

**University of Nairobi**

Department of Pharmacology and Pharmacognosy

**Effects of *Chinsaga* (*Gynandropsis gynandra*)  
on Haematological Profile and Markers of Iron  
Metabolism in Kenyan Breastfeeding Women**



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**A thesis submitted in fulfillment for the award of the degree of Doctor  
of Philosophy in Pharmaceutical Sciences of the University of Nairobi**

**2014**

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I hereby declare that this research thesis is my original work and has not been presented to any other academic institution for evaluation for research and examination.

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# Foreword

The genesis of this work was not a result of painstaking innovation as an individual but rather a product of ideas shared and experiences gained during my upbringing. The knowledge and selection of this subject for study was motivated by a deep desire to explore the richness of cultural knowledge and the fascination with the diversity of plants used by different communities as interventions during sickness and more importantly, in promotive health. Several ideas were explored eventually leading to selection of the current study as a model for validating our traditional knowledge and practices related to health. Unfortunately, the knowledge gained over centuries and initially passed on by oral tradition is getting eroded by westernization of our societies.

The opportunity for interaction with the Kisii culture and exposure to many others has helped shape my development of a comparative map of the cultural practices among communities in our country. This has provided the basis for identifying similarities and distinctions related to health promotion. My hope is that this work will stimulate and encourage documentation and evaluation of our heritage for future generations.

# Abstract

**Introduction:** *Chinsaga* (*Gynandropsis gynandra* (L.)) is a leafy vegetable indigenous to Africa, and is an important component of the traditional diet of the people of western Kenya such as the *Abagusii*, who refer to it as *Chinsaga*. The *Abagusii* believed *Chinsaga* has powerful blood restorative properties, and is recommended for pregnant and lactating women as a hematinic and immunostimulant.

**Objective:** This study sought to provide scientific validation for the traditional use of *Gynandropsis gynandra* (L.) among the Kisii community (*Abagusii*) in promoting maternal and child nutrition. This was done by assessing the impact of *Gynandropsis gynandra* (*Chinsaga*) consumption on the hematological profile and selected iron metabolism biomarkers among lactating women at Kenyatta National Hospital as indicators of nutritional status. The study also aimed at documenting the socioeconomic value of *Chinsaga* and chromatographic characterization of the plant sourced from Kilgoris and Kisii.

**Methodology:** A cross-sectional survey was undertaken to examine the socioeconomic value and trade of *Chinsaga* in Kisii and Kilgoris followed by plant collection and processing to provide material for a clinical study. A sample of the processed material was subjected to chromatographic characterization using Thin Layer (TLC) and High Performance Liquid (HPLC) Chromatography.

The study was reviewed and granted ethics approval by the Kenyatta National Hospital – University of Nairobi Ethics and Research Committee (KNH-UoN ERC), reference number: KNH-ERC/01/3757. A randomized triple blind controlled study was carried out at Kenyatta National Hospital Maternal Child Health Clinic 17. The study enrolled 119 women below 35 years of age, with a maximum of 4 live births and between their first and

second month after delivery. The two arm study evaluated the effects of *Chinsaga* consumption on hematological profile and on selected markers of iron metabolism. The comparator intervention was dietary supplementation with processed kale (*Brassica carinata*). Participants were followed for 28 days and anthropometrics, demographics, blood and milk specimens were collected. The pre and post treatment hematological laboratory parameters tested included; ferritin, transferritin and lactoferrin as biomarkers of iron metabolism.

**Results:** *Chinsaga* trade has a well structured supply chain with: producers; collectors; wholesalers; retailers and consumers in a variety of combinations. The highest demand is in urban areas. Commercial agriculture of *Chinsaga* has good prospects; however farm sizes are declining.

The plant undergoes three stages of maturation as described by farmers: *Omonyenye* (germination to four weeks); *Amasabore* (week 4 to 8) and *Ekegoko*, (mature stage). The *Amasabore* and *Ekegoko* are recommended for lactating mothers. A chromatographic fingerprint of *Chinsaga* was developed using ethyl acetate: methanol: water (50:20:10) on thin layer chromatography (TLC) and greater resolution achieved using gradient elution on HPLC-UV with acetonitrile, propan-2-ol, water and formic acid (0.4%) in the ratios 85:35:25, adjusted to pH 2.3 as mobile phase on a C18 column.

In the clinical study involving lactating mothers, *Chinsaga* supplementation was associated with significantly higher values of Red Blood Cell Counts (RBC) in the 2nd visit (median 4.79 Interquartile Range (IQR) 4.51 – 5.05, p=0.03) and 3rd visit (median 4.85, (IQR 4.46 – 5.18, p=0.03) compared to the control arm. Similar differences across arms were observed with changes in mean corpuscular volume; (median 27.3, IQR 25.5 – 29, p=0.03) and hemoglobin concentration (median 27.5, IQR 25.85 – 28.85, p=0.05).

However there were no statistically significant differences across arms for hemotocrit, (median 39.2, IQR 33.9 – 40.3, p=0.31) and hemoglobin (median 13.3, IQR 11.7 – 13.85, p=0.42).

On stratified data analysis, the effects of *Chinsaga* are dependent on patient reported duration of iron intake. The effects of *Chinsaga* on RBC count were most prominent in patients who had taken iron supplements for at least 1 month. On bivariable generalized linear regression modeling, the other variables that were positively associated with RBC counts and with  $p < 0.05$  were; duration of stay at the current residence, age of husband, Njahe, *Ugali* and Cocoa. However, on adjusting for confounding, only cocoa consumption had a statistically negative effect on RBC counts (adjusted  $\beta - 6.735 \pm 1.827$  P=0.00).

At a molecular level, there was progressive increase in transferrin gene expression during the three clinic visits. The highest mean was observed in the third visit (2.3, 95% C I (4.49 , 0.170).

**Conclusion and recommendations:** This study confirms the cultural value of *Chinsaga* and its socioeconomic benefit to the community of the Kisii by providing scientific data supporting the folklore use of *Chinsaga* by breastfeeding mothers for blood restoration as evidenced by the improvement of the hematological profile and effects on markers of iron metabolism. *Chinsaga* is a viable commercial crop that should be exploited for the socioeconomic benefit of Kenyans. Studies are needed to examine the effect of *Chinsaga* diet on enhancing breast milk quantity. This study exposes a gap in the knowledge and the need to conduct similar studies on traditional vegetables and foods consumed by various communities in Kenya. Such action will help preserve what is known and promote the incorporation of the traditional vegetables into the diet for the benefit of mankind. In addition, this study has demonstrated feasibility and provided a model for the design and conduct similar studies.

# Dedication

*To Dr. Enoch Bosire Nyanusi and Mrs. Milkah Nyaboke Bosire; Naomi Bosibori, Vanessa Yunuke, Hellen Mokeira, Walter Nyanusi, Edward, Ombeng'i ;Caro, Irene, Emma; Eric, Oscar, Mark and Justo*

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# List of abbreviations

%HYPOm	Percentage of Hypochromic Red Blood Cells
ALVs	African indigenous and traditional leafy vegetables
CEC	Cation exchange capacity
cGMP	Current Good Manufacturing Practices
CHr	Ratio of Hypochromic Red Blood Cells
DCRO	District Health and Records Officer
DNA	Deoxyribonucleic acid
EMEA	European Medicines Agency
FAO	Food and Agricultural Organisation
FFQ	Food frequency questionnaire
Hb	Haemoglobin
Hct	Hematocrit
HDL	High Density Lipoproteins
HES	High-energy supplement
HPLC	High Performance Liquid Chromatography
IDA	Iron Deficiency Anemia
IDDM	Type I insulin dependent diabetes mellitus
IGUR	Intrauterine growth restriction;
IRE	Iron Responsive Element
IRP1	Iron Regulatory Proteins 1
IRP2	Iron Regulatory Proteins 2
IUGR	Intrauterine Growth Retardation
KAVI	Kenya Aids Vaccine Institute for Clinical Research
KNH	Kenyatta National Hopsital
LAM	Lactational amenorrhoea method
LBW	Low birth weight
LC	Liquid Chromatographic system
LDL	Low density Lipoproteins
MAE	Microwave-assisted extraction

MCH	Mean Cell Hemoglobin
MNCH	Maternal Newborn Child Health
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Cell Volume
MLC	Mixed lymphocyte culture
MUAC	Mid upper arm circumference
NAPRALERT	Natural Products Alert Database
PCR	Polymerase Chain Reaction
PROTA	Plant Resources of Tropical Africa
RBC	Red Blood Cells
RDW	Red Cell Distribution Width
RID	Relative infant dose
SHLE	Superheated liquid extraction
SIDS	Sudden infant death syndrome
TfSat	Transferrin
TLC	Thin Layer Chromatography
TOC	Total Organic Carbon
USAE	Ultrasound-assisted extraction
UV	Ultraviolet Light
VAD	Vitamin A deficiency
WHO	World Health Organisation

## CHAPTER ONE

# 1.0 Introduction and Literature Review

## **1.1 Introduction and literature review**

### **1.1.1 Traditional use of *Chinsaga (Gynandropsis gynandra)* in Kenya**

*Gynandropsis gynandra* (L.) Briq. (1914) is a leafy vegetable indigenous to Africa, and is important to the traditional diet of the people of western Kenya such as the *Abagusii*. *G. gynandra* belongs to the family *Capparaceae* and is known by other synonyms including *Cleome gynandra* (L.), *Cleome pentaphylla* (L.) (1763) and *Gynandropsis pentaphylla* (L.) DC. (1824). The *Abagusii* people of western Kenya refer to it to as *Chinsaga* (1).<sup>1</sup>

### **1.1.2 Biological activities of *Gynandropsis gynandra***

*Chinsaga* has been reported to possess a wide range of activities. Notable applications of the plant include use of an infusion of the roots for chest pain, leaves for diarrhea, seeds to kill fish and contents of the glandular trichomes as insect repellent. The plant is planted among *Brassica* species to deter infestations by the diamond back moth larvae and by flower thrips among French beans (2).

*Chinsaga* has been evaluated for antiviral, antibacterial and ant-tumor activities. The ethanol water (1:1) extract was observed to show activity against hepatoma cells in a mouse model with a maximum tolerable dose of 1 g/Kg (3). In the case of rheumatism, the leaves and seeds are rubbed on skin to relieve the pain. The 95% ethanol extract has shown activity against CA-9KB and Melanoma B16 cancer cell lines (4). The 100% ethanol extract has shown anti-inflammatory (Kumar, Sadique, 1987) and antihelmintic activity against

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<sup>1</sup> In this work, the name *Gynandropsis gynandra* and the common name in Kisii, *Chinsaga* are used

*Fasciola gigantica* and *Perithima posthuma* (5) and weak activity against poliovirus. The water extract has been shown to have antioxidant activity (6). The methanol extract has been shown to have anti-yeast and anti-mycobacterium activity (7) and weak antiviral activity (8). If passed on in the breast milk, these components may have anti-infective properties aiding in the healing of cracks on nipples and oral thrush in babies.

The seeds contain the glucosinolates cleomin and glucocapparin, and a volatile oil which are believed to contribute to the distinct flavor and odor when cooked (6). Besides inhibiting growth of *Culex quinquefasciatus* larvae, the carvacrol component in the oil is also associated with repellent and acaricidal properties to larvae, nymphs and adults of the ticks *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (8).

### **1.1.3 Traditional Leafy Vegetables and Human nutrition**

Vegetables are an important part of most diets in Africa and globally. The variety of vegetables found in any locality is determined by ecological and cultural factors. There is a growing awareness of the health promoting and protecting properties of non-nutrient bioactive compounds found in traditional vegetables used mainly in soups or sauces that accompany starch staples like yam, maize, cassava and millet (9).

African indigenous and traditional leafy vegetables (ALVs) play a significant role in providing nutritional needs within Sub-Saharan Africa. The joint FAO/WHO 2003 Consultation on Diet, Nutrition and the Prevention of Chronic Diseases, recommended a minimum daily intake of 400g of fruits and vegetables (10). In concurrence, one year later, the WHO and FAO joint Kobe workshop on fruit and vegetables for health, proposed increased production, access and greater consumption of fruits and vegetables (11)

For definition, African Leafy Vegetables are indigenous to the continent and are included in traditional diet. The ALVs have their natural habitat on sub-Saharan Africa. However the traditional leafy vegetables include vegetables introduced over a century ago and due to long use, they have become part of the food culture in the continent. There are over 6,300 useful indigenous African plants of which 397 are listed as vegetables in the Plant Resources of Tropical Africa – PROTA (12). In the April 2005 Issue of *Spore*, it was argued that books and the internet are awash with information on the African leafy vegetables but is “often scattered like leaves in the wind” (13). The distribution is summarized in table 1.1.

Quite a large number of African indigenous leafy vegetables have long been known to have health protecting properties and uses (14–16). Several of these indigenous leafy vegetables are also associated with prophylactic and therapeutic uses by rural communities which form a strong basis for their promotion and protection. ALVs also contain non-nutrient bioactive phytochemicals. However, most of this information about the vegetables is anecdotal. There is very little published information or data on their production, use and benefits when consumed by humans. This information would be useful in developing food policy initiatives on the continent (17).

**Table 1.1:** Regional distribution of some commonly found Leafy Vegetables (Adopted from (10))

<p><b>All over the Subcontinent:</b>  <i>Abelmoschus esculentus</i>  <i>Amaranthus cruentus</i>  <i>Corchorus olerius</i>  <i>Cucurbita maxima</i>  <i>Vigna unguiculata</i>  <i>Solanum macrocarpon</i></p>	<p><b>West/East &amp; Central Africa</b>  <i>Basella alba</i>  <i>Citrullus lunatus</i>  <i>Colocasia esculenta</i>  <i>Hibiscus sabdariffa</i>  <i>Ipomea batatas</i>  <i>Manihot esculenta</i>  <i>Solanum aethiopicum</i>  <i>Solanum scarbrum</i>  <i>Talinium triangulare</i>  <i>Vernonia amygdalina</i>  <i>Moringa oleifera</i></p>
<p><b>East/Central and Southern Africa:</b>  <i>Solanum nigrum</i>  <i>Bidens pilosa</i>  <i>Cleome gynandra</i></p>	<p><b>West and Southern Africa</b>  <i>Amaranthus caudatus</i>  <i>Amaranthus hybridus</i>  <i>Portulaca oleracea</i></p>

For sustainable food security and health there is need to mobilize local biodiversity including use of the African Leafy Vegetables. However, trends in the use of ALVs are varied. Some are declining while others are thought to be increasing in popularity. It may be of interest to evaluate this information in relation to urbanization of populations (18,19).

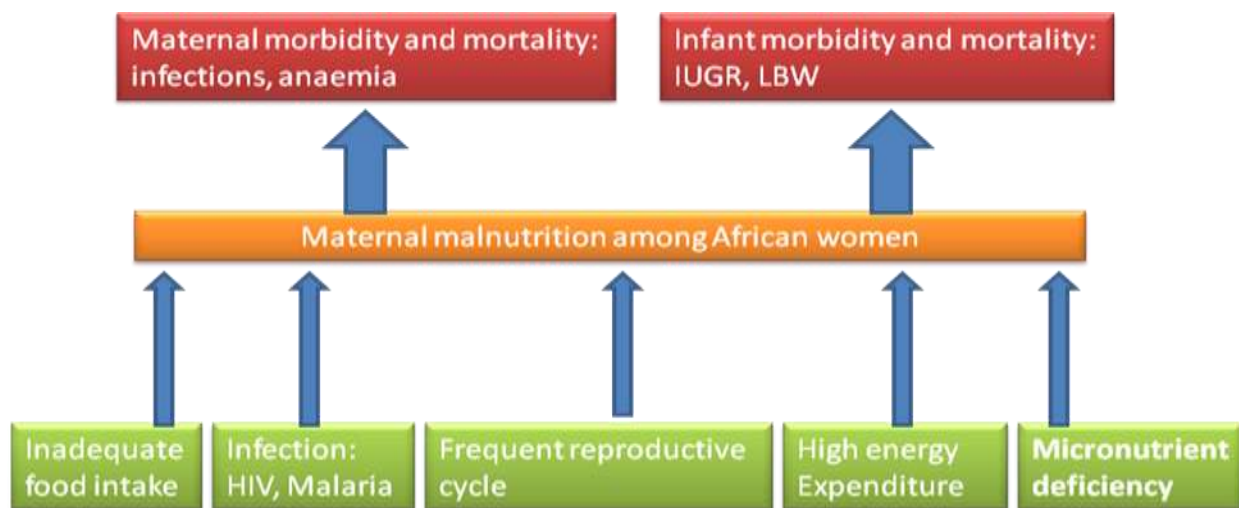
Estimates of per capita consumption per day of ALVs average about 80g in Senegal and Burkina Faso. Within country variations are also observed. An example is Mauritania with 65g/day (urban) and 16g/day in rural areas (14). Consumption also varies with season. In Uganda, the rate is about 160g/ day during the rainy season. In western and Eastern Nigeria, the rate is placed at about 65g/day. In spite of the abundance of African indigenous and traditional leafy vegetables, they remain under-exploited and under-utilized due to various constraints (20,21) and this is partly attributed to constraints during production, processing, distribution, marketing and lack of information for both the farmer and potential consumers.

Indigenous vegetables are more drought and heat tolerant than commonly grown exotic vegetables. Cowpea is the most drought-tolerant crop, followed by

nightshade, pumpkin and tsamma melon. Amaranth is observed as the most heat-tolerant crop. Water requirements for optimum growth of the African leafy vegetables range between 240 mm and 463 mm for a full growing season (22).

In sub-Saharan Africa, women, especially pregnant and breastfeeding women, infants and young children are among the most nutritionally vulnerable groups. There is an increase in nutrient requirements during pregnancy, breastfeeding and rapid growth and development in infants(23).

IUGR, intrauterine growth restriction; LBW, low birth weight.



**Figure 1.1** Effect of maternal malnutrition on maternal and infant health (Modified from (24) )

High poverty levels and heavy workload affect the quality of the diet (25). This inadequate nutrition is aggravated by frequent and short reproductive cycles without replenishing body nutrient stores and frequent infections among children less than five years of age. As a result, there is high frequency of low weight gain during pregnancy, low birth weight among 14 % of infants, life-threatening situation during delivery (26) and HIV infection (27). (**Figure 1.1**)

It is important to address the nutritional needs of adolescent girls in preparation for reproductive roles in adulthood. Similarly women need adequate nutrition before and during pregnancy for the developing fetus (28).

Maternal nutrition education should be given during antenatal and postnatal care. During the complementary feeding, growth faltering is frequent hence supplementary feeding programs should be integrated early (29). Across the continent the prevalence of anemia ranges from 21 to 80 %, with similarly high values for both vitamin A and zinc deficiency. Fortification of staple foods, direct fortification of commercial complementary foods,(30,31) and direct addition of micronutrients as sprinkled powder, crushable nutritabs or nutributter, to home-prepared complementary foods have all been applied with varying success.

Indigenous vegetables are more drought and heat tolerant than commonly grown exotic vegetables. Cowpea is the most drought-tolerant crop, followed by nightshade, pumpkin and tsamma melon. Amaranth is observed as the most heat-tolerant crop. For optimum growth, water requirements for the African leafy vegetables studied for a full growing season range between 240 mm and 463 mm (22).

In relation to their nutritional value, some provide more than 50% of the recommended daily allowance for Vitamin A, and at least 30% of the estimated average requirement. They also provide varying amounts of other nutrients, such as protein, mineral elements, and fiber. They are mostly gathered, few cultivated and more as mixed cropping system in kitchen gardens. Traditional leafy vegetables are easier to produce and less resources demanding yet rich in micronutrients like iron and Vitamin A (31).

To avert malnutrition, humans need to eat diverse foods. However, high rates of micronutrient malnutrition are present despite global efforts to reduce the

levels of Iron deficiency, which affects an estimated 2.0 billion people, mainly women and children in the poorer segments of the population in developing countries. Vitamin A deficiency (VAD) affects more than 200 million people and is the major cause of preventable visual impairment and blindness (32).

Promoting eating of traditional vegetables is a sustainable way of mitigating deficiencies in resource-poor communities. These vegetables grow more easily, are resistant to pests and diseases and are readily acceptable. Leafy vegetables and fruit vegetables form a significant part of the traditional diets of agricultural communities. In Kenya about 200 indigenous plant species of leafy vegetables are included in human diet. A small number are fully domesticated, more are semi-domesticated while the most grow wild (33). Cowpea leaves (*Vigna unguiculata*), pumpkin leaf (*Cucurbita maschata*), amaranth (*Amaranthus blitum*), jute mallow (*Corchorus olitorius*), and mushrooms are popular (31).

In a study in Shurugwi District, Zimbabwe 21 edible weeds belonging to 11 families and 15 genera were studied. They represented *Amaranthaceae* (19%), *Asteraceae* and *Tiliaceae* (14.3%), *Capparaceae*, *Cucurbitaceae* and *Solanaceae* (9.5% each). Majority (52.4%) were indigenous edible weeds semi-cultivated or growing naturally as weeds. Most (81%) of them were used as leafy vegetables. Coincidentally, the most cited edible weeds were *Gynandropsis gynandra* (93.9%), followed by *Cucumis metuliferus* (90.5%), *Cucumis anguria* (87.8%), *Corchorus tridens* (50.3%) and *Amaranthus hybridus* (39.5%). An additional vegetable was *Moringa oleifera* and all were used at different times of the year. The edible weeds are an important component in providing food security and nutrition (34).

While most populations are tending towards less variety in food sources, these alternatives are often less nutritional compared to the traditional foods. With increasing urbanization and movement of the younger populations away from

rural areas, there is a diminishing number of people involved in propagating the foods. The traditional vegetables are often better adapted to the local conditions and require less care (35). It is also observed that majority of those who are involved in production are often women. They therefore contribute to both food security and income for their families. Consequently, a shift away from these foods will further exacerbate poverty and the state of food insecurity (36). There is great urgency to involve researchers and extension services in support of the traditional leafy vegetables as a strategy in food security for households. Additionally, this will have an influence of the cultural environment of the women who are involved in the production system. These factors play a significant role in the women's ability to produce and maintain household food security (35).

Inadvertently, the shift in consumer behavior leads to loss of available income sources in local food systems, loss of jobs and increased uncertainties in the food supply as communities shift to non local staples.

Kenya has many species of edible leafy vegetables yet accessibility remains low. Other indigenous vegetables like African nightshades (*Solanum scabrum*), spider plant (*Cleome gynandra*), African kale (*Brassica carinata*) Slender leaf (*Crotalaria brevidens*), Jute mallow (*Corchorus olitorius*) and pumpkin leaves (*Cucurbita moschata*) and 'Enderema' (*Basella alba*) (27) are slowly increasing in availability in the local markets.

## **1.2 Human lactation**

Breastfeeding has been shown as a means to save lives, reduce illness and protect the environment. Policy makers are increasingly aware of its role in reducing healthcare costs and enhancing maternal and infant well-being. Breastfeeding promotion and support has been recognized as a healthcare priority by the World Health Organization (27), numerous other institutions

and organizations concerned with preventive medicine and the healthcare of mothers and infants. WHO recommends exclusive breastfeeding for the first six months and its continuation as long as both mother and infant wish well past the first year of life. After six months, breastfeeding is complemented by appropriate introduction of other foods (27).

With the extensive research now available on the benefits of breast milk and the risks of artificial milks, doctors need to be able to support their breastfeeding patients. Unfortunately not many healthcare workers currently in practice have adequate education to support mothers on breastfeeding physiology and practice despite the existence of well-researched basic principles and guidelines. In addition to well-educated lactation professionals and mother to mother resources, more needs to be done to promote breastfeeding knowledge and practice.

### **1.2.1 Normal lactation physiology**

Ordinarily, milk supply is established during the first few days and weeks after the birth of the baby. Nursing early (within the first half-hour), and frequently (on demand, or 8 - 12 times per day), allows the mother to nurse comfortably and efficiently. It usually takes less than 1 minute for an infant to stimulate the milk ejection reflex with little or no discomfort or pain when breast feeding appropriately.

Within 6 - 8 weeks, milk supply will adjust to the baby's needs. Before that time, the breasts may feel either too full or empty. Frequent, comfortable feedings will maintain milk supply which will increase or decrease based on the baby's hunger and energetic sucking (milk demand or use). Changes in milk supply will occur within 1 - 3 days after changes in milk demand or use (37).

### **1.2.2 Importance of Breastfeeding for infants**

Human milk is specially suited for human infants as it is easy to digest and contains all the nutritional needs of the baby in the early months of life (37). It provides enzymes to optimally digest and absorb the nutrients in the milk before infants are capable of digesting by themselves. It contains multiple growth, maturation factors and antibodies specific to illnesses encountered by both the mother and baby (38) that protect infants from a wide variety of illnesses (39). Research suggests that fatty acids, unique to human milk, play a role in infant brain and visual development (40). Higher cognitive and neurological performance is associated with breastfed babies over artificially fed infants (41). Lack of breastfeeding is a risk factor for sudden infant death syndrome (SIDS) (42), and human milk seems to protect the premature infants from life-threatening gastrointestinal disease and other illnesses (14,38,43).

On the whole, breastfed infants are healthier. Those who are exclusively breastfed for at least four months are less likely to have ear infections in the first year of life and suffer less severe bacterial infections such as meningitis, lower respiratory infections, bacteraemia and urinary tract infections (29,43)(44). They also have lower incidence of infant botulism(45), a lower risk of "baby-bottle tooth decay" (46), less diarrhea and evidence suggests that exclusive breastfeeding for at least two months protects susceptible children from Type I insulin dependent diabetes mellitus (IDDM) (47). Breastfeeding may reduce the risk of subsequent inflammatory bowel disease and childhood lymphoma (48,49) allergy, illnesses such as heart disease, stroke, hypertension and autoimmune diseases in adulthood (41).

### **1.2.3 Benefits of Breastfeeding to Mothers**

Breastfeeding help's mothers recover from childbirth by accelerating uterine contraction to its pre-pregnancy state thus reducing the amount of blood lost

after delivery (50). It also shortens the period of return to pre-pregnant weight compared to bottle-feeding mothers. Breastfeeding mothers usually resume their menstrual cycles 20 to 30 weeks later than bottle-feeding women acting as a natural form of 'contraception'. (51) Breastfeeding is believed to keep women healthier throughout their lives as an important factor in child spacing among women who do not using contraceptives.(38) It is proposed to reduce the risk of breast and ovarian cancer and development of multiple effects on glucose and lipid metabolism in women with gestational diabetes (52). Breastfeeding may offer a practical, low-cost intervention that helps reduce or delay the risk of subsequent diabetes in women with prior risk of gestational diabetes and osteoporosis (53). During lactation, total cholesterol, LDL, cholesterol, and triglyceride levels decline while the beneficial HDL level remains high and improved (54,55). Breastfeeding plays a salient yet key role in promoting maternal confidence (55).

#### **1.2.4 Importance of Breastfeeding to Society**

From an economic perspective, breastfeeding is cheaper than artificial baby milk, reduces healthcare costs, less gastrointestinal and respiratory infections with less hospitalization (56) right through to the adolescent and adult population. This results in less parent absenteeism from work.

Breastfeeding does not require manufacturing or preparation and represents the most efficient conversion of plant material into an ideal high-protein and energy food for infants while averting nearly as many births as all other modern contraceptive methods combined (56).

Most breastfeeding women say they chose to breastfeed because of the health benefits for their infants. Yet, despite the evidence that breastfed babies are healthier, only a fraction of new mothers exclusively breastfeed at one month after their baby's birth (39). If the healthcare benefits of breastfeeding are to be

realized, further education and support of mothers, families, communities and health care professionals is imperative. Breastfeeding is not just a lifestyle choice, it is a health issue. Every baby has a right to breastfeed (44).

Some herbal preparations have been indicated to increase breast milk. These include fenugreek (*Trigonella foenum-graecum*), blessed thistle (*Cnicus benedictus*), aniseed (*Pimpinella anisum*), caraway (*Carum carvi*) and dill (*Anethum graveolus*). They are believed to aid digestion by promoting nutrient absorption combined with galactagogue properties (57). There is a common habit among some mothers to take large amounts of tea or drinking chocolate to increase breast milk. It is important to note that infants poorly eliminate caffeine. Larger amounts of caffeine may cause insomnia in children.

#### **1.2.5 Nutrition and milk production**

The nutritional status of individuals is considered to be the most significant contributor to health outcomes. In Kenya, like in many developing countries, poverty and resultant inadequate nutrition has played a major role in contributing to the poor health outcomes. This is significant in the under five mortality within the poor communities resulting from poor nutrition. There is tremendous effort and resources put in vector control, drug and vaccine development, however, not much attention has been given to the likely impact of the traditional diet of the inhabitants in disease endemic areas on the overall immunity and health.

A study conducted by Gonzalez CossyBo (58) sought to evaluate whether milk production can be improved by increasing food intake. The results demonstrated a positive effect of long-term food supplementation on lactation performance among undernourished women increasing infant milk intake and infant milk energy intake. The difference was more evident when adjusted for

potential confounding determinants of milk volume or energy content especially within malnourished mothers.

The results obtained underscored the importance of coupling nutritional programs to malnourished lactating women with programs that promote exclusive breastfeeding at 4-6 months of age because a highly nutritious food continues to be provided with a reduction in contaminated weaning foods. It was also clear that improvement of lactation performance of malnourished mothers can be improved by appropriately designing and targeting nutritional programs for the mothers.

Research into beneficial plants has revealed wide range of potential applications in improving health. There are reports on the immunomodulating effects of two Echinacea species, *E. purpurea* and *E. angustifolia* and Larch arabinogalactan extracted from *Larix occidentalis* (59). In another report, standardized Milk Thistle extract was studied in murine lymphocyte proliferation tests using Concanavalin A (ConA). It was found that Milk Thistle is immunostimulatory *in vitro*. It increased lymphocyte proliferation in both mitogen and MLC assays. These effects of Milk Thistle were associated with an increase in interferon gamma, interleukin (IL)-4 and IL-10 cytokines in the MLC. This immunostimulatory effect increased in response to increasing doses of Milk Thistle. The study uncovered a novel effect of milk thistle on the immune system. This immunostimulatory effect may be of benefit in increasing the immunity to infectious diseases (59).

### **1.3 Study rationale and justification**

The use of *Chinsaga* is widespread in the western regions of Kenya and has spread to other parts of Kenya. Use is associated with a variety of health claims but the most common use is linked to the hematinic and lactation benefits in women. To our knowledge, no study related to these applications has been conducted and this forms the basis of this investigation.

Traditionally the local communities give specific dietary recommendations to pregnant and lactating mothers to improve lactation, blood levels, and general immunity. The Kisii recommend use of *Chinsaga*.

This study explores the scientific merits of using *Chinsaga* in promoting maternal and child health by evaluating changes in hematology, expression of selected iron metabolism biomarkers in blood and lactoferrin in breast milk. No such investigation in Kenya or in published works has been conducted.

### **1.4 Study objective**

#### **1.4.1 Broad objective**

To determine effects of *Chinsaga* supplementation on the hematological profile and selected iron metabolism biomarkers among lactating women at Kenyatta National Hospital.

#### **1.4.2 Specific objectives**

1. To document the botany and socioeconomics of *Chinsaga* in Kisii-Kilgoris area.
2. To develop a suitable chromatographic method for fingerprinting *Chinsaga*.

3. To assess the dietary knowledge and practices among breast feeding women attending Kenyatta National Hospital.
4. To evaluate effect of *Chinsaga* supplementation on hematological profile of breastfeeding women.
5. To evaluate effects of *Chinsaga* on the expression of transferrin, ferritin in blood and lactoferrin in breast milk.

Each of the objectives is compiled in a chapter describing the methodology, results and discussion of the findings. A final chapter summarises the observations providing a proposed general overview of the work covered in this thesis.

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## CHAPTER TWO

# 2.0 Socioeconomic Botany of *Gynandropsis gynandra* Cultivation and Trade

## 2.1 Background and literature review

In this study essential preliminary information about *Gynandropsis gynandra* was obtained to enable standardization of the cultivation, processing and was obtained to enable standardization of the cultivation, processing and packaging of the material to be used in the clinical trial evaluating the effects in hematology and markers of iron metabolism among breastfeeding mothers.

The plant *Gynandropsis gynandra* (L.) Briq. (1914) belongs to the family *Capparaceae* (APG: *Brassicaceae*). It is also known by other synonyms including *Cleome gynandra* (L.), *Cleome pentaphylla* (L.) (1763), *Gynandropsis pentaphylla* (L.) DC. (1824). In Kenya the plant is locally known as *Chinsaga* (Kisii), *saget* (Kalenjin), *dek* (Luo) and *tsisaka* (Luhya). Examples of vernacular names beyond Kenya include spider plant, cat's whiskers, spider flower and bastard mustard (English); *Caya blanc*, *brède caya* and *mouzambé* (French); *Musambe* (Portuguese); *Mgagani*, *mkabili*, *mkabilishemsi* and *mwangani mgange* (Tanzania) (1).

