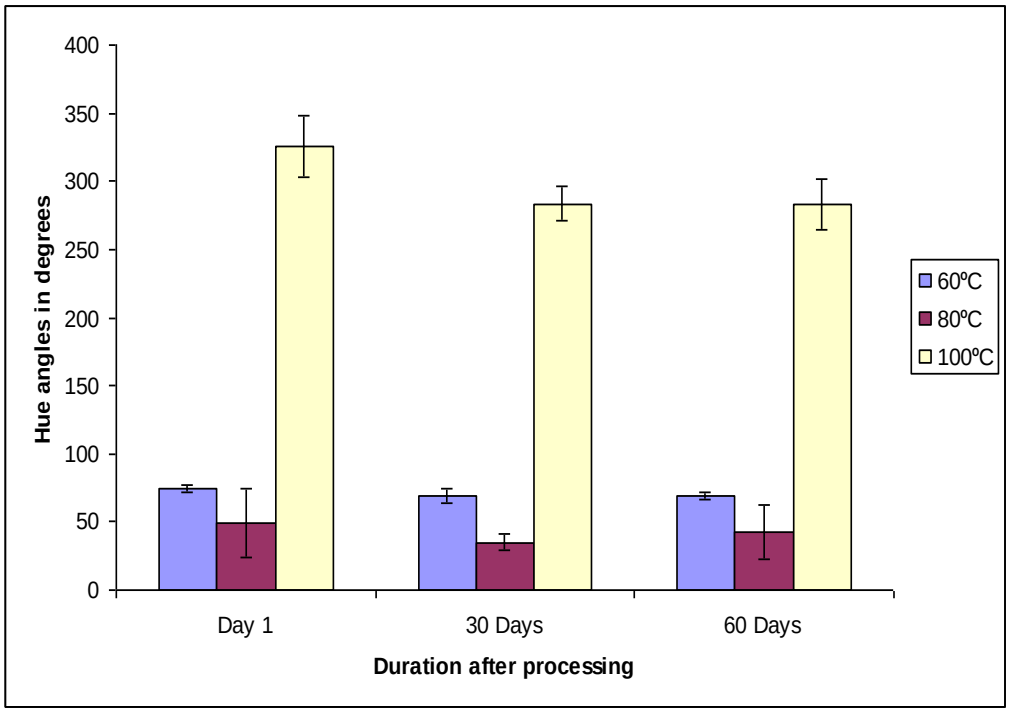

Figure 10: Hue angle of the roselle drink processed and stored

Figure 11 shows the saturation of the corresponding hue (color of roselle after processing and subsequent storage over time

The chroma values increased with increase in processing temperature and duration of storage. Visually the colour changed intensity increased corresponding to the hes in shown in Figure 10. These results are consistent with the assertion by Wrolstad *et al.* (2005)

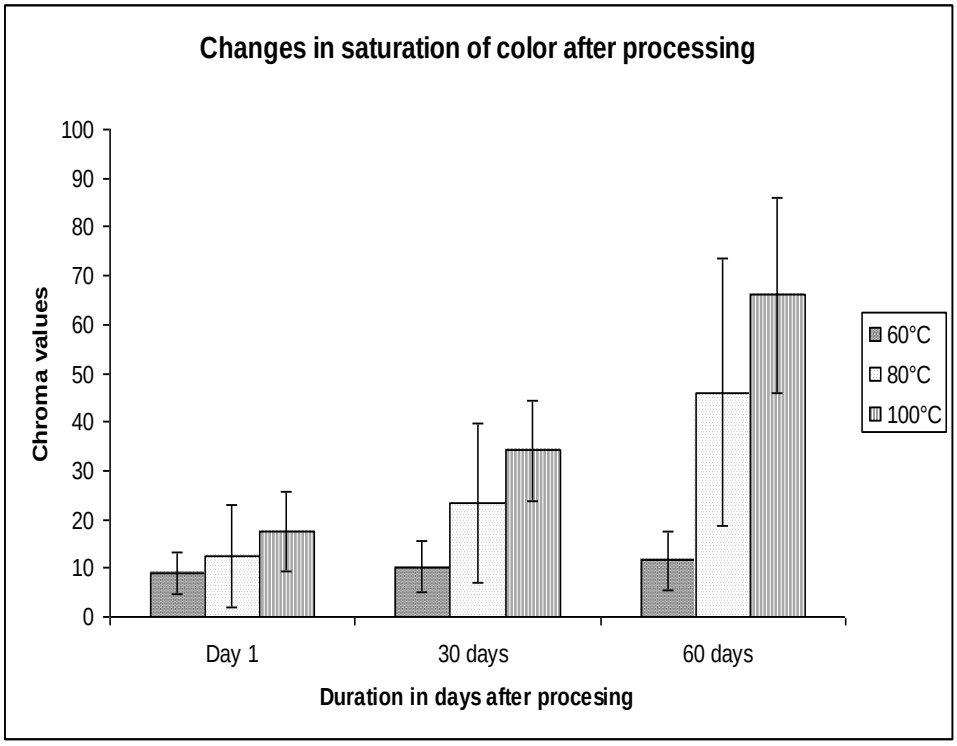

Figure 11: Chroma values for the roselle beverages processed and stored

A confounding phenomon regarding chroma, is that it will increase with pigment concentration to a maximum, and then decrease as the color darkens as shown in Figure 12. Thus a pink and dark red colour can have identical values for chroma as it was also confirmed in a study by (Wrolstad *et al.*, 2005).

4.7 Quality of Roselle Beverages

4.7.2 Sensory Evaluation

The results of sensory analysis of the various formulations for ROD, RAD and RMD are presented in Tables 8, 9 and 10 respectively.

There was no significant preference in the formulations of ROD in sensory parameters of taste, aroma consistency, flavour and general acceptability. However, there was significant preference for appearance/color of the product. In this category 100% roselle drink was most preferred in terms of colour. This is possibly well established because the rest of the formulations had 50%, 40% 1nd 25% fruit suspensions of the orange juice. There was no significant preference in terms of general acceptability for the formulations of ROD.

In RAD, there was no significant preference for sensorial properties of taste, aroma, consistency and flavor. In this category 100% roselle drink was most preferred in terms of colour. This is possibly well established because the rest of the formulations had 50%, 40% 1nd 25% fruit suspensions of the apple juice. There was no significant preference in terms of general acceptability for the formulations of RAD.

