EFFICACY OF MUTHOKOI POUNDED WITH GREEN LEAFY VEGETABLES IN IMPROVING THE VITAMIN A STATUS USING RATS

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## DEPARTMENT OF FOOD SCIENCE, NUTRITION & TECHNOLOGY

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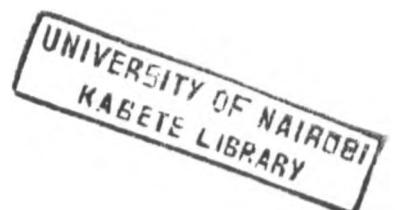
DECLARATION

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

Sign................................. Date........30/11/12..............................

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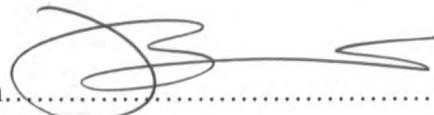
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DEDICATION

To my **ALMIGHTY** God,

My loving husband **Dr. G. G. Mukora**, thank you for your unconditional support,

Daughter **Tiffany Wanjiru**,

Dear parents **Symon & Esther Maina,**

Thank you!

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ABBREVIATIONS/ ACRONYMS

AAS	Atomic Absorption Spectrophotometery
AOAC	Association of Analytical Chemistry
FAO	Food and Agricultural Organization
HPLC	High Pressure Liquid Chromatography
KDHS	Kenya Demographic and Health Survey
MI	Micronutrient Initiative
MOPHS	Ministry of Public Health and Sanitation
NFE	Nitrogen Free Extract
PHPT	Dept. of Public Health, Pharmacology and Toxicology, (University of Nairobi)
RE	Retinol Equivalent
RBP	Retinol-binding protein
RDAT	Rapid Dark Adaptation Test
RDR	Relative Dose Response
UV	Ultra Violet Radiation
VAD	Vitamin A Deficiency
VADD	Vitamin A Deficiency Disorders
VAS	Vitamin A Supplementation
WHO	World Health Organization

ABSTRACT

Majority of Kenyan families especially those from lower socio-economic class feed their children with cereal-based foods sometimes with little vitamin A. Green leafy vegetables are sometimes incorporated, but though containing high levels of vitamin A, do not effectively release the vitamin to the body due to interaction with the leaf matrix. Decortication of maize to produce *Muthokoi* by pounding in a mortar with pestle sometimes incorporates green leafy vegetables resulting in a greenish yellow product due to infused β-carotene. The β-carotene is presumed to be better available from this *Muthokoi* because it is only physically bound.

This study was designed to assess the efficacy of *Muthokoi* pounded with pumpkin leaves in improving the vitamin A status of rats with view to relating the results to humans. Different proportions of maize and the pumpkin leaves were pounded and the most appropriate *Muthokoi* determined. The *Muthokoi* was analyzed for proximate composition, β-carotene, iron and calcium.

The *Muthokoi* was milled into meal and fed to laboratory rats together with whole maize meal as control. A total of ten rats were used, with five rats as the test group and five rats as the control group. The test group was first induced with vitamin A deficiency to the level of $0.696 \pm 0.075\mu mol/L$ from serum retinol level of $1.87 \pm 1.60\mu mol/L$, after which they were fed with β-carotene enriched *Muthokoi* for a period of three weeks. The control group was not induced with the deficiency but were fed on the whole maize meal for a period of three weeks. Both the test group and the control had their basal serum retinol levels determined and thereafter the serum retinol levels were determined every three weeks using High Pressure Liquid Chromatography.

The proportion of maize and pumpkin leaves that was selected for the study was one part of maize pounded with two parts of pumpkin leaves which resulted to *Muthokoi* with a β-carotene content of $465.3 \pm 9.20 \mu g/100g$. After pounding maize with pumpkin leaves, it was observed that the β-carotene content of the maize was significantly increased from $26.87 \mu g/100g$ to 465.3 $\mu g/100g$. The *Muthokoi* was also enriched with calcium and iron to levels of 21.2 ± 5.3 $17.3 \pm 8.24 mg/100g$ respectively.

The serum retinol levels of the control and test groups before deficiency induction did not show significant difference ($p > 0.05$) between them. The mean levels of serum retinol among the control group of rats did not change significantly ($p > 0.05$) after the three weeks of feeding, the serum retinol during the three weeks feeding period changed from $1.85 \pm 0.214 \mu mol/L$ to $1.86 \pm 0.207 \mu mol/L$.

The serum retinol levels of the test group after deficiency induction was 0.696 ± 0.075 and the content after the intervention was 1.496 ± 0.069. This showed a significant increase ($p < 0.05$) in the serum retinol levels due to feeding with enriched *Muthokoi*.

The study showed that maize meal from *Muthokoi* pounded with pumpkin leaves is effective in increasing the vitamin A status of vitamin A deficient rats.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Vitamin A occurs in foods in two different forms. In animal foods, the vitamin occurs as preformed retinol and is the most readily absorbable in the body. In plant foods, the vitamin occurs as very closely related provitamin carotenoids. These compounds are hydrolysed in the body to yield molecules of retinol. However, it is alleged that the availability of vitamin A from plant foods is usually low because the compounds are tenaciously bound to the matrices of the plant and the efficiency of the gastro-intestinal enzymes to hydrolyze them in such enviroment is not very efficient. The rich plant sources of vitamin A include pumpkins, dark green leaves, carrots, ripe pawpaw and yellow or orange sweet potatoes (GOK, 2011).

Vitamin A deficiency has become a global problem in the recent past due to the increased cases of blindness in children as a result of the deficiency. In the early 1990s, WHO estimated that about 3 million children in India, parts of South East Asia and Sub-Saharan Africa had some form of xerophthalmia annually and, another 250 million were clinically deficient (Zempleni et al., 2007).

Vitamin A deficiency is generally estimated to affect approximately one third of children under the age of five around the world. It is estimated to claim the lives of 670,000 children under five annually. Approximately 250,000–500,000 children in developing countries become blind each year owing to vitamin A deficiency, with the highest prevalence in Southeast Asia and Africa. Night blindness is one of the first signs of vitamin A deficiency. Xerophthalmia and complete blindness can also occur since Vitamin A has a major role in photo-transduction. The prevalence

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of night blindness due to vitamin A deficiency is also high among pregnant women in many developing countries. Vitamin A deficiency therefore contributes to maternal mortality and other poor outcomes in pregnancy and lactation (Catharine, 2007).

According to Kenya Demographic and Health Survey 2008/2009 (KNBS, 2010) the proportion of children consuming vitamin A rich foods in Kenya increases with age, from 49 percent at 6-8 months to 86 percent at 24-35 months. At provincial level, children in Central (88 percent) and Western (87 percent) provinces are the most likely to consume vitamin A-rich foods and those in North Eastern province the least likely (27 percent).

The country exhibits an alarmingly high prevalence of vitamin A deficiency. In 1999, at the national level, 84% of children under 6 years had low serum retinol ($<20\mu g/dL$), indicating sub-clinical vitamin A deficiency. Children from Coastal region were the most affected (FAO, 2005).

A commonly used intervention among the Under Fives is the supplementation with vitamin A capsules. Since 1998, Micronutrient Initiative (MI) has been supporting twice-yearly preventative vitamin A supplementation because it is also one of the most cost-effective, established programs that significantly improve child survival. By 2004, almost 60% global coverage had been achieved. MI and partners in the Global Vitamin A Effort continue working for higher coverage with the aim that every child under 5 who is at risk of vitamin A deficiency can receive a high-strength vitamin A supplement (VAS) every 6 months (Zempleni et. Al., 2007).

Food based approach is a vitamin A intervention that encompasses dietary diversification and is the most fundamental and sustainable. This approach is based on increasing selection and consumption of foods rich in dietary vitamin A activity to bring the habitual intake into the range of recommended intakes. Culture, economic limitations and cuisine interfere with achieving targets in low-income societies (Bowman, 2006).

Muthokoi is a Kamba term used to describe decorticated maize. Muthokoi is mainly prepared and consumed by the Kamba community, but also by the Meru and Embu communities. In fact, its origins can be traced to Eastern Province where the Kamba hail from. Traditionally, *Muthokoi* is prepared by pounding wetted maize in a mortar with pestle. Sometimes, supposedly to cushion the brittle maize from breakage, the wet maize is pounded in admixture with green leaves. The wetting of the maize loosens the seed coat, which through the pounding is removed by abrasion. *Muthokoi* is desired to have only the seed coat removed while preferably leave the germ on the kernel. The most commonly used leaves for pounding are the pumpkin leaves. *Muthokoi* prepared in this way is usually greenish-yellow in color, indicating absorbed carotenoids and chlorophyll. Other nutrients from the leaves are also absorbed by the kernels in the process.

Preliminary studies carried out in the laboratories of the department of Food Science, Nutrition and Technology (University of Nairobi) where *Muthokoi* was pounded with four green leafy vegetables showed pumpkin leaves to be the most effective in enriching *Muthokoi* with vitamin A beta-carotene. Although bio-availability studies were not conducted, it was presumed that the beta-carotene absorbed by the *Muthokoi* was only physically bound and would therefore be more readily bio-available than from the leafy vegetables (Imungi, 2002).

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1.2 PROBLEM STATEMENT

Provitamin A carotenoids in foods are often bound within plant matrices of indigestible polysaccharides, fibers and phenolic compounds which reduce bioavailability (Zempleni et al., 2007). Green leafy vegetables contain high levels of beta-carotene, the most potent pro-vitamin athough the beta-carotene is chemically bound within the leaves matrix which makes its availability poor.