Based on folklore and historical records, the plant is thought to have its origins in Africa or Southern Asia but this remain unconfirmed (2). *Chinsaaga* is found throughout most of the tropics and subtropics. It is associated mainly with human settlement as traced by migrations and geographic distribution (3).

There are between 150 and 200 species that make up the genus *Gynandropsis* (or *Cleome*) in the subfamily Cleomoideae with about a third growing in Africa. *Gynandropsis* species of Africa that are used as vegetables do not have a distinct androgynophore. The stamens and ovary are well beyond the corolla like those of the spiderplant. Review of an initial distinguishing factor based on the connection of the staminal base with the gynophore to form an androgynophore has led to the merging of the subfamily *Gynandropsis* with *Cleome* (4).

*Gynandropsis gynandra* grows on a wide range of soils. The plant grows best in deep, well drained sandy to clayey loam soils of pH 5.5–7.0 with high organic matter and adequate mineral reserves. *Gynandropsis gynandra* can be found growing from sea level to 2400 m at temperatures above 15°C. It does not thrive well in humid places but shows some tolerance to drought. Maturity and senescence is quickened under water stress (2).

### **2.1.1 Morphological appearance**

Most plants observed in the field range from an average height of about 10 to about 150 cm. Depending on the method of harvesting, the plant may grow many branches. The plant has a long taproot with few secondary branches. The stem is heavily covered with glandular trichomes which are also found along the petiole of the leaves. The leaves are alternate, palmately compound with between three to seven leaflets. The leaves do not have stipules and petioles are 2–10 cm long. The leaflets are almost sessile, obovate to elliptical or lanceolate, 2–10 cm × 1–4 cm, cuneate at base, rounded to obtuse, acute or acuminate at apex. The leaf margins are finely toothed (5).

The inflorescence comprises a terminal raceme up to 30 cm long, bracteate. The flowers are bisexual, white or tinged with purple pedicels of 1.5–2.5 cm long **Figure 2.1**. The flower consists of 4 free sepals, ovate to lanceolate in shape and 3 to 8 mm long. It also has 4 elliptical to obovate clawed petals with a length up to 1.5 cm. The androgynophore is 1–1.5 cm long. It has six purple stamens; the ovary is stalked, 2-celled and superior. The fruit is a stalked narrow long beaked cylinder about 3 to 12 cm, green or yellow in color. It is dehiscent from below with 2 valves and many-seeded. The spherical seeds are subglobose of 1–1.5 mm diameter, grey to black and irregularly ribbed. At germination the seedling grows a trifoliate of oblong cotyledons (6).



**Figure 2.1:** Flower shoots of *Chinsaga*.

### **2.1.2 Agronomy of *Chinsaga***

In Kenya, there is increasing awareness of *Chinsaga* as a vegetable and supplies are now available in the major supermarkets and nearly all farmer markets in the larger towns. In East and southern Africa, it is sold in rural and in urban markets during the rainy season.

### **2.1.3 Study area and community**

This ethno-botanical study was conducted in Kilgoris area of Transmara District in Kenya. Interviews were conducted among members of the Kisii community in Kisii town and Nairobi city. The surveys were carried out following reconnaissance surveys conducted between December 2007 and March 2008. Kisii and Kilgoris areas both located about 200Km to the west of Nairobi and share a common administrative boundary. The selection of Kilgoris for supply and Kisii for market and distribution aspects of the study was informed by the observations made during the reconnaissance visit. It was established that Kilgoris and south western parts of Kisii were important in the supply chain of *Chinsaga* distributed to other towns. Traditionally, *Chinsaga* is

a vegetable used among the Kisii community but due to increasing land pressure, farmers have moved to Kilgoris where fertile land is still available for leasing.

#### 2.1.4 Profile of Transmara District

Transmara district is located in the south western part of Rift Valley province, **Figure 2.2**. It consists of five administrative divisions namely: Kilgoris, Pirrar, Lolgorian, Keyian and Kirindon. Maasai Mara game reserve occupies 312 square kilometers. In total it covers an area of approximately 2900 Km<sup>2</sup>. The district lies between latitudes 0° 50' South and 1° 50' North and longitudes 34° 35' East and 35° 14' West. It borders the republic of Tanzania to the South, Migori and Kuria districts to the West, Gucha, Nyamira and Bomet districts to the North and Narok district to the East. The population of the district during the 2009 census was 180,417 with a population density of 58 persons per square kilometer. The district has over 32,000 households (7).



**Figure 2. 2:** Map of Transmara District\*.

*\*Image of Kilgoris area was kindly provided to the author by the District Health and Records Officer (DCRO) Kilgoris District Mr. Joel Omiti.*

The population density is higher in and around urban centers such as Kilgoris, Lolgorian, Nkararo, Enoosaen, Emurua Dikrr, Njipship and Murgan trading centers. Population density is influenced by nature of land use, and is greater in areas where livestock farming is practiced compared to crop farming.

### **2.1.5 Topography and climate of Transmara**

Transmara district consists mainly of highlands between 2200 and 2500 m above sea level as well as a plateau which rises from 1500 m to 2200 m above sea level, (**Figure 2.3**). The dominant elevations are between 1800 m to 1950 m interrupted by rocky eroded hills. The lowest elevation is found toward the Migori border while the highest point is found towards the east of Kilgoris division (8).



**Figure 2. 3:** Typical landscape picture of farming areas of Kilgoris

Annual temperature ranges from 14.8 to 20.3 °C, with the highest temperatures occurring between January to March and the lowest from June to August (10.5 to 15.5 °C). The district receives a bimodal rainfall pattern which is well distributed throughout in normal years with peaks in April (long rains)

and December (short rains). Rainfall averages about 1500 mm annually with a range between a high of 2300 mm and low of 700 mm. Hailstones are occasionally reported in the west and the highland in the north of the district. Decline in the total rainfall amount received over the recent years is attributed to indiscriminate felling of natural vegetation for crop growing, wood fuel and building materials (8).

The district is suitable for livestock production and arable agriculture. Currently, the dominant activities include beef livestock rearing and maize farming. Other enterprises with potential include; dairy farming, quarrying and mining, sand harvesting, bee keeping and cash crops such as tea. The Maasai Mara Game Reserve is also an important income generating tourism resource for the Transmara County Council.

#### **2.1.6 Soil types in Transmara**

The two major soil types found in Kilgoris are phaeozems and nitisols. Phaeozoms are dark colored soils rich in organic matter. The topsoil referred to as mollic A-horizon is relatively high in organic matter and is non-acid. The base saturation of the topsoil is over 50%. These soils usually have an ABC sequence of horizons. The subsoil (B-horizon) usually has a well developed blocky structure with high porosity. As observed in the area where *Chinsaga* is planted, the high level of organic matter confers high natural fertility and an abundant supply of mineral nutrients. A major disadvantage of these soils is the high potential for erosion by wind and water if left exposed (9).

Nitisols are the deep, red friable clays observed in some areas. They accommodate soils more than 150 cm deep. They exhibit clay movement with a visible shiny red surface throughout the subsoil B-horizon. They have a clay texture, friable or vey friable and are porous throughout. The chemical properties of these soils vary widely. The organic matter content, cation

exchange capacity (CEC) and percentage base saturation range from low to high. Nitisols are highly productive soils found in humid tropics with a high degree of phosphorus sorption. Unlike the phaeozoms, they are quite resistant to erosion (10).

Regardless of the soil type found in the farming area, the Total Organic Carbon (TOC) ranges from a low 0.81% to an adequate level of 2.81%. The soil pH ranges from strongly acid (4.5) to slightly acid (6.65). These areas have an average altitude of about 2000 m above sea level and fairly cool temperatures most of the year. The soil is of moderate fertility for growth of maize, cabbage, tomatoes, spinach and kales among others crops. The soils for production of *Chinsaga* in Kilgoris were observed to respond well to nitrogen, phosphorus and micro nutrient supplementation (9–11).

#### **2.1.7 Growth, maturation and harvesting of *Chinsaga***

The germination rates of the *Chinsaga* (*Gynandropsis gynandra*) seeds are variable and usually takes about one week to sprout. Plant growth appears strongest after the first month with an equal increase in auxiliary shoots due to regular harvest of the apices. This growth of branches is important as it increases the vegetable foliage. Flowering is often seen towards the end of the rainy season but may also be triggered by stress. After about two to three months, the fruits dry releasing seeds into the surrounding ground (12).

### **2.1.8 Objectives**

This chapter focuses on documenting the botany and socioeconomics of *Chinsaga* cultivation and trade.

### **2.1.9 Specific objectives**

These included documentation of:

1. The cultivation of *Chinsaga* in Kilgoris-Kisii area
2. The distribution and marketing of *Chinsaga* from Kilgoris
3. The challenges and opportunities experienced in the supply chain of *Chinsaga*.

## **2.2 Methodology**

Twelve farms, approximately 300 m apart within Mosocho area of Kilgoris were purposively sampled. The supply chain was traced from the farmers market in Mosocho through Kilgoris town to Kisii and eventually to Nairobi. Information about the activities of the middle-men was obtained from key informants at the major trading points of Kilgoris, Kisii and Nairobi. Farmers, middle men, transporters, traders and individuals that purchased from the retail vendors in the market were also interviewed.

Other interviewees included community leaders and businesswomen that participate in the *Chinsaga* trade and supply chain. Semi-structured discussions directed by the researcher were conducted with a total of 32 participants of all age groups and gender on separate occasions. The responses

were collated and thematically analyzed. Research was conducted by a series of field trips that included collection of qualitative and quantitative data from December 2007 to July 2008. Plant voucher specimens were collected and deposited at the Herbarium of the School of Pharmacy, Department of Pharmacology and Pharmacognosy, University of Nairobi.

### **2.2.1 Ethical Considerations**

Permission for the study was obtained from Kenyatta National Hospital – University of Nairobi Ethics and Research Committee (KNH-UON ERC/01/3757). Prior informed consent was also obtained from each participant and only those who consented to the request and expressed interest in the research participated in interviews.

## **2.3 Results**

### **2.3.1 Folklore use of *Chinsaga***

Through key informant interviews, it was revealed the *Abagusii* (Kisii people) recommend the eating of boiled leaves of *Chinsaga* for treating anemia and by mothers before and after delivery of a child. Firstly, it was believed to enable quick recovery from the blood loss experienced during parturition and secondly, to hasten milk let-down shortly after delivery.

The vegetable is cooked fresh most of the time. When larger quantities are available, the vegetable is blanched and sundried for preservation. The dried form is then packed into small bags and may be stored for cooking later.

During preparation, the vegetable is boiled for at least one and a half hours. Milk, cream or groundnut paste may be added to the second cooking to help mask the bitter taste often associated with the mature form of the vegetable. In other situations, it may be mixed with other vegetables such as pumpkin

leaves, amaranth or *terere*. Traditionally, the *Abagusii* do not blend *Chinsaga* with *Solanum nigrum* (*managu*) as may be found elsewhere.

### 2.3.2 Agronomy and commerce of *Chinsaga* in Kilgoris

It was observed that farming and commercial trade in *Chinsaga* is widely practiced in Kisii and Kilgoris town areas during the field study. *Chinsaga* is widely consumed by the Kisii. The vegetable is grown in many farms in Kisii and parts of Kilgoris and large commercial farms are found in leased farms to the south of Kisii along the Kilgoris-Kisii boundary areas where the population is less dense.

### 2.3.3 Sizes of Farms in Kilgoris

The farmers including natives from Kilgoris area who are Maasai and large number of migrants from Kisii now lease out land and grow the crop. Pieces of land used for farming ranged from small gardens about an eighth to about one acre. The most frequent size observed was half an acre, **Table 2.1**.

**Table 2 1:** *Distribution of land sizes under Chinsaga cultivation in Mosoch area of Kilgoris*

Land Size	Frequency	Proportion%
Less than ¼ acre	2	17
¼ acre	3	25
½ acre	6	50
More than ½ acre	1	8



**Figure 2. 4:** A farmer and his family outside the kitchen house.

#### **2.3.4 Sources of seeds**

Many farmers were observed to have a portion of a garden near their houses used as the seed bank, **Figure 2.4**. In this garden, the crop is allowed to mature till it flowers and gives fruits. The pods from these plants are harvested and dried in the sun. When dry, the pods are rubbed to release the seeds used for the next crop. The garden also serves as a domestic source of vegetable for the home during the earlier stages of growth before maturing. In Kenya, seeds referred to locally as *Saget*, are available from Kenya Seeds Company. Farmers also use seeds from their own cultivars. Majority 26 (81%) of the farmers interviewed in Kilgoris reported that they used their own seeds for cultivation of *Chinsaga*.

#### **2.3.5 Drivers for commercialization of *Chinsaga* farming**

Based on interviews with key informants, it was established that production was originally carried out at subsistence level in small gardens. However, gradual reduction in land sizes, increasing demand in urban centers and

growing populations away from the rural areas raised the demand to exceed level of production by subsistence methods.

### **2.3.6 Soils used to grow *Chinsaga***

Application of compost mature was important to sustenance of the high fertility. It was further observed that vegetable farming is practised in the lower highland extending from Kisii. In the same areas, maize, wheat and pyrethrum were also grown. On occasion application of micronutrients as foliar fertilizers was seen to greatly enhance growth of crops.

### **2.3.7 Stages of growth of *Chinsaga***

Among the Kisii, *Chinsaga* is harvested in three distinct phases with the highest yields occurring in the seventh week. Farmers reported that *Chinsaga* requires about four weeks from planting to the first harvest of the tender sprouts, **Figure 2.5**. The plants are grown in rows about one foot apart. The first three harvests served to prune the numbers leaving better spacing between plants. This first three crop harvests are locally referred to as *Omonyenye*. The vegetable at this stage is of mild and gentle taste. The *Omonyenye* stage is about four week old crop on a farm. The plants at this stage will have already undergone the first harvesting during which some plants are uprooted to allow for optimal spacing. Pruning also allows for the farmer to select out the poorer plants leaving those that appear to be thriving better.

*Chinsaga* two to three months old is described as the *Amasabore* stage, **Figure 2.6**. Leaves from the stems and sturdier shoots are harvested weekly. The stage of the vegetable has a mild acerbic to bitter taste. After the *Amasabore* stage,

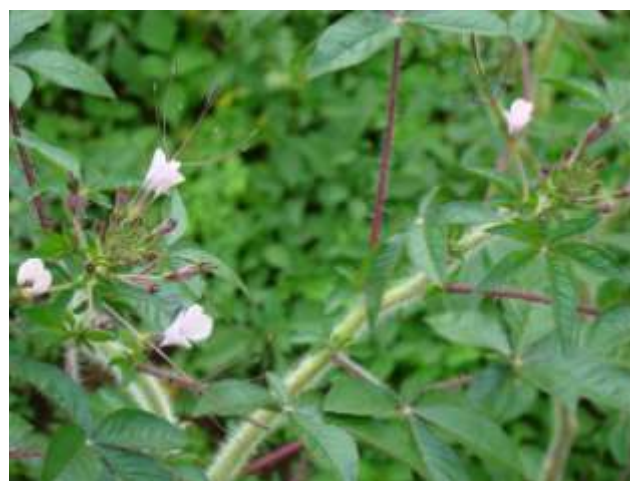
the plant begins to flower, and the stage is referred to as *Ekegoko*, **Figure 2.7**. *Ekegoko* has a bitter taste and is preferred by the older people of the Kisii community. The vegetable is relished at all stages but preferences are mainly determined individually. The bitterness of the plant when cooked varies by variety and appears to be influenced by soil type and the pattern of rain at different stages of plant growth.



**Figure 2. 5:** *Omonyenye*



**Figure 2. 6:** *Amasabore*



**Figure 2. 7:** *Ekegoko*

Overall, well watered crops in fertile soils with plenty of manure will give the best crop with a moderate level of bitterness palatable to most people. However, this degree of bitterness is much more in areas with drier hotter and less fertile soils. The plants will be more stunted and have tougher stalks that make the vegetable more fibrous to chew. This is probably the least favored form of the vegetable in diet.

## **2.4 Production, supply and marketing of *Chinsaga***

The practice of growing *Chinsaga* in Kilgoris was introduced by the Kisii since the Maasai traditionally lived mainly by raising livestock. Upon establishment of better returns from farming of vegetables, some Maasai have also begun to take a keen interest in the farming. However, the main traders and consumers are Kisii and this has helped to retain a fairly controlled supply chain by Kisii traders.

### **2.4.1 *Chinsaga* planting, harvesting and processing**

Vegetables production in Kilgoris and Southern Kisii was based on rain fed system. Single cropping is the most popularly practiced production system. Farmers growing *Chinsaga* are the main actors in the supply chain. They contribute most of the value chain functions. These include ploughing, planting, soil fertilization, weeding, pest and disease controlling, harvesting and post harvest handling. The favorable weather conditions make growing *Chinsaga* economical and high yielding.

The farms are divided into about 5 portions and planting done at different times to ensure there was a crop to be harvested at different stages of maturity. Each section would have a harvest done twice a week giving an average of about 10 to 15 sacks. The weight of sacks ranged from 12 Kilograms to 22 Kilograms with the mode about 16 Kilograms. Crops grown on a new farm will

have good production for two cycles and thereafter the land must be left fallow or crops changed to have legumes and revert to grazing land for over six months.

The vegetables are best harvested from the field early in the morning. As the plants produce a sticky gum as the day progresses and this complicates the handling while harvesting. Labourers are paid per sack of the crop harvested by the buyer and the farmer gets money for each sack at a pre-negotiated price depending on the prevailing market prices. After harvesting, the crop is packed in gunny bags with an approximate weight of about 18 Kilograms. The measurement is by visual assessment and by experience.

The market price will vary depending on availability of the vegetable and tends to be lower in rainy season and higher in the dry season. However, the farmers have lower pricing power often getting less than optimal returns for their crop in the markets, **Figure 2.8**. In addition, they bear a portion of the post-harvest losses.



**Figure 2. 8:** Group of farmers waiting for pay by the middle men they supply regularly

Post harvest handling includes sorting, grading, packing, storing, transportation, loading and unloading. This is done by the farmers or by traders or brokers. As was observed in many farms, vegetables are sold at the

farm. In this case the post-harvesting handling is carried out by the traders or broker. Most of the farmers, traders or brokers use sacks and ground outside their residential house. High postharvest losses were observed to occur mainly as a result of improper harvesting, handling, packaging and poor facilities to market. A proportion of the *Chinsaga* gets spoilt before it reaches the market.

#### **2.4.2 Transport of *Chinsaga* from the farms to markets**

It was observed that means of transportation varied widely. Farmers deliver the vegetables to the producers' market in the late morning. Predominately producers use donkeys, **Figure 2.9**, and motorcycles. Over shorter distances porters are used, **Figure 2.10**. Most farmers transported their vegetables to the nearby market centers such as Mosoch. These market centers may be termed producers' markets where farmers negotiate price, traders make purchases and eventually transport most *Chinsaga* to urban markets. Vegetable supply is often spread in heaps on the ground. They are aired by frequently turning the heap prior to repacking in yellow or green polythene bags.

The producers' market are more remotely located compared to the more reknown retailer markets. The retail markets are more accessible in the larger centers along the main roads. The local county government levies fees on *Chinsaga* sale at each level of market, **Figure 2.11**. Those interviewed felt the action of levying charges for the growing and supply of these traditional vegetables was a disincentive by the local government.



**Figure 2. 9:** Typical means of transport using donkey. Note the vegetables being aired in the background prior to repacking.



**Figure 2. 10:** Transport by a porter for those that are nearer the centers or over steep rough terrain.



**Figure 2. 11:** County council staff from Kilgoris negotiating with a farmer over the fee to be charged.



**Figure 2. 12:** Typical producers' center.

### 2.4.3 Collection and assembly of *Chinsaga* from the producers

The collectors or assemblers are the traders in assembly markets who collect vegetables from farmers in village markets and from farms for the purpose of reselling it to wholesalers

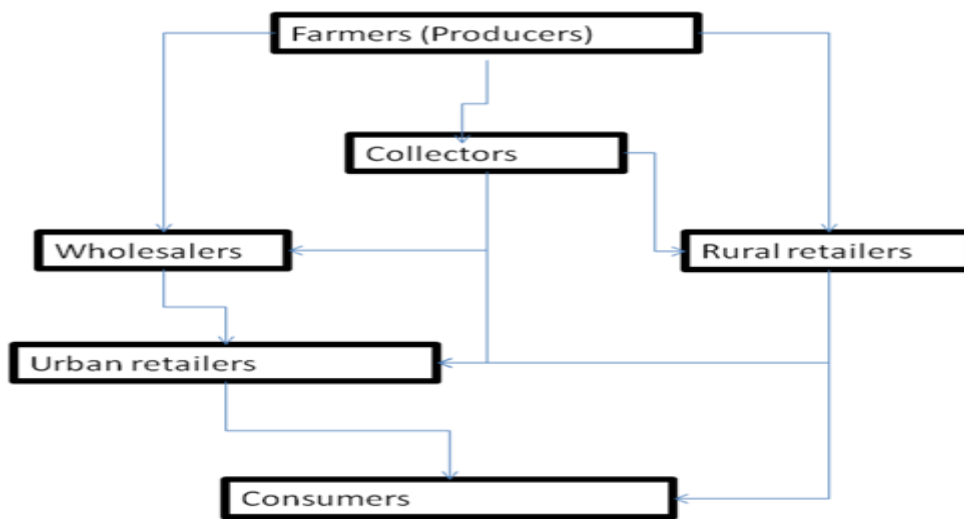
It was observed that once the vegetables are delivered to the center, they are removed from the gunny bag and allowed to air, **Figure 2.12**. This helps to reduce heating and damage that result from compaction after harvesting and during transportation. The aired vegetables are then packed into sacks again or into yellow polythene bags with a capacity of about 10 Kg or into green polythene bags with a typical capacity ranging from 5-6 Kg. The bags are punched with holes to allow for aeration and prevent spoilage due to heating and continued fermentation of the materials.



**Figure 2. 13:** Commercial vehicle hired to transport *Chinsaga* at Mosoch market.

*Chinsaga* is transported to Kilgoris or other towns in Kisii using minivans, **Figure 2.13** from where it is repackaged in smaller bags for onward transport to larger towns like Nairobi. The market channel identified by observation and through interviews gave several alternate pathways, **Figure 2.14**

Producers → Consumers  
 Producers → Retailers → Consumers  
 Producers → Wholesalers → Consumers  
 Producers → Wholesalers → Retailers → Consumers  
 Producers → Collectors → Retailers → Consumers  
 Producers → Collectors → Wholesalers → Consumers  
 Producers → Collectors → Wholesalers → Retailers → Consumers



**Figure 2. 14:** Illustration of the Chinsaga market channel

The proportions of each of these components of the market channel for *Chinsaga* could not be quantified within the context of this study.

#### **2.4.4 Collection and retailing of *Chinsaga***

Collectors and retailers are persons that take the role of collecting and retailing of *Chinsaga* supplied from the producers’ market. When interviewed respondents reported that collectors and retailers are expected to be witty and deeply versed with the knowledge about the producers, their field harvesting capacities and personalities of each of their suppliers. The collector and retailers often apply cunning tactics to maximize on their limited finances to pool together as much supply of *Chinsaga* as possible. They must know their niche including where they can obtain large quantities at lower prices and therefore are important in the *Chinsaga* value chain. They primarily buy, pool, sort, repack, transport and supply the wholesale markets.

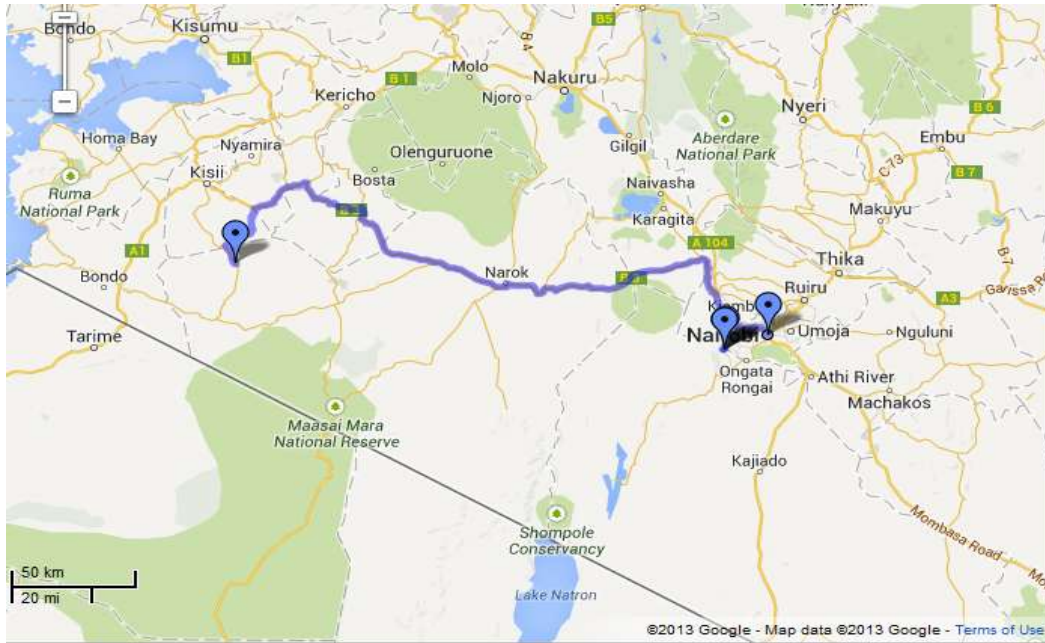
#### 2.4.5 Structure of the supply chain

The brokers and middlemen are important in linking farmers to market and others in the *Chinsaga* supply chain. The brokers were observed to exert power over pricing. They appear to set the price. There were brokers that resided in Kilgoris and others commuted from Kisii. Most of those from Kisii handled the market in urban areas. Brokers in the rural areas are important mediators between the farmers and the urban retailers. Others act on an agency basis on behalf of urban brokers or retailers.

Brokers are divided into village level brokers, urban brokers and commission agents. Brokers at village level enable engagement with producers. They are often focused on getting the best quality in large amounts.

It was observed that there was a small group of traders that bought vegetables from farmers and collectors in larger quantities and could be termed the wholesalers. They link many farmers with retailers and consumers. They had greater capacity for longer storage of the *Chinsaga* to cover the transit duration or in the event of glut in supply. Wholesalers often shoulder a portion of the post harvest losses. The wholesale market is often at Kilgoris and Kisii towns. They are more capitalized to carry out this storage and transportation. Nearly all wholesalers are from Kisii town.

At the time of the field visit to Transmara area, Kilgoris was the major center involved in the supply of the vegetables was determined as Kilgoris town. The vegetables are packed into sacks or polythene bags at Kilgoris town and loaded onto small vans (*Nissans*) and transported to Kisii. Other markets included Magena and Ogembo on the road to Kisii. Once the vegetables reached Kisii, they were repacked into smaller packets of about 4 to 6 kilograms. The packets are then perforated to allow aeration. The packaged material is then loaded onto buses for transport to markets in Nairobi, **Figure 2.15**.



**Figure 2. 15:** Distribution of Chinsaga from Kilgoris/ Kisii to Nairobi.

The packed vegetables often have designated vendors who receive them in Nairobi. Once in the city, the first vendors often wholesale to other vendors that supply the various markets in the city. Others supply clients directly within offices and homes within the environs of Nairobi reaching as far as Kitengela, Ngong, Rongai, Ruai and areas along Thika road.

The increase in demand has helped in establishment of commercial viability and a supply chain that is now almost fully structured. There are fairly well established farmers, middle men and market suppliers from the growing areas in Kisii - Kilgoris areas to its main markets in Nairobi and its environs.

The largest part of postharvest management of the vegetables is carried out by the retailers. They buy and transport the *Chinsaga* to retail points. They also grade, displaying and sell the vegetables to consumers. The retailers represent the final front in the supply as well as value chain. They are important players in maintaining the consistency of demand that is eventually fed by the producers.

Most retailers buy from wholesalers and focus on supply to urban consumers. Some retailers buy *Chinsaga* directly from the farmers but this is less common. Majority of consumers get their vegetables from the urban retailers who carry out some additional sorting and cleaning of the vegetables. This helps to improve the appeal of the vegetables especially by the removal of stiffer stems. Rural retailers often hold much less stock and have lower turnover compared to their urban counterparts.

It was noted that consumers of *Chinsaga* were mainly of three types. The first type was domestic consumers, who bought *Chinsaga* for home use. The domestic consumers were the majority. The second type of consumers purchased for restaurants and other food outlets. Last were institutional consumers such as training institutions and schools. These represented the least mode of consumption.

The majority of clients purchasing the vegetables for restaurants and institutions had established relationships with the vendors. This loyalty was common in the supply chain of the *Chinsaga*. Its role was most often an important component in guaranteeing supply even during the lean months of the year. The relationship was also found to play an important part in protecting the price variations seen seasonally. The regular buyers often got better bargains. A common practice observed was the addition of vegetables to the customers as part of this relationship.

Direct purchases by consumers from wholesalers was not observed but may have happened rarely. Some of the features most sought were consistency of the heap and its cleanliness. The more tender forms were slightly more popular and often got finished faster among the retailers.

The major role of value chain actors is to support the market of the *Chinsaga* keeping a steady supply and moderation of pricing. The value chain actors help

develop the rules of engagement by those in the mainstream trade of *Chinsaga*. It was observed that brokers and retailers played the greatest role in setting the supply and pricing. Much less was observed among the wholesalers. Lack of formal structures may explain the lack of information and lower price setting power observed among the farmers. Horizontal linkages of communication were not clearly in place. Most transactions were cash based indicating low levels of trust among the players. On the other hand, the retailers often faced the risk of high losses if they did not sell all the supply within a short time. This tension between the various market factors often resulted in selling price variations within the day. Vendors tended to offer vegetables at lower prices towards close of the day.

Often the purchasers would bargain to get the lowest price possible leaving the farmers feeling underpriced. The farmers are thus seen as price-takers. In reaction, farmers were observed to include more stems in the heaps as this increased the weight and volume of the sack. The purchasers, on the other hand, desired more leaf hence putting pressure on retailers to cleave off most of the stems as part of the process of enhancing the appeal of the vegetables to the consumers. This inadvertently results in substantial changes in volume and weight of the vegetables with a collated increase in price.

## **2.5 Discussion and Conclusion**

*Chinsaga* in Transmara is grown as both a subsistence and commercial crop. The consumption of *Chinsaga* in urban areas like Nairobi has contributed immensely to the growing commercial demand for the vegetable. Ambrose- Oji has attributed the growth of commercial farming of *G. gynandra* and other traditional vegetables in Africa to expansion of the middle class especially in the urban areas (13). The overall effect in Kenya is the increase in the land utilized for growing the vegetable in Kisii and now on leased land within Kilgoris as observed during the field visits.

The expansion of “supermarket culture” has also driven commercial farming of *Chinsaga*. Ngugi *et al* (14), identifies the integration of traditional vegetable sales in the supermarket as a major driver of increased demand for indigenous vegetables. In this study we determined that more families in rural towns have access to ready packaged traditional vegetables including *Chinsaga*. Further it was noted that vegetable vendors are a key source of fresh vegetables for many homes in the towns. One of the biggest challenges in the trade of the traditional vegetables include requirement for standardization of product for it to be acceptable in the international market. Most growers of crops like *Chinsaga* would not be prepared to meet these requirements and the supply systems may not be financially capable of investing to achieve them. Indirectly, international trade policies may be a barrier to growth of the *Chinsaga* market(15).

Land availability may be another barrier to expansion of commercial farming of *Chinsaga*. Acreage under farming of *Chinsaga* could be another barrier to expansion of commercial farming. In this study, most commercial farmers had half acre dedicated to *Chinsaga* farming. Other studies in Western Kenya show that the mean size of land under indigenous crop per farmer has been continuously decreasing since 1960s(16).

Our interviews revealed that the nature of *Chinsaga* supply chain is complex. Actors in the supply chain included farmers, collectors, wholesalers, retailers and consumers. The typology of the supply chain was however not unique for *Chinsaga* and parallels supply chains described for other East African indigenous vegetables (17).

When inquiries were made to explain the apparent high demand and focus on supply from Kisii and its environs by clients in the city, key factors were noted. Most clients in the city believed that *Chinsaga* from Kisii or Kilgoris were of superior quality to any other that was brought to the market. There was also a strong trust system associated with the supply chain. Clients were often keen

to know the origins of the vegetables they bought. Upon establishing a trust relationship with vendors, many would maintain such relationships as their primary source. Many believed that vegetables from elsewhere grown under different conditions would not have the same benefits. Similar importance of loyalty has been described in Costa Rica for supply of indigenous vegetables (18).

Modern farming has often resulted in monoculture. Traditional vegetables such as *Chinsaga* are often grown along with other crops in mixed cultures. This has the advantage of providing a wider range of crops as sources of food and doubles up as a strategy that conserves the soil by providing cover throughout the year reducing soil erosion (19). Observations made in Kilgoris indicate a trend towards monocultures even for the traditional vegetables on farms that have been prepared almost purely for commercial goals. The pricing for the vegetables has reached sufficient levels stimulating specialization by some of the growers to meet this demand as a mechanism to provide family income.