RMD scored low ratings for taste, aroma, consistency, and general acceptability. The preference for all the sensory attributes was not significant for the different formulations. Similar preference for 100% roselle drink was exhibited in this

category too. One striking observation was noted with RMD containing less than 75% roselle extract: the panelists expressed high degrees of dislike with scores less than 5. This is implied that the product is unacceptable. The natural aroma of water melon juice could have been objectionable to many panelists

Generally all product categories of 100% Roselle drink, ROD and RAD formulations were acceptable with scores above 6.3 to consumers except RMD with < 75% roselle which was rated below 6/9.

The highly rated (in terms of hedonics score) products were 100% roselle drink and ROD with 50% roselle extract. These two product categories were then considered for consumer acceptability testing during the Nairobi International Trade Fair of October, 2010 as presented in Table 12.

Table 6: Sensory evaluation of different formulations of roselle orange drink.

% of roselle in Product	Appearance	Taste	Aroma	Consistency	Flavour	General Acceptability
100	7.7^{a}	5.7^{b}	6.7^{a}	6.1^{a}	6.3^{a}	6.2^{a}
50	6.2^{b}	6.7^{a}	6.5^{a}	6.8^{a}	6.2^{a}	6.4^{a}
75	6.9^{ab}	6.7^{a}	6.4^{a}	6.4^{a}	6.4^{a}	6.8^{a}
60	6.6^{b}	7.1^{a}	6.2^{a}	6.4^{a}	6.1^{a}	6.7^{a}

NB/=Means in columns with the same letters are not significantly different ($p>0.05$)

Table 7: Sensory evaluation of different formulations of roselle apple drink.

% of roselle in product	Appearance	Taste	Aroma	Consistency	Flavour	General Acceptability
100	7.7[a]	6.5[a]	6.4[a]	6.7[a]	6.8[a]	6.9[a]
50	6.1[b]	6.9[a]	6.3[a]	6.0[a]	7.1[a]	6.9[a]
75	6.3[b]	6.7[a]	5.7[a]	5.3[a]	6.5[a]	6.8[a]
60	6.0[b]	6.8[a]	6.6[a]	6.1[a]	6.8[a]	6.3[a]

NB/=Means in columns with the same letters are not significantly different ($p>0.05$)

Table 8: Sensory evaluation of different formulations of roselle melon drink.

Products ratios (roselle: melon)	Appearance	Taste	Aroma	Consistency	Flavour	General Acceptability
100	7.3[a]	6.0[a]	6.0[a]	5.7[a]	6.0[a]	5.8[a]
50	5.6[b]	4.0[a]	4.1[a]	4.8[a]	4.8[a]	4.8[a]
75	6.1[b]	4.5[a]	5.7[a]	4.9[a]	5.8[a]	5.3[a]
60	6.2[b]	4.8[a]	6.6[a]	5.0[a]	4.9[a]	5.2[a]

NB/=Means in columns with the same letters are not significantly different ($p>0.05$)

4.7.2 Microbial load

Tables 9 and 10 show the results of microbial analysis of the products during storage. Total microbial counts were undetectable from week 0 to week 8. Microbial loads were detected in the roselle drink at week 10 although the levels were low and within the acceptable limits of microbial standards on fruit flavored products (ICMSF, 1996).

From week 12-18 there was a steady increase in the total number of microorganisms. The levels ranged from 0.10 log CFU/ml, 0.24 log CFU/ml for and 0.14 log CFU/ml for RD, ROD and RAD respectively but these values were within the acceptable limits (10^3g/Kg) provided for soft beverages and water based beverages (KEBS, 1996).

The levels remained within the acceptable standard levels. The stability of the beverages in regard to microbial load could be attributed to the acidity of roselle, thermal processing and the aseptic packaging of the products.

Table 9: Microbial Load: total plate count on selected roselle beverages

(log_{10} CFU/ml)

Week	RD	ROD	RAD
0	*ND	*ND	*ND
2	*	*	*
4	*	*	*
6	*	*	*
8	*	*	*
10	*	0.18	*
12	*	0.20	0.02
14	0.02	0.12	0.02
16	0.10	0.21	0.02
18	0.10	0.24	0.14

Values=Mean of 2 replicates (log_{10} CFU/ml) *ND=Not detected.

RD-Pure roselle drink, ROD-Roselle orange drink and RAD –Roselle apple drink

Table 10: Microbial load: yeast and moulds on the three roselle products (Log_{10} CFU/ml) to ascertain GMP

Week	RD	ROD	RAD
0	ND*	ND*	ND*
2	*	*	*
4	*	*	*
6	*	*	*
8	*	*	*
10	*	*	*
12	*	0.12	*
14	*	0.12	*
16	0.12	0.24	0.12
18	0.12	0.40	0.14

Values=Mean of 2 replicates*ND=Not detected.

RD-Pure roselle drink, ROD-Roselle orange drink and RAD –Roselle apple drink

4.7.3 Nutrition composition of the Roselle beverages

Table 11 presents the important product information that is required for product labeling. Though not significantly different, the most acceptable roselle: fruit ratio for RAD, ROD in comparison to pure roselle beverages.

Table 11: Nutritional composition of the most preferred roselle beverages

% of roselle	Percentage (%)						Mg/100ml			
in product	MC	CHO	Protein	Fibre	Ash	Fat	Vit.C	Vit. B6	Iron	Calcium
RD 100	89.6	6.4	0.3	1.3	2.3	1.1	3.1	1.1	8.5	14.4
ROD 75	87.7	6.3	0.6	1.5	0.4	0.1	4.1	1.4	18.5	74.8
RAD 50	84.2	11.8	0.5	1.4	0.4	0.1	5.4	1.3	20.5	76.9
RMD 60	88.6	8.7	0.9	1.6	0.3	0.7	5.2	1.3	16.4	60.6

MC- moisture content, RAD – Roselle apple drink, CHO – carbohydrate, RD- roselle drink, ROD – roselle orange drink, RMD- roselle melon drink.

There was reduction in the macronutrients of the roselle developed products generally as compared to its content in the roselle calyces. However, roselle fruit drink fortification with apples, oranges and melon lead to increase in vitamins C, B6, Iron and calcium. The total increase in the vitamins was between 20-25 %. Vitamin C which is very labile and decreases with thermal processing was improved by incorporation of the fruits in roselle significantly at $p<0.05$

4.7.4 Consumer acceptability tests

The results of the 300 untrained consumer-type panelists were obtained using the Questionnaire in appendix (V) were statistically analyzed and their mean values obtained as shown in Table 12. The product having the highest means on the parameters analyzed were considered the most preferred, though the difference in preference was not significant (P>0.05)

Among the 10-19 age groups, there was no significant difference in terms of the product acceptability where they rated 7.7 and 7.5 for RD and ROD respectively. Colour was a striking parameter that exhibited significant differences in this age group whereby the Roselle Orange Drink was more preferred to RD.