Children in Kenya are mainly fed cereal based starchy foods with green vegetables. These foods though containing reasonable levels of pro-vitamin A carotenes, do not release it sufficiently to the body due to the tenacity with which the compounds are bound in the tissue structures. Most cereals with the exception of the yellow maize are very poor in vitamin A, the little present only being found in the germ most of which is removed during the milling process.

Current vitamin A deficiency interventions include food fortification and supplementation which have significant impact in regions where they are administered but the interventions are not sustainable in the long run and may not reach resource-poor communities. This is because resource-poor households consume insignificant amounts of processed foods, limiting the use of food fortification. In addition, they tend to be situated in remote areas characterized by poor infrastructure, inadequate health care, and insufficient public funds. This situation limits the use of supplementation as a sustainable intervention (Mwaniki, 2007).

Vitamin A is found in large amounts in relatively few foods that are often highly seasonal, such as orange-coloured fruits and dark green leafy vegetables. Although vitamin A is stored in the liver, the amounts ingested during the season of abundance may not be sufficient to maintain

adequate vitamin A status throughout the year, especially when foods with poor availability are consumed. Food based approaches are however, still the most sustainable interventions in such areas.

1.3 JUSTIFICATION FOR THE STUDY

Dietary diversification is still one of the best way to alleviate malnutrition and micronutrient deficiencies. It aims at ensuring that the available diet is adequate in every nutrient. Dietary diversification is a long term objective, and it provides some indicators about what strategies may be sustainable (Mwaniki, 2007). It is also a cheap method of overcoming malnutrition compared to other interventions for vitamin A deficiency, since nutrient-rich foods that are native to the diets and livelihoods of the people are utilized. Food strategy interventions need to be used since they are more sustainable and offer long-term solutions to vitamin A deficiency.

The pounding of *Muthokoi* with green leaves is an accepted tradition in communities which include the Kamba, Meru and Embu where *Muthokoi* is a common food. The pounding leads to infusion of the maize kernel with beta-carotenes. A study in the Department of Food Science, Nutrition and Technology, University of Nairobi showed that pounding with pumpkin leaves resulted in up to 7 times increase in the beta-carotene of the kernel (Imungi, 2002). It is presumed that the nutrients are only physically bound on the kernel and therefore will be more readily available than from the leafy vegetables in a meal. The pounding which leads to infusion of the kernel with other nutrients such as vitamins and minerals. In particular the kernel absorbs niacin from the leaves and helps to control pellagra in communities with predominantly maize diet.

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Maize is the preferred staple food for almost all communities in Kenya. The *Muthokoi* is therefore an appropriate choice of vehicle for this bio-fortification, since the *Muthokoi* can be promoted to other communities. The *Muthokoi* can be further dried and milled into flour which can be used for making porridge (for infants and children) and *Ugali*, a common maize-meal dish in Kenya.

This food strategy can be adopted in other parts of the country where *Muthokoi* is not consumed as a means to improve the vitamin A status of the vulnerable groups. Nutrition education to communities on the importance of adoption of this indigenous practice which is capable of improving the people's vitamin A status will be necessary for its adoption.

Most people in Kenya derive their dietary vitamin A from plant foods. The green leafy vegetables including the traditional African leafy vegetables such as pumpkin leaves are the most important sources of vitamin A for those whom consume them. The traditional leafy vegetables are very affordable; hence even the resource-poor individuals can afford to purchase or grow them. Thus, there is a need to develop techniques to preserve provitamin A-rich foods in order to ensure adequate supply through seasons of lower availability and to reduce postharvest losses (Marie, 2000). Since the pounding of Muthokoi with the leaves is a tradition in the community who have it as a staple, this would form an entry point for intervention in vitamin A deficiency. Moreover, the brisk inedible leaves can be used for the pounding, leaving the tender ones to be used for vegetables.

Studies done at the University of Wisconsin (Howe, 2006) using beta-carotene rich maize resulted in the increase of the vitamin A status of gerbils under a study aimed at tackling vitamin

A deficiency. From the results it was reported that if humans responded similarly, it would have important implications for vitamin A depleted populations consuming maize as a staple food crop. The report went on to say that such a venture could potentially eliminate costly and most often ineffective supplementation programs, improve morbidity and mortality of mothers and infants, and reduce the occurrence of night-blindness in adults and blindness in children. It was concluded that the biofortified maize adequately maintained vitamin A status of Mongolian gerbils and was as efficacious as beta-carotene supplementation (Howe, 2006). From the above study it is clear that studies done on rats concerning deficiency in vitamin A can be applied to human studies to determine the efficacy of enriching maize with beta-carotene.

1.4 OBJECTIVES

1.4.1 Overall Objective

To assess the efficacy of *Muthokoi* pounded with green leafy vegetables in improving the vitamin A status using rats.

1.4.2 Specific Objectives

1) To develop appropriate beta-carotene enriched *Muthokoi* using pumpkin leaves

2) To determine the proximate composition, beta-carotene, calcium and iron contents of *Muthokoi* and its products

3) To assess the serum retinol levels of laboratory rats before and after feeding with *Muthokoi* products

1.5 HYPOTHESIS

H_0: " The Beta-carotene enriched Muthokoi and products will not improve the vitamin A status of laboratory rats"

CHAPTER TWO: LITERATURE REVIEW

2.1 CURRENT GLOBAL STATUS OF VITAMIN A DEFICIENCY (VAD)

Vitamin A deficiency affects over 250 million people worldwide and is one of the most prevalent nutritional deficiencies in developing countries, resulting in significant socio-economic losses. Balanced diets are not accessible for a large proportion of the world's population, particularly those who live in developing countries. Many populations or subgroups of populations subsist on staple plant-based diets that often lack diversity (and also quantity sometimes), which may result in micronutrient deficiencies (Ruel, 2000).

The World Health Organization (WHO) estimates that over four million children under five years suffer from by vitamin A deficiency, resulting in increased vulnerability to common childhood diseases (such as measles, diarrhea, and malaria) and leading to the deaths of one million children each year.

Vitamin A deficiency is among the most common and serious of all nutritional deficiency diseases. But it is also the one for which there is the greatest hope for control and prevention within the foreseeable future (WHO, 1996).

Risks of vitamin A deficiency occur when the level of vitamin A deficiency is "subclinical" – that is to say, there is no clinical disease and the deficiency can be revealed only by carrying out biochemical or other laboratory tests. This subclinical deficiency is much more common and widespread than clinical deficiency, such as Xerophthalmia. It affects mainly young children and their mothers during pregnancy and lactation. This means that the greatest emphasis needs to move away from blindness and the eye, to the area of Maternal and Child Health (Mclaren, 2001).

2.2 PREVALENCE OF VITAMIN A DEFICIENCY

2.2.1 Global Prevalence

Globally, 5.2 million preschool-aged children and 9.8 million pregnant women are reported to be vitamin A deficient, which corresponds to 0.9% and 7.8% of the population at risk of VAD, respectively. An estimated 190 million preschool-aged children and 19.1 million pregnant women have low serum retinol levels. This corresponds to 33.3% of the preschool-age population and 15.3% of pregnant women in populations at risk of VAD. Africa and South East Asia have the highest proportions of both preschool-aged children and pregnant women with VAD (Sight and Life Magazine, 2009).

2.2.2 Prevalence In Sub-Saharan

Sub-Saharan Africa is the only region in the world where the nutritional status of children is worsening and where infant and child mortality rates continue to rise. Vitamin A deficiency (VAD) across the region is extremely high, varying between 20 and 80% (Sight and Life Magazine, 2009).

2.2.3 Vitamin A Status in Kenya

Kenya exhibits an alarmingly high prevalence of vitamin A deficiency. In 1999, at the national level, 84% of children under 6 years had low serum retinol ($<20\mu g/dL$), indicating sub-clinical vitamin A deficiency. Children of Coastal region were the most affected (FAO, 2005). According to the current micronutrient status, 2004, the estimated % of children under six with sub-clinical vitamin A deficiency was 70%.

2.3 NATURE AND OCCURRENCE OF VITAMIN A IN FOOD

Vitamin A was discovered in the 1900s by McCollum and colleagues at the University of Wisconsin and independently by Osborne and Mendel at Yale (Catharine, 2007).

Vitamin A is a term used to describe group of compounds having similar biologic activity and they include **retinol**, **retinal** and **retinoic acid**. It is soluble in fat and ordinary fat solvents. Dietary vitamin A is ingested in 2 main forms:

1) Preformed vitamin A (retinyl esters and retinol)

2) Provitamin A i.e. carotenoids

Provitamin A carotenoids are provided in the diet by green and yellow or orange vegetables and some fruits. Provitamin A carotenoids include beta-carotene, alpha-carotene and beta-cryptoxanthin with beta-carotene having the highest vitamin A activity and being the most widely distributed in plant products compared to the rest. Foods rich in provitamin A include yellow and orange fruits, dark green leafy vegetables, pumpkin, carrots, sweet potatoes (orange-fleshed). In most Kenyan communities children under 5 years are fed on pumpkin leaves in combination with other starchy roots and tubers which form a major part of their diet. Infants in sub-Saharan Africa rely on maize or millet porridge as a complimentary feed during the weaning period, hence enrichment of maize with beta-carotene may play a big role in alleviation of VAD especially among the under fives.

Preformed vitamin A is present in foods of animal origin mainly organ meats such as liver, meats, eggs and dairy products. These foods are however, not commonly consumed by the majority of house-holds in developing countries and especially sub-Saharan Africa. VAD is not a problem in the developed countries because the diet contain reasonably large amount of animal foods for example about 70% of vitamin A in a typical North American diet comes from animal sources (Zempleni et. al., 2007).