During the field visits, no use of chemicals was noted. This is a key observation considering the belief among most consumers is that the production of the vegetables in Kisii and its environs is largely “organic”. The vegetables are highly priced as an introductory diet by women shortly after birth in the Kisii culture. They are also widely believed to be the best vegetable for those that are unwell. The use of pesticides and fertilizers will greatly alter this property of purity associated with this source of the vegetable. This may also explain the low preference for use of the same vegetables from other areas of Kenya or the environs of Nairobi in the open market. The members of the Kisii community even in urban areas still preferred to obtain their vegetables from sources they knew. Other studies in Italy showed that consumers are sensitive to the use of chemicals in the production of their foods (20).

Dixon (21) identified seven factors that strongly compel focus on traditional vegetables like *Chinsaga*. Among the seven, include the decline in national food self-sufficiency resulting from a reduction in agricultural extension support especially for peasant farming in favor of commercial models using intensive methods such as green houses. There is also competing needs for the land as families subdivide pieces into smaller sizes hence larger proportions lost to the homestead area. In addition, more land is getting consumed to allow urban infrastructure and real estate development for human settlement.

### **2.5.1 Conclusion**

In conclusion, *Chinsaga* is cultivated both for subsistence and commerce by small holder farmers in Kilgoris. It contributes significantly to the livelihood of families in Kilgoris and parts of Kisii. *Chinsaga* has positive impacts as a nutritional and commercial product for communities in Kisii and Transmara counties, and its demand is increasing in the urban areas. This implies commercial agriculture of *Chinsaga* has good prospects; however declining farm sizes under traditional vegetables may constrain the farming of *Chinsaga*.

### **2.5.2 Recommendations**

We recommend further surveys to establish proportion of producers carrying out sorting and grading of vegetables before they are taken to the market as part of value addition. The survey may help establish the major sources of losses along the supply chain.

During the field study, no clear formal structures of support were observed. It would have been expected that training, extension services, farming and market information, financial services or research should have been made available considering the importance of the vegetable to the Kisii community.

These services would greatly enhance the success of the supply chain for *Chinsaga*.

In addition, we recommend that the national and county government adopt positive planning models that promote production of traditional vegetables such as *Chinsaga*. For example, the charging of levies may need to be addressed if use of traditional foods to combat food insecurity and malnutrition is to be realized.

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## CHAPTER THREE

### 3.0 Phytochemical and chromatographic fingerprint of *Gynandropsis gynandra*

### 3.1 Introduction

In preparation for the clinical trial, a method to characterize the *Gynandropsis gynandra* (*Chinsaga*) sourced from Kilgoris area was required. Thin layer and high performance chromatographic methods were explored for the advantage of being versatile in quality assurance of the study material and other studies such as pharmacokinetic assays of *Chinsaga*.

Botanicals such as *Chinsaga* contain complex mixtures of natural compounds called secondary metabolites. In characterizing natural extracts from plant sources, analysts have to determine a method for separating and identifying constituent parts of the mixture of compounds. During this process, compounds unique to the plant may be identified and these will often be used to develop a chromatographic profile that can help to distinguish the plant from others. This is often referred to as the “fingerprint” of the specific plant (1).

The technique of chromatography is often exploited for separation, isolation and identification procedures. Components of the plant are extracted and run through chromatographic separation under standardized conditions. The results of a run remain fairly consistent and can therefore be used to identify *Chinsaga* and other plants.

A simple technique such as Thin Layer Chromatography (TLC) with a variety of modifications can be sensitive and reproducible. One example is the use of High Performance TLC to confirm identity of *Actea* species such as *A. racemosa* L. native to North America from Asian *A. cimicifuga*, *A. simplex* and *A. dahurica* (2) using cimifugin. All the Asian species contain cimifugin not present in the North American “authentic” *A. racemosa*. (3). In instances, such methods may have an additional visual characteristic such as the blue fluorescent zone in the TLC profile of cimifugin “imitations” of black cohosh (4). (5).

TLC also has additional applications in current Good Manufacturing Practices (cGMP) regulations for botanicals in which identity testing for raw materials is required as a safety precaution. Chromatographic profiling may also distinguish closely related herbs. Plants are classified primarily along family, genus and species. Each family of plants is further subdivided into genera (plural of genus). Members of a genus (species) share many anatomical and phytochemical characteristics differentiating them from other genera in the same family. The phytochemical components present in a plant can be used to characterise plants used as herbal medicines.

Phytochemical fingerprinting has been demonstrated to be a powerful technique for the quality control of herbal medicines (6). A phytochemical fingerprint is a unique pattern that indicates the presence of multiple chemical markers within a sample. The quantity of a chemical marker can be an indicator of the quality of a herbal medicine.

The European Medicines Agency (EMA) defines chemical markers as chemically defined constituents or groups of constituents of a herbal medicinal product which are of interest for quality control purposes regardless whether they possess any therapeutic activity (6). Ideally, chemical markers should be unique components that contribute to the therapeutic effects of a herbal medicine. As only a small number of chemical compounds have clear pharmacological actions, other chemical components are also used as markers (7).

The EMA categorizes chemical markers into analytical markers and active markers. Analytical markers are the constituents or groups of constituents that serve solely for analytical purposes, whereas active markers are the constituents or groups of constituents that contribute to therapeutic activities.

There are other classifications of chemical markers. For example, Srinivasan (8) proposed the following four categories: active principles, active markers, analytical markers and negative markers. Active principles possess known clinical activities; active markers contribute to clinical efficacy; analytical markers have no clinical or pharmacological activities; and negative markers demonstrate allergenic or toxic properties. All markers may contribute to the evaluation, standardization and safety assessment of herbal medicines.

Visualization in Thin Layer chromatography (TLC) is simplified for colored components such as flavanoids and carotenoids but other components require additional techniques. Chromatographic results (fingerprint profile) can provide a powerful tool for monitoring plant extracts (9).

### **3.2 Objectives**

The main objective of this study was to develop a fingerprintTLC and HPLC chromatogram of *Gynandropsis gynandra* which can be used for qualitative and quantitative purposes.

The specific objectives were to:

1. Develop a method of extraction of *Chinsaga*
2. Develop a TLC fingerprint of *Chinsaga*
3. Develop a finger print HPLC profile of the extract of *Chinsaga*
4. Compare the HPLC profile of *Chinsaga* at various stages of maturity.

### 3.2.1 Review of the phytochemistry of *Chinsaga*

Several compounds have been isolated from *Chinsaga* (*Gynandropsis gynandra*). These include chromones, flavones, flavonols, lipids, oxazolidines, steroids, sulfur compounds and triterpenes. **Table 3.1** is a summary of the chemical components obtained from a search on the NAPRALERT database which can be found at: <http://napralert.org/>.

**Table 3 1:** Compounds isolated from *Gynandropsis gynandra* (Capparidaceae)

Compound	Compound Class	Reference
Cleomin	Oxazolidine	(10)
Sitosterol,Beta:	Steroid	(11)
Chromone,5-7-Dihydroxy	Chromone	(12)
Luteolin	Flavone	(13)
Centaureidin	Flavonol	(13)
Flavone,5-Hydroxy-3-4'-7-Trimethoxy	Flavonol	(13)
Kaempferol	Flavonol	(13)
Kaempferol-3-O-Beta-D-Glucosyl-		(13)
Glucoside	Flavonol	
Myricitrin	Flavonol	(13)
Quercitrin	Flavonol	(13)
Rutin	Flavonol	(13)
Glucobrassicin	Sulphur compounds	(14)
Glucobrassicin,Neo	Sulphur compounds	(14)
Glucocapparin	Sulphur compounds	(14)
Glucoiberin	Sulphur compounds	(14)
Thiocyanate,Iso: Methyl	Sulphur compounds	(14)
Amyrin,Beta	Triterpenes	(15)
Amyrin,Alpha	Triterpenes	(15)
Cleogynol	Triterpenes	(15)
Lupeol	Triterpenes	(15)
Taraxasterol	Triterpenes	(15)

Nine lipids have been isolated from *Chinsaga* including; Arachidic Acid, Capric Acid, Lauric Acid, Linoleic Acid, Myristic Acid, Oleic Acid, Palmitic Acid, Palmitoleic Acid and , Stearic Acid (16).

### **3.3 Materials and Methods**

#### **3.3.1 Procurement and preparation of the *Chinsaga* and Kale**

Approximately 660 Kg (80 bags) of *Chinsaga* was bought in June 2009 from the commercial centers located in the farming areas of Kilgoris, about 230 Km west of Nairobi City. *Chinsaga* was transported to Kisii High School where the vegetables were sorted out to remove extraneous materials and excess stems after which they were washed to remove soil and dust. The washed vegetable was allowed to drip to reduce wetness before loading into the large cooking pots. The vegetable was steamed without addition of water for one hour with regular checking to avoid charring. Charring adversely affects the flavor of the whole cooking rendering the vegetable bitter and unpalatable.

After steaming, the material was then transported in pots to Nyankoba tea factory 23 Km from Kisii for spreading and drying on beds. In the tea factory, the vegetables were blown with warm air and turned regularly till they attained near crisp dryness. This pre-drying stage was critical to preserve the material during transportation to Nairobi to the School of Pharmacy where it was dried further till completely crisp with an average moisture reduction of about 94% compared to the raw material. The dried material was then packed into well aired porous polyester sacks which had been washed and dried. It was later emptied into and milled using a hammer mill (Makiga, Kariobangi, Kenya) at the School of Pharmacy. The powdered material was packed into a single sack with a total weight of about 62 kilograms.

Kale (*Sukuma wiki*), (1200 kg) for use in the study was purchased from the local market in Kisii. The vegetables were transported to Kisii School kitchen

where they were cooked and partially dried before transportation to the tea factory for further drying. The pre-dried material was also transported to Nairobi and dried till crisp. The dried material was milled and sent for packaging at the same site as the *Chinsaga*.

### **3.3.2 Packaging of the milled dried *Chinsaga* and Kale**

Sachets were identified as the most feasible and reasonably priced packing material. The sachets were also aesthetically appealing and easy to use. The dried milled *Chinsaga* would also receive adequate protection from elements in the environment during an estimated 12 months expected for use in the study when stored at room temperature. Each sachet (**Figure 3.1**) had an average weight of 9 grams equivalent to 180 grams of the cooked wet material.



**Figure 3 1:** Sample Sachet of powdered vegetables

A serving on a regular eating plate averages between 150 to about 250 grams arrived at after making servings of the cooked material on typical plates used to serve food. Packing was done at Bespack Foods Company located in Nairobi and regularly contracted by other food industries to package food products.

### **3.3.3 Extraction and analytical profile using chromatography**

#### **3.3.3.1 Materials**

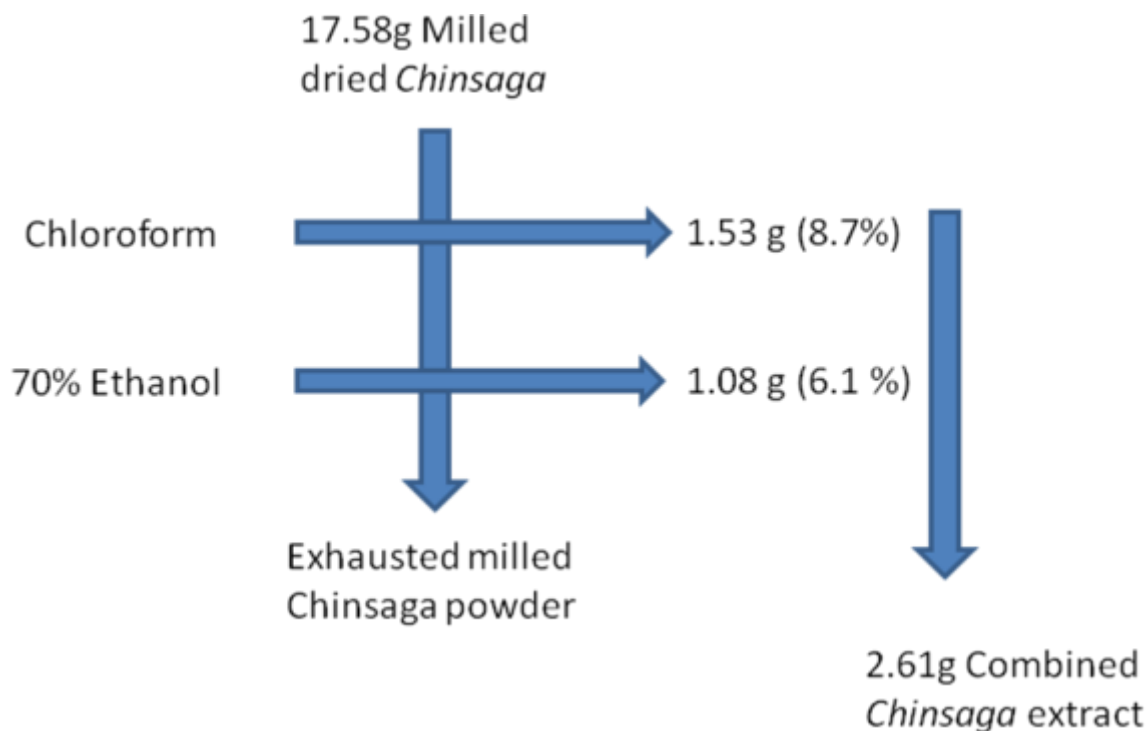
Analytical grade triethanolamine (BDH Laboratory Supplies, Poole, England), anhydrous sodium acetate (Fluka Chemie GmbH, Buchs, Switzerland), ammonium acetate (Loba Chemie PVT. Ltd) and glacial acetic acid (Sigma-Aldrich Laborchemikalein GmbH, Seelze, Germany) were used during method development. Methanol (Fischer Scientific U.K. Ltd.) and acetonitrile (VWR International Ltd., Poole, England) used in preparation of all chromatography mobile phases were of HPLC grade.

All aqueous solutions used in the study were prepared using purified water obtained through reverse osmosis treatment and ultra filtration through successive 0.45  $\mu\text{m}$  and 0.2  $\mu\text{m}$  membrane filters using a combined Arium 61316 RO and Arium 611 VF water system (Sartorius AG, Göttingen, Germany).

#### **3.3.3.2 Extraction of Chinsaga**

Dried ground material was used for development of phytochemical and chromatographic profile of *Chinsaga*. Working standard solution was prepared by accurately weighing 17.58g and sequentially extracting to exhaustion a sample of milled *Chinsaga* with chloroform and 70% ethanol. The milled powder was weighed and loaded into a silk pouch. The pouch was tied to seal the opening and placed in a glass bottle into which solvent for extraction was added. The bottle was then placed in the sonication bath, run for 15 minutes and drained to recover the extract. Divided portions of the fresh solvent was added and the extraction procedure repeated until there was no detectable change in readings for extract obtained as measured using UV at the 220 nm. This procedure was carried out for both solvents. Chloroform and 70% ethanol extracts were combined to give a single *Chinsaga* extract (**Figure 3.2**)

which was reduced to using a rotary evaporator (Buchi Labortechnik AG, Flawil Switzerland)



**Figure 3 2** Extraction of milled dried *Chinsaga*

The thick gummy residue obtained by re-dissolving and combining the chloroform and 70% ethanol extracts (*Chinsaga* extract) was then used to carry out solubility tests.

### 3.3.4 Assessment of solubility

Solubility of the *Chinsaga* extract was tested by applying a drop on a white ceramic plate placed at a slight tilt. A drop of solvent at a time was then applied to a location just above it on the slant and allowed to wash down through the extract spot. An ideal universal solvent for the extract was identified by the extent of wash achieved. In testing for solubility using solvation in a vial, a small amount of the *Chinsaga* extract was loaded at the

bottom of a vial and small amounts of solvent added with intermittent sonication.

Solvent was added in 0.5 ml portions till there was no observable change in the material that remains insoluble at which point it is presumed no further solvation is taking place. Visual assessment was made on the volume of material that remains and this was used to gauge the best solvent of mixture of solvents to use. This was vital to ensure safety even for chromatographic columns

### **3.4 Chromatographic profiling of *Chinsaga* extract**

#### **3.4.1 High Performance Liquid chromatography (LC) instrumentation**

The LC apparatus consisted of a Merck Hitachi LaChrom HPLC System (Hitachi Ltd, Tokyo, Japan) incorporating the following components: a quaternary low pressure gradient pump model L7100, a variable wavelength UV detector model L7400, a variable injection volume autosampler model L7200 supported by Merck-Hitachi Model D-7000 Chromatography data station software - HSM Manager Version 4.1 (Merck KGaA, Darmstadt, Germany and Hitachi Instruments Inc., San Jose, USA). Mobile phase preparations were degassed by Organic modifier and sonication of the general mobile phase preparation using a Sonorex Super RK103H ultrasonic bath (Bandelin Electronic, Berlin, Germany) for 15 min. The variable injection volume auto sampler allowed sample solutions to be injected into the chromatographic system without using a sample loop of fixed volume. This facilitates variation in volume of injected sample without having to replace the loop physically.

The chromatography column was a reversed-phase Phenomenex Gemini-NX<sup>®</sup> C18 column of dimensions 250 mm length and 4.6 mm internal diameter with particle size 5 $\mu$ m and pore size 110Å (Phenomenex, Torrance, California, USA).

The temperature was controlled using a thermostatically controlled Clifton unstirred water bath (Nickel-Electro Ltd., Weston-S-Mare, Somerset, England). Mobile phases were pumped at a constant flow rate of 1.5 mL/min throughout method development. Column back pressures were maintained below 200 bar. The upper limit was set at 250 bar.

The wavelength for use in analysis was established by carrying out a spectral run from 200nm to 700 nm against a neat solution of 70% ethanol for background correction. Regions of  $\lambda$  maxima and  $\lambda$  minima absorption were established.

#### **3.4.2 Working standard solution of *Chinsaga* extract**

A 70% ethanol in water was selected as the solvent of choice. The concentrations of the analyte compounds were calculated in terms of the weight of dry material (g)/total volume of extract (ml). The combined chloroform and 70% ethanol extract (*Chinsaga* extract) constituted the primary standard used to generate other concentrations.

#### **3.4.3 Mobile phase composition**

The different mobile phases prepared during method development comprised varying the proportions of water, acetonitrile, methanol, propan-2-ol and formic acid. In some cases ammonium acetate at different pH values was also tested to determine the effects of pH on separation. Mobile phases were prepared by mixing appropriate volumes of the salts solutions with water before adjusting pH to the desired value using the molar equivalent solution of the parent acid or buffer salt.

The volume of propan-2-ol, methanol or acetonitrile required to yield the desired proportions of the different mobile phase components was achieved

using programming available in the Merck Hitachi HPLC system equipped with a four channel solvent inlet solenoid valve control. All solutions were degassed with an ultra-sonic water bath prior to use (**Table 3.2**).

**Table 3 2:** Solvents used as mobile phase in developing of a fingerprint chromatogram of *Chinsaga*

Component	Composition
A	Acetonitrile 50% in water
B	Methanol 50% in water
C	Formic Acid 0.4% in water

The first mobile phase consisted of a mixture made using A: B: C, (**Table 3.2**), in the ratios 80: 10: 10. (% v/v). Working standard solution (20  $\mu$ L) was injected into the LC system.

## 3.5 Results

### 3.5.1 Extraction of cooked *Chinsaga*

Dried milled material weighing 17.58g was sequentially extracted to exhaustion using chloroform and 70% ethanol. Extraction with chloroform yielded 1.53 g (8.7%) while 70% Ethanol gave 1.08 g (6.1 %). The two were combined to give a single extract

### 3.5.2 Assessment of solubility

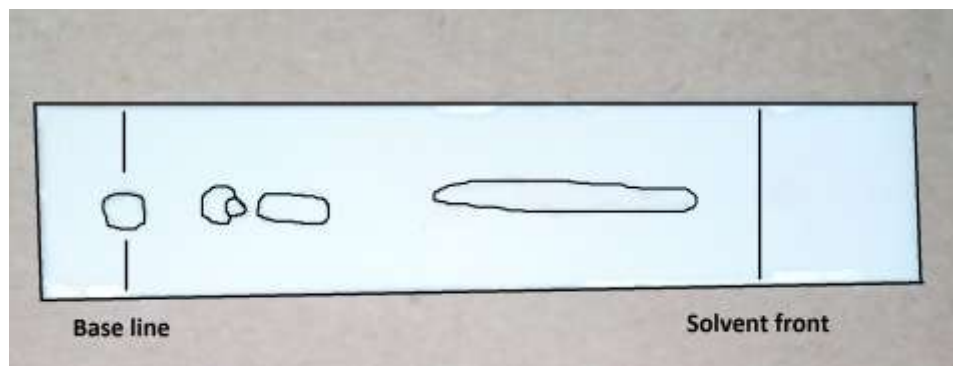
The best solubility for the *Chinsaga* extract was achieved with 70% ethanol and the lowest solubility was seen in ethyl acetate and 50% methanol. Acetone, acetonitrile, acetonitrile with 1-3% formic acid, 50% acetonitrile in water and methanol gave intermediate solubility (**Table 3.3**).

**Table 3 3: Solubility of the water extract of milled Chinsaga**

<b>Solvent</b>	<b>Observations</b>	<b>Conclusion (Solubility)</b>
Acetone	Very good washing away of the green matter and leaves slightly less of the grey mass.	Fair
Acetonitrile	It dissolves small components of the green constituent leaving a green cake like friable matter at the bottom of the vial. It appears to overlap with methanol. The prominent whitish cake like mass is either dissolved or is masked by the green undissolved pigment.	Fair
Acetonitrile 50% in Water	Appears to dissolve most materials well and sends the undissolved matter into a very fine suspension. It dissolves most of the matter.	Fair
Acetonitrile with 1-3% Formic acid	Similar wash to acetonitrile with a brownish residue remaining in suspension.	Fair
Ethanol	Dissolves the green matter and white precipitate at the bottom very well but still has a refractive portion of green near the walls of the vial.	Good
Ethanol 70-80%	Very good solution of all materials. The whole extract is suspended including the white matter and no refractive precipitate is noted on the bottom edges of the vial.	Very good
Ethyl acetate	Good solution of the green color and matter but leaves behind the grey mass similar to methanol.	Poor
Methanol	Dissolves all matter except for a refractive white cake that comes off the bottom of the vial. No further solution occurs even on warming. Addition of equal volume of water (1.0 ml) only serves to make the solution more cloudy but dissolves the white precipitate. Addition of more methanol (0.5 ml) does not change the cloudiness and still leaves a greener color precipitate compared to 70-80% ethanol solution	Fair
Methanol 50%	Poor solution of the materials. Most remains in the residue	Poor
Water	Able to dissolve all the other matter except the green pigment. This remains as a distinct particle that appears unlikely to dissolve despite addition of more solvent. The resultant solution has a pale orange brown color.	Fair

### 3.5.3 TLC profile of *Chinsaga* extract

A solution of the *Chinsaga* extract was spotted onto a normal phase silica coated plate and run using 100% Hexane. No spot was observed in the development of the plate. Using ethyl acetate, a single red colored spot at an Rf of 0.8 visible at UV 320 nm was observed. A mixture of ethyl acetate: methanol: water in the ratios of 50:20:10, yielded the highest separation visible to the naked eye and UV light (**Figure 3.3**).



**Figure 3 3:** TLC of the 70% ethanol extract developed on normal phase silica plate using ethyl acetate: methanol: water in the ratio of 50:20:10.

### 3.5.4 Spectral analysis of ethanolic solution of *Chinsaga* extract

An absorbance  $\lambda$  maxima was identified at about 255nm and was used in method development.

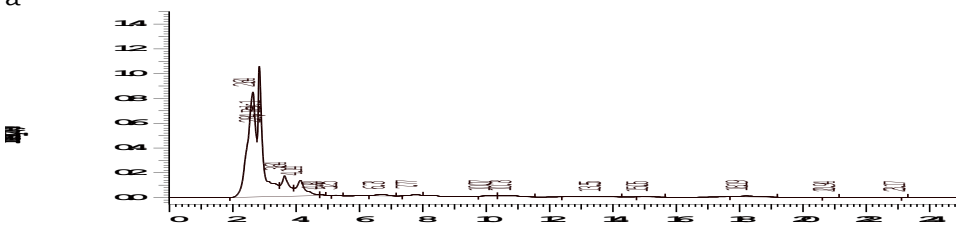
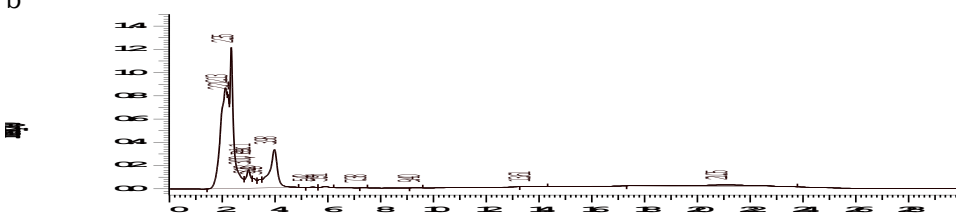
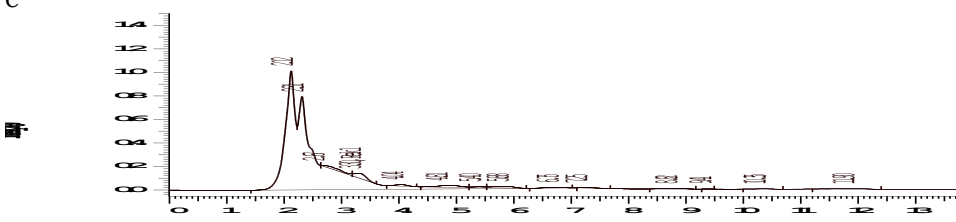
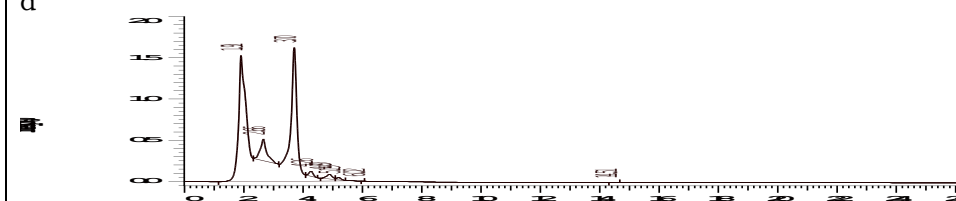
## 3.6 Optimization of fingerprint HPLC fingerprint chromatogram

### 3.6.1 Effects of organic modifiers on separation of components

Using the mixture of acetonitrile 50% in water (A), methanol 50% in water (B), formic Acid 0.4% in water (C), here referred to as A: B: C (**Table 3.2**) in the ratios 80: 10: 10. (% v/v) as mobile phase, a working standard solution (20  $\mu$ L) was injected into the LC system. Very poor separation of the analyte compounds was achieved (**Figure 3.4**), with no distinct peak and almost all components eluting close to the solvent front.

In the next step, the proportion of the organic phases was reduced to give a composition (A:B:C) of 75:15:10. In this run, there was partial separation of some of the peaks from the solvent front. There appeared to be first four probable bands as indicated in the **Figure 3.4** showing the effects of reducing the amount of acetonitrile and increasing the ratio of methanol. The ratio A:B:C of 65:30:5 % v/v resulted in greater tailing of components probably part of the solvent front. The four peaks initially separated were reduced to two. It was not clear if they merged or eluted among the wash out in the tailing from the solvent front **Figure 3.4**. The addition of methanol was seen to cause merging of the peaks and increase tailing.

To confirm this, the ratio of methanol was reduced and that of acetonitrile increased, **Figure 3.4d**, to give a mixture in the ratio A: B: C of 40:20: 40 (% v/v). The result was clearer separation of the first three peaks within four minutes but there appeared tailing effect persistent till the end of the first few minutes overlapping with about two other peaks eluting after four minutes **Figure 3.4(d)**. Initial adjustments in components of the mobile phase targeted only variation of the organic modifiers.

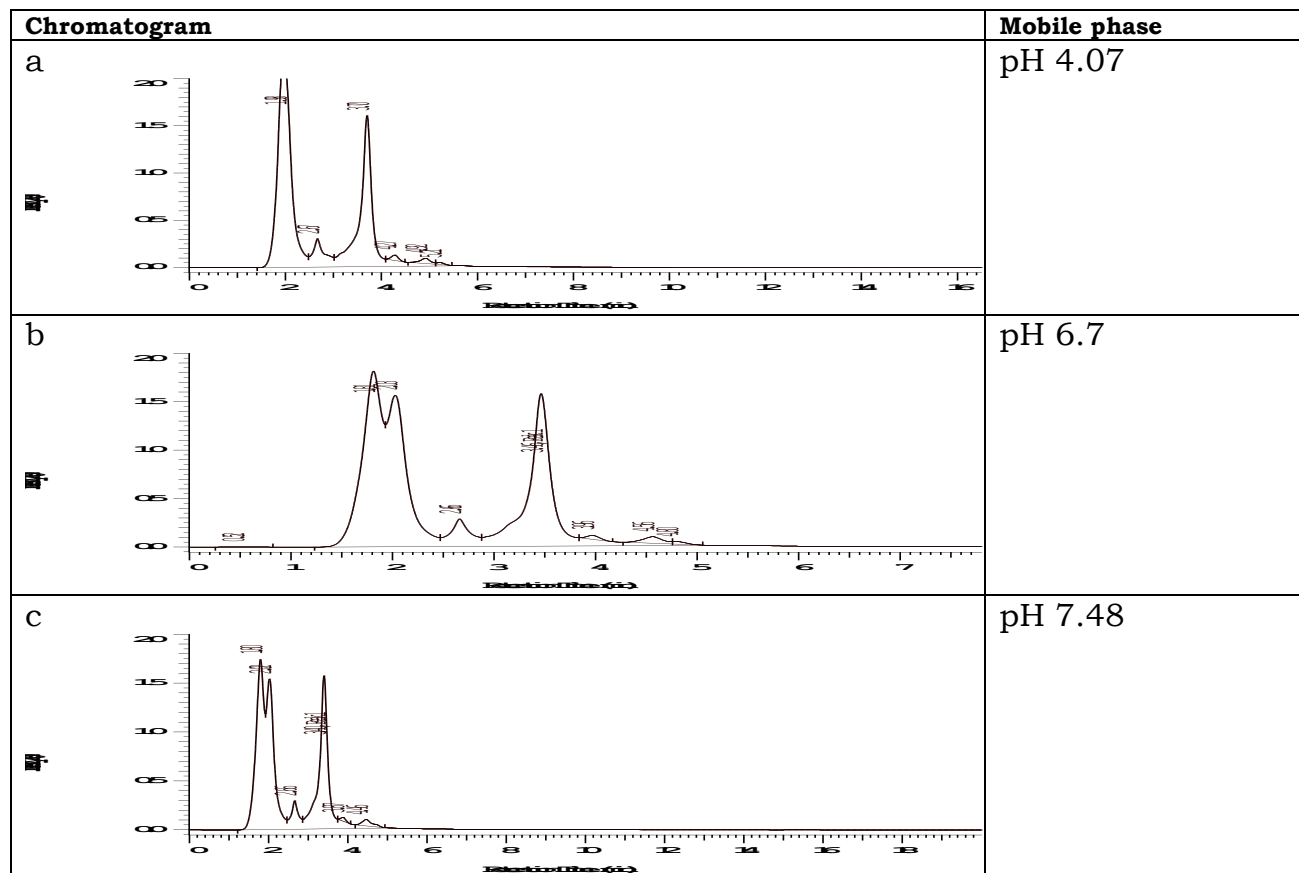
Chromatogram	Mobile phase	
a 	Ratio of mobile phase(A:B:C) 80: 10: 10	
b 	75:15:10	
c 	65:30:5	
d 	40:20:40	Ratio of (A:B:C) 40:20 : 40

**Figure 3 4:** Effect of methanol on separation of ethanolic extract of Chinsaga

### 3.6.2 Effects of pH on separation

The pH of the solution was also changed in the subsequent injections (series), **Figure 3.5**, by addition of ammonium acetate to a pH of 4.07. This had very little effect in the chromatogram but may have slightly reduced the extent of tailing of the components from the solvent front as indicated in the chromatogram **Figure 3.5(a)** using a Phenomenex Gemini-NX 5  $\mu$ m Column, temperature of 40 °C, Mobile phase components: A: methanol (50%), B: acetonitrile (50%), C: formic acid (0.4%) adjusted to pH 4.07 using 0.02 mM

ammonium acetate. Mixture made of the ratio of A: B: C of 80: 10: 10 (% v/v). Flow rate: 1.20 mL/min. Detection: 255 nm. Injection volume: 10  $\mu$ L.