Table 12: Consumer acceptability testing of roselle products.

Age group	Product category	Colour	Taste	Overall consumer acceptability
10-19 years	RD	7.2^{a}	7.7^{a}	7.7^{a}
	ROD	7.7^{b}	7.5^{a}	7.5^{a}
20-29 years	RD	7.0^{b}	7.8^{a}	7.5^{a}
	ROD	7.9^{a}	7.5^{a}	7.3^{a}
>30 years	RD	7.6^{a}	7.9^{a}	7.8^{a}
	ROD	7.9^{a}	7.5^{a}	7.7^{a}

RD-roselle drink, ROD- roselle orange drink, NB/=Means in columns for each age group with the same letters are not significantly different (p>0.05)

In the age group of 20-29 years, they scored 7.5 and 7.3 in terms of general acceptability for RD and ROD respectively. The colour of ROD was more preferred by this age group than that of RD.

Significant differences were noted for the product colour and insignificant for the taste and general acceptability.

The group aged above 30 years expressed more liking for RD than ROD in overall acceptability and taste but not in colour. Similar reasons for colour preference could be true, where a consumer judges a product by appearance. The psychological basis for this judgment is well established.

Figure 12: Roselle beverage products

The packaging and labeling of the products was subjectively attractive according to the consumers' responses where 80% liked very much. The groups as represented by age category had varying preferences, 10-19 years had more preference for ROD similarly, and the 20-29 years had synonymous preferences.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The findings suggested that roselle calyces' nutritional composition include proximate composition, major organic acids, vitamins, minerals and some phytochemicals. It was found that the proximate composition of roselle was constituted mainly by soluble carbohydrate at 66.3%. The minerals that were K, Na, Mg, Ca, Fe, Mn, and Cu. The water soluble vitamins were seven including thiamin, niacin, pyridoxine, folic acid, ascorbic acid and panthothenic acid. The major organic acids were malic, tartaric, citric and oxalic. The major Phytochemicals present in significant amounts were total polyphenols, flavonoids and tannins.

Novel Functional beverages were developed, Roselle Drink, Roselle Orange Drink, Roselle Apple Drink, and Roselle Melon Drink. Quality changes during Storage at 5^0C provided greater retention in phytochemicals as compared to 27^0C. These Phytochemicals exhibited **antioxidant activity, that is,** capacity to scavenge for free radicals and therefore showing the potential of functional properties.

Shelf stability was established and clearly microbial load was within safe limits (KEBS) after 90 days of storage. **Sensory evaluation and** tests showed no significant difference in overall acceptability for the beverages made from roselle calyces alone, and those mixed with Orange, Apple whereby they all had scores above 6.3. However for Roselle melon drink (RMD) the average preference score was below 5, hence they were deemed unacceptable.

Consumer acceptability showed that the roselle novel beverages were generally highly acceptable and this is a pointer of success on market scenario.

In conclusion, the results showed that the Roselle calyces could therefore find applications in the production of acceptable refreshing soft drink and other tropical fortified beverages for instance in blends with apple juice, orange juice and water melon juice . However, with melon juice, the acceptable blend contains down to 75% roselle drink while with two other juices, the roselle proposition can be as slow as 50%.

5.2 Recommendations

The following are the recommendations

i. Further research in elucidation of the functional properties of roselle and the beneficial individual Phytochemicals present.
ii. The Ministry of Agriculture (MOA) and JKUAT to collaboratively upscale the production of roselle using elite cultivars to enhance commercial utilization in Kenya.
iii. The ministry of Industrialization through KIRDI to support further development of other convenient products from roselle for application in other industries e.g. pharmaceutical and textile
iv. Further research studies should be done to ascertain the linkage of flavonoids to health benefits of roselle

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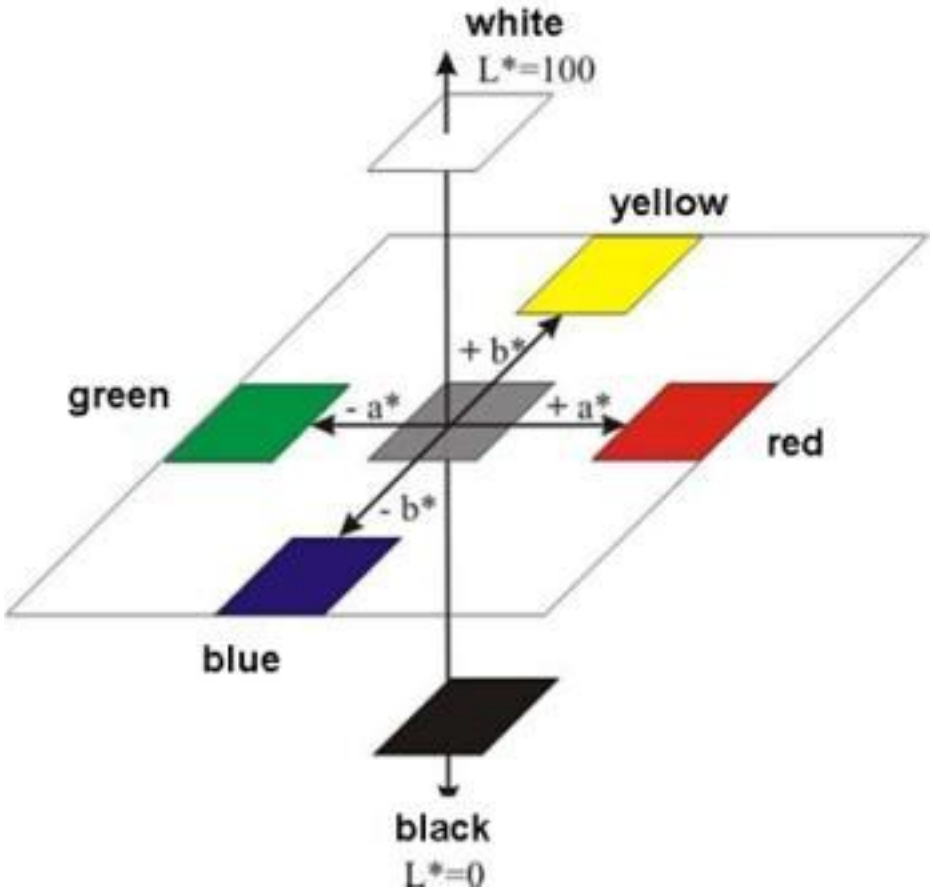
APPENDICES