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2.3.1 Vitamin A Requirements for Children aged 0-6 years

The recommended nutrient intake of children aged 0-6 years is as shown in table 1 below:

Table 1: Recommended Vitamin A Intake by Children

Group	Vitamin A (RE/day)
INFANTS	
0-6 months	375
7-12 months	400
CHILDREN	
1-3 years	400
4-6 years	450

Source: Adapted from WHO/FAO, 2004. Vitamin and Mineral requirements in Human Nutrition, 2nd edition.

2.3.2 Units of Conversion of Retinol to β-carotene

Vitamin A levels in blood are conventionally expressed in μg/dL or μmol/L of all-trans-retinol.

Retinol equivalent (Re) is used to express the vitamin A activity of carotenoids in diets and the

following relationships among food sources of vitamin A have been established

(www.wikipedia.com).

   1 μg retinol = 1 RE

   1 μg beta-carotene = 0.167 μg RE

   1 μg other provitamin A carotenoids = 0.084 μcg RE

2.4 FUNCTIONS OF VITAMIN A IN THE BODY

Vitamin A (retinol) is an essential micronutrient for all vertebrates. It is required for normal

vision, reproduction, embryonic development, cell and tissue differentiation and immune

function (Gibson, 2005).

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2.4.1 Vision

Vitamin A as retinal is needed in the retinal of the eye to turn visual light into nerve signals to the brain. The absorption of light by rhodopsin in the photoreceptor cells results in the instantaneous isomerization of its 11-*cis*-retinal moiety to all-*trans*-retinal, and this photoisomerization event initiates a signal cascade to nearby retinal ganglion cells, which is propagated to the visual cortex of the brain. When retinal tissue is deprived of vitamin A, rod and cone function is impaired (Gibson, 2005).

2.4.2 Maintenance of Healthy Epithelial Tissue

Vitamin A is necessary to build and maintain healthy epithelial tissue, which provides our primary barrier to infections. Vitamin A is required for the integrity of epithelial cells throughout the body, via the regulatory action of retinoic acid at the level of the gene (Gibson, 2005). Without vitamin A the epithelial cells become dry and flat and undergo keratinization. This is observed in the eye cornea, respiratory tract, skin, genitourinary tract etc.

2.4.3 Growth and Development

Vitamin A (retinoic acid) is vital in embryonic development where it's important for the growth of bones and soft tissues. Its lack in early pregnancy results in birth defects and fetal mortality. Advances in molecular biology and retinoic acid receptor research have significantly contributed to the understanding of the role of vitamin A during vertebrate development. Vitamin A requirement begins at the time of formation of the primitive heart, circulation and specification of hindbrain. The lack of vitamin A at this critical time results in gross abnormalities and early embryonic death (Gibson, 2005).

2.4.4 Immune Function

Vitamin A as retinoic acid is important for immune system functions. The ability of vitamin A to reduce mortality is widely thought to be due to effects on the immune system, which collectively may reduce the severity of disease and increase the likelihood of survival. Studies indicate that diets high in carotenoid-rich fruits and vegetable may decrease risk of certain eye diseases, cancers and cardiovascular disease. Studies have shown increased incidence of infection as one of the first symptom of vitamin A deficiency (Gibson, 2005).

2.4.5 Antioxidant Capacity

The provitamin A, beta-carotene has been shown to have antioxidant capacity, raising interest in protecting persons as they grow older from cell damage caused by free radicals (Gibson, 2005).

2.5 VITAMIN A DEFICIENCY (VAD)

Vitamin A deficiency is the primary cause of preventable childhood blindness and a major contributor to severe morbidity and mortality from infections, especially among the poor in low and middle income countries (Sight and Life Magazine, 2009).

Almost one third of children in developing countries are affected to some degree by vitamin A deficiency, which impairs their growth, development, vision and immune function, and in extreme cases leads to blindness and death.

Vitamin A deficiency is not easily defined. World Health Organization (WHO) defines it as tissue concentrations of vitamin A low enough to have adverse health consequences even if there is no evidence of clinical xerophthalmia (WHO, 1996). VAD can occur in individuals of any age.

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However, it is a disabling fatal public health problem for children under 6 years of age. This is due to the high requirements for vitamin A to support rapid growth (WHO/FAO, 2004).

A large number of foods contain substantial amounts of either vitamin A or carotenoids, and many of these foods are widespread and inexpensive even to the very poor. Thus, in a logical sense, there is no reason exists for vitamin A

deficiency to be a world-wide problem. Nonetheless, it is a world-wide problem. In the absence of dietary vitamin A, children grow poorly and develop signs of deficiency, of which Xerophthalmia and night blindness are the most characteristic and specific (Olson, 1994).

The term Vitamin A Deficiency (VAD) embraces all forms and degrees of deficiency, including the most severe, in which the function and structure of the eye are affected. All stages of the eye changes are covered by the term Xerophthalmia (Mclaren, 2001).

Vitamin A deficiency compromises immunity, resulting in major morbidity and mortality. The link between clinical vitamin A deficiency (night blindness and Xerophthalmia) and infectious disease morbidity and mortality has been known for hundreds of years (Semba, 1998).

2.6 SOURCES OF DIETARY VITAMIN A

Vitamin A is provided in the diet either as preformed vitamin A, from animal sources or provitamin A carotenoids, from plant sources. The most concentrated sources of preformed vitamin A are: liver, fortified margarine, milk and dairy products, oily fish, egg yolk and fish liver oils; whereas the most concentrated sources of provitamin A carotenoids are: dark green leafy vegetables, carrots, red pepper, tomatoes and yellow fruits.

In developing countries, most of the vitamin A is ingested from fruits and vegetables. Estimates suggest that more than 80 percent of dietary vitamin A intake in Africa and South East Asia, for example, is from pro-vitamin A carotenoids (WHO, 1996). The main sources of provitamin A are yellow and orange fruits, orange roots—carrots in particular, and some sweet potato varieties, dark green leafy vegetables and palm oil (Ruel, 2000).

2.6.1 Plant Sources of Vitamin A

The vitamin A that is found in plant sources must be converted by the body into vitamin A, or retinol, before they can be used. This conversion takes place in the upper intestinal tract. Once the carotenes enter the upper intestinal tract, they are converted to vitamin A by the actions of fat splitting enzymes and bile salts. This form of vitamin A is water soluble and cannot be stored by the body. Of all of the various carotenes, beta-carotene is the one that is most easily converted to vitamin A. Plant sources of vitamin A include many fruits and vegetables that have rich, deep, intense color. The more intense the color of the vegetable or fruit, the more beta-carotene it contains.

Below is table 2 showing the beta-carotene content of foods commonly consumed by children less than 5 years of age in Kenya.

Table 2: Beta-carotene content of foods commonly consumed by children less than 5 years of age

Food Type	β-carotene (μg)
Maize white, flour, 60-80% extraction	0
Millet, finger, flour	25
Rice, milled, polished	0
Plantain, ripe, raw	390
Potato, raw	12
Sweet potato, yellow, raw	1800
Amaranth leaves, raw	2300
Amaranth leaves, cooked	1700
Carrots, raw	6000
Cassava leaves	3000
Pumpkin, squash, raw	1200
Pumpkin, leaves, raw	1000
Banana, ripe, raw	90
Mango, ripe, raw without skin	2400
Avocado, raw	400
Papaya, raw	300

Source: adapted from West et. al., 1998

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2.6.2 Animal Sources of Vitamin A

Dietary sources of vitamin A from animal products is well absorbed and used more efficiently by the body than other sources of the vitamin. The rich animal sources of vitamin A include the following, liver, oily fish and fish liver oils, dairy products, kidney and eggs.

2.6.3 Bioavailability of Vitamin A from Plant Foods

Carotenoids are the most widespread of all groups of naturally occurring pigments. They are red, orange or yellow in color and are found in many plants and animals. The attractive colors of many red and yellow fruits and vegetables are also attributable to their carotenoid content. No animal is able to synthesize carotenoids.

Dark green leafy vegetables, yellow fruits, orange roots – mainly carrots – and the oils of palms are the main sources of provitamin A. Among leaves only those that are dark green are really good sources. This is because their carotenoid content in chloroplasts is roughly proportional to the concentration of chlorophyll with which they are associated for photosynthesis. Edible dark green leaves are readily available in most areas where vitamin A deficiency disorders (VADD) is a problem (Mclaren, 2001).

The advances that are taking place in our understanding of the bioavailability of provitamin A carotenoids is having a profound impact on the approaches that are being adopted to the control of VADD. The naturally occurring configuration of carotenoids in plants is usually the all-trans isomer. It is more readily absorbed in man than the 9-cis form. A significant proportion of 9-cis beta-carotene is converted to the all-trans form before entering the blood stream (Mclaren, 2001). In green leaves carotenoids exist within chloroplasts as pigment-protein complexes which require disruption of the cells for the carotenoid to be released. In other vegetables and fruits carotenoids are sometimes found in lipid droplets from which they may be readily released.

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Cooking assists in the release, but if excessive may lead to oxidative destruction of the carotenoid. Fiber, chlorophyll and non-provitamin carotenoids, which are commonly present in the diet, tend to reduce bioavailability. Absorption of carotenoids is influenced by vitamin A status. If it is low, conversion of carotenoids to vitamin A is likely to be increased. There is evidence that zinc deficiency impairs the efficiency of beta-carotene conversion to vitamin A. Meanwhile, efforts to promote the consumption of green leaves and yellow fruits remain a major element of control programs (Mclaren, 2001).