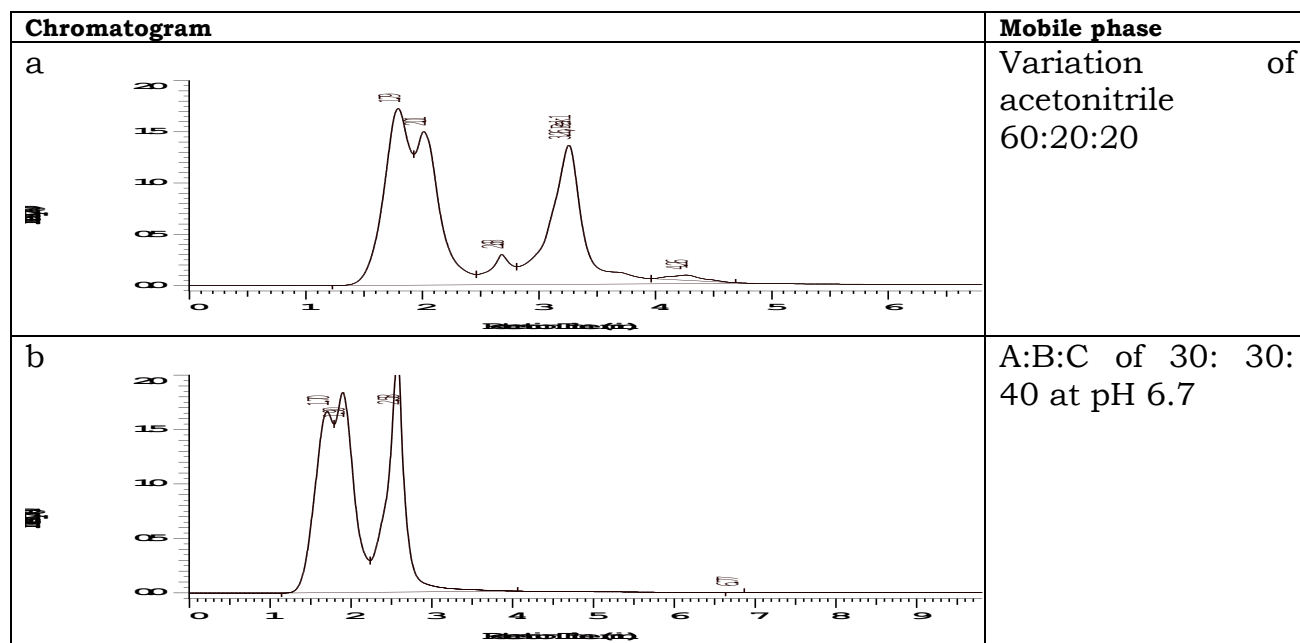


**Figure 3 5:** Effect of pH on separation of 70% ethanol extract of Chinsaga

The pH of the solution was further raised to 6.7, **Figure 3.5** and **Figure 3.6**, using a solution of 0.02 M ammonium acetate. There appeared to a greater reduction in the tailing effect observed earlier and the peaks had a better separation profile as shown in the chromatogram (a). Further work was done replacing formic acid 0.4% solution with 20mM ammonium acetate solution adjusted to a pH of 6.7. The changes observed were not significant as indicated in the **Figure 3.5** (b).

The pH was finally raised to 7.48 and the chromatogram obtained showed only a slight reduction in elution time especially for the third peak but the general

profile was similar to that obtained at pH 6.7. The print out is as indicated in **Figure 3.5 (d)**.

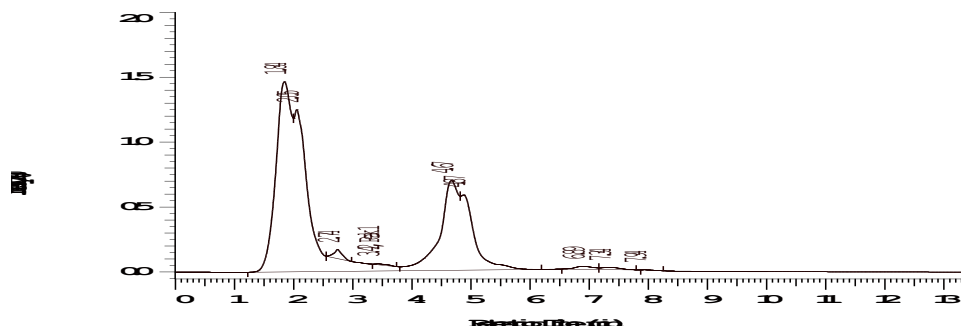


**Figure 3 6:** Effect of variation of acetonitrile on separation of 70% Ethanol extract of Chinsaga at pH 7.48 and replacement of formic acid with ammonium acetate buffer pH 6.7.

With no significant change observed at pH of 7.48, the buffer was adjusted back to pH 6.7 and the component of A (Acetonitrile 50%) increased to 60% reducing that of the buffer 0.02 mM Ammonium acetate to 20%. This resulted in a significant reduction in the amount of tailing observed earlier **Figure 3.6(a)**. The peaks that were eluting after four minutes also appeared to have merged with the first peaks eluting before 3 minutes resulting in a chromatogram of three main components. This shift was thought to suggest that acetonitrile played a key role in the overall elution of components of the extract and could help sharpen the peaks (b).

To confirm these effects, acetonitrile ratio was reduced and that of methanol increased to give a mixture A:B:C of 30: 30: 40 at pH 6.7 and this resulted in a much further separation of the two main peaks. However, it was noted that the

two main peaks each also appeared to consist of two unresolved components as seen in the **Figure 3.7**.



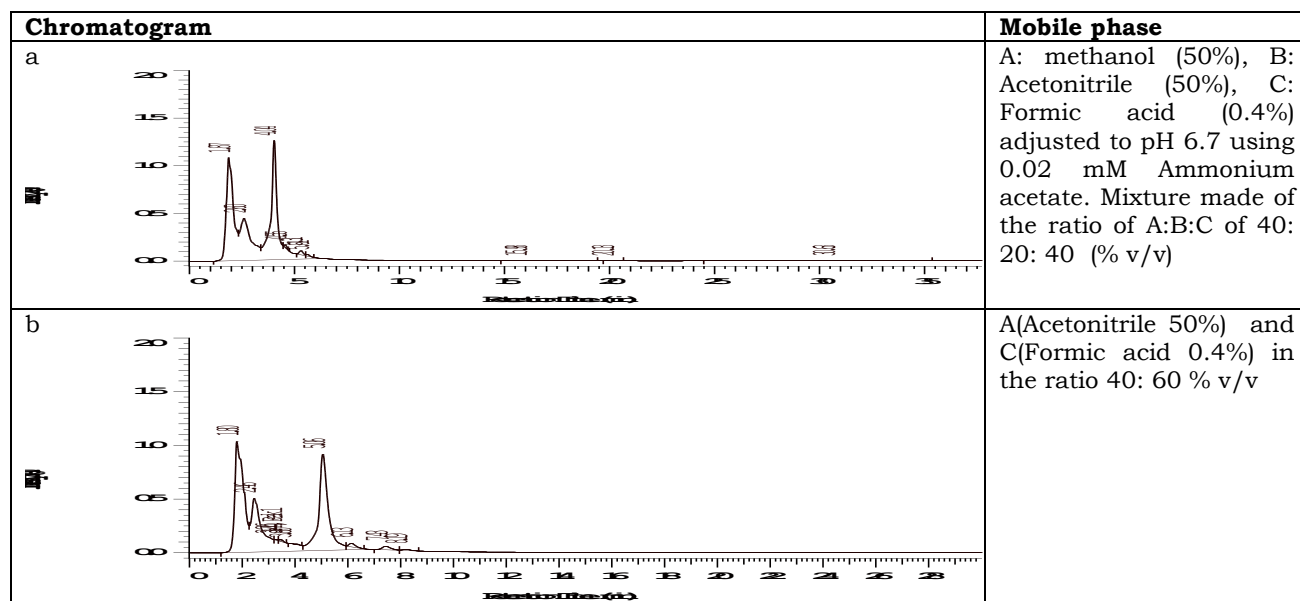
**Figure 3 7:** Effect of increasing methanol to acetonitrile ratio on separation of Chinsaga ethanolic extract.

### 3.6.3 Simplification of mobile phase components

To minimize the complexity in preparation of mobile phase, effort was made to explore the effects of only adjusting organic modifier components while maintaining a simple solution of formic acid. The mobile phase was run with the components: A: methanol (50%), B: acetonitrile (50%), C: formic acid (0.4%) adjusted to pH 6.7 using 0.02 mM ammonium acetate. Mobile phase mixture made of the ratio of A:B:C of 40: 20: 40 (% v/v). Flow rate: 1.20 mL/min. Detection: 255 nm. Injection volume: 10  $\mu$ L, **Figure 3.8 (a)**.

To understand the contribution of methanol, a run was made in which the methanol component was eliminated altogether giving a mixture consisting of A (Acetonitrile 50%) and C (Formic acid 0.4%) in the ratio 40: 60 % v/v. The chromatogram obtained, **Figure 3.8 (b)** indicated that there was better peak shape however the tailing effect on the solvent front was seen to have increased.

The second peak was however much sharper and clearer than obtained previously. Following this observation, effort was made to replace methanol in subsequent trials with other organic modifiers to improve the separation.



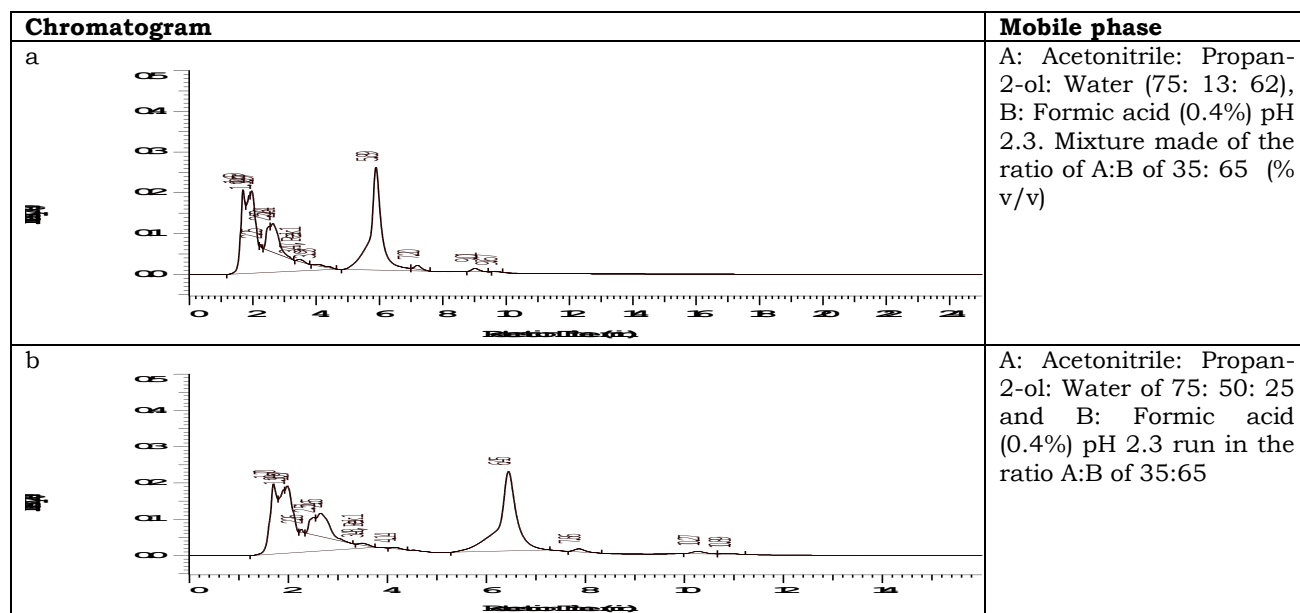
**Figure 3 8:** Effect of acetonitrile and methanol based mobile phase mixtures on separation of ethanolic extract of Chinsaga.

### 3.6.4 Effects of propan-2-ol on separation

A solution of propan-2-ol was added to the mobile phase to give a mixture consisting of acetonitrile: propan-2-ol : water of (11: 1 : 10) as component A and the solution of Formic acid 0.4% was run as B in the ratio (A:B) of 65:35 % v/v. The result was an increased separation of the early peaks as indicated in the chromatogram in **Figure 3.9**.

The addition of propan-2-ol appears to have improved separation of components at the solvent front. To investigate this effect further, the composition of A was varied to from A: acetonitrile: propan-2-ol: water (75: 13: 62), B: formic acid (0.4%) pH 2.3. Mixture made of the ratio of A:B of 35: 65 (% v/v) which was a reversal of the previous ratio. The result showed an improvement in the separation of early components in the chromatogram (a).

A further increase in the composition of propan-2-ol in the mobile phase was made to give a mixture in A: acetonitrile: propan-2-ol: water of 75: 50: 25 and B: formic acid (0.4%) pH 2.3 run in the ratio A:B of 35:65.



**Figure 3 9:** Effect of propan-2-ol of separation of ethanolic extract of *Chinsaga*

The result showed an increased merging of the solutes and loss of the initial separation. It suggested that increase in propan-2-ol in the mobile phase would reduce the separation of the components as seen in chromatogram (b).

### 3.6.5 Effect of gradient elution on separation

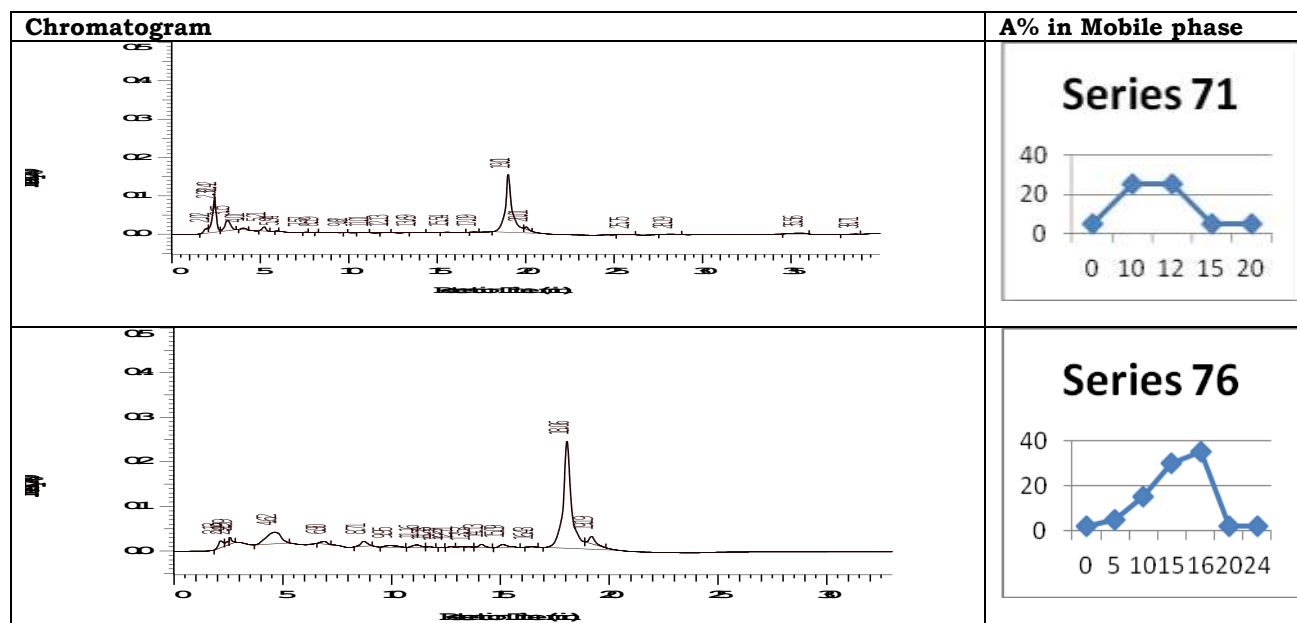
The change in elution time and improved resolution in the early components suggested that effort could be made to explore using a gradient approach to separate elements eluting below five minutes. A mobile phase consisting of a gradient was run as shown in the **Table 3.10**.

**Table 3.4** is a summary of the mobile phase program used in the development of the chromatographic method for the *Chinsaga* extract on column: Phenomenex Gemini-NX 5  $\mu$ m. Column temperature: 40  $^{\circ}$ C. Mobile phase components: A: Acetonitrile: Propan-2-ol: Water (70:50:25), B: Formic acid (0.4%) pH 2.3. Mixture made of the ratio of A:B of 35: 65 then 20:80 (% v/v). Flow rate: 1.20 mL/min. Detection: 255 nm. Injection volume: 10  $\mu$ L.

**Table 3 4:** Mobile phase program used for components: A: Acetonitrile: Propan-2-ol: Water (70:50:25) in the development of a chromatographic profile of Chinsaga extract.

Injection (Series) 71		Injection (Series) 76	
Time	A (%)	Time	A (%)
0	5	0	2
10	25	5	5
12	25	10	15
15	5	15	30
20	5	16	35
		20	2

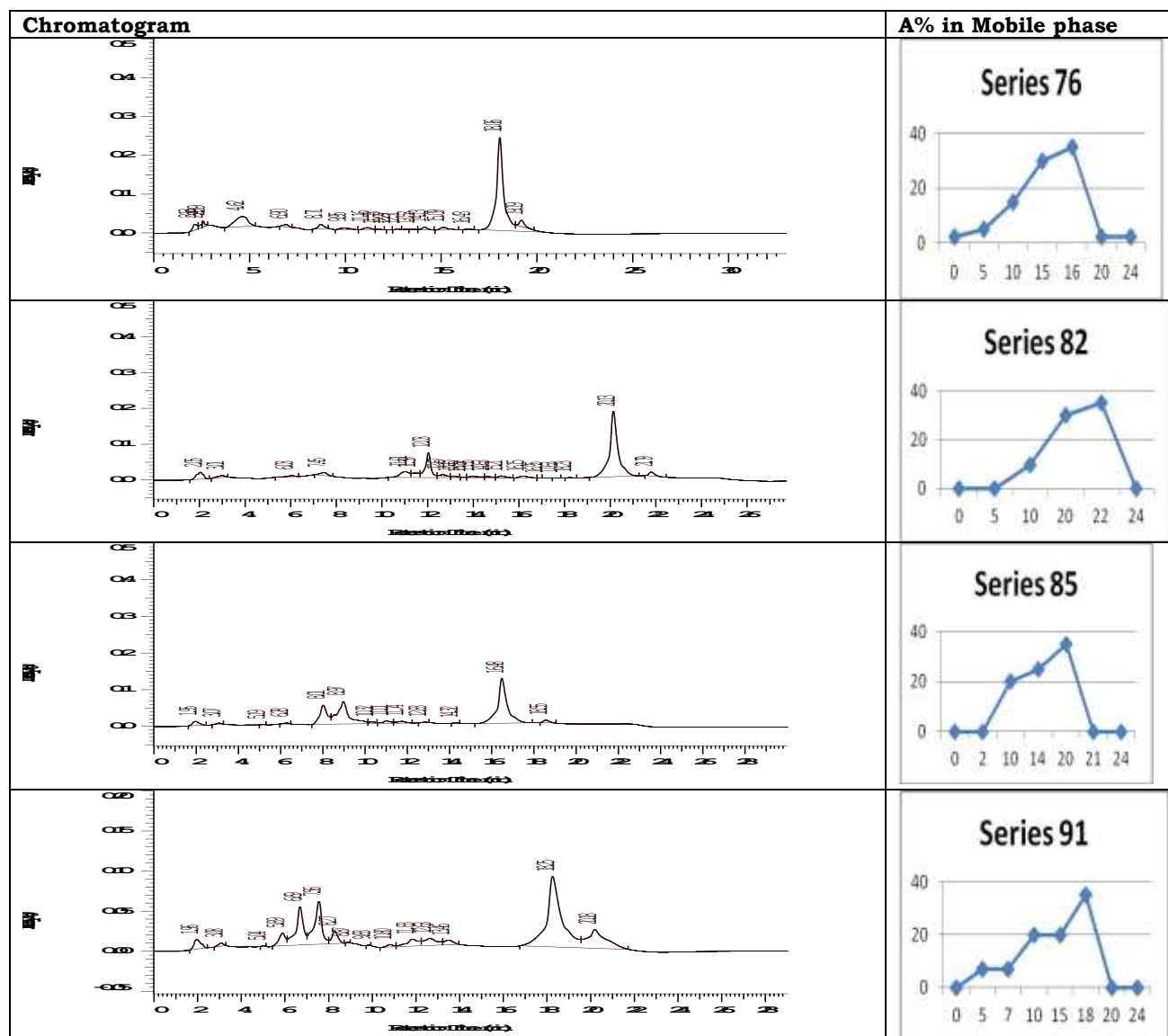
The chromatographic outcome was as shown in **Figure 3.10**.



**Figure 3 10:** Effect of gradient elution on separation of ethanolic extract of Chinsaga. Series refers to the electronic serial record of the injection of the sample.

There was improved resolution in the components of the extract and this was the first time components were not concentrated at the solvent front. This was the most important phase in the development of the method and gave indication that good resolution could be achieved by using a gradient of

solvents in the method. Subsequent efforts were made to refine it. Some of the tested alterations were as indicated in 3.11.

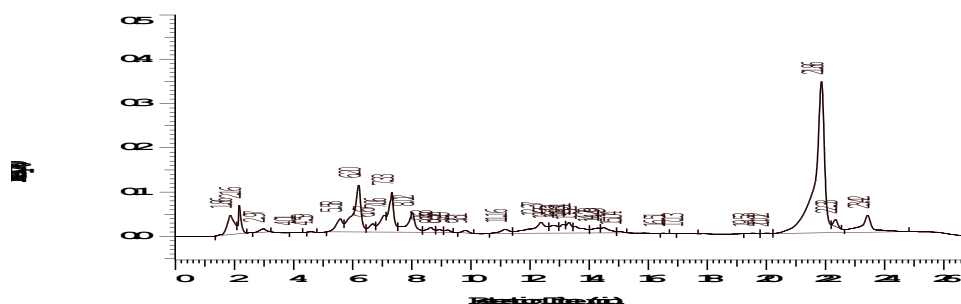


**Figure 3 11:** Effect of gradient program variation on separation of components of ethanolic extract of Chinsaga.

After maximizing the separation of components under the fixed ratio of 70:50:25, slight variations in this composition were introduced and effects analysed. In the first instance, the ratio of acetonitrile was increased from 70 to 75 in method series 101. The chromatogram appeared to have better peak sharpness (**Figure 3.12**). Components eluting between 5 to 10 minutes

appeared more clearly separated and an area between 12 and 16 minutes had a set of poorly separated compounds. However, there was some distinction achieved for compound close to the peaks eluting just after 21 minutes.

Method series 101

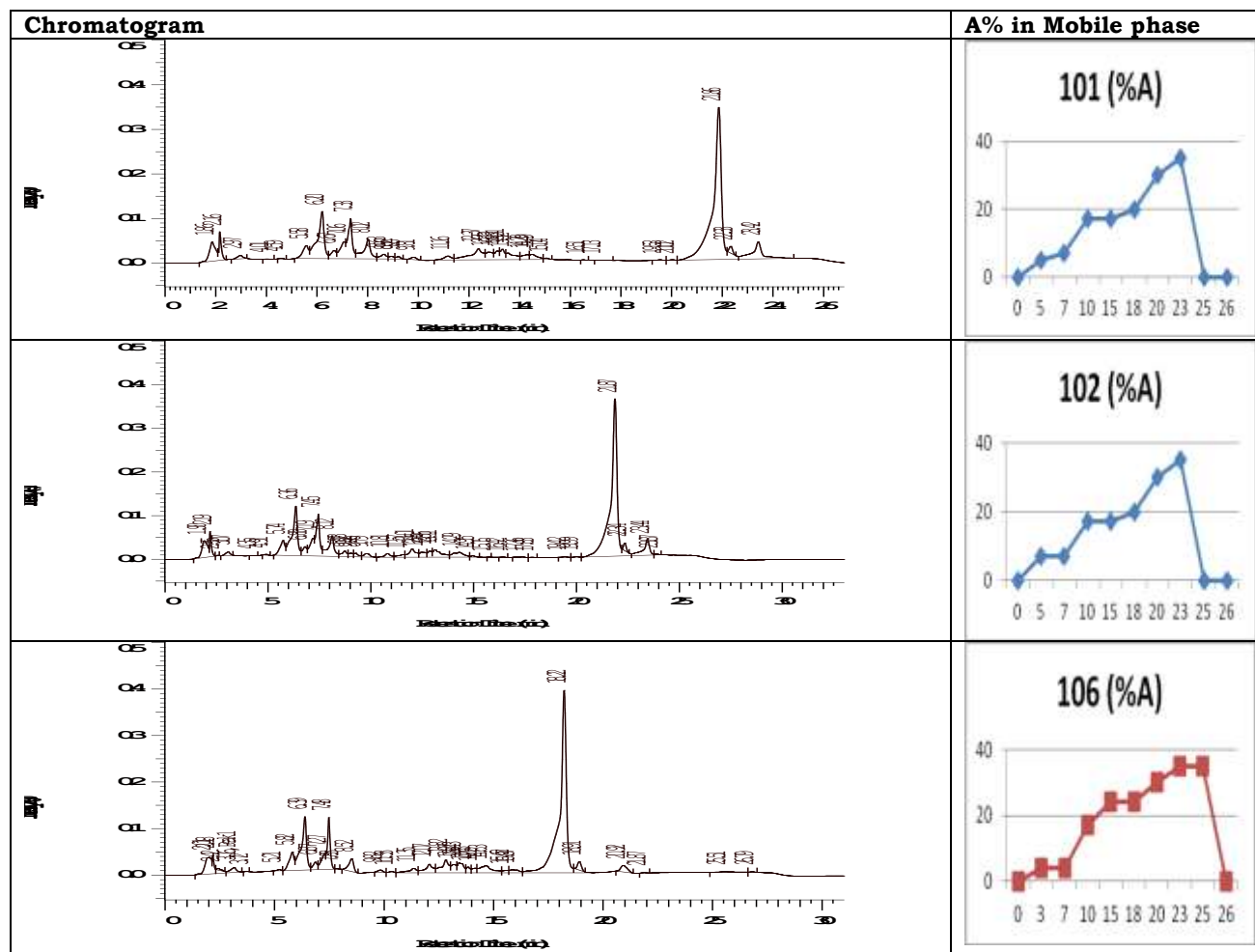


**Figure 3 12:** *Optimized separation of components of ethanolic extract of Chinsaga.*

Mobile phase: A: Acetonitrile: Propan-2-ol: Water (75:50:25), B: Formic acid (0.4%) pH 2.3. Mixture made of the ratio of A:B of 35: 65 then 20:80 (% v/v). Flow rate: 1.20 mL/min. Detection: 254nm.

### **3.6.6 Improving peak sharpness**

To increase peak sharpness, an addition of acetonitrile and reduction in propan-2-ol was made from 75:50:25 (101) to 85:35:25 (102) and the effects were as shown in **Figure 3.13**. The mobile phase components were: A: acetonitrile: propan-2-ol: water (70:50:25), B: formic acid (0.4%) pH 2.3 with a mixture made of the ratio of A:B of 35: 65 then 20:80 (% v/v) and a flow rate of 1.2ml/min.



**Figure 3 13:** Optimization of peak resolution of ethanolic extract of Chinsaga.

The changes in chromatogram series 106 suggested some increased separation of the components between 5 and 10 minutes, better sharpening of peaks between 11 and 16 minutes and some increase in resolution between the key peak at 21 minutes and those that follow at 21.6 and 22.8.

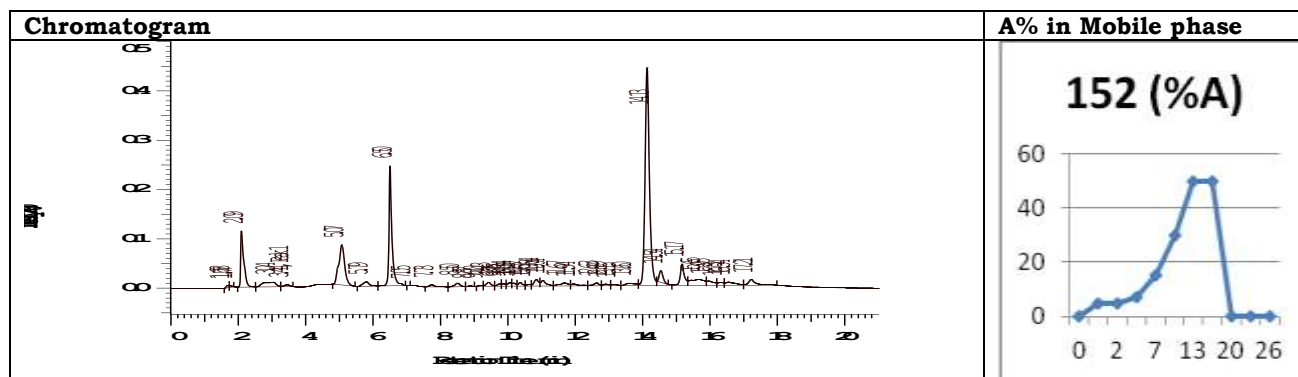
Achieving any further separation would now be very difficult without increasing the run time and application of other detection strategies such as diode array or Mass spectroscopy to help resolve peak purities. Refinement of the method using a newer column and the gradient only needed slight tuning to improve peak resolutions from Chromatogram series 122 to 157 using A: acetonitrile:



### 3.6.7 Chromatography of different stages of *Chinsaga* maturity

The method developed to this point was then applied for use to compare the three stages of the plant (*Omonyenye*, *Amasabore* and *Ekegoko*) and to see if there was any significant difference in the ratios of the resolved peaks.

Extracts were obtained using 70% ethanol in water and run to compare the chromatographic profiles. The results showed no visible differences in pattern under these conditions Column: Phenomenex Gemini-NX 5  $\mu$ m, Column temperature: 40 °C, Mobile phase components: A: acetonitrile: propan-2-ol: water (85:35:25), B: formic acid (0.4%) pH 2.3, Flow rate: 1.20 mL/m). The chromatogram for *Omonyenye*, *Amasabore* and *Ekegoko* was as illustrated in **Figure 3.15**.



**Figure 3 15:** Chromatogram of different stages of *Chinsaga* 70% Ethanol extract.

### 3.7 Discussion and Conclusion

A chromatographic method for HPLC fingerprint analysis was designed, developed and optimized for dried milled *Chinsaga*. The method can be used to identify *Gynandropsis gynandra*. During the processing of the study materials, it was observed that the materials lost averagely 93% weight during the drying step. Other studies suggest 83.3 to 86.6 %. Other components found in *Chinsaga* include 4.8% protein g, 0.4% fat, 5.2% carbohydrate and 1.2 % fiber. Mineral composition was claimed to be Ca 288 mg, P 111 mg, Fe 6.0 mg, ascorbic acid 13 mg (16–18).

During this study, chloroform followed by 70% ethanol in water was established as the optimal solvent system. Investigation of plant materials has been done using different solvents or mixtures to obtain optimal yields. In other instances, serial extraction may be applied from the least polar to the polar solvents and later combined. The goal is to obtain the highest extractive rate of all possible components of interest such as extraction of high-priced compounds from vineyard and wine byproducts from grape seeds and skins (19).

Extraction of phenolic compounds from vine shoots using superheated liquid extraction (SHLE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (USAE) were compared. Use of 80% (v/v) aqueous ethanol at pH 3; 180 °C, and 60 min for SHLE; 140 W and 5 min microwave irradiation for MAE; and 280 W, 50% duty cycle, and 7.5 min extraction for USAE yielded the best values. SHLE reported better extraction efficiencies compared to the other approaches(19).

However, ethanol is not always the most optimal solvent for extraction hence the process of determination of the best mix involving others such as methanol (5). In the case of hesperidin, an abundant bioflavonoid in Pengan (*Citrus*

*reticulata*) peel, with various pharmacological properties was extracted best using ultrasonic extraction with methanol (20).

Chromatographic profile of ethanol extracts has been used for differentiating closely related plant species. *Fabiana* species (Solanaceae family) extracts have long been used in Argentinean traditional medicine as anti-inflammatory, antiseptic, bone fractures and others diseases. Aqueous and ethanolic extracts of four *Fabiana* species (*Fabiana bryoides* Phil., *Fabiana punensis* A.C. Arroyo, *Fabiana densa* J. Remy and *Fabiana patagonica* Speg.) were compared by HPLC. It was observed they had different phytochemical patterns, which also showed different potencies in biological activities when tested (21).

The development of the extraction and chromatographic method was adopted from similar applications used for quality control for plant extracts and applied in differentiating botanical species by High Performance Chromatography (HPLC) (22).

Guided by the solubility observed, 70% ethanol was chosen as the best compared to the other solvents tested. The goal was to ensure that solvents and equipment used would be those that may be readily available in settings where use of the procedure may be adopted as a standard method in quality assurance or quality control technique at a commercial scale (23).