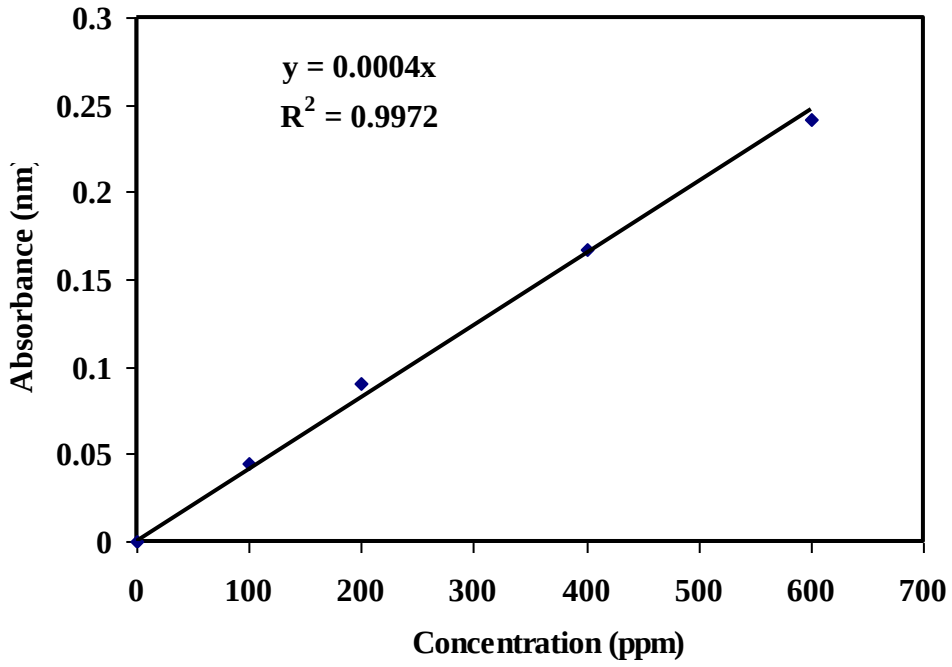
Appendix I: Tri stimulus colour code match table



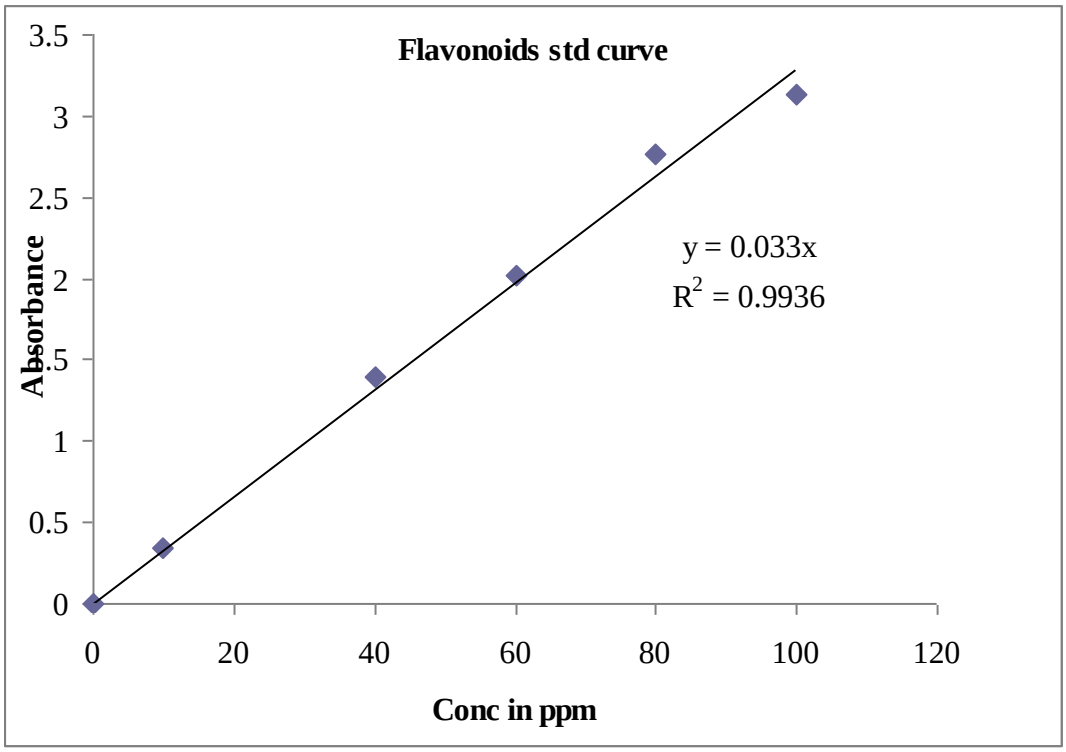
Code	Meaning	Range	Trend
L*(+)	Lightness	0-100	Lightness increases from 0-100.
a* (-)	Green	0 Downwards	Greenness increases with negativity.
a* (+)	Red	0 Upwards	Redness increases with increase in numerical value.
b* (-)	Blue	0 Downwards	Blueness increases with negativity
b* (+)	Yellow	0 Upwards	Yellowness increases withl value.