2.7 VITAMIN A DEFICIENCY INTERVENTIONS

2.7.1 General Health Interventions

A healthy host is better able to absorb, retain and utilize vitamin A. Sanitation and hygienic measures that reduce diarrhea and control intestinal parasites optimizes intestinal capacity to take up the vitamin from the diet (Bredbenner, 2009).

2.7.2 Food Based Approach

Dietary diversification is the most fundamental and sustainable form to prevent hypovitaminosis A. It's based on increasing selection and consumption of foods rich in dietary vitamin A activity to bring the habitual intake into the range of recommended intakes. Culture, economic limitations, cuisine interfere with achieving targets in low-income societies (Bredbenner, 2009). In Kenya, addition of milk/meat to the school snack (vegetable stew) improved overall vitamin A intake (Hongo, 2003).

It is also referred to as dietary modifications and it encompasses a wide variety of interventions that aim at:

1) Increasing the production, availability, accessibility to food rich in vitamin A.

2) Increasing consumption of foods rich in these micronutrients.

3) Increasing the bioavailability of vitamin A in the diet e.g. food combinations that increase the bioavailability of certain micronutrients (also called food-to-food fortification strategies).

Food-based strategies are often described as a sustainable approach because the process empowers individuals and households to take ultimate responsibility over the quality of their diet through own-production of nutrient rich foods and informed consumption choices. Food-based strategies are also appealing because they can address multiple nutrients simultaneously, including calories, proteins and various micronutrients, without the risk of antagonistic nutrient interactions or overload (Ruel, 2000).

Other strategies recognized as more sustainable, long-term solutions have received less attention, and their potential has not been evaluated. Such strategies aim to increase dietary intake of vitamin A and/or decrease physiologic requirements; these strategies include horticulture, public health, socioeconomic improvement, and nutrition and health education measures (Underwood, 1990).

2.7.3 Food Fortification with Vitamin A and Provitamin A

Addition of vitamin A to foods began in the 1930s with enrichment of margarine. Foods that are commonly fortified with vitamin A include cooking fats and oils, margarine, sugar and skim milk powder..

Sugar and *cooking oil* are currently leading vehicle in fortification of staple foods. Sugar is fortified with *retinyl palmitate* in four of the five republics of Central America and Zambia.

Vitamin A fortification can be targeted to a specific segment of the population. Industrial fortification of commercial foods is adding more vitamin A to diets (Bredbenner, 2009).

2.7.4 Bio-fortification

Bio-fortification is the breeding of crops to increase their nutritional value. This can be done through conventional selective breeding or through genetic engineering (Wikipedia, 2012). Bio-fortification differs from the ordinary fortification because it focuses on making the plant foods more nutritious as they grow, rather than having nutrients added to the foods when they are being processed. It is a more efficient means of providing nutritious foods to the rural poor whom often than not are not able to access commercially fortified foods (Wikipedia, 2012).

There has been a lot of research into bio-fortification of plants to explore ways of increasing the vitamin A status. Bio-fortification of crops is focused on breeding crops to contain high concentrations of pro-vitamin A carotenoid, beta-carotene, which is subsequently converted to vitamin A (retinol) in the human body (www.harvestplus.org).

2.7.5 Vitamin A supplementation

Target is neonates of HIV+ mothers, children under 5 years of age and women of reproductive age. The Government of Kenya through the Ministry of Public Health and Sanitation has made it routine for mothers attending well baby clinics to be educated on the importance of supplementation with vitamin A capsules twice a year of children under the age of five years.

2.7.6.1 Mineral content of maize

According to the Food and Agricultural Organization (FAO) the mineral content of maize carried out on five samples is as represented in table 3:

Table 3: Mineral Content of Whole Maize (Average of five samples)

Mineral	Concentration (mg/100 g)
P	299.6 ± 57.8
K	324.8 ± 33.9
Ca	48.3 ± 12.3
Mg	107.9 ± 9.4
Na	59.2 ± 4.1
Fe	4.8 ± 1.9
Cu	1.3 ± 0.2
Mn	1.0 ± 0.2
Zn	4.6 ± 1.2

Source: Bressani, Breuner and Ortiz, 1989

2.7.6.2 Vitamin A content of Maize

Maize generally has a low content of vitamin A with the little being concentrated in the germ of the maize kernel which is removed during processing of the maize to maize flour. White maize only has 2 IU of vitamin A which is quite insignificant (www.livestrong.com). However, yellow maize has a considerable amount of carotenoids whose levels can be genetically manipulated.

2.8 METHODS OF VAD ASSESSMENT

Most of the vitamin A in the body is stored in the form of retinyl ester in the liver. Therefore, a measure of liver vitamin A stores is the best index of vitamin A nutriture. The conventional definition of vitamin A deficiency is when liver stores of retinol are below 0.07 micromol/g (Rosalind, 2005). Total serum vitamin A, or more recently, serum retinol concentrations are more often determined since liver biopsies are impractical in population studies. Various methods are used to assess the VAD in an individual and they include:

2.8.1 Serum Retinol

Vitamin A in the serum circulates largely in the form of a 1:1 complex of retinol and retinol-binding protein (RBP). Serum retinol levels reflect the vitamin A status only when liver vitamin A stores are severely depleted (<0.07micromol/g liver) or excessively high (>1.05 micromol/g liver). Various methods are used to measure the serum retinol and they include (Rosalind, 2005):

1) High-performance liquid chromatography (HPLC)

2) Fluorometric methods

3) Dried blood spots (DBS)

2.8.1.1 High pressure liquid chromatography

Separation of compounds by high-pressure liquid chromatography (HPLC) has been used to eliminate interference from other components in the sample. Reversed phase HPLC (C18 column) followed by UV detection for retinoids is the most common method for analysis (De Leenheer et.al, 1998). Retinol is transported in serum bound to retinol-binding protein (RBP). To analyze serum retinol, RBP is denatured with alcohol or acetonitrile to release retinol for organic-solvent extraction prior to analysis (Solomons, 2001).

2.8.2 Serum Retinol-Binding protein

The vitamin A transport protein is known as the Retinol-binding protein (RBP). During the last stages of vitamin A deficiency the liver becomes depleted in retinol making the RBP toaccumulate in the liver as apo-RBP. This in turn causes the levels of both serum retinol and RBP to decline. The use of RBP to determine vitamin A deficiency is useful in populations where resources and technical support are limited: sample collection and the analytical

procedures are easier and cheaper than for serum retinol, and the analysis can be performed on serum from a finger-prick blood sample (Gibson, 2005).

2.8.3 Relative Dose Response

This test is used to estimate the liver stores of vitamin A therefore identifying individuals with marginal vitamin A deficiency. The test is based on the observation that during vitamin A deficiency, when liver stores are diminished, RBP accumulates in the liver as apo-RBP. RDR is a more sensitive index of marginal vitamin A status than using serum vitamin A levels< 0.70 micromol/L (Gibson, 2005).

2.8.4 Assessment of Night Blindness

Night blindness is the most common manifestation of vitamin A deficiency. Poor dark adaptation resulting in night blindness arises when there is reduced production in the rods of the visual pigment rhodopsin, or opsin protein bound to the retinal form of vitamin A. Determination of night blindness is a simple and easy tool to assess vitamin A deficiency in women of reproductive age. Though this method is subjective compared to other assessment methods, therefore it is not commonly used as a method of determining vitamin A deficiency (Gibson, 2005).

2.8.5 Rapid Dark Adaptation Test

The first stages of night blindness is manifested as disturbances in dark adaptation. The disturbances can be detected by non-invasive tests. The conventional laboratory-based, formal dark adaptometry test is a tedious and time-consuming procedure. A rapid dark adaptation test (RDAT) which is suitable for field conditions has been developed. The RDAT requires a light-proof room, a light source, a dark, non-reflective work surface, a standard X-ray view box, and

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sets of red, blue, and white discs. Measurements of the RDAT are undertaken during the first few minutes of dark adaptation (Gibson, 2005).

2.8.6. Multiple indices

Vitamin A deficiency disorders are not defined with any certainty using a single measure of vitamin A status. WHO (1996) has recommended a combination of biochemical, functional, and clinical indicators for children aged 6-71 months. This requires caution when applying these cutoffs to individuals because of the influence of many confounding factors (Gibson, 2005).

2.9 USE OF RATS IN HUMAN STUDIES

Rats are very similar to humans in terms of the anatomy, physiology and genetics. In addition rats have a short generation time, and a very accelerated life span. Furthermore their brains are strikingly similar to the human brain hence the reason why a lot of research is carried out using rat models (www.danerwin.com). Researchers use the information they have gathered using rat research and find ways to perform such research in humans.

Selection of an appropriate animal model is as important as the outcome of an experiment because selection of a wrong animal model can frustrate and mislead the researcher. It therefore is important to select animals which are closest to humans in its biological systems and hence the use of rat models in human studies (Rajesh, 2002).

The other reason why rats are useful in human studies is because both rats and humans are mammals therefore share many similarities in structure and function (www.chrcrm.com). The small size of rats, low cost, ease in handling, and ability to breed in captivity make rats ideal for laboratory experiments (www.chrcrm.com). Animal models have been used to study provitamin A activity. A series of studies were carried out using Mongolian gerbils to determine bio-

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conversion factors of provitamin A carotenoids to vitamin A of several foods
(www.harvestplus.org). This goes to show that use of rats in research is an invaluable tool.

CHAPTER THREE: STUDY SETTING AND STUDY METHODOLOGY

3.1 STUDY SETTING

The study was carried out in the Department of Public Health, Pharmacology and Toxicology (PHPT) and the Department of Food Science, Nutrition and Technology, University of Nairobi-Upper Kabete Campus.