The mobile phase and chromatographic column Phenomenex Gemini-NX 5  $\mu\text{m}$  was selected for use in the development of the HPLC method since it is widely used and available in analytical laboratories. The column has a wide pH stability range between pH 1 to 12. In this column, ethyl bridges are used to connect vicinal free silanol groups improving resilience to chemical challenge at the pH extremes compared to ordinary C18 columns (24).

Given the wide range of chemical components expected in a typical plant extract, it was also ideal for the separation of constituents of the plant and giving sharp peaks (22). Multiple peaks were observed in the chromatogram of the extract of *Chinsaga*.

Similarly, the detection wavelength used in the development process was fixed at 255 nm since at this wavelength most compounds would be visible. Another consideration made in selecting this wavelength was to allow the method developed to be successfully applied using HPLC instrumentation equipped with fixed wavelength UV detectors, typically set at 254 nm (25), without any need for further adjustments if adopted as a national standard method.

The principle in establishing a suitable mixture of solvents for the mobile phase was compatibility to UV and mass spectrometry detectors. This narrowed the options to propan-2-ol, acetonitrile and other volatile buffers based on ammonium or acetate (26). It was also important that they should be fairly easy to obtain locally.

### **3.7.1 Conclusion**

In conclusion, using the solubility profile, a mobile phase composed of acetonitrile, propan-2-ol, water and formic acid was formulated. The separation of extract components showed propan-2-ol was the most suitable combination with acetonitrile improving peak shapes compared to those based on methanol. The extract from the *Chinsaga* used in this study showed poor solubility in pure solvents. A graded series of chloroform followed by 70% ethanol was established as the most optimal method. A solution of 70% Ethanol achieved the best solubility profile for the extract. A finger print HPLC chromatogram the extract of dried milled *Chinsaga* was developed to compare similar materials and serve as a method of determining similarities of other *Chinsaga* samples.

### 3.7.2 Recommendation

The recommendations from this study would include application of the method developed in the assessment of *Chinsaga* as part of the food analysis protocols at the Kenya Bureau of standards. Both the method and fingerprint chromatogram can be refined as a *Chinsaga* standard locally. The method can also be useful in assaying during pharmacokinetic studies of *Chinsaga*.

### 3.7.3 Study Limitations

Identity of peaks will require a mass spectrometer or use of standards along with the analysis. The lack of a MS and the standards limits the identification of the major components in the fingerprint chromatogram.

## 3.8 References

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## CHAPTER FOUR

# 4.0 Baseline Nutrition and Demographic Characteristics of Study Participants in the *Chinsaga* Clinical Trial

## 4.0 Background

In this chapter we report on the demographic data, nutrition practices and breast feeding that may potentially relate to lactational and hematological outcomes among postnatal mothers enrolled into the *Chinsaga* clinical trial at Kenyatta National Hospital.

Worldwide, about 800 women die daily due to pregnancy related causes (1). Nearly all occur in developing countries and more than half are sub-Saharan Africa. The majority of deaths happen around the time of delivery. Recent national surveys in Kenya have estimated that approximately 6500 women die from childbirth-related causes each year (450–500 deaths per 100 000 live births)(1). Factors associated with high maternal mortality are maternal nutritional status, age, parity, education and marital status (2).

The city of Nairobi is faced with inadequate housing, supply of clean and safe water, sanitation and heavy reliance on food supplies from rural areas. Concurrently, reduction in agricultural productivity in areas within and near the city renders about 47% of the population food-insecure (3). Most of such households spend about three-quarters of their income on food. Like in many other cities in Africa, food is usually available but about a third of households may not attain a nutritionally adequate diet due to cost. Anemia and vitamin A deficiencies are prevalent among children and women (3). The value of fruits and vegetables is associated with better health outcomes and reduced risk of diseases.

Frequency of consumption of fresh fruits and vegetables is high in Kenya. Markets serve as the main supply of fresh fruits and vegetables and about three quarters of rural households grow some for sale (4). Access to fresh fruits and vegetables is a global challenge. While WHO/FAO recommend 400g daily, it has not been fully achieved (5). In Nairobi, consumption is highly correlated

with wealth status. Most fruits and vegetables exhibit seasonality in availability. In addition, local markets in rural areas are more restricted in the variety of fruits and vegetables available for purchase. The **Table 4.1** adapted from Ayieko *et al* summarizes some of the fresh fruits and vegetables consumed by households in Nairobi (6).

**Table 4 1:** Weighted Household Purchases of Major Fresh Fruits and Vegetables in Nairobi. Monthly purchases of fruits and vegetables (Adapted from Ayieko *et al* (5).

Item	% of Households purchasing	Mean(Kg)	Average monthly expenditure (Ksh)
Tomatoes	96%	9.8	229
Onions	94%	4.5	114
Sukuma wiki (Kales)	82%	12.7	99
Cabbage	77%	3.8	68
Irish potatoes	77%	22.7	144
Carrots	67%	5.0	61
Cooking bananas	35%	13.3	48
Sweet potatoes	24%	6.7	23
French beans	16%	3.9	13
Average total monthly purchases of vegetables over all households is Ksh 779			
Item	% of Households purchasing	Mean(Kg)	Average monthly expenditure (Ksh)
Bananas	77%	9.1	146
Oranges	74%	4.2	112
Mangoes	53%	5.4	65
Avocado	48%	5.1	45
Pawpaw	40%	10.7	64
Average total monthly purchases of fruits over all households (Ksh) 431			

#### 4.1.1 Breastfeeding and its role in reproductive health

The ability of breastfeeding to delay return of fertility is well known. (7,8). In 1988, the Bellagio Consensus Conference proposed guidelines promoting the use of lactational amenorrhea method (LAM) for family planning. In the practice of LAM, the woman fully or nearly fully breastfeeding remain amenorrhagic the first 6 months postpartum. Cumulative 6-month life-table pregnancy rate as low as 0.45% has been demonstrated among women who

relied on LAM as their only family planning method (one woman pregnant in month 6). Other advantages include high acceptance and efficacy, viability, ease of initiation and safety as a starting method for breastfeeding women (9).

The over 98 % efficacy of LAM has been widely demonstrated and results suggest possible utility beyond 6 months. It is highly effective as an introductory postpartum method in a variety of cultures, health care settings, socioeconomic strata, and industrial and developing country settings (10–12).

Other studies have been carried out to determine correlating factors to success or failure of LAM. In one study maternal nutritional status was assessed to play an independent role in the return of ovulation after delivery (13). Mothers in urban settings may also experience differences in effectiveness of LAM and experience lower efficacy (14).

#### **4.1.2 Main objective**

The purpose of this study was to establish the baseline characteristics of the study participants in relation to; demographic data, behavior related to nutrition and breast feeding in mothers recruited into the *Chinsaga* clinical trial at Kenyatta National Hospital.

#### **4.1.3 Specific Objectives**

Specific objectives were to:

1. Describe the nutritional practices with regard to intake of fruits, vegetables and animal products;
2. Identify foods eaten by lactating mothers that are believed to increase milk output;

3. Measure the prevalence of pica habit and use of herbs that are believed to promote lactation.

#### **4.1.4 Study justification**

It has been observed that many women have beliefs that guide what they can eat during pregnancy and/or breast-feeding. Some are based on folklore associated with eating some types of food. It is still possible that some of the myths may not support improvements in nutritional outcomes.(15). This study was designed to identify poor nutrition practices that may be related to postnatal hematological and lactational outcomes. This will help in the design of interventions to promote good postnatal nutrition.

## **4.2 Materials and Methods**

### **4.2.1 Ethical considerations**

The study was conducted with approval from Kenyatta National Hospital – University of Nairobi Ethics and Research Committee (KNH-UON ERC/01/3757) and the Kenyatta National Hospital clinical services department. Only code numbers were used in the records of the participants. No names and identifiers were used in the files. Informed consent was obtained from all participants and confidentiality maintained by use of codes instead of patients' names. Primary and secondary data was safely stored in a locked cupboard and a password protected computer accessible only to the study investigator.

### **4.2.2 Study Design and location**

This assessment of the baseline characteristics as a cross sectional study carried out as part of the randomized placebo controlled triple blind study. The

study was carried out at Kenyatta National Hospital. The hospital is the largest teaching and referral hospital in Kenya serving mainly women from Nairobi and its environs.

#### **4.2.3 Study duration and time frame**

This study was conducted over a period of four months between August and November 2008 enrolling mothers who met the inclusion criteria.

#### **4.2.4 Participant's inclusion criteria:**

Patients were included if they met the following criteria:

1. Women below 35 years of age and in their seventh to eighth month of current pregnancy attending KNH postnatal clinic
2. Healthy (free from chronic disease with exception of asymptomatic HIV positive mothers)
3. Not smoking
4. Not been on *Chinsaga* 4 weeks prior to enrolment
5. Willing to consent to participate in the study
6. Available for the 28 days follow-up period within Nairobi or its environs.

#### **4.2.5 Participant's exclusion criteria:**

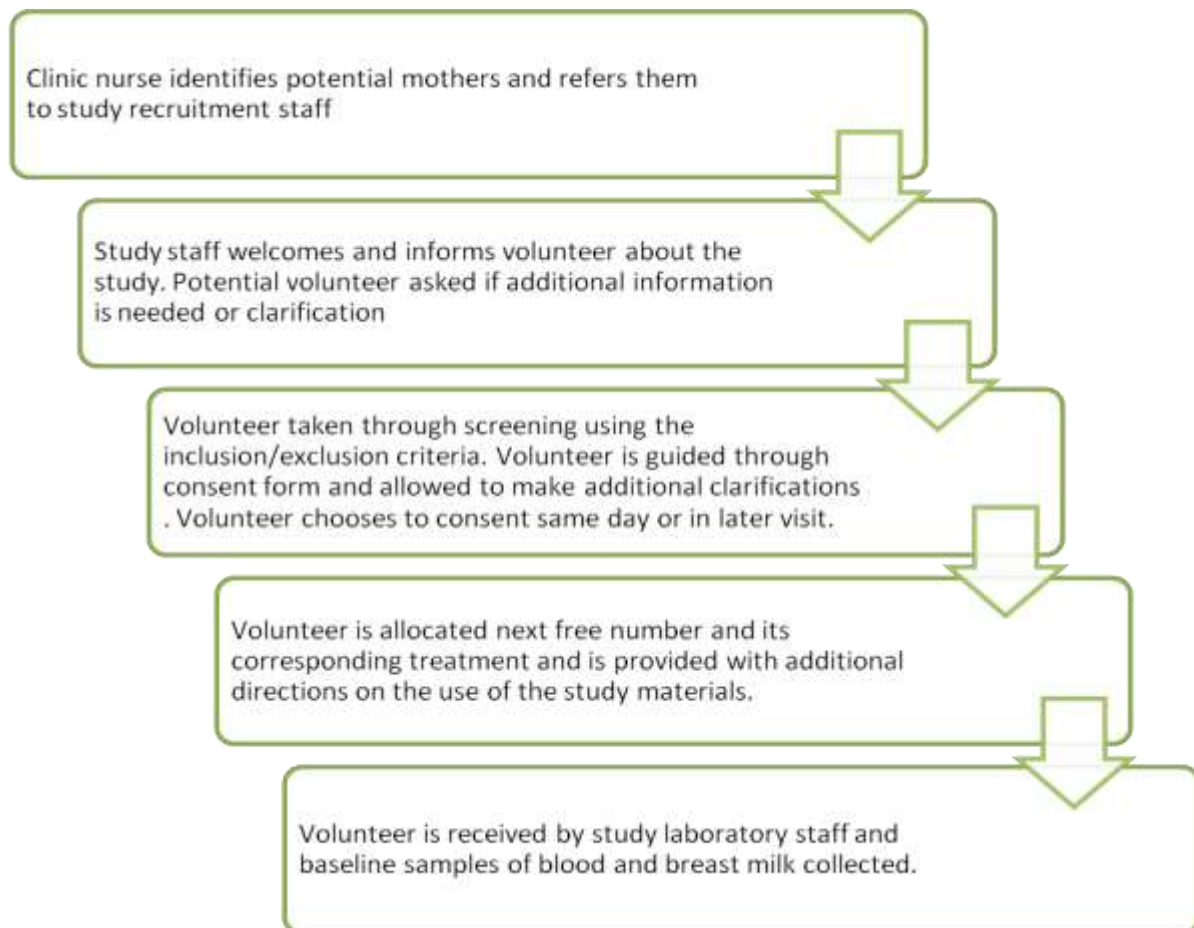
Patients were excluded if they:

1. Were smokers
2. Were instructed not to breastfeed for any reason
3. Had a chronic diseases such as inflammatory diseases (rheumatoid arthritis, Crohn's disease, colitis ulcerosa), diabetes, asthma and hypertension.
4. Used prescription medication or nonsteroidal anti-inflammatory drugs on a regular basis
5. Used of vitamin or non iron mineral supplements for the last 1 month
6. Used corticosteroid treatment and immunostimulators for the last 4 wks.
7. Had hemoglobin concentration below 8g/dL

#### 4.2.6 Sample size considerations

The overall aim was to determine if *Chinsaga* is biologically active or inactive in altering iron metabolism in lactating women shortly after delivery using surrogate makers as endpoints. The baseline characteristics of the participants in the *Chinsaga* clinical trial study involved all 119 mothers as described in this chapter. Effect estimations are described in chapters 5 and 6.

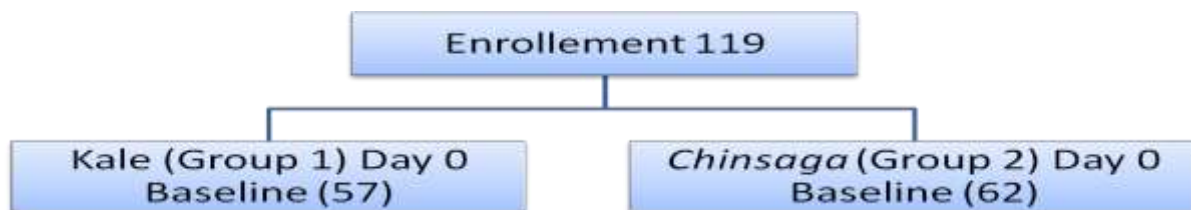
#### 4.2.7 Recruitment procedures



**Figure 4 1:** Flow diagram of recruitment process at Kenyatta National Hospital clinic 17 for *Chinsaga* study.

The **Figure** 4.1 indicates the steps of the recruitment process to enrolment of volunteers at clinic 18 of Kenyatta National Hospital. Mothers who attended

and anticipated delivering at the Maternal Child Health (MCH) clinic 18 were informed about the study by nurse officers at the clinic. The potential volunteers would then be directed to the recruitment officer and asked if they are interested in taking part in the study. Those that accepted to join the study were screened for eligibility by checking through the inclusion and exclusion criteria **Figure 4.2**.



*Figure 4 2 Consort diagram for enrollement of study volunteers into kale (control) and Chinsaga (Treatment) arms at KNH at visit one (days 0).*

Those that qualified for recruitment were adequately informed of the study and again asked if they still wished to join the study. Additionally, they would be asked if any more information was needed for them to make an informed decision to participate. Those that agreed to join the study were required to give informed and documented consent. They were allowed to make this decision at another visit if unable to do so on the first day .

#### **4.2.8 Case definition of Pica habit**

Pica habit was defined as regular consumption of one of the following: stone, soil, soap or any other non-food materials. Poor nutrition practices is consumption of fruits and vegetables less than once a week, meat less than once a week, of foods rich in calcium and low consumption of iron rich foods.

#### **4.2.9 Data collection**

Volunteers were asked to complete a questionnaire which was designed to obtain information on lactation beliefs and nutritional habits (**Appendix 3**). In

addition, information on demographic characteristics such as sex and age were obtained. The volunteers were required to complete the questionnaire in the premises of the clinic where they were encouraged to seek help for sections they did not understand. Since the focus of the study was on food intake rather than portion sizes for nutrient calculations, the food frequency questionnaire (FFQ) was used to obtain information on the type and frequency of foods consumed by study volunteers in the preceding 7 days. Various foods from different food groups were read out to the respondent, who in return was required to state the number of times she had consumed this food within the past week.

#### **4.2.10 Data Management**

All data was entered into an Excel spread sheet within 24 hours of data collection. The data was regularly checked for inconsistencies. When identified, the researchers reviewed patient records and interviewed patients on subsequent visits to reconcile the inconsistencies. The data was backed up every week and copies kept at a separate site. All patient identifying information was kept under lock and key by the principal investigator. All data was password protected.

#### **4.2.11 Data analysis**

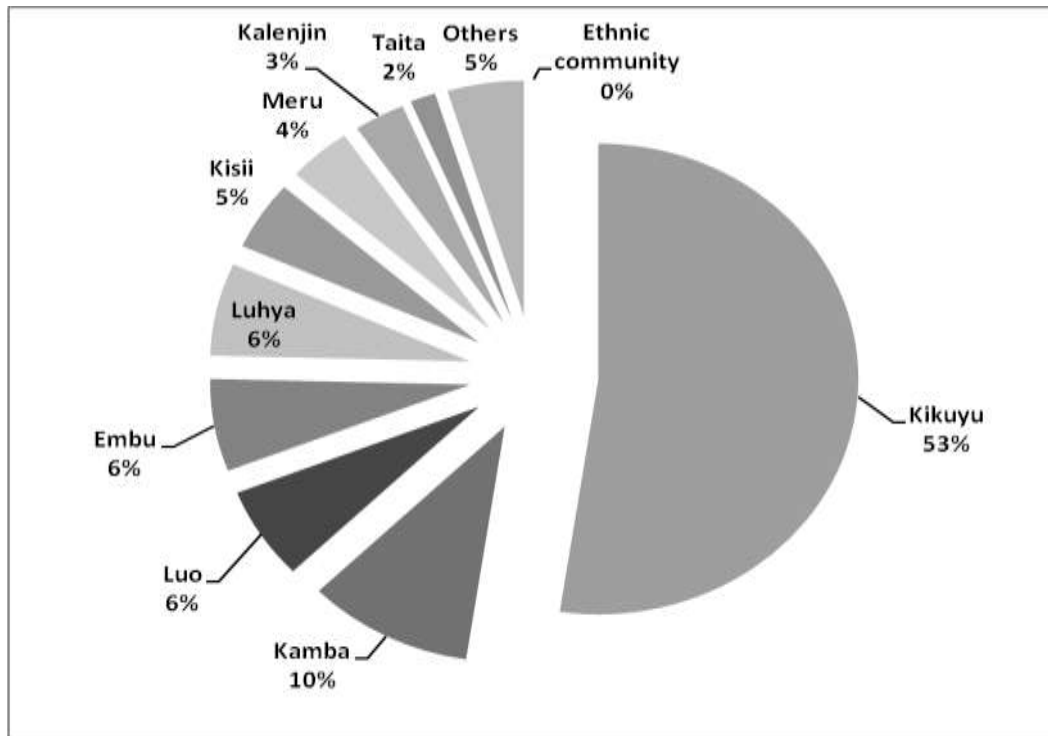
The key outcomes of interest were the pica habit, frequency of breastfeeding, maternal beliefs and poor maternal nutritional practices. Since the study was mainly cross sectional, most of the analysis was restricted to descriptive data analysis. Continuous variables were summarized as the median and inter-quartile range. Categorical variables were summarized as proportions. In addition, exploratory data analysis was carried out to determine if there were significant associations and correlations. The Chi square test was used to test for the distribution of categorical variables across groups. The Kruskal Wallis

and the unpaired t-tests were used to test for the distribution of the continuous variables across groups. P-values of less than 0.05 were considered to be statistically significant.

## **4.3 Results**

### **4.3.1 Baseline Socio demographic profile of the Study participants**

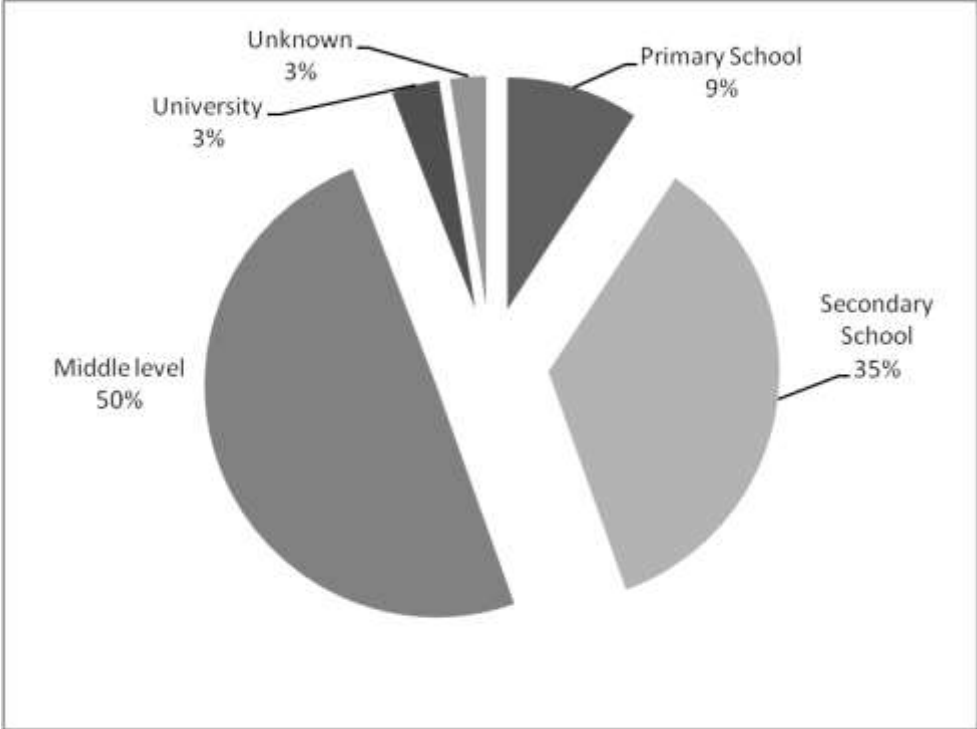
There were a total of 119 female volunteers. They represented 11 of the 42 ethnic communities of Kenya (**Figure** 4.3). The large majority (n=66) of volunteers were from the Kikuyu ethnic community which forms the larger proportion of the city's inhabitants. Others included Kamba (n= 13), Luo (n=8), Embu (n=8), Luhya (n=8), Kisii (n=6), Meru (n=5), Kalenjin (n=4), Taita (n=2) and one each from the Somali, Maasai and Nduruma ethnic groups. Some (n=4) were unclassified. Only two of the 119 volunteers were of Muslim faith and one allocated to each treatment group purely by the randomization process.



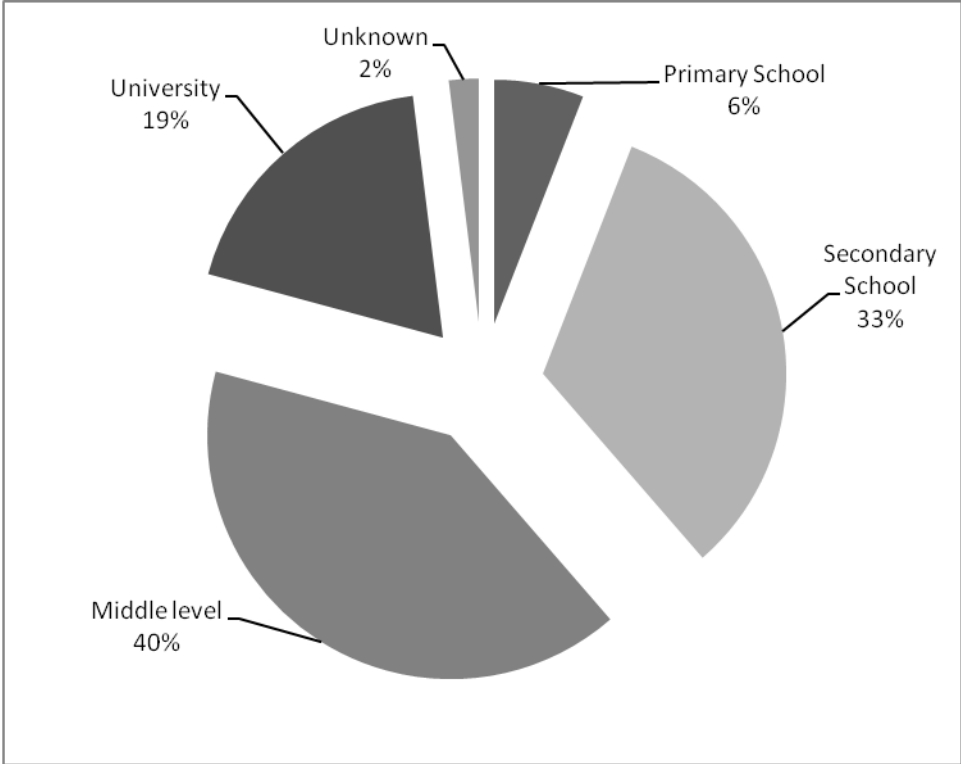
**Figure 4 3:** Ethnic distribution of study participants

The majority of volunteers were married (n=102). A large number (n=50) of women joined the study after their first pregnancy, 36 after the second, 21 after the third and 5 after the fourth pregnancy. Seven of them did not have this data collected. Many (n=48) had lived in their current area of residence for less than three years. This was expected considering that majority were young couples probably still establishing their livelihoods in the city.

In relation to attainment in education (**Figure 4.4**), 59 volunteers had middle level college, 42 secondary, 11 primary school, 4 university education and 3 had not indicated the level of exposure to schooling which tallies closely with the national distribution. In contrast, their husbands had higher attainment in education with more (n=19) at University and middle level college (n=41) in **Figure 4.4**.



**Figure 4 4:** Education profile of study participants

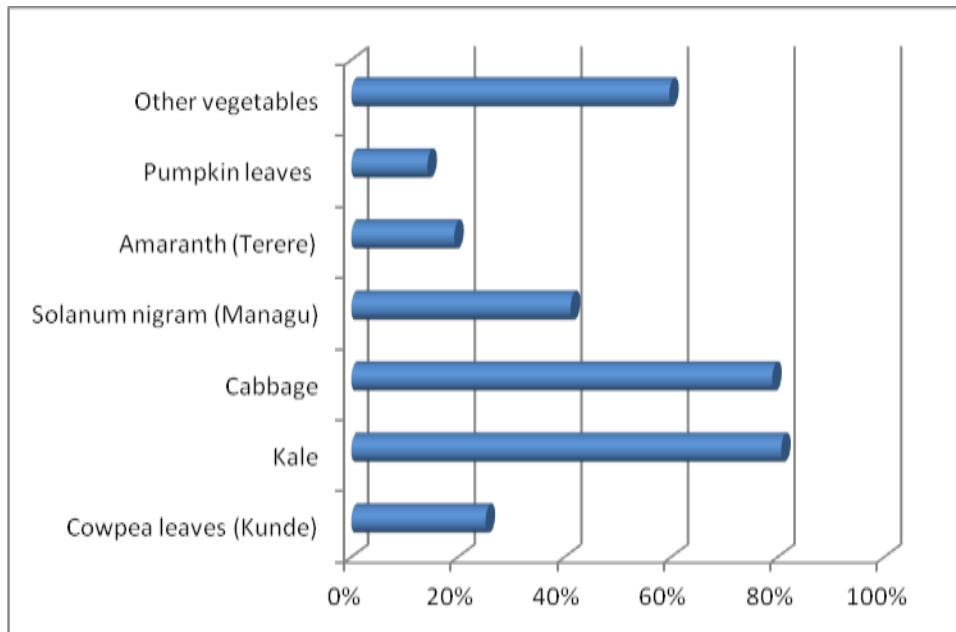


**Figure 4 5:** Education profile of spouses to study participants

### 4.3.2 Nutritional practices of the study subjects

#### 4.3.2.1 Vegetable consumption among mothers

Popular vegetables among the women was kale leaves 81% (n= 96) and cabbage 79% (n=94). With respect to other popular vegetables in Kenya, cow peas (*Kunde*) was much less frequently eaten by only 25% (n=30), Managu leaves (*Solanum nigrum*) 41% (n=49) and Amaranth (*terere*) 19% (n=23) **Figure 4.6**. The least popular traditional leafy vegetable was pumpkin leaves which was consumed by 14 % (n=17). A very significant proportion consumed other vegetables that were not listed amongst the most common vegetables in the Kenya market.



**Figure 4.6 a**

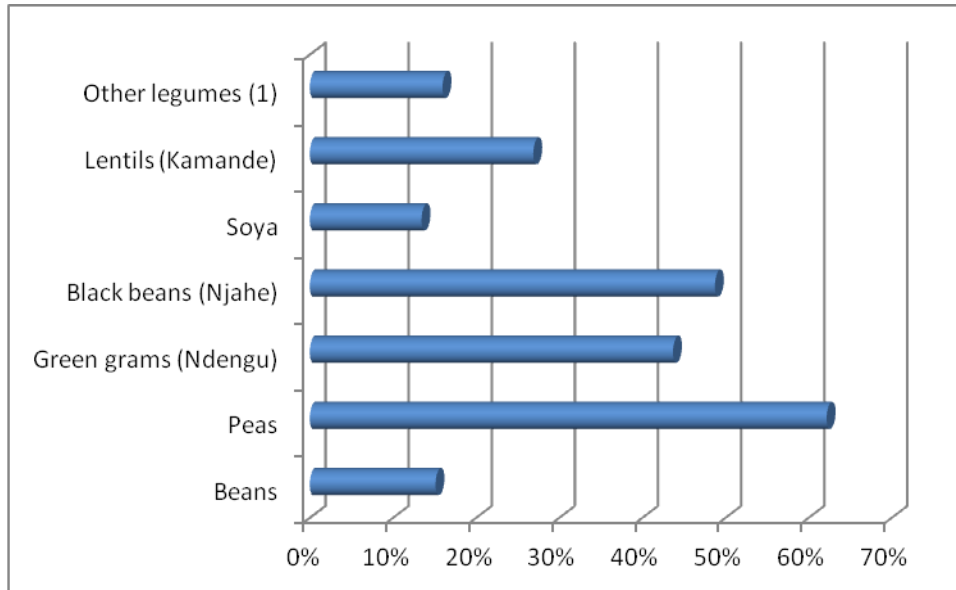
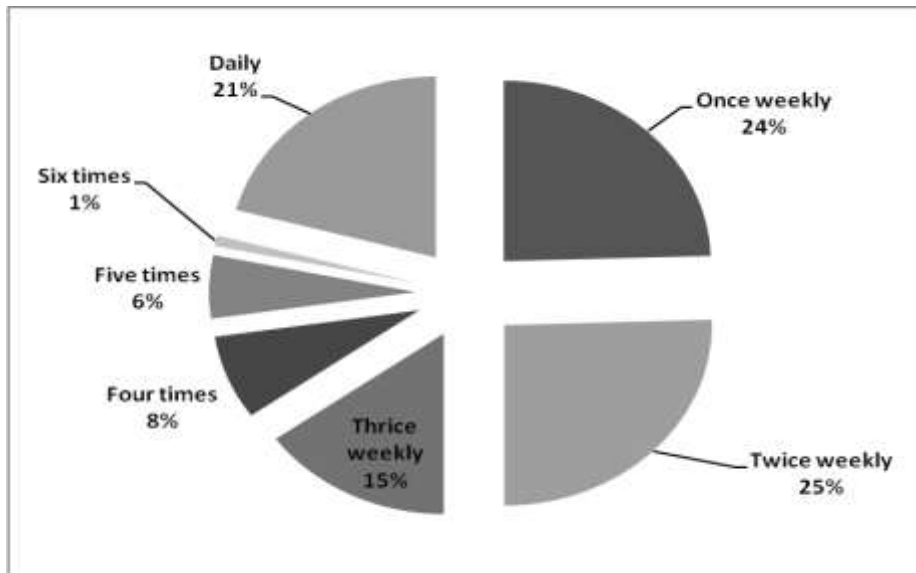


Figure 4.6 b

**Figure 4 6:** Vegetable and legume preferences among breastfeeding women at Kenyatta National Hospital

#### 4.3.2.2 Sources of protein

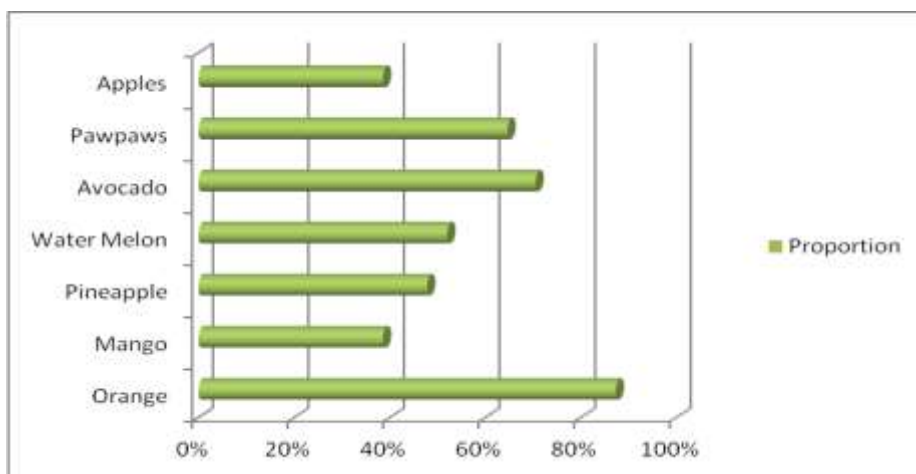
The main sources of vegetable protein included; peas (*Pisum sativum*) 62% (n=74), green grams (*ndengu*) 44% (n=52), black beans (*Njahi*) 49% (n=58), brown lentils (*Kamande*) 27% (n=32) while beans were reported by 82% (n=98) of the women (**Figure 4.6**). Meat was eaten between 1-3 times a week by 60% (n=76) of study participants **Figure 4.7**



**Figure 4 7;** Frequency of meat consumption among breastfeeding women visiting Kenyatta National Hospital

#### 4.3.2.3 Fruit consumption

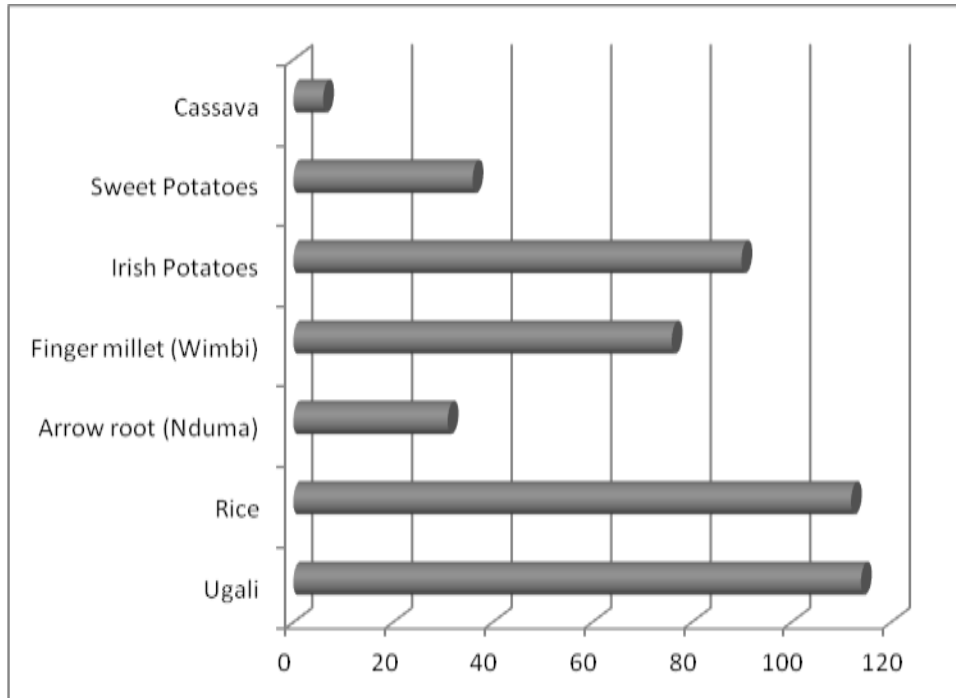
Fruit eating habits were also gathered indicating a high frequency in the consumption of oranges 87% (n=104), avocado 71% (n=84) and lower for mango 39% (n=46), pineapple 48% (n=57), melon 52% (n=62), apple 39% (n=38) and pawpaw 31% (n= 37). This may be attributed to seasonal availability and cost (**Figure 4.7**).