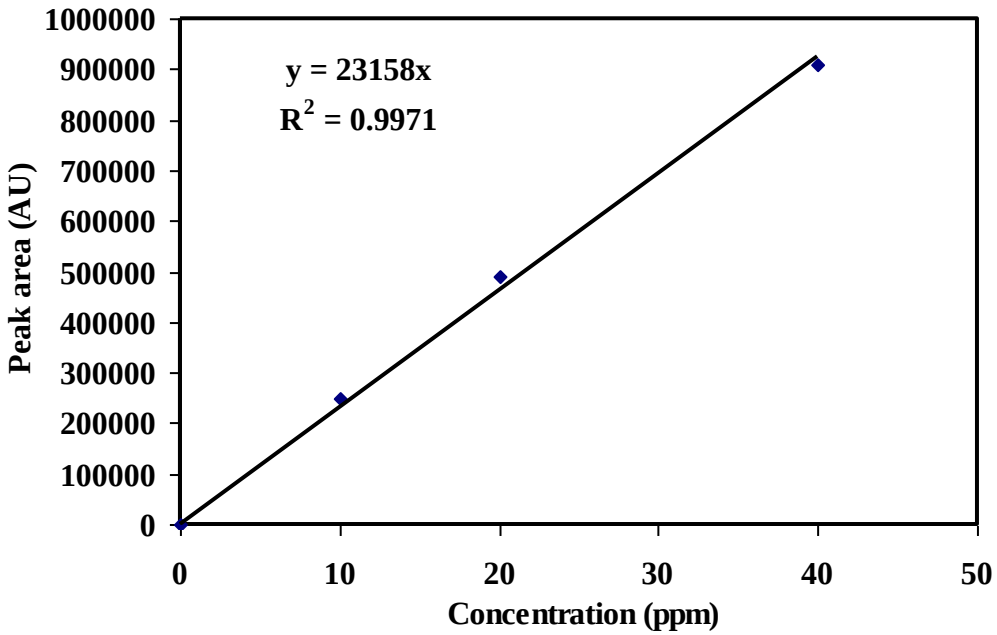
Appendix II: Standard curves

a) Tannins

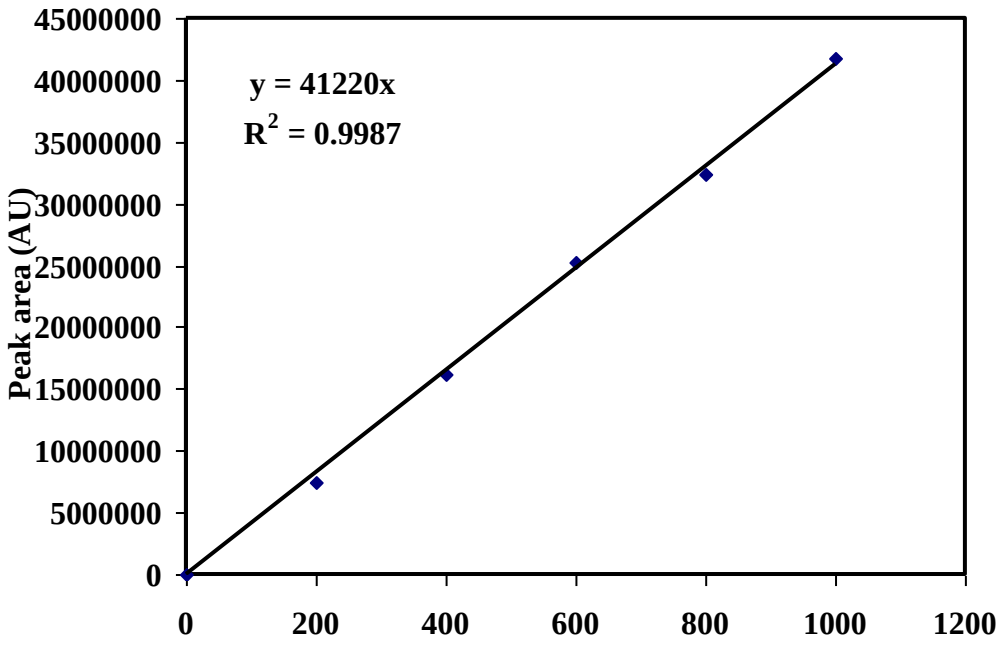


(b) Tannin standard curve

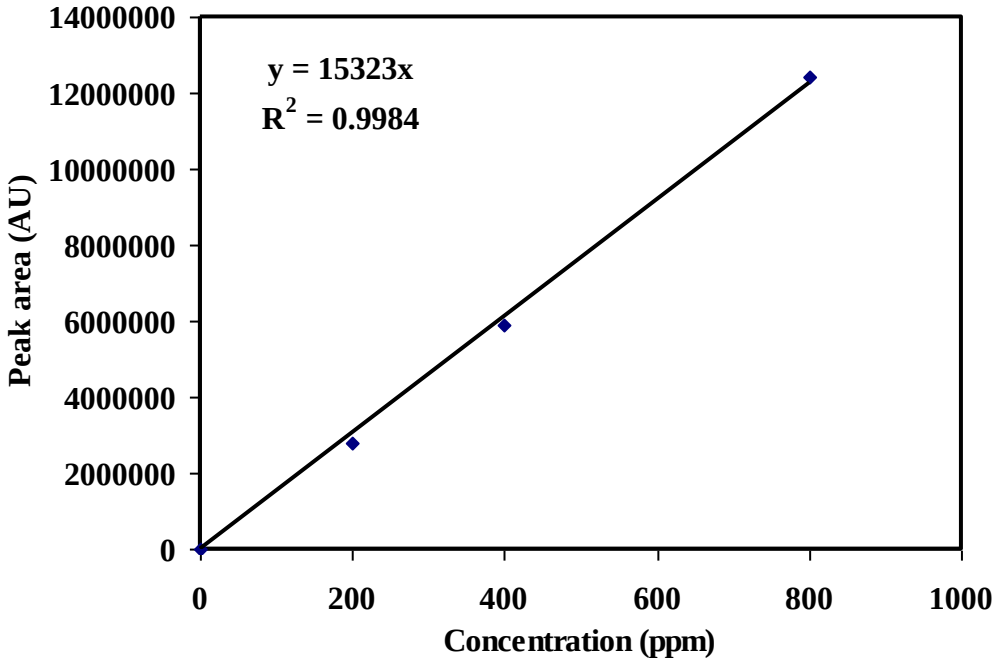