3.2 STUDY DESIGN

The study used a Quasi-experimental design at the Department of Public Health, Pharmacology and Toxicology (PHPT) University of Nairobi . The first phase of the study involved production of the beta-carotene rich *Muthokoi* at the Pilot plant, Department of Food Science, Nutrition and Technology and chemical analysis carried of the raw materials and the end products. This was followed by particle size determination of the *Muthokoi* in comparison with maize meal that is available in retail outlets. The other phase of the study involved feeding trials using laboratory rats which were bred, born and stored in the animal store, Department of Public Health, Pharmacology and Toxicology. The rats that qualified for the study involved five test groups which were then fed with the intervention feed over a pre-determined period of time and observations against ordinary rats which were not fed the intervention but instead were allowed to feed on the normal rodent diet of maize in form of whole maize meal.

3.3 STUDY POPULATION AND SAMPLING FRAME

The study involved the use of laboratory rats at the animal store, Department of Public Health Pharmacology and Toxicology. The rats used for the study were bred in the animal store and their sex determined at birth and the females were selected for the study. In total 10 female rats

were selected for the study. The 10 rats were marked at their tails to differentiate them from the rest of the litter as they had to be housed together with their mother until weaned at the age of three weeks. After three weeks the 10 weanlings that were selected for the study were separated from their mothers and the rest of the litter and housed in two different metal cages. They were divided into two groups consisting of five female weanlings in each group. Each group was placed in a metallic cage; one cage housed the test group while the other housed the ordinary rats. All the ten rats were provided with water through a bottle fitted within the cage and were fed with the different feeds using Petri-dishes and were fed ad-libitum.

3.4 STUDY METHODOLOGY

3.4.1 Rat Study Experiment

Three weeks after the birth of the rats, the rats were weaned off and separated from their mother.

3.4.1.1 Test group

The test group consisted of five 3 week old female weanlings which were housed in the same cage. Their blood was drawn by a qualified laboratory technician from the PHPT Department from the rat sinuses using a heparinized capillary to prevent blood clotting. This was done to determine the basal serum retinol content of the test rats. Vitamin A deficiency was induced to the rats by feeding them with vitamin A free diet (maize meal 80% extraction rate) for 3 weeks. After the three weeks, blood was drawn from the rats to determine if the deficiency induction was successful and intervention was done by feeding the rats with beta-carotene enriched *Muthokoi* for 3 weeks. Feeding was done using Petri-dishes and the rats fed adlib. At the end of the intervention period blood was drawn from the rats for analysis to determine whether their

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serum retinol levels had improved after the intervention. Serum retinol analysis was carried out using a High Pressure Liquid Chromatography (Shimadzu, UV-Vis Detector, Japan).

3.4.1.2 Ordinary rats

They consisted of five 3 week old female weanlings which were housed in one cage. The basal serum retinol content of the control group was done by drawing blood from the rats though the sinuses using heparinized capillary tubes. The blood was collected in vaccuettes. Drawing of the blood from the rats was done by a qualified laboratory technician from the PHPT Department. After determination of the basal serum retinol content of the control group, the weanlings were fed with whole maize meal for a period of 3 weeks after which their blood was drawn to determine the serum retinol content after the feeding trial. The study design is shown in Figure 1.

```
                    ┌─────────────────────────┐
                    │  3-week old weanlings    │
                    │  (10 rats)               │
                    └─────────────────────────┘
                              ⇓
        ┌───────────────────────────────────────────────┐
        │  Determination of basal serum retinol using HPLC │
        └───────────────────────────────────────────────┘
              ⇓                                  ⇓
┌──────────────────────────┐        ┌──────────────────────────┐
│ Ordinary rats (5 female  │        │ Test group (5 female rats)│
│ rats)                    │        │                          │
└──────────────────────────┘        └──────────────────────────┘
              ⇓                                  ⇓
┌──────────────────────────┐        ┌──────────────────────────┐
│ Feeding with whole maize │        │ Feeding with vitamin A   │
│ meal for 3 weeks         │        │ free diet for 3 weeks to │
│                          │        │ induce vitamin A         │
└──────────────────────────┘        └──────────────────────────┘
              ⇓                                  ⇓
┌──────────────────────────┐        ┌──────────────────────────┐
│ Determination of serum   │        │ Determination of serum   │
│ retinol content after    │        │ retinol after induction  │
│ feeding trial            │        │ of the deficiency        │
└──────────────────────────┘        └──────────────────────────┘
                                                 ⇓
                                    ┌──────────────────────────┐
                                    │ Feeding with beta-carotene│
                                    │ enriched Muthokoi for 3   │
                                    │ weeks                    │
                                    └──────────────────────────┘
                                                 ⇓
                                    ┌──────────────────────────┐
                                    │ Determination of serum   │
                                    │ retinol after            │
                                    │ intervention             │
                                    └──────────────────────────┘
```

Figure 1: The Study Design

3.4.2 Development of Beta-carotene Enriched *Muthokoi*

Pumpkin leaves, maize cereal were purchased from the local market (Kangemi market) and transported to the Pilot Plant of the Department of Food Science, Nutrition and Technology for processing. The pumpkin leaves were washed to remove any extraneous matter then pounded together with the washed, clean maize using a mortar and pestle. The production of the β-carotene enriched *Muthokoi* is as shown in Figure 2. After pounding, enriched maize kernels were dried at 60^0C in the oven to an approximate moisture content of 4%. After drying, the maize kernels were milled to flour using Norris and Hammer mill (Germany). Samples for proximate analysis were drawn from the enriched *Muthokoi* flour. To determine the most appropriate ratio of the pumpkin leaves and maize, blending was done with five different ratios and their beta-carotene content determined. The blending ratios are presented in the Table 4 below:

Table 4: Different blending ratios of maize to pumpkin leaves

Raw Materials/ Blending Ratios Maize (grams) : Pumpkin leaves (grams)	Treatment Label
100g : 100g	1
100g : 200g	2
100g : 300g	3
100g : 400g	4
100g : 500g	5

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                    ┌─────────────────────┐
                    │   Maize (1Kg)       │
                    └─────────────────────┘
                              │
                              ▼
          ┌─────────────────────────────────┐
          │ Cleaning (winnowing, removal of │
          │ debris)                         │
          └─────────────────────────────────┘
                              │              ┌──────────────────────────────┐
                              │◄─────────────│ Addition of pumpkin leaves   │
                              │              │ (2Kgs)                       │
                              ▼              └──────────────────────────────┘
             ┌─────────────────────────────┐
             │ Pounding in mortar and      │
             │ pestle                      │
             └─────────────────────────────┘
                              │
                              ▼
             ┌─────────────────────────────┐
             │ Beta-carotene enriched      │
             │ Muthokoi                    │
             └─────────────────────────────┘
                              │
                              ▼
                    ┌─────────────────────┐
                    │ Drying              │
                    └─────────────────────┘
                              │
                              ▼
       ┌──────────────────────────────────────┐
       │ Milling using hammer mill to         │
       │ particle size: 63.5% were 500µm      │
       └──────────────────────────────────────┘
                              │
                              ▼
          ┌─────────────────────────────────┐
          │ Beta-carotene determination     │
          └─────────────────────────────────┘
                              │
                              ▼
          ┌─────────────────────────────────┐
          │ Feeding to the test group       │
          └─────────────────────────────────┘
```
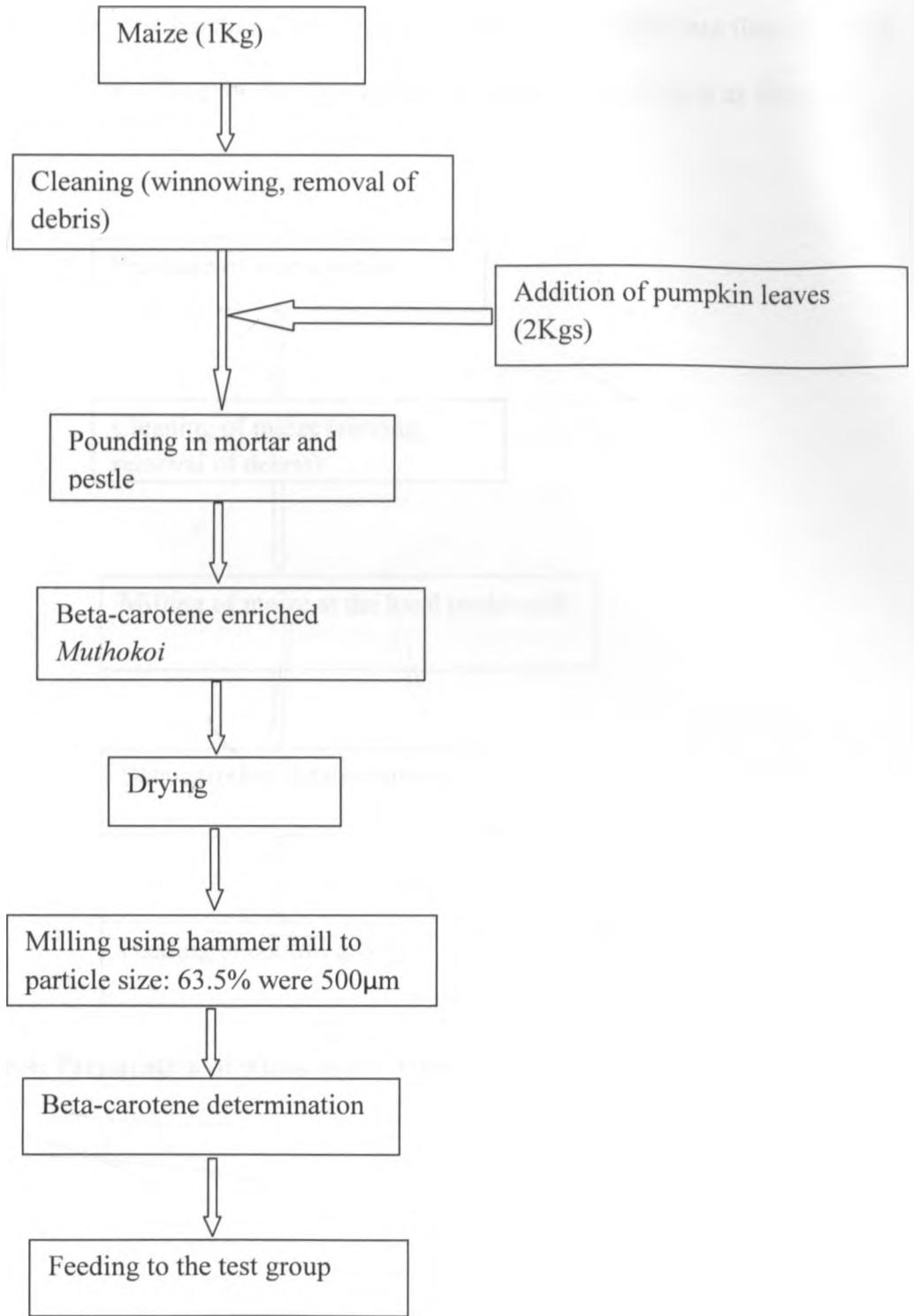
Figure 3: Procedure for Production of β-carotene enriched *Muthokoi*

3.4.3 Preparation of Whole Maize Meal

Whole, dry maize grains were purchased from Kangemi market and milled into flour at a local posho mill (in Ndumbo-ini). The process of preparing the whole maize meal is as shown in Figure 4 below:

```
┌─────────────────────────────┐
│  Purchase of whole maize    │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  Cleaning of maize (sieving,│
│  removal of debris)         │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  Milling of maize at the local posho mill │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  Beta-carotene determination│
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  Feeding to control group   │
└─────────────────────────────┘
```
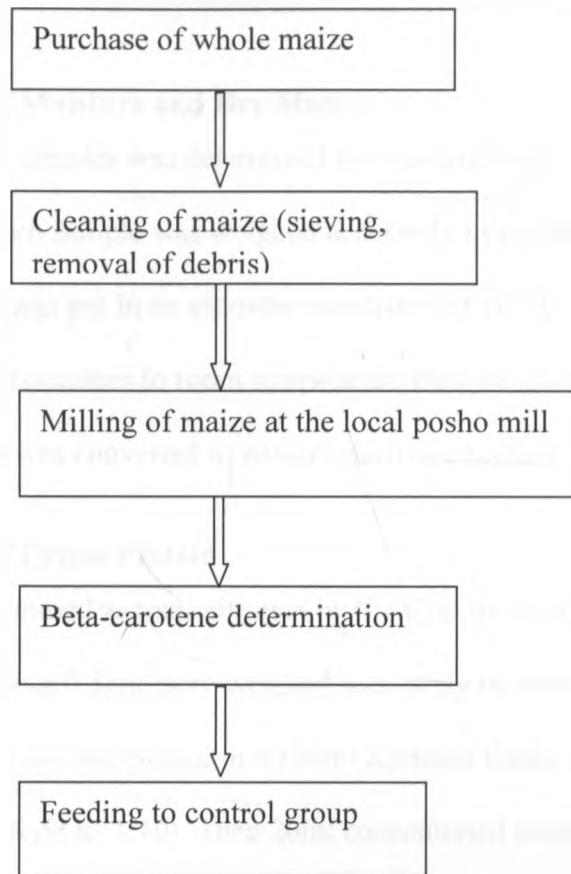
Figure 4: Preparation of whole maize meal

3.6 ANALYTICAL METHODS

A sample of 50gm was drawn randomly from the beta-carotene enriched *Muthokoi* and products which were analyzed for proximate composition, beta carotene, iron and calcium. The proximate composition analysis involved analysis of the total moisture and dry matter, crude fiber, crude protein, total ash and soluble carbohydrates according to AOAC methods (AOAC, 1999).

3.6.1 Determination of Moisture and Dry Matter

Moisture content of the samples was determined by standard analytical AOAC methods (AOAC, 1999). About 5gm of each sample was weighed accurately in triplicates into aluminium dishes. Each dish and contents was put in an air oven maintained at 105^0C and dried for 4 hours. The dishes were cooled in desiccators to room temperature then weighed and results recorded. Loss of weight due to drying was converted to percent moisture content.

3.6.2 Determination of Crude Protein

Crude protein was determined as total nitrogen by the Kjeldahl method (AOAC, 1999). Triplicate samples of about 0.5gm were weighed accurately in nitrogen free filter paper. Each sample was folded carefully and placed in a 100ml Kjeldahl flasks followed by 5.5gm Kjeldahl catalyst tablet ($CuSO_4$: K_2SO_4=1:10). Then 20ml concentrated sulphuric acid was added. A blank analysis was carried out without the sample at the same time. The flasks were heated on the Kjeldahl heating assembly until all frothing stopped and a clear blue obtained. After cooling, distilled water was added to have the liquid fill ¾ of the flask. Some drops of phenolphthalein indicator were added. Four hundred (400) ml conical flasks containing 50ml of 0.1N HCl solution and some drops of methyl orange indicator were placed under the outlet of Kjeltic system 1002 distilling unit. The diluted digest was mixed with 40% NaOH to make sufficient

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alkaline then steam distilled up to a volume of 200ml. The ammonia in the distillate was determined by back titrating with 0.1N HCl. Percent crude proteins were calculated as:

Nitrogen %= $(v_1 - v_2)$ x N x f x 0.014x100/s, where,

V_1= quantity for the sample (ml)

V_2= quantity for the blank (ml)

S= weight of the sample taken in grams

N=normality of the standard HCl solution=0.1

Protein %= nitrogen x protein factor (6.25)

3.6.3 Determination of Crude Lipids

Crude lipid was determined by standard AOAC methods (AOAC, 1999). About 5gm of sample was weighed accurately in triplicate into cellulose extraction thimbles and extracted with analytical grade petroleum ether of boiling point 55^0C in soxhlet extraction unit for 16 hours. The ether extract was transferred to 300ml flat bottomed flasks that had been previously washed and dried in an oven at 105^0C cooled in a desiccator and weighed. The excess petroleum ether was evaporated and residual extract dried in an oven at 80^0C to constant weight. The residue was calculated as percentage crude lipids.

3.6.4 Determination of Crude Fiber

Crude fiber determination was carried out according to standard AOAC method (AOAC, 1999). Triplicate samples of about 2gm were weighed accurately into a graduated 500ml beaker. Small amount of boiling distilled water together with 25ml of 2.04N Sulphuric acid solution were added. The content was adjusted to 200ml with boiling distilled water and maintained at this

volume while boiling gently on a hot plate for 30 minutes. The content was then filtered using a Buchner funnel slightly packed with glass wool and then the residual was washed three times using boiling distilled water.

The residues together with glass wool were transferred quantitatively to the beaker. Small amount of water was added as well as 1.73N KOH solution. The volume was made to 200ml with distilled water and this volume was maintained as the contents boiled on a hot plate for 30 minutes. The contents were filtered using glass wool and the residue was washed as above. The residue was again washed three times with small amount of ethanol. The residue was transferred quantitatively to a porcelain dish dried in an air oven for 2 hours, weighed then ignited the dish and content at 550^0C to constant weight.

After cooling in a dessicator, the weight of each was taken. The weight of the fiber content was be calculated as follows:

Fiber %= (w_1-w_2)/sample weight) x 100

3.6.5 Determination of Total Ash

Total ash was determined by standard AOAC method (AOAC, 1999). About 2gm of samples was weighed accurately in triplicate into porcelain ashing dishes, which was cleaned and dried in an air oven at 105^0C cooled in a desiccator and weighed. The dishes and the content were held in a muffle furnace at 550^0C overnight. They were then removed, cooled to room temperature and weighed. Percent ash was calculated as follows:

Ash %= (weight of ash remained/weight of sample) x 100

3.6.6 Determination of Soluble Carbohydrates

Total carbohydrates were determined by difference;

100-(crude fiber + crude protein + crude fat + total ash + moisture content)

3.6.7 Determination of β-carotene

About 2gm of the sample was subjected to color extraction using a mortar and pestle with small portions of acetone until the residual was colorless. All the extracts were combined into a 100ml volumetric flask and evaporated to dryness in a Rotary evaporator at about 60^0C. To the dry sample 1 ml of petroleum spirit was added so as to dissolve the beta-carotene.

The beta-carotene was eluted through a packed column and the orange or yellow pigment received into a 25ml volumetric flask. The absorbance was read at 450nm and the beta-carotene calculated from the beta-carotene standard curve.

3.6.8 Determination of Iron and Calcium

A small sample was taken; it was ashed and dissolved in a small quantity of 20% HCl. The contents were filled to the mark then taken to the Atomic Absorption Spectrophotometer (Burke Scientific, United States of America) for analysis.

3.6.9 Determination of Serum Retinol

Serum retinol analysis was carried out using a reverse phase High Pressure Liquid Chromatography using C18 column. A quantity of $100\mu mol$ of Internal standard ($5\mu L$ of $20\mu M$ retinyl acetate in ethanol) was added to each sample (blood). 10 ml of Hexane was added to the aqueous ethanol phase. The samples were vortexed and centrifuged for 1-3 minutes at 60 rpm in a centrifuge to facilitate phase separation. The hexane (top) phase containing non polar retinoids was removed. Retinol extracts were resuspended in $120\mu L$ acetonitrile. The retinol was resolved

by reverse phase chromatography on a HPLC system and quantified by UV absorbance at 325 nm. The mobile phase consisted of 78% acetonitrile, 22% deionized water and 0.19% triethylamine. 20μL of the supernatant was injected into the HPLC for analysis. The HPLC (Shimadzu, UV-Vis Detector, Japan) system was maintained at a flowrate of 0.9 ml/min, retention time of 4 min, oven temperature 35^0C and detection made using UV at 325 nm.

3.6.10 Particle size determination

A sample of 100g meal was accurately weighed and quantitatively transferred to a Vibrator/ Shaker (Fritsch, Germany). The shaker was switched on and allowed to vibrate for 15 minutes. The shaker was filled with six sieves of different sizes ranging from 500 μm to 20μm. The sieves were put apart and the contents quantitatively transferred to a clean piece of paper and weighed to determine the particle distribution in each sieve.

3.7 DATA COLLECTION AND MANAGEMENT

Data obtained from the above mentioned chemical analysis of the food products used in the feeding trials was recorded in an MS-excel worksheet. Blood analysis for serum retinol levels was done at the start of the study and after three weeks of feeding.

3.7.1 Quality Control

There was regular follow up by the investigator to the PHPT laboratory to ensure that the different groups of the study sample were receiving the designated feeds.

3.7.2 Data Analysis

Data collected during the laboratory experiments and feeding trials was analyzed using Statistical Packages for Social Sciences (SPSS). The statistical tests that were carried out including central measures of tendencies and parametric tests which included:

1) Means and standard deviation of β-carotene levels, proximate composition analysis, mineral levels, particle size and serum retinol levels.

2) Analysis of Variances (ANOVA) of the β-carotene levels of processed *Muthokoi* and serum retinol levels.

3) Paired t-tests of the serum retinol levels of the control and test groups.

4) Independent t-tests of the serum retinol levels of the rats before inducing with the deficiency.

4.1 PARTICLE SIZE DISTRIBUTION

The particle size of the enriched *Muthokoi* meal and maize meal is as shown in Table 5:

Table 5: Particle size distribution of maize meal and enriched *Muthokoi*

SIEVE SIZE (μM)	MAIZE MEAL (80% EXTRACTION) %	B-CAROTENE ENRICHED MUTHOKOI %
500	59.4 ±12.5	63.5 ±14.9
250	16.2 ±6.3	10.9 ±3.8