**Figure 4 8:** Fruit consumption among breastfeeding mothers at Kenyatta National Hospital

#### 4.3.2.4 Sources of Starch

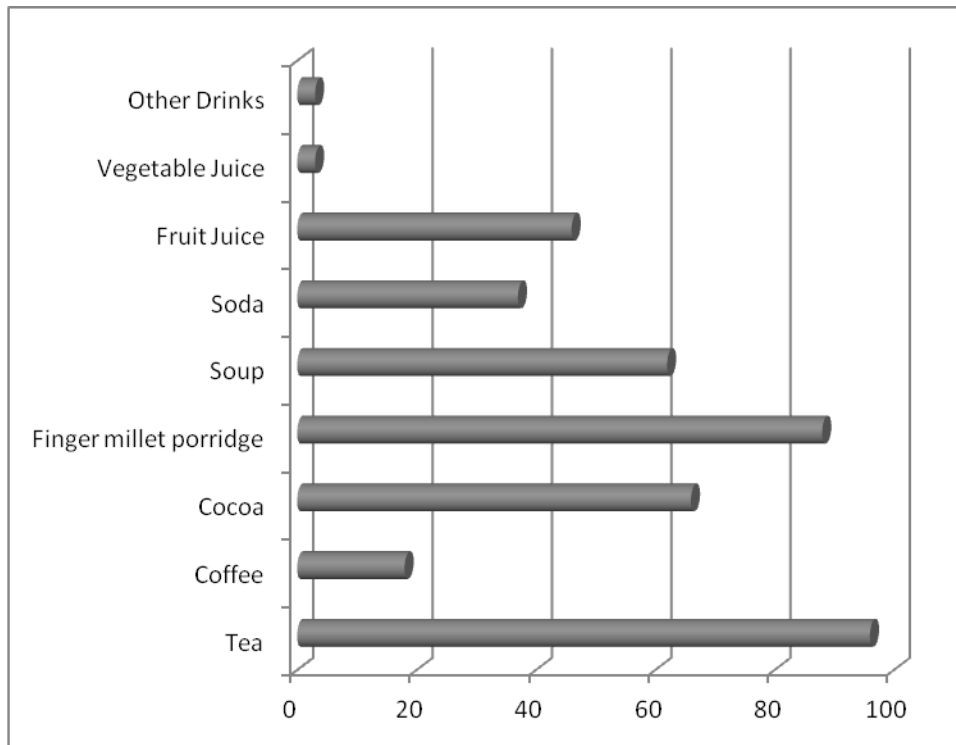
Popular sources of starch were Maize meal (*Ugali*) 96% (n=114), rice 94% (n=112), Irish potatoes 75% (n=90) and finger millet 64% (n=76). Other sources of starch included; sweet potato 30% (n=36), *Nduma* (Mannihot) 26% (N=31) and cassava 5% (n=6) **Figure 4.9**.



**Figure 4.9:** Starchy food preferences among breastfeeding mothers at Kenyatta National Hospital.

#### 4.3.2.5 Beverage preferences

When asked about beverage preferences, tea was most popular 81% (n=96) followed by porridge 74% (n=88), cocoa 55% (n=66), soup 52% (n=62) and unspecified fruit juice 39% (n=46). In contrast there appeared to be an aversion to coffee with only 15% (n=18) reporting using coffee within the previous week (**Figure 4.10**).



**Figure 4 10:** Beverage preferences among breastfeeding mothers at Kenyatta National Hospital.

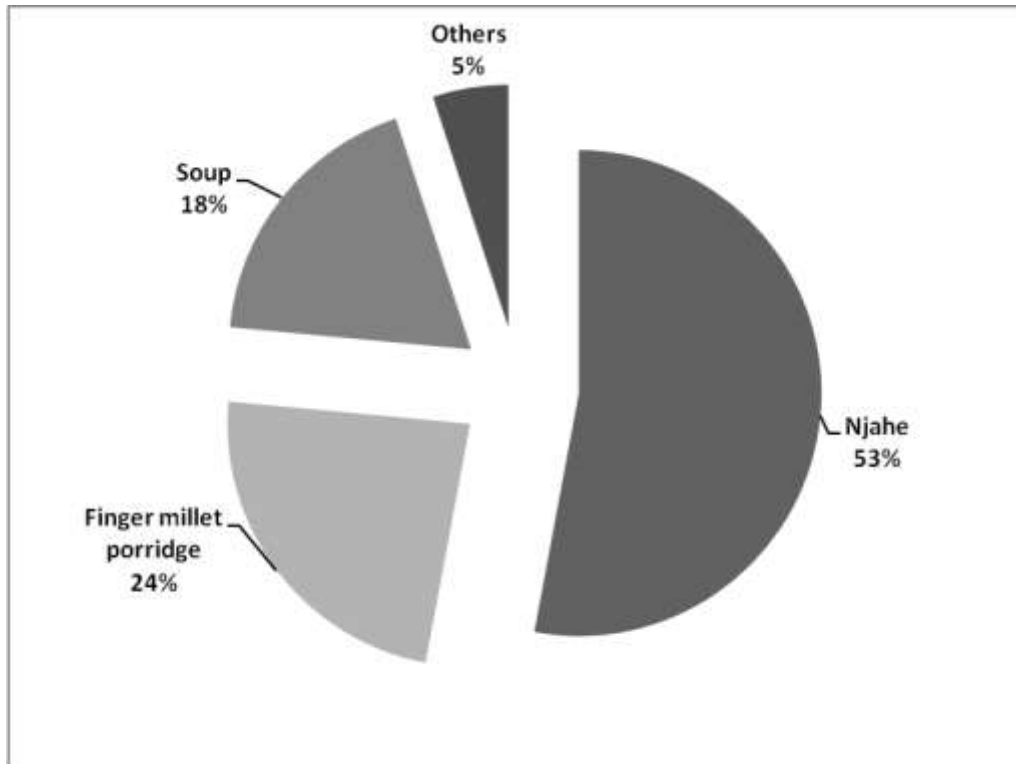
#### **4.3.2.6 Mothers knowledge, beliefs and practices with regard to breastfeeding**

Out of 53 women, 48 (90%) reported sharing the bed with the baby. Out of 52 responding to the inquiry over the number of times the baby suckled at night and 34 (65%) reported suckling more than four times at night. This parameter was later found to have a significant relationship with maternal RBC count as analyzed in chapter 5 of this report.

#### **4.3.2.7 Foods believed to increase milk output**

Many communities have traditions associated with certain foods in relation to pregnancy and after delivery. When asked about foods they believed were beneficial to mothers during lactation, a large number gave black beans (Njahe)

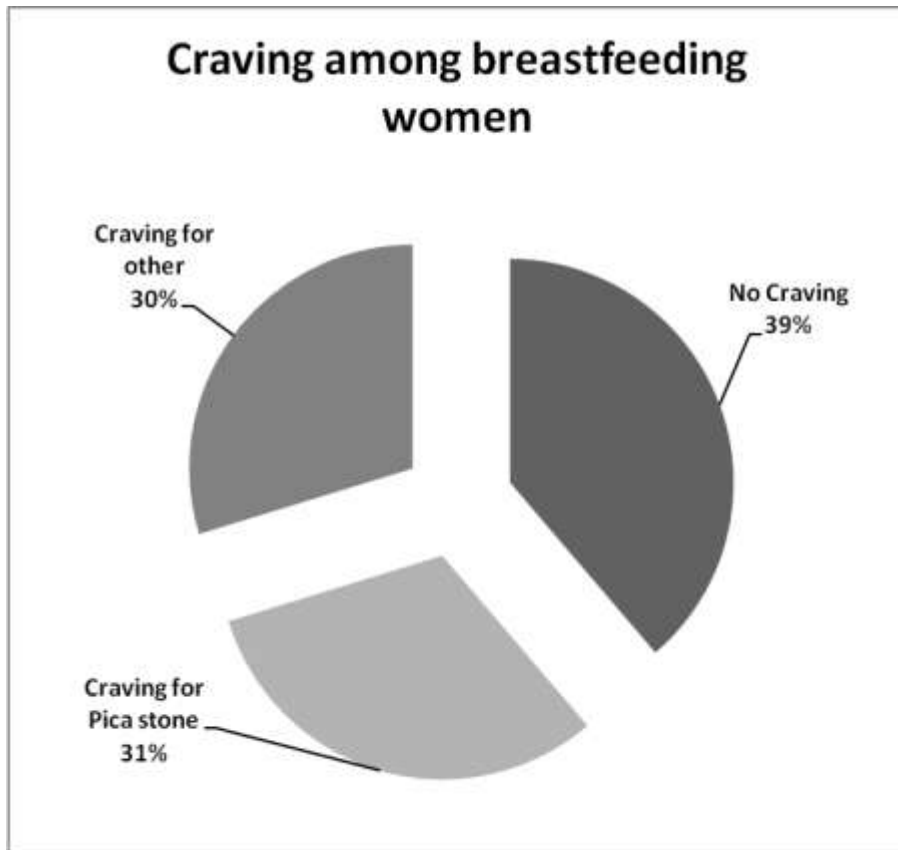
53% (n=63), finger millet porridge 24% (n=28) and some bone soup 18% (n=22) (**Figure 4.11**).



**Figure 4.11:** Food believed to have lactagogue effect among breastfeeding mothers at Kenyatta National Hospital.

#### **4.3.2.8 Prevalence of the pica habit**

Exploration on pica habit revealed that 57% (n=68) had developed a craving during the pregnancy with 31% (n=35) for pica stone (**Figure 4.12**).



**Figure 4 12:** Food craving and pica habit among breastfeeding mothers at Kenyatta National Hospital

#### **4.3.2.9 Use of complementary medicines**

At enrolment, 22% (n=26) out of the 119 indicated they were aware of herbs that are useful in managing infant illnesses.

#### **4.3.2.10 Link between Lactational amenorrhea and breastfeeding**

When mothers were asked if they knew about lactational amenorrhea, equal numbers (n=26) in each arm responded yes but majority (n=63) had not heard about it. Curiously, many (n=84) did not know where to get information about lactational amenorrhoea method. Kenyatta National hospital is the country's top referral facility and this result indicates need to promote awareness among its clientele.

More mothers 68% (n=81) made one to two antenatal visits. This is an important factor in determining the quality of monitoring during pregnancy to help identify those likely to develop complications during delivery.

#### **4.3.2.11 Use of Iron supplements**

Iron was taken by 58% (n=69) of the mothers, while 49% (n=58) took folic acid prior to joining the study. This is further analyzed in chapter 5.

The information gathered on use of herbs was found to have a very strong association with the maternal hematology. This is also analyzed in chapter 5 of this report.

### **4.4 Discussion and conclusion**

This baseline of the study participants in the *Chinsaga* clinical trial sought to describe the demographics and nutritional behavior of mothers attending the postnatal clinic at Kenyatta National Hospital that may have a bearing on the hematological and lactational outcomes of this study.

Not many peer reviewed publications are available on the fruit and vegetable eating habits of Kenyans in Nairobi but one report by Ayieko *et al* (6) attempts to provide some information and is used as a comparator to data collected in this study.

Mothers in the population mostly consumed Kale and cabbage as the vegetable component of their diet. Tomatoes and onions are universal while kale, cabbage carrots and bananas follow in decreasing order (6). *Sukuma wiki* (Kale) is the most consumed leafy vegetables justifying its use as the control in this study. Among fruits, bananas and oranges are the most consumed followed by mangoes, papaws and avocados in decreasing order of frequency. About a

third of household food expenditure is on dairy products, meat and eggs; another third on fresh produce that constitute staples and a quarter on fresh fruits and vegetables.

Ayieko *et al* (6) also showed a range from 15% for the lowest quintile to 6% for the highest quintile in the expenditure of households on basic foods with most going to dairy products and within this milk was the prime item. Lower income groups were found to spend more on maize. The expenditure on maize diminishes with rise in income as wheat, rice, beef, mutton and poultry products become more popular among the high income groups.

Studies have been conducted elsewhere to evaluate nutritional practices. One such study looked into consumption of fruits and vegetables among school children of Asian countries and found Thai school children had the highest rate of fruit and vegetable consumption. It is thought that availability and income may be the key determinants of vegetable consumption and this collated with findings by others in the same country (16) . It is important to note that, the Thai government instituted a national policy promoting consumption of fruits and vegetables resulting in steady rise in the rate of consumption of fruits and vegetables since 1986 and may be worth emulating in Kenya.

The highest consumed fruits among mothers in this study were oranges 87% (n=104) and avocado 71% (n=84) Fruit consumption is determined mainly by family income, preferences and market prices(6). Preferences were affected by composition of the household, its member's knowledge education, habits and cultural norms, personal experience and biological drivers of hunger. Fruits and vegetables are considered a pricier source of energy especially among poor households. At these levels, larger amounts of starchy foods and grains were consumed.

Fruits and vegetables are a rich source of provitamin A (carotenoids). The relationship between carotenoids taken in from plant foods and the status of vitamin A was tested in breastfeeding women in rural Vietnam. Changes in blood and breast-milk retinol concentrations suggested lower bioavailability of carotenoids from vegetables and fruit than previously assumed (17).

Considering the survey was conducted in Nairobi, the food eating patterns are highly influenced by multiple cultures and key among them are communities from central Kenya. Thus the foods seen in the survey are very similar to those collected in the present study conducted among women attended at Kenyatta National Hospital. One example in the consistency of Irish potatoes in the diet seen across all income groups. The proportion of budget spent on vegetables is expected to decline as one climbs up the income groups. This is probably because the absolute expenditure may rise but less so in proportion to the total income (18).

Diet rich in fruits is generally pricy in Nairobi. Ayeiko *et al* (6) also observed food spendings on dairy products, vegetables, beef, wheat products, maize products, and fruits similar to findings in this study at Kenyatta National Hospital. Most participants responded positively for these items in the food questionnaire. Rise in incomes, were also noted to lead to more meats, animal products and fruits incluion diet.

According to Ayieko *et al* (6), there was association between educational level of the head of household and expenditure on fresh produce. Female headed households tended to have more fruit and fresh vegetables despite income levels. This finding may be an indicator of economic independence among women with higher incomes, and probably also reflects different decision making processes regarding consumption in households where a woman is the primary decision maker.

There was no systematic pattern between age of the head of a household and income but Ayieko *et al* (6) noted decreased expenditure on fresh fruits and vegetables in homes with more children which is expected. However in this study, the measure of RBC discussed in chapter 5 indicated a higher RBC count with increasing age of husband.

In a study carried out across several African countries, the percentage of households consuming fruits was found to vary from about 20% in Ethiopia, Mozambique to about 50% in Rwanda, Kenya and Malawi, and about 75% for Tanzania and Guinea. When results for vegetables are included, the percentages are well over 90%. It is notable that among the countries surveyed, including Ethiopia, Burundi, Malawi, Mozambique, Tanzania, Rwanda, Uganda, Ghana and Guinea, Kenya had the highest consumption per capita per year of 25.8 Kg/person/year for fruits and 88.3 Kg/person/year for vegetables. It was also one of the countries that exceeded the minimum recommended level of 146Kg/person/ year. It was also observed that many families spend on fruits and vegetables ranging from 43% in Guinea to 73% in Burundi. Diets in urban areas are generally more diverse than those in rural areas (19). This is associated with greater availability of supply converging to the urban markets. A rich variety of fruits were listed for consumption among mothers involved in this study.

Differences between rural and urban consumption was observed to be largest in Kenya and Burundi. Income differentials were also observed to affect percentages within countries. Fruit and vegetable consumption tended to increase with income (19). Family size was observed to have a negative association with proportion of income spent on fruits and fresh vegetables. Surprisingly, in this study presence of a household member with higher than secondary education, was associated with lower allocation to fruits and vegetables. Valuable information may be derived from a study to assess the effect of such a person on the decision making processes and resource

allocation resulting in this disparity. Part of this may be the expectation that such a person may be the leader in the home and the major breadwinner.

From the recent past, consumers have become increasingly aware of the nutritional and medicinal value of African indigenous vegetables in bridging the gap of micronutrients among African households (20). African indigenous vegetables are known for their importance in providing nutritious food, both in rural and urban areas. The vegetables play a crucial role in income generation and subsistence. Some of them have been attributed with having medicinal-value properties and are grown for home consumption (21). They are considered traditional crops because some of the vegetables were planted; others were readily available and harvested in their habitat appearing as volunteer crops or weeds. Others had been consumed for countless generations signifying their value and importance in local cultures (22).

Increasing popularity of traditional leafy vegetables has caused a rise in demand especially in major urban centers. The supply of these vegetables has however not matched this growing demand (23). Most farmers are semi commercially oriented poor farmers, are not organized, and lack inputs and skills to enable them to satisfy the dynamic market requirements. They are not able to access high value markets such as supermarkets and are often exploited by middlemen responding to the changing consumption patterns and market opportunities occasioned by the growing demand for these vegetables in the urban centers (4,24). Consumption of traditional vegetables and grains was established to be lower than kale and cabbage among the participants

In conclusion, foods and diet items vary with culture. Across Kenya and in Africa, maize, sweet potato, sorghum, millet and cassava are among the most popular foods. Vegetables used traditionally are also rich sources of vitamins such as pro-vitamin A from *Chinsaga*. Promoting the use of such vegetables is

an important part of a multipronged approach to address the gap in supply of such nutrients.

Recommendations from this study include public awareness creation, active government policy to facilitate and promote consumption of fruits and vegetables with a greater emphasis on production and distribution of traditional varieties. The Ministry of Health needs to carry out public re-education on the importance of breastfeeding and its contribution as a natural form of family planning.

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## CHAPTER FIVE

### 5.0 Effects of *Chinsaga* (*Gynandropsis gynandra*) on hematological indices

## **5.1 Introduction and literature review**

### **5.1.1 Role of Iron in Red Blood Cell Physiology**

Iron is essential for oxygen transport and cell growth and survival. The typical adult human body contains an average of 3.5 g of iron (approximately 4 g for males and 3 g for females). Most of the iron within the body is used in haemoglobin (2.1 g) synthesis. A small amount is devoted to cellular protein synthesis (myoglobin, cytochromes) or circulates through plasma bound to transferrin (1). Iron homeostasis is closely regulated via intestinal absorption and by recycling of iron already present in the body. This element has the particularity that once absorbed; there is no physiologic mechanism for its excretion from the body. Males lose less iron (1 mg/day) compared to an average of two mg/day by women during their menses.

To maintain adequate supplies of iron for heme synthesis, 20 mg of iron is recycled daily, going from senescent red cells that are removed from the circulation to new cells in the bone marrow (2). Iron from older cells is loaded onto transferrin by macrophages for delivery to the bone marrow. The diet provides 10–20 mg per day of iron requirement, as heme (mainly in red meat) and non-heme (white meat, vegetables, and cereals). Healthy adults absorb approximately 10 to 15% of this iron in their diet, but absorption is influenced by the body's iron stores, the type of iron in the diet (heme and non-heme), and other dietary factors that may increase or reduce the absorption of iron. Heme iron is absorbed very efficiently by the body whereas only 1 to 7% of non-heme iron is absorbed (3).

### **5.1.2 Anemia in Pregnant and Lactating women**

Prevalence and complications of Iron deficiency anaemia (IDA) are the most common type of mortalities associated with anaemia arising when there is insufficient iron to support normal red cell production. According to the World Health Organisation (4), iron deficiency is the most common form of malnutrition in the world, affecting around 2 billion people (25% global population) worldwide.

It is of particular concern among women and children. Pregnancy and increased bleeding during menstruation confer women a higher risk of developing IDA leading to high morbidity and mortalities. Mothers with anemia have an increased risk of complications during pregnancy and delivery. Maternal anemia has been considered as a risk factor for an undesirable pregnancy outcome (5).

### **5.1.3 Diagnosis of Anaemia in Pregnant and Lactating mothers**

Many women remain undiagnosed due to the reliance on conventional parameters, such as the post-delivery serum ferritin. As an inflammatory protein, ferritin is artificially raised after delivery (6). This enhances the potential for false results. It is therefore advisable to test for post-delivery serum ferritin at least four weeks after delivery.

Judgment about clinical iron status in pregnancy is often clouded by factors such as hemo-dilution and the observation that even apparently high Hb, mothers may be suffering iron deficiency.

Hematocrit is found to provide a better estimate than hemoglobin to get a measure of the red blood cell volume to total blood volume. Besides hematocrit, other parameters that affect rheology of blood include blood cells

concentration, dimensions of blood vessels and flow and plasma concentration. Changes in these parameters may therefore serve as indicators of possible danger during pregnancy (7).

Considering the high prevalence of anemia during pregnancy worldwide, one study was carried out to compare detection of iron deficiency during pregnancy with hemoglobin (Hb) and serum measurements considering the effects of hemodilution or accelerated erythropoiesis. It was to determine whether cell indices will give a more reliable measure of iron deficiency in pregnant women at term (8).

According to the Krafft study (9), results provided by the cell indices alone of Percentage of Hypochromic Red Blood Cells (%HYPOm or CHr) were in good agreement with the results based on the combination of three commonly used tests (Hb, MCV, ferritin). These findings of their study suggested use of cell indices such as CHr and %HYPOm provided by the automated hematological analyzer as the most practical way to diagnose iron deficiency in pregnant women at term but would need additional work to determine their utility in monitoring trends helpful in diagnosis of iron deficiency during the course of pregnancy.

#### **5.1.4 Debate over benefits of iron and folic acid supplementation**

Suggestions have been made to have iron supplementation for pregnant women, young children and adolescents universal in countries where the prevalence of anaemia in the population is over 40% because of the presumed challenge in providing sufficient amounts through the regular diet.

The benefits of iron supplementation in pregnancy and lactation remain a debatable issue in perinatal medicine in reducing the prevalence of iron

deficiency anaemia. The results of supplementation programmes have often proved disappointing, especially in developing countries, (10,11), despite the evidence of benefit in terms of red cell and iron parameters in pregnancy and lactation (12). What is lacking, however, is proof of the benefit of postpartum iron supplementation in the women with non-anaemic iron deficiency at term that form a substantial proportion of the obstetric population, even in industrialised countries.

The 1999 Cochrane Database found no case for routine iron supplementation in developed countries with a balanced dietary and micronutrient intake (13). In countries with poorer antenatal care, compliance to iron supplementation is low. In Brazil, Trugo (14) found that 80–160 mg/day of iron failed to lower the incidence of third trimester iron deficiency in non-anaemic healthy women compared with controls, concluding that supplemental iron served to supply the red cells, fetus and placental tissue rather than replenish the iron stores.

#### **5.1.5 Iron formulations and salts**

Various iron formulations are commercially available and their efficacy and tolerability have been tested in several studies. The most common way to correct for iron deficiency anaemia, is to replenish the iron usually by oral administration of ferrous formulations. Intravenous administration has the risk of causing the undesirable state of iron overload. Unfortunately, most formulation of either ferrous (divalent) or ferric iron have erratic bioavailability especially the ferric types and this reduces compliance among most patients. The most common side effects of oral iron therapy are gastrointestinal disturbances including constipation, diarrhea nausea, heartburn and gastric pain.

Divalent iron is more commonly prescribed as the ferrous salt, gluconate or at times, fumarate. They are generally better absorbed than the trivalent ferric

preparations due to poor solubility of ferric ions in the alkaline conditions of the small intestines. In addition ferric iron must be transformed into ferrous iron to be absorbed across the gut mucosa. One major drawback to use of iron formulations is the nausea and intestinal discomfort associated with their use. Most manufacturers have attempted to improve the formulations to reduce these effects.

Studies have been made (15) evaluating quick versus slow release iron and found minimal difference in effect after 21 days. They observed that haemoglobin levels increased to near baseline values after two months of treatment in both treatment groups.

Iron coupled with sugar complexes has been compared to ferrous formulations in several studies and found to have very low efficacy contrary to expectations. Trivalent iron is believed to be more stable and easier to carry across the intestinal mucosa. In 2003, Mehta's (16) report of 27 patients who failed to respond to iron coupled with sugar complexes given for 4 to 52 weeks but later responded to ferrous fumarate for 4 to 13 weeks. Ruiz-Argüelles *et al* (17) showed a 31% failure rate with better response when switched to ferrous fumarate.

A study (15) using  $^{59}\text{Fe}$  III hydroxide-polymaltose showed a near 40 fold difference in absorption in the fasted state. The difference reduced when the iron was taken with food. In another study (18), haemoglobin levels failed to increase in 9 patients receiving 100 to 300 mg of ferric polymaltose complex over a four week period. At week 9 the difference between both groups was not statistically significant.

### **5.1.6 Association between maternal iron levels and farming practices.**

One study conducted among the Pokot in Kenya (19), Etyyang et al found women from the farming community in West Pokot, Kenya, had lower hemoglobin and serum ferritin concentrations in the third trimester of pregnancy and four months postpartum than women in the pastoral community. However, no differences were observed in serum retinol levels. Notably, the farming community had a significantly higher proportion of newborns weighed less than 2.5 kg compared to the pastoral community.

### **5.1.7 Hematological biomarkers of iron metabolism**

The hematological indices that are related to erythrocytes are: RBC; hemotocrit; hemoglobin levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), Red blood cell distribution (RDW); and Mean Corpuscular Hemoglobin Concentration (MCHC). MCHC is diminished in microcytic anemia. It is a more reliable indicator of anaemia because levels remain low even though the hemoglobin and MCH levels are high. The reference values for MCHC range from 32 to 36 g/dL or 4.9 – 5.5 mmol/L. It is calculated by dividing MCH with the RBC.

Mean corpuscular volume is obtained by dividing hemotocrit by the RBC. RDW is an additional measure of red blood cell volume. The usual size of the human red blood cell is 0 to 8 micrometers. The reference range of RDW values in humans is 11 – 15%. Both MCV and RDW are used to determine the cause of anemia. In macrocytic anemia caused by Vitamin B12 or folic acid deficiency, RDW values are high. MCV is obtained by dividing the hemotocrit with the RBC. The reference range for MCV is 80 – 100 fL.

RDW is calculated using the formula below:

$RDW = (\text{Standard deviation of MCV} / \text{Mean MCV}) \times 100$

Population values of hemoglobin range from 12 to 16 grams per deciliter for women of childbearing age with the minimum about 11 grams per deciliter in the first and third trimester of the pregnancy and 10.5 grams per deciliter in the second trimester of pregnancy (21).

Anemia is the leading cause of low values of hemoglobin while erythrocytosis triggered by chronic obstructive pulmonary disease myeloproliferative disorders and other hypoxic lung conditions may lead to abnormally raised Figures during tests. It may be prudent to include socioeconomic and anthropological factors in the forecast when making such interpretations especially in a varied society as that found in Kenya.

Plasma volume is an important factor in determining hemoglobin levels and hematocrit which requires fresh whole blood for determination.

#### **5.1.8 Problem statement**

In many African communities there are beliefs that some plants promote lactation and aid recovery from post-delivery anemia. However there is lack of clinical evidence about these claims. This may therefore lead to unsupported use of these plants. If indeed such plants are beneficial, this knowledge should be made widely available as a strategy to combat postnatal complications such as anemia. *Chinsaga* is widely used by the Kisii for its claimed benefits in improving lactational and pregnancy outcomes. This study therefore sought to determine if use of this vegetable is clinically beneficial.

### **5.1.9 The Null hypothesis**

There is no difference on the hematological profile of breastfeeding mothers taking *Chinsaga* (*Gynandropsis gynandra*) compared to those on Kale over 28 days.

### **5.1.10 Research Objectives**

The main objective was to compare the effects of *Chinsaga* (*Gynandropsis gynandra*) to Kale (*Brassica carinata*) on haematological profile of breastfeeding women.

### **5.1.11 Specific objectives**

1. To compare the effects of *Chinsaga* (*Gynandropsis gynandra*) and Kale on the selected hematological parameters such as hemotocrit, mean corpuscular hemoglobin and hemoglobin levels.
2. To identify which hematological parameter is most affected by supplementing the diet of mothers with *Chinsaga*
3. To determine if there is biological interaction between iron supplementation and use of Kale or *Chinsaga*.

### **5.1.12 Study Justification**

The findings of this study are expected to guide policy makers on optimal local nutritional interventions that may reduce the incidence of post-natal anaemia,

## **5.2 Materials and methods**

### **5.2.1 Ethical considerations**

The study was conducted with approval from Kenyatta National Hospital – University of Nairobi Ethics and Research Committee (KNH-UON ERC/01/3757) and the Kenyatta National Hospital clinical services department as mentioned in Chapter 4.

Mothers were informed about the study and again asked if they still wish to join the study. Participants were required to give informed and documented consent. They were allowed to make this decision at another visit if unable to do so on the first day. During all aspects of sample handling, only numbers were used to track the records of the participants.

### **5.2.2 Study design**

This study was a randomized triple blinded placebo controlled study involving lactating mothers attending Kenyatta National Hospital Maternal Newborn Child Health (MNCH) clinic. There were two study arms. One arm was treated with placebo which was cooked dried kale and the second arm was treated with *Chinsaga*.

### **5.2.3 Study site and duration**

The study was conducted in Kenyatta National Hospital which serves mainly women from Nairobi and its environs. Participants were recruited from the Maternal Newborn Child Health (MNCH) clinic. This study was conducted over a period of four months, July to November 2008.

#### **5.2.4 Inclusion and Exclusion criteria**

The study targeted healthy women, free from chronic disease with exception of asymptomatic HIV positive mothers. They should not have been on *Chinsaga* at least four weeks prior to enrollment, willing to give consent, available for six months within the environs of Nairobi city and should not be smoking cigarettes.