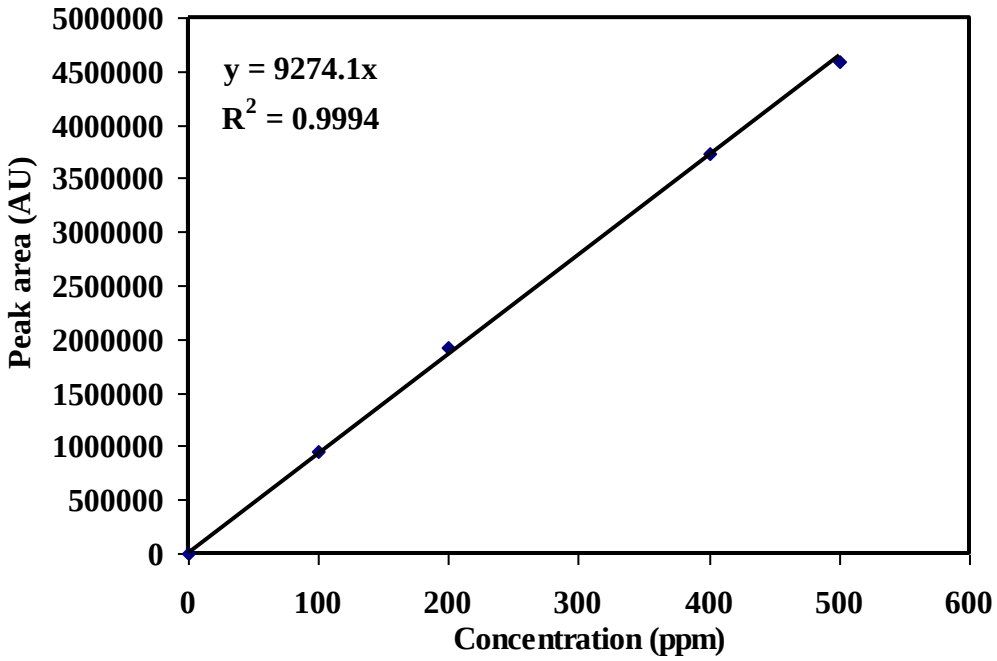
(c) Folic acid standard curve



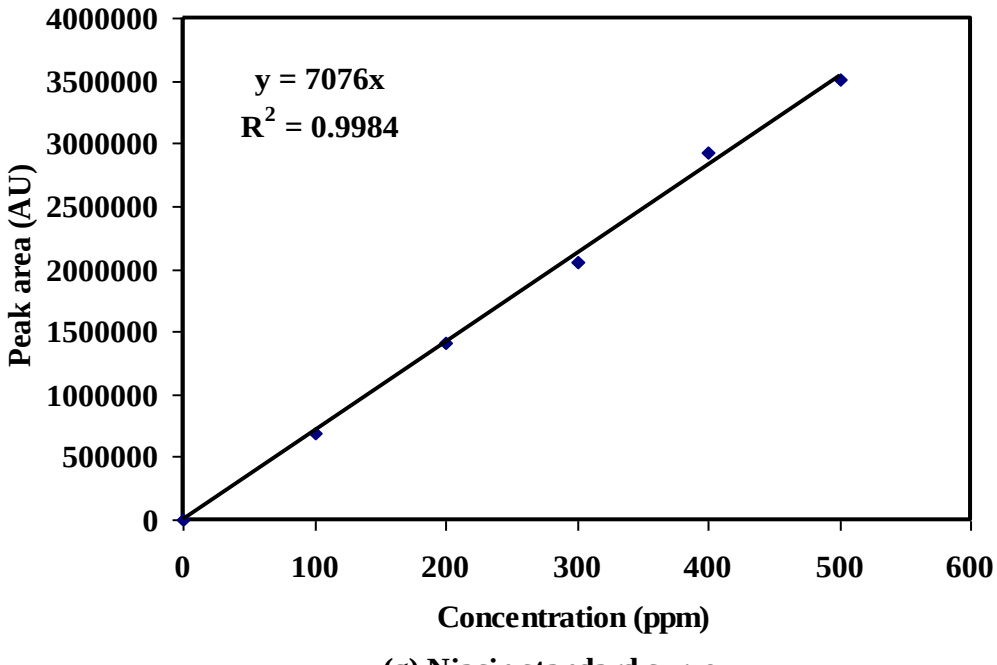
(d) Thiamin hydrochloride standard curve