150	20.9 ±5.7	23.2 ±10.4
125	0.7 ±2.5	0.5 ±3.6
32	0.1 ±4.7	-
20	-	-

4.2 β-CAROTENE LEVELS OF ENRICHED *MUTHOKOI* MEAL

Preparation of the β-carotene enriched *Muthokoi* underwent different treatments to determine which treatment would give the highest beta-carotene content. The β-carotene levels of the different treatments are as shown in Table 6.

The pumpkin leaves blend that was selected as the intervention feed to the vitamin A deficient rats was the regime that consisted of one part of maize to two parts of pumpkin leaves, treatment number 2.

Table 6: β-carotene content of Muthokoi produced from pounding maize with pumpkin leaves

TREATMENT NUMBER	MAIZE: PUMPKIN LEAVES (grams)	Mean β-carotene content (μg/100g)
1	100g:100g	151.4±10.54
2	100g:200g	465.3±9.20
3	100g:300g	741.7±8.53
4	100g:400g	923.6±11.33
5	100g:500g	1241.7±12.57

Values are averages of duplicate analytical replicate

The retinol equivalent (RE) of the intervention feed was as is shown in the following calculation:

Retinol equivalent= Beta-carotene content/6

= 465.4/6

= **77.6 RE**

4.2.1 β-Carotene Content of Maize and Pumpkin Leaves

Analysis of the raw materials was done to determine their beta-carotene content. Whole maize had beta-carotene content of 26.87μg/100g ± 2.17 while that of pumpkin leaves (fresh) was 2172.95μg/100g ± 0.99.

4.2.2 Effect of processing on the β-carotene content of the enriched *Muthokoi*

The effect of processing on the β-carotene content of the enriched *Muthokoi* meal is as shown in Table 7:

Table 7: The effect of processing on the β-carotene level of the enriched Muthokoi

Process	Mean β-carotene content	p-value
Drying	398.6 ± 29.26	
Milling (Quantitatively)	373.4 ± 14.62	0.267
Cooking	367.3 ± 13.66	

Values are averages of triplicate analytical replicate

4.3 PROXIMATE COMPOSITION AND MINERAL CONTENTS OF *MUTHOKOI*

The proximate composition and mineral contents of Muthokoi are shown in Table 8:

Table 8: Proximate composition and Mineral contents of dried *Muthokoi*

Dry matter %	96.1 ± 10.55
Crude Protein %	8.6 ± 3.15
Crude Fat %	4.3 ± 2.78
Crude Fiber %	3.1 ± 1.9
Ash %	1.4 ± 0.21
Nitrogen Free Extract (NFE)	82.6 ± 19.88
Iron (mg/100g)	17.3 ± 8.24
Calcium (mg/100g)	21.2 ± 5.3

Values are averages of duplicate analytical replicate

Pumpkin leaves (edible plus inedible portions) are rich in calcium and from analysis the pumpkin leaves were found to have calcium content of 346.4±48.7 mg/100g.

4.4 SERUM RETINOL LEVELS

The serum retinol level of the laboratory rats was determined and the results obtained are shown in Tables 9 and 10:

4.4.1 Basal Serum Retinol Levels

The basal serum retinol levels of the laboratory rats were statistically analyzed using an independent t-test and the results are shown in table 7 below:

Table 9: Basal serum retinol of the control and test laboratory rats

GROUP	MEAN SERUM RETINOL (µmol/L)	P-VALUE
Test rats	1.87 ±1.60	0.859
Ordinary rats	1.85 ±0.21	

4.4.2 Serum Retinol Before and After the Feeding Trials

The serum retinol levels of the laboratory rats after the feeding trials are as shown in Table 10:

Table 10: Serum retinol contents of the rats before and after the feeding trials

FEEDING TRIAL	MEAN SERUM RETINOL (µmol/L)	P-VALUE
	ORDINARY RATS	
Before feeding	1.85 ±0.214	0.178
After feeding	1.86 ±0.207	
	TEST GROUP	
Before feeding	0.696 ±0.075	0.00
After feeding	1.496 ± 0.069	

These results can be hypothetically related to consumption of the beta-carotene enriched *Muthokoi* in form of porridge to children. Assuming that the amount of flour that would be used to make a 250ml cup of porridge would be say 15% of the flour. If a child were to consume 2 cups of porridge a day it would result to 500ml of porridge per day. The total amount of flour used would be:

$$= 0.15* 500ml= 75g \text{ of flour (96.1\% DM)}$$

$$= 72g \text{ flour}$$

The retinol equivalent in the enriched *Muthokoi* after cooking it (humans can only consume cooked *Muthokoi*) is as shown below:

$$\text{Retinol equivalent} = \text{Beta-carotene content}/6$$

$$= 367.3/6$$

$$= \mathbf{61.22 \; RE}$$

The RDA of vitamin A for children 7- 36 months is 400 µg RE/day.

The enriched Muthokoi would provide: $\underline{61.22} * 100$

400

$$= \underline{\mathbf{15.3\%}}$$

CHAPTER FIVE: DISCUSSION

5.1 β-CAROTENE CONTENTS OF MAIZE KERNELS POUNDED WITH LEAFY VEGETABLES

In Kenya white maize is the staple food to majority of the households unlike the yellow maize, though being rich in vitamin A the Kenyan population did not embrace it when the government tried introducing it to the local market in the 1990s. White maize is still the accepted staple and therefore enriched it with nutrients would be a step towards mitigating micronutrient deficiencies.

The treatment regime that gave the highest beta carotene content was the blending which comprised of 1 part of maize pounded with 5 parts of pumpkin leaves. This blending regime would not be feasible to a majority of households since it requires a lot of the green vegetables which can be quite expensive especially during the dry seasons. Therefore, due to economic considerations and ensuring the sustainability of such a Food-based approach on alleviating the deficiency, the blend that would give a suitable level of β-carotene and still be economical was the blend that consisted of 1 part of maize to 2 parts of pumpkin leaves. This blend was used as the intervention for the study.

HarvestPlus is developing high-vitamin A maize through conventional breeding methods to provide vitamin A to millions of poor consumers through the diet, especially in Africa. Therefore, this indigenous technique of enriching maize, which is a staple to most of the people in sub-Saharan Africa can be adopted alongside other methods since it is cost effective and can be employed even in the most remote areas of the country and the continent as a whole.

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Studies supported by HarvestPlus showed that a team of scientists had discovered rare variations of a maize gene (crtRB1) that could lead to an 18-fold increase in beta-carotene content of maize in an academic research setting. Plant breeders have started to use these naturally occurring genetic variations to breed maize that can provide more beta-carotene to malnourished people. The first trials of the conventionally bred vitamin A maize was to be released in Zambia this year (Yan, 2010). Pounding of maize with pumpkin leaves resulted to the β-carotene content of maize being drastically increased using simple, local and affordable techniques.

From the results it is clear to see that pumpkin leaves are very rich in β- Carotene compared to maize which is deficient in this nutrient. Therefore, this study sought to use enrich maize with β-carotene from the pumpkin leaves. The β-carotene levels of the pumpkin leaves used for the study differ slightly from a study done in South Africa where the β-carotene content of pumpkin leaves was found to be 1695µg/100g and this could be attributed to genetic differences and differences in the soil profile of the two countries (Schönfeldt, 2011). It is a fact that plant foods provide much of the vitamin A intake of poorer individuals living in developing countries. White maize has little or no carotenoid content. Carotenoids are found mainly in yellow maize in amounts that are genetically controlled.

5.1.1 Effect of Processing on the β-carotene Content of the Enriched *Muthokoi*

There was no significant difference ($p > 0.05$) in the β-carotene content of the milled and cooked Muthokoi. Vitamin A is unstable when exposed to air, light and heat (Bauernfeind, 1991) hence the reason why there was a decrease in the vitamin A content of the enriched Muthokoi during cooking. The laboratory rats were fed on the milled *Muthokoi* without cooking it due to convenience in feeding the rats. This was convenient for the study since rats being rodents

consume raw maize and therefore administering the intervention to the rats posed no challenge. There is a slight decrease in the β-carotene content during the cooking process and the loss is attributable to oxidative destruction of total β-carotene (Schönfeldt, 2011). Since there was no significant difference in the βcarotene levels between the milled and cooked enriched *Muthokoi*, this implies that once cooked in whatever form, be it porridge or Ugali, humans will still attain the still attain the β-carotene since we cannot consume raw foods. This goes to show that cooking has no significant effect on the β-carotene content of the enriched *Muthokoi*.

5.2 PROXIMATE COMPOSITION AND MINERAL CONTENTS OF ENRICHED *MUTHOKOI*

Maize, as most cereal grains, is low in calcium and trace minerals. Therefore, the pounding of the maize with the pumpkin leaves resulted to the maize being enriched with minerals which included calcium and iron. The enriched *Muthokoi* was significantly enriched with both iron and calcium as seen above. In her research Sehmi (1993), established that the iron and calcium contents of maize obtained from Central province was 5.0 mg/100g and 10.2 mg/100g respectively and as observed from the results, enriched *Muthokoi* was not only enriched with β-carotene but with calcium and iron too. Calcium and iron are critical nutrients for the growth and development of children. Iron deficiency and rickets among children has been a major concern in the country and so enriching the *Muthokoi* with these nutrients will not only help deal with vitamin A deficiencies but with other deficiencies as well. This could be a great step in fighting micronutrient deficiencies by using affordable and sustainable food approach strategies.