Mothers were excluded from the study if they were not breastfeeding, smoked cigarettes, and suffered chronic illness such as Rheumatoid arthritis, Crohn's disease, colitis ulcerosa, diabetes, asthma and hypertension. They were also excluded if they were on prescription medication or non-steroidal anti-inflammatory drugs on a regular basis, using vitamins or mineral supplements for the last one month, corticosteroids or on immunostimulators for the last 4 weeks or their hemoglobin concentration was below 8g/dL. Positive HIV status was not an exclusion criterion.

#### **5.2.5 Sample size considerations**

One of the major benefits expected in the *Chinsaga* treatment group is a rapid improvement in hemoglobin levels. Sample size was calculated using the proof of concept model. A major benefits expected in the *Chinsaga* treatment group was a more rapid improvement in hemoglobin levels. With normal hemoglobin concentration range from 9.5-15.8 g/dL (22), difference of 1.5 g/dL between the Kale (Group 1) and *Chinsaga* (Group 2) and variance of 1.5 g/dL, power of 80%, confidence level of 95%, and correcting for a 25% dropout rate, a sample size of 22 was calculated as the minimum to differentiate between the experimental and control groups, we would require 22 women participants in each of the two groups.

### **5.2.6 Participant recruitment**

Mothers attending the Maternal Newborn Child Health (MNCH) clinics were informed about the study and asked if they are interested in taking part in the study. Those that accepted to join the study were screened for eligibility. Those that qualified for recruitment were adequately informed of the study as outlined in Chapter 4. About 115 healthy subjects were enrolled after screening for eligibility with 37 completing the follow up for 28 days thereafter.

### **5.2.7 Randomization and treatment allocation**

The randomization scheme was generated by a volunteer working as an IT staff within a clinical trial independent from the investigator and the list printed and copies made for use during the work by using the Web site Randomization.com (23) (24) (<http://www.randomization.com>) by Gerard E. Dallal and last updated on 07/16/2008 05:07:20.

The volunteer mothers were divided into two treatment groups: *Chinsaga* and a control group on Kale (*Brassica oleracea*). A block randomization (5 subjects in each block) was used to assign women into either the kale - placebo (group one) or the *Chinsaga* – test (group two). Study volunteers were allocated to treatment groups according to the list and after consenting to participating in the study. Each volunteer was sequentially assigned a number and the corresponding treatment given using the randomization list.

### **5.2.8 Blinding and Allocation concealment**

In order to achieve maximal level of blinding, several measures were taken to achieve triple blinding. During the preparation, materials for the study were packed in identical sachets. The sachets were all marked with the single digit 1 which whose position was varied slightly depending on the two treatments. The

distinction between both packets was adequately masked unless one was given prior information. The study staff would only know the treatment group but were blinded as to which of these was intervention or control.

The treatment material was packed in sachets that were similarly marked and practically indistinguishable unless prior information was given to those participating in administering or taking the materials. The mark to distinguish the two materials was kept sealed from the investigators. This was important to prevent confusion during packing the study sets and allow cross-checking during the dispensing.

Information about the meaning of the codes, treatment or control arms was kept from the data analyst until data analysis was complete. Directions to help disclosure were sealed except to workers at the firm packing the treatment sets. A closed envelope was provided for use during un-blinding to the supervisor of the study for safekeeping until the end of the data analysis of the study. At this point, disclosure was sought to help determine effect of the intervention. Directions for disclosure were sealed except to workers of the packing firm for the treatment sets.

#### **5.2.9 Data collection**

The study staff that facilitated the conduct of the research were recruited and trained appropriately. They were formally introduced to the clinic and other hospital staff, and were involved in a study conducting preliminary pilot review of work and fine tuning of documentation and study procedures.

### **5.2.10 Administration of Study products**

Study participants were given sachets of prepared *Chinsaga* daily for the test group and Kale for the control group for 4 weeks. Each member of the Intervention group was given a 9gram sachets made from dried *Chinsaga* to be taken taken for 28 days from the day of recruitment. Each member of the control group was given a 9 gram placebo sachets made from dried kale taken for 28 days.

Participants were given directions on how to take the study products with their main meal and to report if they encountered any challenges or problems during the entire intervention period. The participants were required to make at least three scheduled study visits at screening and enrolment at enrollment (day 0), first follow up at day 14 and the final follow up at day 28.

### **5.2.11 Collection and analysis of biological specimens**

Blood samples, 5ml, were collected in EDTA tubes (Vacutainer™, Becton Dickinson Vacutainer Systems, Plymouth, UK) for a complete blood cell count, including analysis of red blood cell and reticulocyte parameters obtained with an Act 5 B Hematology Becton Dickinson System. The serum samples, collected in serum tubes (Vacutainer™, Becton Dickinson Vacutainer Systems), were centrifuged and the serum was separated and stored frozen at  $-70^{\circ}\text{C}$ . Transferrin, ferritin and lactoferrin from breast milk samples were analysed as detailed in chapter 6 of this report.

### **5.2.12 Data analysis**

Data analysis was conducted on all variables to generate summaries of findings. Kruskal wallis test was used to test for differences across arms because most of the outcome variables were not normally distributed. Sub-

group analysis was done in which the baseline characteristics of participants who came for Day 14 was compared. This was done to rule out the fact that there could have been systemic differences at baseline values in individuals who completed the study.

In order to select which outcome variable was affected by treatment, the outcome variables were subjected to generalized linear regression analysis with treatment group as the dependent variable and time of visit as the independent variable. Variables where the p value of the treatment variable was less than 0.05 were subjected to modeling using a forward step wise approach. Generalised linear modeling was done to control for confounding. The variables were first transformed so that they could be normally distributed.

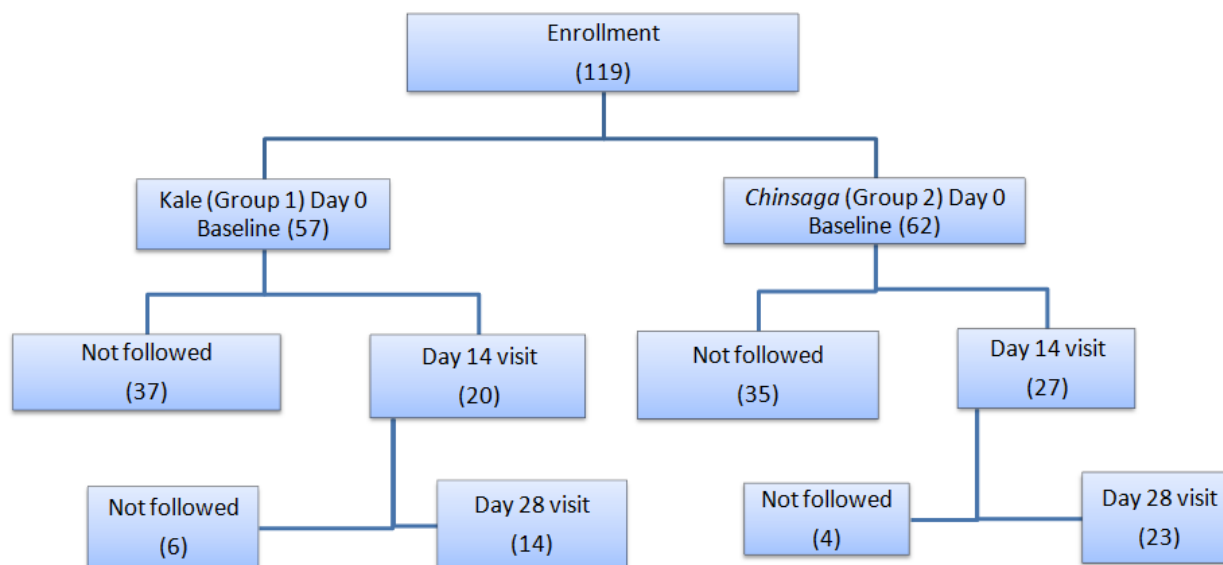
An additional component of data analysis sought to determine if the effect of treatment assignment were confounded and if there was biological interaction with known hematinic agents and other socio-demographic and nutritional factors. The presence of confounding was evaluated by generating a regression model that had treatment assignment and a putative confounder as the only covariate variables. A variable was considered to be a confounder if the percentage change in the crude beta coefficient of the treatment variable was greater (10%).

In order to determine that there was statistical interaction between treatment and other covariates, an interaction term was generated from the product of treatment and a covariate. If the interaction term was statistically significant, statistical interaction was deemed to be present.

## 5.3 Results

### 5.3.1 Randomization of study participants

Study participants were randomized as illustrated in the consort diagram Treatment 1 (Kale), Treatment 2 (*Chinsaga*) (**Figure 5.1**).



**Figure 5 1:** Consort diagram of randomisation and follow up of study volunteers into kale (Control) and Chinsaga (Test) at KNH on days 0, 14 and 28.

The baseline characteristics across the study arms are summarized in chapter 4 of this thesis

### 5.3.2 Effects of Chinsaga on selected Red Bloodcell Count related parameters

The focus of analysis was mainly on the effects of *Chinsaga* treatment on hematological indices related to erythrocytes. The variables that were used as markers of iron metabolism were: hematocrit (HC), hemoglobin levels (Hb), MCHC, MCV, RDW and RBC counts. **Table 5.1** summarizes the differences across arms during the 3 study visits.

**Table 5 1:** Differences in selected hematological parameters in patients treated with *Chinsaga* and *Kale* over 28 days

Blood parameter	Duration	Kale	Chinsaga	P
HEMATOCRIT	Baseline	37.3 (IQR 33.4–40.3)	37.4 (IQR 34.1–39.5)	0.75
	Day 14	40.25 (IQR 33.0–42.15)	39.7 (IQR 35.3 – 41.1)	0.91
	Day 28	37.8 (IQR 31.8 – 40)	39.9 (IQR 36.8 – 40.3)	0.31
HEMOGLOBIN	Baseline	12.7 (IQR 11.4 – 13.7)	12.5 (IQR 11.5 – 13.6)	0.33
	Day 14	13.6 (IQR 10.75–14.55)	13.4 (IQR 11.0 – 13.8)	0.60
	Day 28	12.6 (IQR 10.6 – 13.6)	13.3 (IQR 12.4 – 13.9)	0.42
RDW	Baseline	13.5 (IQR 12.5 – 15.1 )	13.4 (IQR 12.6 – 14.8 )	0.86
	Day 14	13.6 (IQR 13.05 – 14.6)	14.8 (IQR 13.05 – 16.9)	0.08
	Day 28	13.7 (IQR 11.7 – 14.8)	14.6 (IQR 13.5 – 16.3)	<b>0.05</b>
MCV	Baseline	85 (IQR 78 - 88)	82 (IQR 78 – 88)	0.11
	Day 14	84 (IQR 78.5 – 88)	80 (IQR 76 – 83)	0.07
	Day 28	85 (IQR 77 – 89)	79 (IQR 77 – 83)	0.08
MCHC	Baseline	34 (IQR 33.5 – 34.5)	33.7 (IQR 33.2 – 34.3)	0.05
	Day 14	34.05 (IQR 33.15–34.6)	33.7 (IQR 33.2 – 34.2)	0.11
	Day 28	34 (IQR 33.4 – 34.4)	33.4 (IQR 33.2 – 34.4)	0.22
MCH	Baseline	28.2 (IQR 26.4 – 30.2)	27.8 (IQR 25.8 – 29.1)	0.09
	Day 14	28.65 (IQR 26.4 – 30.4)	27 (IQR 25 – 28.4)	<b>0.03</b>
	Day 28	29 (IQR 26.6 – 30.3)	27.3 (IQR 25.5 – 28.6)	<b>0.05</b>
RBC Count	Baseline	4.38 (IQR 4.18 – 4.98)	4.56 (IQR 3.79 – 5.26)	0.25
	Day 14	4.6 (IQR 4.69 – 5.76)	4.91 (IQR 4.69 – 5.08)	<b>0.03</b>
	Day 28	4.5 (IQR 4.37 – 4.81)	4.91 (IQR 4.8 – 5.08)	<b>0.03</b>

The only parameters for which there was no statistically significant difference across study arms were: haematocrit; hemoglobin; and MCHC. The parameters for which there was a statistically significant difference across study arms by Day 28 were: RBC count; MCH; and RDW. In the case of RBC counts and MCH, the median values of patients treated with *Chinsaga* were statistically

higher than the values of patients treated with Kale by day 14 and later on day 28 of the study.

The Kale-treated arm had higher MCV values and lower RDW values by Day 28. In addition, the Kale treated arm had higher median MCHC and MCH values compared to the *Chinsaga* treated arm by Day 28. High MCV and RDW values are indicative of macrocytic anemia caused by deficiency of Vitamin B12 and/or folic acid. Hematocrit of the *Chinsaga* treated arm was also higher in the third visit

### 5.3.3 Longitudinal changes in Hematological parameters

#### 5.3.3.1 Changes in Hemotocrit and Hemoglobin levels

As previously stated, there was no statistically significant difference in the hemotocrit and hemoglobin levels by Day 28 compared to the control. The median levels and IQR for these two parameters at each of the visits are illustrated in **Figure 5.2** As can be seen, both the values of both these parameters increased during the course of the study compared to the baseline value.

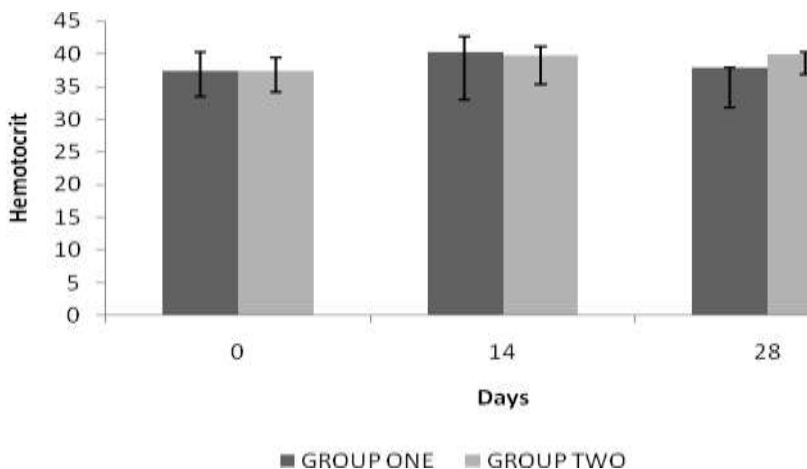


Figure 5.2a Group one (Kale), Group two (Chinsaga).

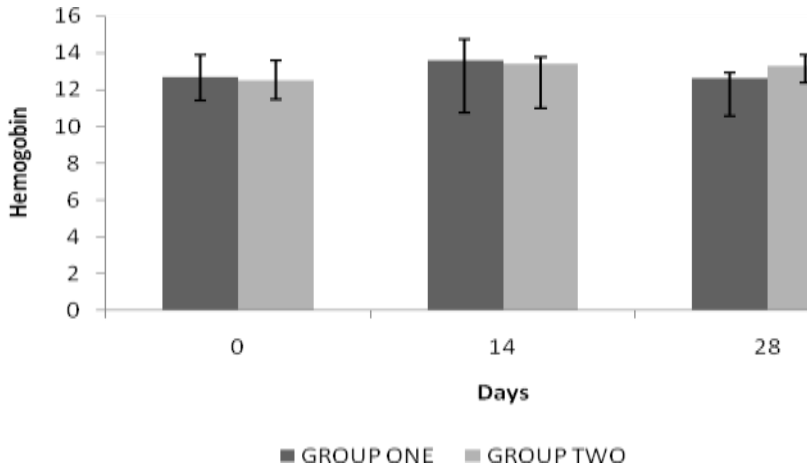
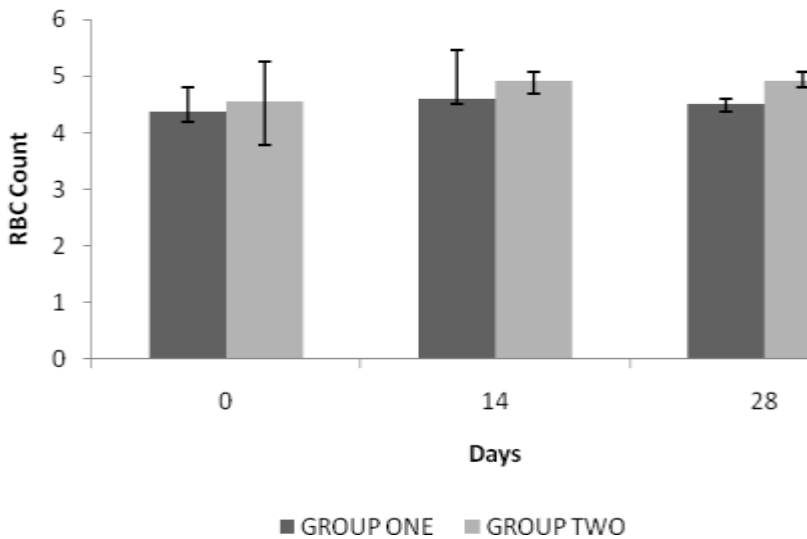


Figure 5.2 b Group one (Kale), Group two (Chinsaga).

**Figure 5 2:** Median changes in Hemotocrit and Hemoglobin levels Kale (Group one), Chinsaga (Group two).

### 5.3.3.2 Longitudinal changes in Red Bloodcell Count



**Figure 5 3:** Longitudinal changes in the Red Blood Cell count (Group one - Kale, Group two - Chinsaga).

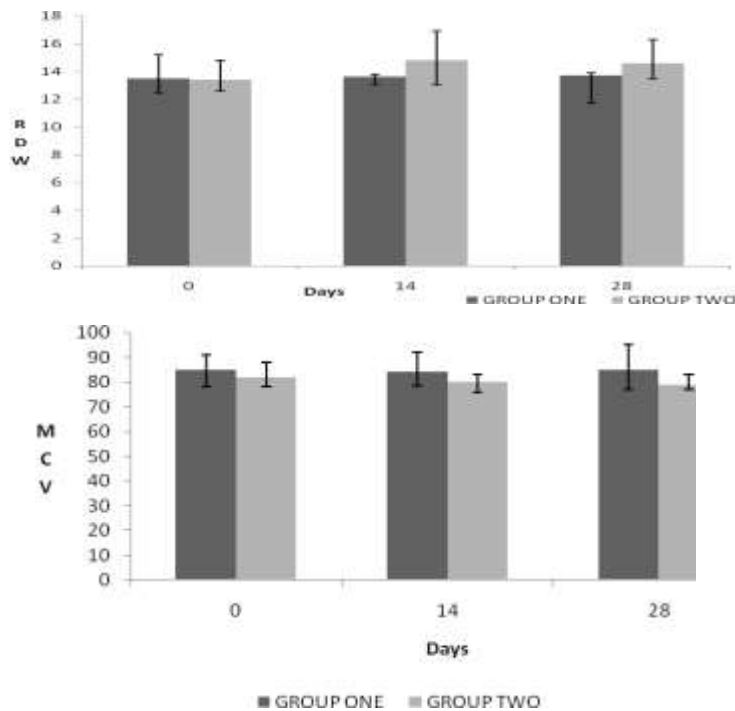
In the *Chinsaga* treated arm, there was a noticeable increase in the RBC count from baseline. An increase was also noted for the Kale treated arm but the rate of increase seemed slower, **Figure 5.3**.

### 5.3.3.3 Longitudinal changes in the Mean Copurscular volume and Red Bloodcell diameter

These two parameters were considered together because they are indicators of cause of anemia. High values are indicative of macrocytic anemia caused by deficiency of either Vitamin B12 and/or folic acid. **Figure 5.4** presents the changes in these two parameters.

For the rest of the variables (RBC, MCV, RDW, HC) changes occurred between groups.

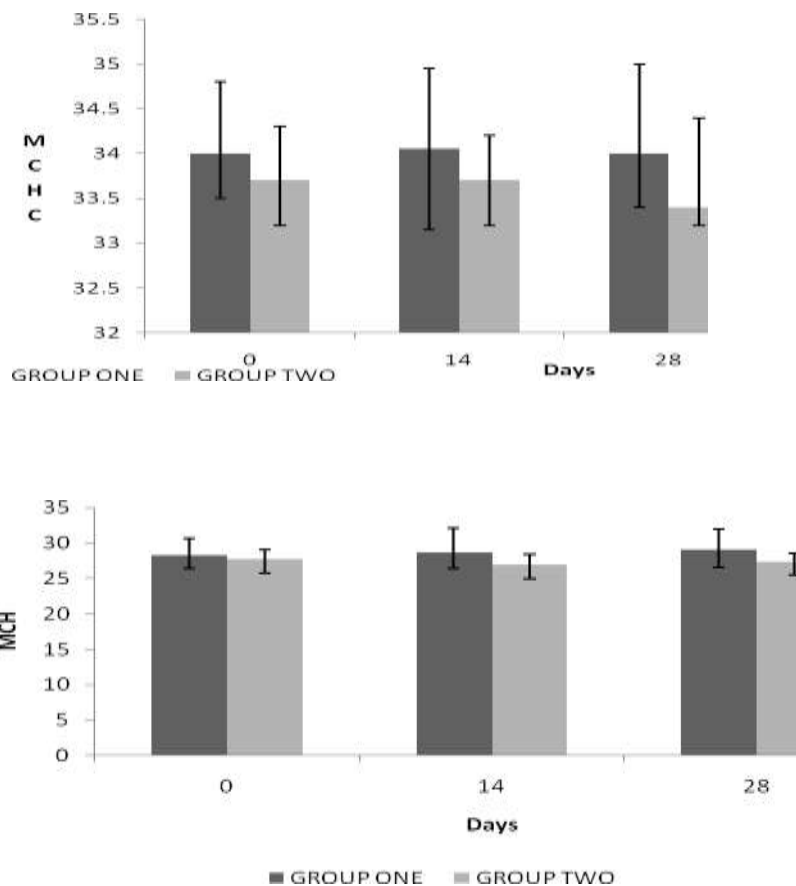
MCV decreased with time and this was particularly noticeable for participants treated with *Chinsaga*. Contrary to the expectation the RDW increased with time unlike the decrease observed with MCV during the four weeks. By Day 28, there was a statistically significant difference across arms.



**Figure 5 4:** Changes in the levels of MCV and RDW. Group one (Kale), Group two (Chinsaga).

### 5.3.3.4 Longitudinal changes in Mean Corpuscular Hemoglobin and Mean Copurscular Hemoglobin Concentration

MCHC and MCH declined with time in the *Chinsaga* treated arm compared to Kale treated arm where it remained fairly constant, **Figure 5.5**. This decrease could be attributed to the rapid increase in the RBC count in patients treated with *Chinsaga* without a corresponding increase in Hb. MCHC is calculated by dividing MCH with the RBC and is the most reliable measure of anaemia. In the case of MCH, the difference between the two arms was not as obvious as the difference showed by MCHC.



**Figure 5 5:** Longitudinal changes in MCH and MCHC. Group one (Kale), Group two (*Chinsaga*).

### 5.3.3.5 Effects of loss to follow up on hematological parameters

To determine if differential loss to follow may have caused systemic differences across the two arms, the baseline hematological parameters of participants who came for Day 14 were compared. There was no statistically significant difference as shown in **Table 5.2**. The only exception to this observation was MCHC for which there were differences across arms as was seen for entire group of participants. This showed that the differences across arms in Day 14 could not be attributed to differential loss to follow but rather to the effects of the two treatments.

**Table 5 2:** Comparison of baseline hematological parameters across treatment arms for patients who come for the Day 14

Blood parameter	Treatment One: Kale (N= 18)	Treatment Two: Chinsaga (N= 25)	Pooled (Kale+Chinsaga) (N= 43)	P
	Median (IQR)			
Hematocrit	35.3 ( 30.5 ,42.9)	38.5 ( 34.5, 41.1)	38.1 (32.9 , 41.2)	0.77
Hemoglobin		12.9 (11.6, 13.8)	12.9 ( 11.1, 13.8)	0.94
RBC	4.275 (3.79, 4.98)	4.58 ( 4.48 , 4.89)	4.54 (4.22, 4.98)	0.09
MCV	85.5 (79, 89)	81 (80, 85)	83 (79, 89)	0.08
RDW	13.55 (13.9, 15.9)	13.4 (12.8 , 15.5)	13.5 (12.8, 15.6)	0.50
MCH	29.3 (26.4, 30.5)	27.8 (26.7, 28.6)	28 (26.4, 29.5)	0.06
MCHC	34 (33.6, 34.6)	31 (33.2 – 33.8)	33.6 ( 33.3, 34.3)	<b>0.02</b>

### 5.3.3.6 Bivariable and Multivariable analysis of the effects of treatment by Generalised Linear Model

Bivariable analysis of the effects of *Chinsaga* treatment on selected hematological parameters was carried out using generalized linear modeling. The following variables were selected for more detailed analysis because the differences were statistically significant across arms during the treatment visits: RBC; MCH; RDW and MCHC.

Shapiro-wilk test showed that not all of the outcome variables were normally distributed. Consequently, they were transformed. The only explanatory variables that were considered was treatment assignment and duration of treatment. In addition interaction between treatment duration was evaluated. The output of regression analysis is presented in **Table 5.3**.

The findings were similar to the previous observations that treatment assignment had no statistically significant effect on hemoglobin levels and haematocrit. Unlike the previous analysis, there was no effect on RDW. Treatment assignment had statistically significant effects on RBCs and Mean Corpuscular Haemoglobin (MCH).

**Table 5 3:** *Generalized Linear Modeling – Bivariable analysis of the effects of treatment on selected hematological parameters*

<b>Blood Parameter</b>	<b>Transformation</b>	<b>P value of the treatment variable (MODEL ONE)</b>	<b>P value of interaction term between Duration and Treatment. MODEL TWO</b>
Hemoglobin	Cubic	0.64	P<0.01
Hematocrit	Cubic	0.23	
RBC	Cubic	0.02	
MCV	None	No convergence	
RDW	Reciprocal of square	0.10	
MCH	Cubic	<0.01	
MCHC	None	No convergence	-

Model One has only treatment group as the dependent variable. Model Two has treatment, visit and an interaction term between visit and treatment. The outcome variable, RBC was selected for further analysis because there were statistically significant differences across treatment groups and was unaffected by changes in corpuscular Haemoglobin. The association between this variable and other potential predictors was evaluated by bivariable generalized linear regression modeling. All variable with P values was less than 0.1 were used to adjust for confounding in multivariable analysis. The results are presented in **Table 5.4**.

On bivariable analysis, socio-demographic and nutritional foods were assessed for positive or negative association with the RBC. The socio-demographic variables for which there was a statistically significant positive association with outcome variable were: time lived in the current residence and age of the husband. On bivariable analysis, there was a statistically significant negative association between the marital status and RBCs (P=0.05). The foods for which there was a statistically significant positive association with RBCs were: Black beans (*Njahi*); pineapples, avocado, maize meal (*Ugali*) and cocoa. On adjusting for confounding, most of these variables were not statistically significant. The only variables that remained statistically significant on multivariable analysis were: cocoa, number of visits and the interaction term between treatment and number of visits. There was a negative association between cocoa consumption and RBCs.

**Table 5 4:** Bivariable and multivariable generalized linear regression analysis of the effects of covariates on the RBCs of lactating mothers on Chinsaga and Kale over 28 days treatment

Variable	Bivariable Analysis			Multivariable analysis		
	Coefficient	SE	P-value	Coefficient	SE	P-value
Treatment	1.794	0.745	0.02	30.646	30.275	0.31
Visit	0.944	0.481	0.05	5.112	2.304	<b>0.03</b>
Interaction term between visit and treatment	-	-	-	-2.767	1.250	<b>0.03</b>
Duration current residence	5.829	0.315	<0.01	1.834	1.524	0.23
Marital status	-2.307	1.161	0.05	-	-	-
Age of husband	0.241	0.078	<0.01	-1.134	0.982	0.25
Took iron tablets	-0.036	0.755	0.96	-	-	-
Duration iron intake	-1.483	0.732	0.04	10.565	21.023	0.62
Shares bed with baby	-6.347	1.68	<0.01	5.166	4.381	0.24
Black beans (Njahe)	1.476	0.758	0.05	7.200	6.110	0.24
Pineapple	1.417	0.768	0.07	7.659	7.332	0.30
Avocado	1.720	0.841	0.04	-3.866	10.385	0.71
Melon	1.881	0.759	0.01	-3.230	5.429	0.55
<i>Ugali</i> (Maize meal)	9.154	1.698	<0.01	-	-	-
Beneficial food 9	5.127	2.262	0.02	3.801	4.077	0.35
Tea	0.714	0.921	0.44	-	-	-
Coffee	1.716	1.002	0.09	14.565	10.101	0.15
Cocoa	1.824518	0.744	0.01	-6.735	1.827	<b>0.00</b>
Uji (Finger millet porridge)	-1.587606	0.867	0.07	1.977	2.945	0.50
Soup	-1.568408	0.757	0.04	-8.419	4.939	0.09
Herb 12	-9.054	5.042	0.07	-	-	-

For the following variables, the P values were greater than 0.05 but less than 0.1: Amaranth (*terere*) (0.07), pumpkin leaves (0.08), pineapple (0.07) and coffee (0.09).

### 5.3.4 Assessment of the Biological interaction between iron and *Chinsaga* consumption

The first covariate that was considered as a potential interaction variable was patient reported duration of iron intake. This variable was given priority because it is a known hematinic agents and it was plausible that there was biological interaction between the effects of *Chinsaga* and iron intake. On forward stepwise model building it was a key determinant of changes in RBC count, **Table 5.5**.

**Table 5 5:** Evaluation of the interaction between the duration of iron intake and treatment with *Chinsaga*

Variable	Parsimonious model without interaction term			Parsimonious model with interaction term		
	Beta coefficient	SE	P-value	Beta coefficient	SE	P-value
Treatment	0.834	1.195	0.49	-5.820	3.411	0.09
Number of visits	1.815	0.755	0.02	1.709	0.741	0.02
Duration of treatment with iron	-1.424	0.715	0.05	-6,148	2.380	0.01
Interaction term*	-	-	-	2.967	1.429	0.04

\*Interaction between duration of iron intake and treatment with *Chinsaga*

Clearly there was confounding and statistical interaction between *Chinsaga* and duration of intake of iron. The interaction was considered to be biological because statistical interaction in a linear model was additive which is one of the pre-requisites for demonstration of biological interaction. This interaction was qualitative because direction of association between treatments with *Chinsaga* changed with duration of treatment with iron.

One-way sensitivity analysis was conducted to determine the threshold duration of iron intake at which *Chinsaga* began to have a beneficial effect **Table 5.6** . The beta coefficient of the treatment variable was negative if patient reported duration of iron intake was less than about 1.5 to 2 months.

**Table 5 6:** *One way sensitivity analysis of the effect of patient reported duration of intake of iron supplements on the beta coefficient of the effect of treatment with Chinsaga on RBC*

Duration on Iron supplements (months)	Beta coefficient of the Treatment variable
>3	83.20
3	3.0820
2	0.115
1	-2.85
0	-5.820

In addition, confounding and interaction with other covariates was explored and the results are presented in **Table 5.7**. Analysis was restricted to variables for which there was a statistical significant association with RBC count on bivariable analysis. The variables marital status, *Ugali*, traditional herbs, coffee, *uji* displayed collinearity and were therefore dropped.

The only variables that caused at least 10 % change in the crude beta coefficient of the association between treatment and RBC counts were the use of bone soup and water melons. The apparent confounding effect of water melons and avocados was attributed to selection bias because when the consumption of various foods across arms was conducted more participants in the *Chinsaga* arm reported past consumption of water melons (P=0.01) and avocados (P=0.03) compared to those in the Kale arm. The sample size was therefore too small to make any conclusive inferences.

It was also difficult to determine whether the effect of soup was due to selection bias because difference in consumption across arms was almost statistically

significant (P=0.08). Consequently patient reported consumption soup seemed to be only confounder in the association between treatment with *Chinsaga* and RBC.

**Table 5 7:** Evaluation for biological interaction and confounding between *Chinsaga* and other covariate use

Second variable	Coefficient	SE	P value	P-value of interaction term
Treatment	1.653	0.745	0.03	0.13
Visit	0.828	0.479	0.08	
Treatment	1.684	0.749	0.03	collinearity
Herb 12	-8.077	5.005	0.11	
Treatment	1.725	0.736	0.02	0.74
Cocoa	1.757	0.736	0.02	
Treatment	1.621	0.754	0.03	0.23
Coffee	1.342	1.008	0.18	
Treatment	2.108	0.744	0.01	<b>0.05</b>
Soup	-1.921	0.753	0.01	
Treatment	1.650	0.747	0.03	0.92
Uji (Porridge)	-1.353	0.864	0.12	
Treatment	1.276	0.817	0.12	0.97
Melon	1.435	0.808	0.08	
Treatment	1.916	0.752	0.01	0.16
Black beans (Njahi)	1.499	0.747	0.05	
Treatment	1.879	0.690	P<0.01	0.11
Ugali (Maize meal)	9.255	1.669	P<0.01	
Treatment	1.890	0.740	0.01	0.13
Amaranth ( <i>terere</i> )	-1.833	0.921	0.05	
Treatment	1.525	0.783	0.05	0.44
Avocado	1.372	0.853	0.11	

When the duration of iron consumption was added as the third explanatory to the models presented in **Table 5.7**, all other variables lost statistical significance.