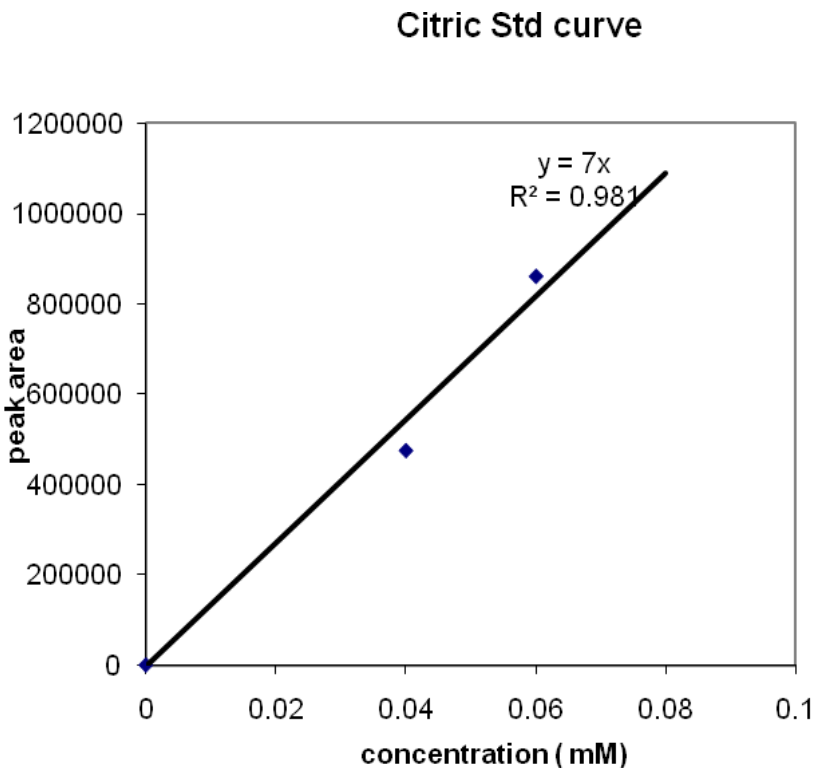
(e) Riboflavin standard curve



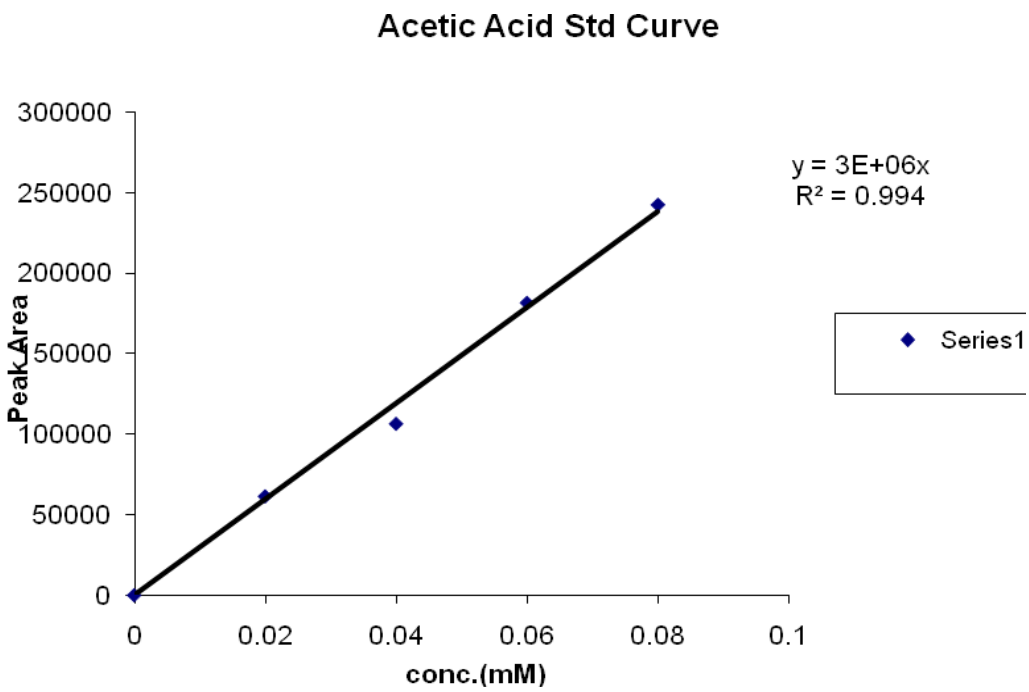
(f) Pyridoxine standard curve

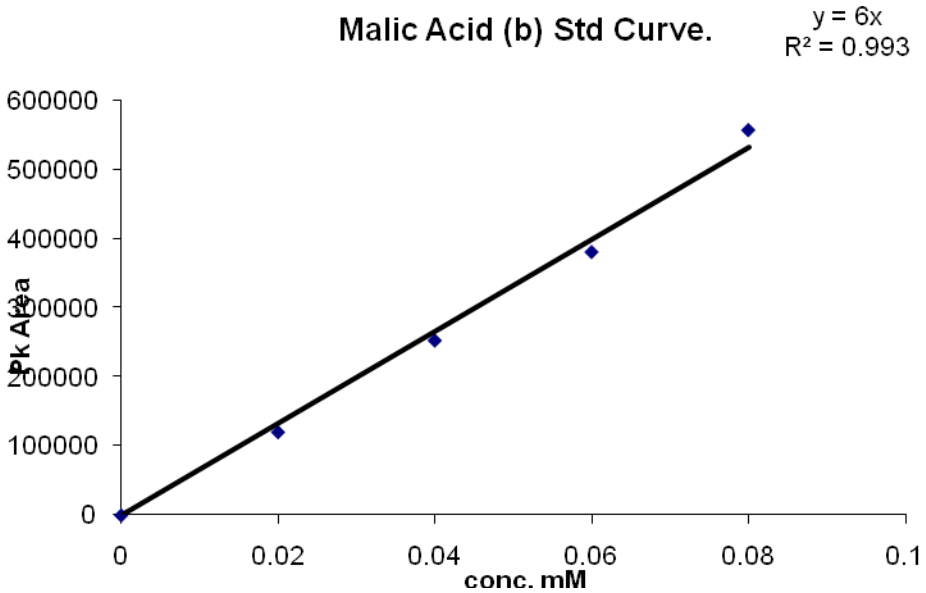


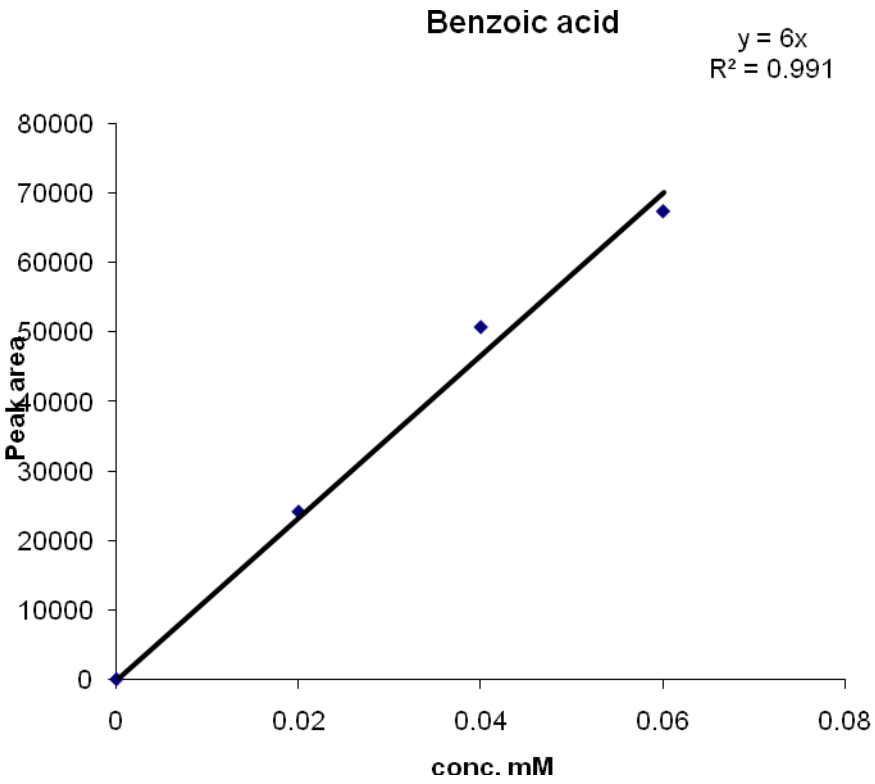
(g) Niacin standard curve

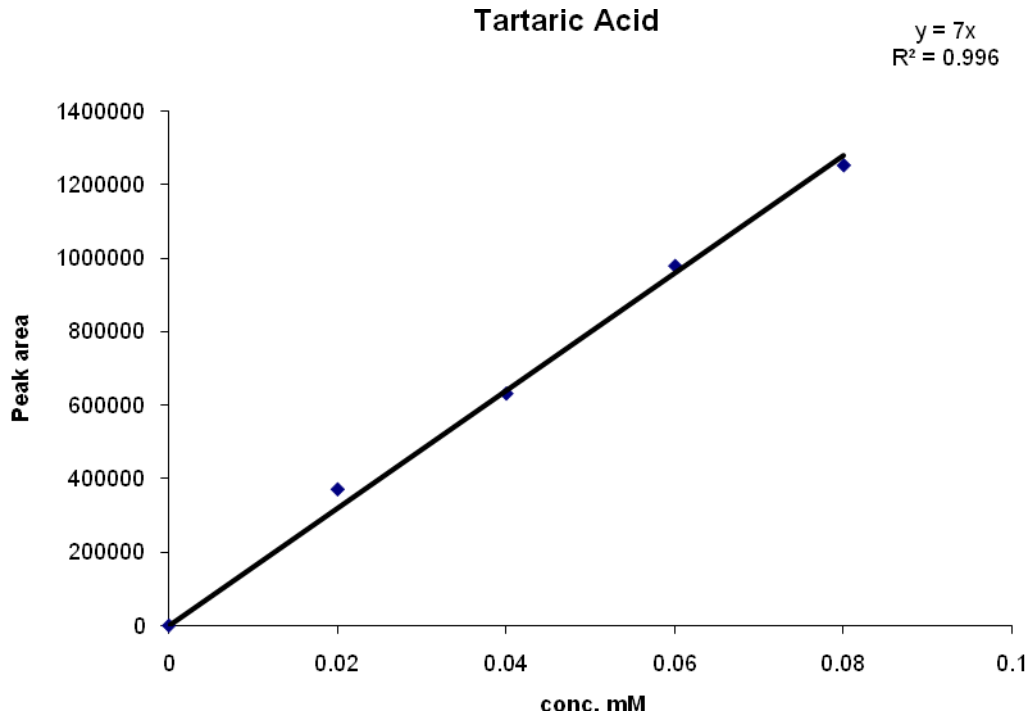


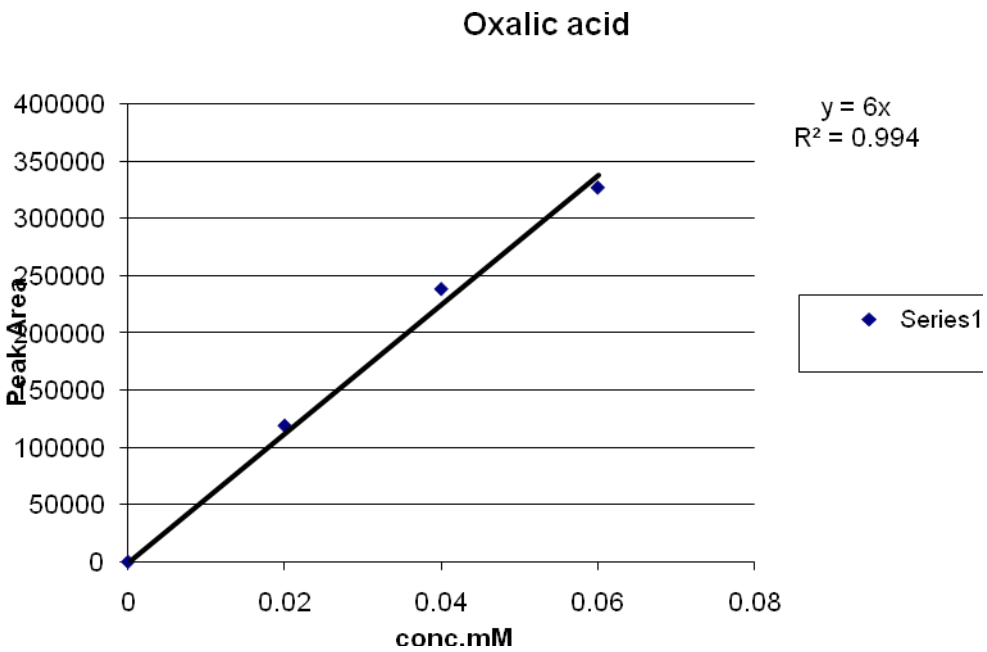
h) Citric acid


Acetic Acid Std Curve
y = 3E+06x
R² = 0.994
Series1
Peak Area
300000
250000
200000
150000
100000
50000
0
0
0.02
0.04
0.06
0.08
0.1
conc.(mM)


Malic Acid (b) Std Curve.
y = 6x
R² = 0.993
600000
500000
400000
300000
200000
100000
0
0
0.02
0.04
0.06
0.08
0.1
conc. mM


Benzoic acid
y = 6x
R² = 0.991
80000
70000
60000
50000
40000
30000
20000
10000
0
Peak area
0
0.02
0.04
0.06
0.08
conc. mM


Tartaric Acid
y = 7x
R² = 0.996
1400000
1200000
1000000
800000
600000
400000
200000
0
Peak area
0
0.02
0.04
0.06
0.08
0.1
conc. mM


Oxalic acid
y = 6x
R² = 0.994
Series1
Peak Area
400000
350000
300000
250000
200000
150000
100000
50000
0
0
0.02
0.04
0.06
0.08
conc.mM

Appendix III: Sensory evaluation questionnaire

Date……Time…….

Instructions

You are provided with four batches with four (4*4) different formulations coded samples of fruit beverages to carry out sensory evaluation on them and express how much you like or dislike them. You are also provided with water to rinse your mouth after tasting each sample. Use the scale below to express your attitude towards the product colour, taste, flavor and general acceptability of each of the samples by inserting the appropriate score in the space provided.

You are also requested to give any comments about the products and please try to be as honest as possible. Thank you.

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

Attribute	Samples			
Sample codes	300	301	305	100
Appearance: Color Clarity				
Taste				
Aroma				
Consistency				
Flavour				
General Acceptability				

Remarks

……

……

……

Appendix IV: Product labels and packaging

Figure 13: Samples for packaged Roselle products.

The process of product development was dully followed and sample labels developed**.**

Appendix V: Consumer Product acceptability testing questionnaire

Date…….Time……

Instructions

You are provided with two coded samples of fruit beverages to carry out sensory evaluation on them and express how much you like or dislike them. Use the scale below to express your attitude towards the product' colour, taste, and general acceptability of each of the samples by inserting the appropriate score in the space provided.

You are also requested to give any comments about the products and please try to be as honest as possible. Thank you.

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

Attribute	Product codes	
	201	100
Colour		
Taste		
General Acceptability		

Remarks

……

……

……