The major chemical component of the Maize kernel is carbohydrate. The carbohydrate is composed of the crude fiber and the nitrogen free extract.

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The NFE is an estimate of crude starch and sugar content of a feed. The constituents of NFE include lignin, hemicelluloses, polysaccharides and pectin. The starch in maize is made up of two glucose polymers: Amylose which is linear and amylopectin which is branched. The composition of maize starch is genetically controlled. Crude fiber is a measure of the quantity of the fibrous, poorly digested material in a feed

After starch, the next largest chemical component in maize is protein which most of it is found in the endosperm. Crude protein measures the total nitrogen content of a product and from that, estimates the amount of protein within product. The protein content in maize varies in common varieties from 8-11% of the kernel weight. Most of the protein is found in the endosperm. From the analysis made of the enriched Muthokoi, its protein content falls within the normal protein range. Oil content of the maize kernel is from the endosperm.

The pumpkin leaves used for the study were found to have a higher calcium content compared to a research done by Sehmi who found the calcium content of pumpkin leaves (edible portion only) obtained from Nairobi to be 231 mg/100g (Sehmi, 1993). This may be because Sehmi (1993) only used the edible portion of the leaves, but this study used the entire leaf both the edible and the inedible portions of the leaf. This goes to show that much of the nutrients from pumpkin leaves are lost since people only consume the edible portions. Such an approach that utilizes even the inedible portions is commendable because the nutrients which would otherwise be lost in the inedible portion will still be harnessed and utilized to enrich maize, hence conserving of nutrients.

The particle size distribution of both the enriched *Muthokoi* and the 80% extracted maize meal that is commercially available at the retail outlets showed that majority of the particles were within the 500μm sieve with none being at the 20μm sieve, though, the *Muthokoi* had a greater percentage of particles within the 500μm sieve compared to the 80% extracted maize meal.

5.3 EFFECT OF FEEDING ON THE SERUM RETINOL LEVELS OF THE LABORATORY RATS

5.3.1 Basal Serum Retinol Levels of Test and Ordinary rats

The observed results showed that there was no significant difference between the basal serum retinol levels of the two different groups of rats (p>0.05). This is because the weanlings were from the same mother and were weaned at the same time, hence it was not expected to be a difference in their serum retinol levels.

5.3.2 Serum Retinol Before and After the Feeding Trials

5.3.2.1 Ordinary rats

There was no significant difference (p>0.05) between the serum retinol content of the control rats before and after feeding them with whole maize meal. This may be because maize is limited in β-carotene and so even in feeding the rats for the specified period with the whole maize meal made no significant difference in the serum retinol levels of the rats. This can be supported by the fact that populations that depend on starchy staples as their main source of nutrients and eat very little green vegetables are more susceptible to vitamin A deficiency compared to individuals/ populations that consume adequate green leafy vegetables. The rate of increase of serum retinol was 0.541% over the entire feeding period which was not significant and this could be attributed to the low content of beta-carotene in maize. Therefore, feeding the control rats with whole maize meal did not positively influence the vitamin A status.

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5.3.2.2 Test group

There was a significant difference ($p<0.05$) in the serum retinol content after the induced deficiency and after the intervention. This signifies that the β-carotene enriched *Muthokoi* was able to greatly improve the vitamin A status of the laboratory rats since its β-carotene content was increased from 26.87µg/100g to 465.27 µg/100g β-carotene content of whole maize. The rats had a 53% rate of increase in the serum retinol levels over the entire feeding period. This shows that the intervention was able to significantly increase the serum retinol levels of the vitamin A deficient rats. If the feeding period for the test rats would have taken longer than the allocated three weeks, it is presumed that the serum retinol level of the rats would been taken back to the basal serum retinol level.

Studies done at the University of Wisconsin using beta-carotene rich maize resulted in the increase of the vitamin A status of gerbils under a study aimed at tackling vitamin A deficiency (Yan, 2010). B-carotene enriched maize in whatever form is able to improve the vitamin A status of rats. Hence, if such results could be translated to humans it would be a great achievement towards combating vitamin A deficiency. From the research carried out by Yan (2010), if humans responded similarly, it would have important implications for vitamin A depleted populations consuming maize as a staple food crop. Cross sectional and case-control studies have demonstrated the importance of dietary vitamin A from plant foods in preventing vitamin A deficiency. Several cross-sectional studies in young children have shown an association between increased risk of Xerophthalmia and less frequent consumption of carotene-rich vegetables and fruits (Hess, 2005). Therefore β-carotene is important in the prevention of vitamin A deficiency

since it is more accessible to majority of households compared to preformed vitamin A from animal sources, which poor households cannot afford.

The usual method of rearing deficient rats has been to feed normal weanlings a vitamin A-free diet until growth ceases (Lamb et al, 1974). Lamb further adds that the animals are weaned at three weeks of age then fed a vitamin A-free diet ad libitum for a period of three weeks to induce the deficiency. At this age of the weeks the rats have not accumulated adequate retinol stores in the liver and so inducing them with the deficiency would not be a difficult task. This procedure was effective in inducing the deficiency to the test rats as observed from their reduced serum retinol levels after the 3 weeks of inducing with the deficiency.

The enriched Muthokoi would provide 15.3% of the recommended daily allowance for vitamin A for children. This is a significant contribution to RDA since the children would still be consuming foods rich in vitamin A. For a child to approximately achieve 30-40% of the RDA of vitamin A, the child would be required to consume at least three 250ml cups of porridge made from the enriched *Muthokoi* which will contribute to 45.9% of RDA. Hence, the more a child consumed in terms of cups of porridge per day, the more β-carotene the child consumed implying that such a child is able to meet his/her RDA for vitamin A ensuring that the vitamin deficiency was kept at bay.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Beta-carotene enriched *Muthokoi* can be appropriately developed by pounding maize with pumpkin leaves.

Pumpkin leaves are not only rich in beta-carotene but they are also rich in other nutrients such as calcium and iron which can be translocated to the maize kernel during pounding of maize with pumpkin leaves in the development of a beta-carotene enriched *Muthokoi*.

Supplementation of the laboratory rats with the β-carotene enriched *Muthokoi* improved their serum retinol levels significantly. This can be related to humans since consumption of three 250ml cups of porridge made from the enriched *Muthokoi* contributed to 45.9% of the recommended dietary allowance of vitamin A, implying that the β-carotene enriched Muthokoi is capable of improving the vitamin A status of children, preventing its deficiency. This goes ahead to emphasize that traditional fortification mechanisms can be used to enrich staples such as maize in trying to alleviate the burden of micronutrient deficiency among the vulnerable members of the society.

5.2 RECOMMENDATIONS

The study recommends the following:

1) There is need for promotion of the suitability of the β-carotene enriched *Muthokoi* in addressing vitamin A deficiency especially to the communities that do not consume *Muthokoi*. Since maize is a staple food in Kenya, the nutrition education need not overemphasize on the *Muthokoi* but encourage communities that it is still maize that has been enriched with nutrients hence more nutritious compared to just white maize, which is the most widely, accepted maize in the country. Communities need to be trained on the technology of enriching maize with β-carotene.

2) A comparative study should be carried out comparing the efficacy of the β-carotene enriched *Muthokoi* with *Muthokoi* cooked in admixture with green leafy vegetables to determine which of the two is more efficacious.

3) Further studies should be carried out to see whether the same results would be reflected in children.

4) There is need to conduct further analysis to determine the extent to which the Muthokoi is enriched with other nutrients including niacin, zinc.

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