## 5.4 Discussion

The aim of this study was to establish if there is any improvement of selected hematological parameters by including *Chinsaga* in the diet. The key findings of this study were participants in the arm treated with *Chinsaga* had

statistically significant higher RBC counts on Day 14 and 28 compared to patients in the Kale treated arm. There was qualitative biological interaction between patient reported duration of intake of iron supplements and treatment with *Chinsaga* and was effective in participants who had taken iron for at least one month.

For the outcomes, hematocrit and hemoglobin there were no statistically significant differences across the treatment arms from the baseline to Day 28. For the rest of the variables (RBC; MCV, RDW, HCTC) changes occurred between groups.

Looking at the MCHC, it was observed there was a statistically significant difference across the two arms in the baseline. This difference was reflected in the sub-population that came for Day 14. On Day 28 there was no statistically significant difference across the two arms which suggested that one treatment was curative and improved indices in the arms that systemically had low levels. Mothers with anemia raise the risk of having complications during pregnancy and delivery and undesirable pregnancy outcome.

However, it was noted, time of residence in current location was very highly significance ( $P < 0.01$ ). This suggests that the longer a woman has spent in her current residence the better her values of RBC count. The question arises then, what is it about staying in one location that would have a relationship with the state of a woman's RBC levels?

Several factors may be involved. It may be speculated that the longer one lives in an environment, the more likely they are to have overcome initial challenges which include sourcing of resources and especially where they get affordable foods. In addition, it may be related to the establishment of networks among the women that provide social needs that translate into intangible resources that further support her in such a manner impacting on her health. It may also

be true that living in one location for long also enables one identify protective behavior patterns and minimization of exposure to elements of harm. This may need to be investigated further.

On bivariable analysis, marital status ( $P=0.05$ ) and especially age of husband ( $P<0.01$ ) were positively associated with RBC counts. Social aspects evidently have an important contribution to the welfare of the women. These too may be tied to the benefits of protection and social stability in influencing the health of women. Sharing the bed with the baby and eating maize meal (*Ugali*) were also strongly correlated with RBC count ( $P<0.01$ ).

Studies have been done on the effects of rooming in on mother and babies in hospital settings. Evidence shows that rooming in largely benefits the mother relationship with the baby by allowing more skin contact and easier placing of the baby on the breast by the mother. However, there is also the indication that the mothers tend to have less sleep due to a raised consciousness of the baby losing their depth of sleep as they remain sensitive to turning over the baby.

In the home setting, the mother may have similar challenges as the hospital, but the factors here are different because of the existence of the father of the baby on the same bed. Depending on the size of the bed, the baby may be a source of duress for the mother as she tries to squeeze into the space of the bed

This then raises the question, what does living in an area for long, having a more mature husband, sleeping with the baby; eating melon and drinking cocoa have in common? Are they surrogate indicators of behavior that results in improved maternal RBC count? If so what are they? Again, this is an opportunity to conduct further social investigations on the women using these parameters to determine the common aspects in their environment or behavior that relates with the RBC count.

Surprisingly, education ( $P=0.90$ ), ethnicity ( $0.80$ ), occupation ( $P=0.58$ ), of the woman and less so the husband ( $P=0.3$ ) had no statistically significant association with RBC counts. This was unexpected since these are normally expected to influence nutritional behavior and consequently hematological parameters. Education of women is particularly important in health circles in relation to maternal health outcomes .

Evaluation of the effects of data collected in the study revealed that it is prudent to include socioeconomic and anthropological factors in the forecast during interpretation of data especially in a varied society as that found in Kenya. Results of hemoglobin levels showed much less change over the three visits as observed in the p value. Hemoglobin is often used as simple measure of iron status and is widely used for classification of anemia.

The Shapiro Wilk test showed that none of the outcome variables was normally distributed. Most needed transformation for normality to be achieved. By evaluating the cumulative results of RDW, correlation was observed with other parameters. One advantage with the use of this parameter is the ease of application in rural laboratory settings.

Looking at the treatment groups, the mean Hb value for the *Chinsaga* group appeared to increase more steadily than the Kale group where the value seems to rise and fall back to baseline value within the four weeks. Assuming the effect is consistent over time, this change in MCHC may yet be the most sensitive indicating a diminishing difference in the two groups over time. However, there is also the possibility MCHC may be changing due to a natural tendency for the body among the women to correct for changes in blood levels due to losses after delivery. In this case the effect observed in the study would need to be evaluated in a separate study involving women that are neither pregnant nor well beyond the initial months post delivery.

## **5.5 Conclusion**

Through our present study, the results confirm that *Chinsaga* consumption increases iron uptake during supplementation. It may thereafter be formulated as an adjunct in iron supplementation to postpartum women. Overall, additional studies should be carried out to elucidate the mechanism for the observed effects on RBC count and MCHC and the extent of influence on cellular markers of iron metabolism.

This study therefore recommends the conduct of similar studies on other traditional vegetables consumed by various communities in Kenya as a way of preserving the knowledge and promoting their incorporation into the diet for the benefit of mankind.

In this clinical trial surrogate makers were used as endpoints. Challenges and problems faced were in: recruitment and retention, ambiguous situations, time and resource limitations, laboratory equipment, data management, assessment of safety and dose response determinations translating this clinical trial into a proof of concept study.

## **5.6 Study limitations**

Specific methods of diagnosis of iron deficiency such as ferritin levels was not elucidated in this study but may form a useful adjunct to include in future investigations of the *Chinsaga* effects. Dehydration as well as over-hydration may alter the values of the measurement and were not controlled for in this study.

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## CHAPTER SIX

# 6.0 Effects of *Chinsaga* on Biomarkers of Iron Metabolism in Serum and Breast milk

## **6.1 Introduction and background**

*Gynandropsis gynandra* (Chinsaga) is a rich source of beta carotene ( a pro-vitamin A),with its content, ranging between 6.7 -18.9 g per 100 g of vegetable (1). Vitamin A supplementation has an effect on iron metabolism. Bloem *et al* demonstrated that biochemical indicators of Vitamin A status including retinol and retinol-binding protein correlated with biomarkers of iron metabolism such as serum Iron and percentage saturation of transferrin (%ST). However, this difference in retinol, retinol-binding protein, serum Fe, and % Saturation of Transferrin (%ST) diminished after 4 months (2).

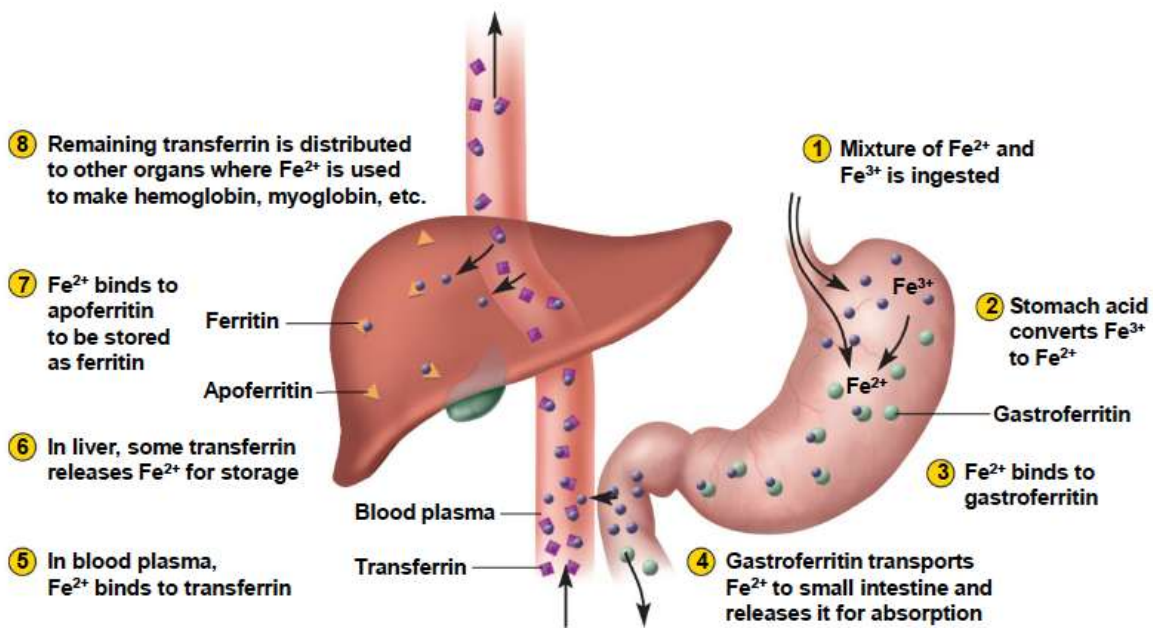
### **6.1.1 Biomarkers of Iron Metabolism**

According to the National Cancer Institute in America, the term biomarker refers to: “a biological molecule found in blood, other body fluids, or tissues that are a sign of a normal or abnormal process or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Biomarkers are also called molecular marker and signature molecule”(3). The US National Institutes of Health defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”(4) .

Biomarkers can therefore be viewed as tools to improve understanding about diseases and process from initial to the final state. They may help monitor progression and management of diseases. Depending on the focus, biomolecules as biomarkers can be classified into: (i) Genomic biomarkers: based on the analysis of DNA (deoxyribonucleic acid), (ii) Transcriptomic biomarkers: based on the analysis of RNA expression profiles, (iii) Proteomic biomarkers: based on the analysis of the protein profiles and (iv) Metabolomic biomarkers: based on the analysis of metabolites. Alternatively, certain

biomolecules may be associated with absence of a state or condition and referred to as “negative biomarker”.

Iron metabolism can be monitored using diverse biomarkers (Figure 6.1). In this study, transferrin, and ferritin were chosen as indicators in the blood and lactoferrin to follow changes in iron metabolism in breastmilk.



**Figure 6 1:** The physiology of iron metabolism in humans (5). Step 1 to 8 shows biomarkers that are indicators of absorption, transport, and storage of iron from diet.

Ferritin and transferrin were chosen as biomarkers for this study on the basis of ease of analysis, significance in the processes of iron metabolism under investigation and cost of analysis. Lactoferrin was also similarly selected to aid in determining effects on iron that reaches the baby through the breast milk. Each of the selected biomarkers shall be described.

### **6.1.2 Transferrin**

Human transferrin is a serum glycoprotein of about 80 kDA which complexes iron, and transports it through the circulatory system. In people with low iron levels, more transferrin is produced by the liver this is thought to be a compensatory reaction, to make more iron available to the body, including the brain. Iron deficiency alters dopamine metabolism in the caudate and putamen. People with high transferrin and lower brain iron might have lower volumes of dopamine containing structures, involved in learning and motor control (6).

In humans, for example, increased serum transferrin is associated with dietary iron deficiency and pregnancy (7). Experimental studies in murine models (8) have suggested that nutritional iron deficiency, thyroxin, glucocorticoids, and estrogen all cause an increase in serum transferrin specifically inducing an increase in the rate of liver transferrin synthesis.

Neither the physiological significance of these changes in serum transferrin nor the mechanism by which the liver responds to hormones and nutritional stimuli is fully understood. Elevated transferrin levels are associated with an increase in iron absorption and mobilization of iron from tissue stores, although the role of transferrin in these changes is unclear. The response to hormones, such as estrogen, may be important during pregnancy when the demand for iron by the fetus is high; transferrin specifically releases iron to the placenta and the increase in circulating transferrin may facilitate this process (9).

The liver is the main site of transferrin synthesis, but other tissues and organs, such as the brain, also produce it. The main role of transferrin is to deliver iron from absorption centers in the duodenum and white blood cell macrophages to all tissues. Transferrin plays a key role where erythropoiesis and active cell

division occur (10). The transferrin receptor helps maintain iron homeostasis in the cells by controlling iron concentrations.

Transferrin is also associated with the innate immune system. It is found in the gut mucosa and binds iron, thus creating an environment low in free iron that impedes bacterial survival in a process called iron withholding. The level of transferrin decreases in inflammation (11).

Transferrin imbalance can have serious health effects for those with low or high serum transferrin levels. A patient with an increased serum transferrin level often suffers from iron deficiency anemia (10). A patient with decreased plasma transferrin can suffer from iron overload diseases and protein malnutrition. An absence of transferrin results from a rare genetic disorder known as atransferrinemia; a condition characterized by anemia and hemosiderosis in the heart and liver that leads to complications, including heart failure. Most recently, transferrin and its receptor have been shown to diminish tumor cells by using the receptor to attract antibodies (12).

Serum transferrin plays a vital role in the transport of iron from intestinal absorption sites and sites of storage (primarily liver, spleen, and bone marrow), to reticulocytes where the iron is incorporated into hemoglobin. Transferrin is synthesized primarily in the liver, although synthesis has also been detected in the spleen and bone marrow (13). The circulating concentration of serum transferrin ranges from 1 to 2.5 mg/ml in mammals and birds; however, this level is regulated by a variety of hormones and nutritional factors.

### **6.1.3 Ferritin**

Ferritin is the major intracellular iron storage protein in all cells. It is composed of 24 subunits of the heavy and light ferritin chains. Variation in ferritin subunit composition may affect the rates of iron uptake and release in

different tissues. A major function of ferritin is the storage of iron in a soluble and nontoxic state. Defects in this light chain ferritin gene are associated with several neurodegenerative diseases and hyperferritinemia-cataract syndrome. This gene has multiple pseudogenes (14).

Increasingly, perturbations in cellular iron and ferritin are emerging as an important element in the pathogenesis of disease. These changes in ferritin are important not only in the classic diseases of iron acquisition, transport, and storage, such as primary hemochromatosis, but also in diseases characterized by inflammation, infection, injury, and repair. Among these are some of the most common diseases that afflict mankind: neurodegenerative diseases such as Parkinson disease and Alzheimer disease, vascular diseases such as cardiac and neuronal ischemia-reperfusion injury, atherosclerosis itself, pulmonary inflammatory states, rheumatoid arthritis, and a variety of premalignant conditions and frank cancers (15).

The link between alteration in ferritin regulation and these diseases is forged through a diverse set of cellular stress pathways that alter ferritin subunit composition and/or content within cells (16).

Ferritin sequesters iron in a nontoxic form, but levels of “labile” iron regulate cellular ferritin levels, protecting cells from damage triggered by excess iron. Iron-mediated, largely posttranscriptional pathways of ferritin regulation have been identified through a series of elegant experiments over the last 15 years (17–19).

The content of cytoplasmic ferritin is regulated by the translation of ferritin H and L mRNAs in response to an intracellular pool of “chelatable” or “labile” iron (20,21). Thus, when iron levels are low, ferritin synthesis is decreased; conversely, when iron levels are high, ferritin synthesis increases. Although in certain circumstances there is an increase in ferritin mRNA in response to iron,

(22) the regulatory response of ferritin to iron is largely posttranscriptional, (23) and is due to the recruitment of stored mRNA from monosomes to polysomes in the presence of iron (21).

This process is mediated by interaction between RNA binding proteins and a region in the 5' untranslated region of ferritin H and L mRNA termed the iron responsive element (IRE) that has a "stem-loop" secondary structure. There are 2 RNA binding proteins, iron regulatory proteins 1 and 2 (IRP1 and IRP2), that bind to this stem loop structure and inhibit mRNA translation. However, the proteins are regulated differently: IRP1 is an iron-sulfur cluster protein that exists in 2 forms. When iron is abundant, it exists as a cytosolic aconitase. When iron is scarce, it assumes an open configuration associated with the loss of iron atoms in the iron-sulfur cluster, and can bind the IRE stem loop, acting as a repressor of ferritin translation. In contrast, IRP2 is regulated by degradation: IRP2 protein is abundant in iron scarcity, but is degraded rapidly in iron excess through targeting of a unique 73 amino acid sequence (24). Although both IRP1 and IRP2 bind the IRE and exert an inhibitory effect on ferritin synthesis, there is evidence that IRP1 and IRP2 may have distinct tissue-specific roles (25).

IRE-IRP binding lengthens transferrin receptor mRNA half-life, ultimately lead to increased transferrin receptor display on the cell surface in situations of iron depletion (26). Thus, similar RNA-protein binding motifs can have strikingly different biologic effects when located in different positions on different genes. In the case of ferritin, IRP binding results in inhibition of translation, whereas in the transferrin receptor, IRP binding increases transferrin receptor mRNA half-life (26). Transcription of the human ferritin H gene is induced in response to both hormones and second messengers, including cAMP. The **cis**-acting elements mediating these responses have mapped to a relatively small region in the proximal promoter of the human ferritin H gene (27).

Thyroid hormone may also regulate ferritin posttranscriptionally: T3 modulates the activity of IRP1, affecting its ability to bind to the ferritin IRE, possibly through induction of signal transduction cascades that result in phosphorylation of IRP1 (28). T3 and TRH also induce the phosphorylation of IRP2 (29).

In addition, the shorter circulation time for reticulocytes of one to two days means the changes in Reticulate Haemoglobin Content (CHr) and percentage of hypochromic red blood cells (%HYPOm) is more responsive to changes in availability of iron for erythropoiesis. However, those based on red blood cell with a lifespan of 120 days are the established markers of iron status over the longer duration (30).

It is worth noting that the various parameters reflect different aspects of iron deficiency. While cellular indices reflect iron deficient erythropoiesis, biochemical markers reflect iron availability for erythropoiesis (TfR), iron stores (ferritin), or circulating iron bound to transferrin (TfSat). It is known that iron and transferrin have biological variability, and ferritin reacts as an acute phase reactant (31). However, %HYPOm has been shown to be unaffected by physiologic inflammatory processes in the postpartum period(32).

Consistent with earlier studies (33), the mean value of ferritin was low in pregnant women at term, yet only a few with low ferritin had produced iron deficient erythrocytes. Therefore, it is likely that the reduction in serum ferritin does not exclusively reflect exhausting iron stores. The increase in TfR concentration during pregnancy is assumed to be a consequence of accelerated erythropoiesis (34), which makes it difficult to judge whether an elevated concentration is due to an increased demand for iron or an enhanced rate of erythropoiesis.

#### 6.1.4 Lactoferrin

Lactoferrin, a non-haem iron-binding glycoprotein, is mainly found in milk. It is a major protein in the secondary granules of neutrophils and is present in many biological secretions including the saliva, tears and semen. The protein has been shown to kill bacteria, play an immunomodulatory role and participate in inflammatory response (35–37). However, its mechanism of action is presently unknown. Lactoferrin expression is up-regulated by both estrogens and epidermal growth factor (EGF) (38).

Estrogen regulation of lactoferrin gene expression in the uterus is well established (38). However, there are inconsistent reports on estrogen regulation of the lactoferrin gene in human endometrium (39–42). Estrogen receptor binds to the imperfect ERE of human lactoferrin gene both in isolation and in the context of the gene promoter region. Human lactoferrin gene is activated in transiently transfected human endometrial carcinoma cells through an ER-mediated process and that the imperfect ERE of the gene is required (40). In addition to the *in vitro* physical interaction between ER and the ERE of the human lactoferrin gene, lactoferrin gene expression in the endometrium fluctuates during the menstrual cycle. In Western blotting experiments, lactoferrin has been detected in the proliferative phase but not in the secretory phase, suggesting that estrogen induces and progesterone suppresses lactoferrin gene expression *in vivo*. This provides a relatively homogeneous cell population.

The lactoferrin of rhesus monkey has high similarity to the human protein in both amino acid composition and carbohydrate moiety (43). Moreover, monkey lactoferrin, like human lactoferrin, has an unusual amino acid sequence at the N-terminus which is essential for binding to bacterial lipopolysaccharide and to the mammalian lactoferrin receptor (37,44), indicating that monkey lactoferrin could function similarly to the human protein. The reproductive physiology of

the female rhesus macaque monkey is also highly comparable to that of women (43). However, the polyclonal antibody produced against human lactoferrin does not cross-react with the monkey milk lactoferrin nor does it detect any immuno-reactive protein in the endometrial tissue.

Little is known about the factors that influence milk lactoferrin levels, although it is known that concentration decreases during lactation (36), Zavaleta *et al*, (45) found the concentration of lactoferrin had decreased by day 30 of breastfeeding. In a study by Fransson *et al*. (1985) (46) , lactoferrin levels were found to be high in severely anemic women; however, the number of subjects was very low. Houghton *et al*, (46) studied lactoferrin levels in Aborigine women in Australia and found lower levels in milk from women with poor nutritional status than in milk for well nourished women.

#### **6.1.5 Objectives**

This study sought to determine the effect of *Chinsaga* consumption on biomarkers of iron metabolism ferritin and transferrin in serum and lactoferrin in breastmilk over a 28 day period.

#### **6.1.6 Specific Objectives**

1. To establish the effect of *Chinsaga* treatment on expression of the Transferrin gene in plasma
2. To establish the effect *Chinsaga* consumption on expression of the Ferritin gene in plasma
3. To establish the effect *Chinsaga* consumption on expression of Lactoferrin gene in breast milk

## **6.2 Materials and Methods**

Blood samples of about 4 ml were drawn from the antecubital vein using a needle 21G and syringe at each visit from each volunteer from *Chinsaga* group and control group. The blood was then emptied into EDTA tubes (Beckton Dickinson, Germany) and delivered to the hematology laboratory at KAVI. This was done at baseline and during the follow up after delivery. As long as the mother was willing to participate and was breast-feeding, blood samples were taken at midmorning (9: 00 to 11:00 AM) during the clinic visits (day 0, 14 and 28). The samples were used to run full haemogram after which the serum was separated. The serum obtained was then divided into two aliquotes for storage at -70 C in serum vials until the time of analysis.

### **6.2.1 Extraction of transferrin mRNA**

Human serum, 50 $\mu$ l, was drawn into a 1.5ml eppendorf tube. Lysis buffer, 100 $\mu$ l, was then added and incubated at 37<sup>0</sup>C for 30 minutes in a thermomixer shaking at 1000rpm. The lysates were homogenized by vortexing briefly. Flow was applied through the RNeasy column, it was then subjected to two washes with the first wash using 100 $\mu$ l of wash buffer I and the second wash using 200 $\mu$ l of wash buffer II. 50 $\mu$ l of RNA was eluted in RNA free water. RNA, 10 $\mu$ l, was subjected to DNaseI digestion by adding 1 $\mu$ l of DNaseI then 1 $\mu$ l of DNase inhibitor.

### **6.2.2 Synthesis of transferrin cDNA**

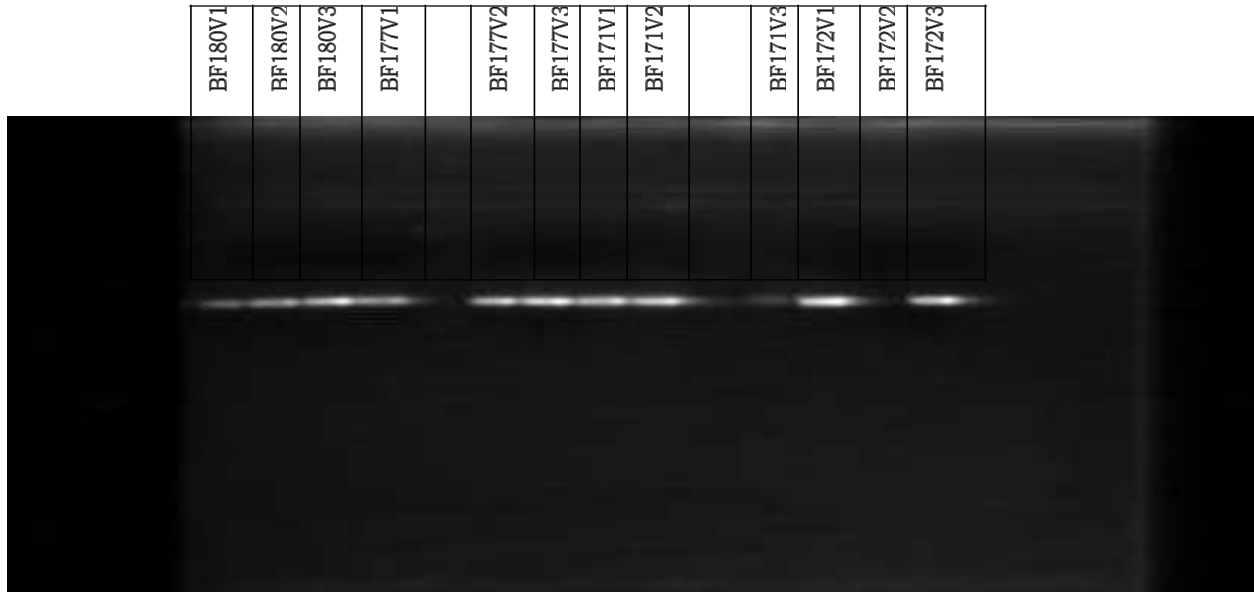
A master mix containing 1 $\mu$ l random primers, 1 $\mu$ l annealing buffer, 10 $\mu$ l RNase free water and 3 $\mu$ l of the sample RNA. This mixture was incubated in a thermocycler machine (BioRad, UK) at 65<sup>0</sup>C for 5 minutes then immediately placed on ice for 1 minute. The contents of the tube were then centrifuged briefly. The 2X first strand reaction mixture containing 10 $\mu$ l superscript III

were then added. RNase out enzyme, 2µl, was then added. The sample mixture was then incubated at 50°C for 50 minutes, then 25°C for 10 minutes and finally 50°C for 50 minutes. The reaction was terminated by incubating the mixture at 85°C for 5 minutes. Amplification reaction mixes consisted of 1–1000 ng of template DNA, 0.5 IU of thermostable DNA polymerase, 0.2 mmol/l dNTPs, 1.5–3.0 mmol/l magnesium chloride, 10–30 pmoles of primers and PCR reaction buffer (20 mmol/l Tris–HCl, pH 8.4 and 50 mM KCl), in a final volume of 20 µl. All samples were initially denatured at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min. A final extension step of 72°C for 5 min was included. All PCR reactions were performed on a pre-programmed automated DNA thermal cycler (BioRad, UK). Using primers with TF1 5' GGG TGG AACT ATACT CCACTG ATG GG 3' as the forward primer and TF2 5'AAA GCGC CCAT GTGA TAGA CC 3' a 419 bp fragment of the transferrin gene from human serum RNA was amplified (47).

### **6.2.3 Results of gel electrophoresis of transferritin**

Nested PCR product, five microlitres, was mixed with a 1-2 µl of 10× loading dye and analyzed by agarose gel electrophoresis on 0.8% agarose gel stained with Ethidium bromide (0.5 µg/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 30 minutes. DNA was visualized by ultraviolet transillumination.

The **Figure 6.2** is an example of a typical gel after development and photography of the results from electrophoresis of the transferrin gene enhanced through PCR. Volunteer numbers are indicated on top of the image.



**Figure 6 2:** Gel after development and visualization of amplified Transferrin genes from human blood using a transilluminator (an ultraviolet lightbox), showing ethidium bromide-stained DNA in gels as bright glowing bands.

### 6.2.3.1 Analysis of Transferrin Gene

Tranferrin gene during baseline, table 6.1 at the beginning of a one month period of using *Chinsaga* in the diet, was generally weakly expressed with the mean being 2.02 at 95% confidence interval of between 3.77 and 0.266 (**Table 6.1** below). Out of the 113 patients whose serum tranferrin levels were analysed, 39 (34.5%) showed no expression, 36 (31.9%) had weak expression of the tranferrin gene, 35 (31.0%) had normal gene expression while 3 (2.6%) showed over expression of the gene. Levels of expression were categorized as none, weakly expressed, normally expressed and over expressed against an intensity scale.

**Table 6 1:** Percentage change in the average gene expression of Transferrin as determined by PCR cDNA template amplification at baseline

<b>Level of tranferrin gene expressed at baseline</b>	<b>Frequency</b>	<b>Percentage</b>	<b>Cumulative Percentage</b>
None (1)	39	34.5	34.5
Weakly Expressed (2)	36	31.8	66.3
Normally Expressed (3)	35	31.0	97.3
Over Expressed (4)	3	2.7	100
TOTAL	113	100	

During the day 14 of using *Chinsaga*, 44 samples were analysed for transferrin expression, **Table 6.2**, most of the patients did not turn up for the follow up even after making effort in persuing them. There was a slight increase in the level of tranferrin gene expression at a mean of 2.25 and 95% confidence interval. The confidence interval ranges from 4.23 to 0.267. Out of the 44 samples analysed, 14 (31.8%) showed no expression, 8 (18.2%) had weak expression of the tranferrin gene, 18 (43.2%) had normal gene expression while 3 (6.8%) showed over expression of the gene.

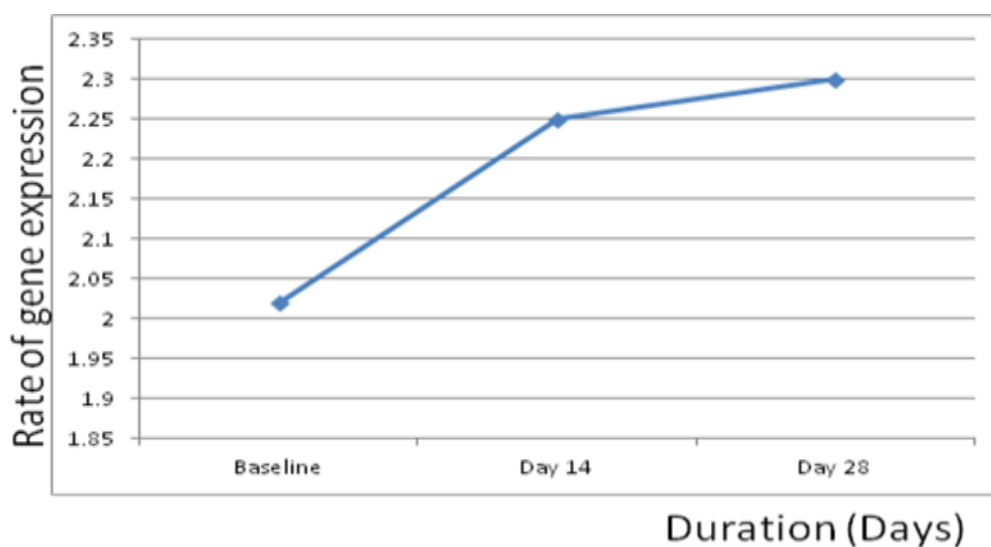
On Day 28, of using *Chinsaga* in the diet, 33 samples were analysed for transferrin expression, **Table 6.3** and **Figure 6.2**, some patients did not turn up for the follow up even after making every effort to persue them. Again a slight increase in the level of tranferrin gene expression was observed at a mean of 2.3 and 95% confidence level. The confidence interval ranges from 4.49 to 0.170. Out of the 33 samples analyzed, 10 (30.3%) showed no expression, 7 (21.2%) had weak expression of the transferrin gene, 11 (33.3%) had normal gene expression while 5 (15.2%) showed over expression of the gene (**Figure 6.3**). The difference was statistically significant with analysis using the F40 -test giving a value of 2.6, a p value of 0.00669. The calculated F40 at 95% confidence level test is greater than the tabulated value which is 1.91.

**Table 6 2:** Percentage change in the average gene expression of transferrin as determined by PCR cDNA template amplification on Day 14.

Level of transferrin gene expressed at Day 14	Frequency	Percentage	Cummulative Percentage
None (1)	14	31.8	31.8
Weakly Expressed (2)	8	18.2	50.0
Normally Expressed (3)	19	43.2	93.2
Over Expressed (4)	3	6.8	100
TOTAL	44	100	

**Table 6 3:** Percentage change in the average gene expression of transferrin as determined by PCR coda template amplification in visit three

Level of transferrin gene expressed at Day 28	Frequency	Percentage	Cumulative Percentage
None (1)	10	30.3	30.3
Weakly Expressed (2)	7	21.2	51.5
Normally Expressed (3)	11	33.3	84.8
Over Expressed (4)	5	15.2	100
TOTAL	33	100	



**Figure 6 3:** Change in the average Transferrin gene expression on days; 0,14 and 28 as determined by PCR cDNA template amplification.

#### **6.2.4 Extraction of ferritin mRNA**

Human serum, 50 $\mu$ l, was drawn into a 1.5ml eppendorf tube. Lyses buffer, 100 $\mu$ l, was then added and incubated at 37 $^{\circ}$ C for 30 minutes in a thermomixer shaking at 1000rpm. The lysates were homogenized by vortexing briefly. Flow was applied through the RNeasy column, it was then subjected to two washes with the first wash using 100 $\mu$ l of wash buffer I and the second wash using 200 $\mu$ l of wash buffer II. 50 $\mu$ l of RNA was eluted in RNA free water. RNA, 10 $\mu$ l, was subjected to DNaseI digestion by adding 1 $\mu$ l of DNaseI then 1 $\mu$ l of DNase inhibitor.

##### **6.2.4.1 Synthesis of ferritin cDNA**

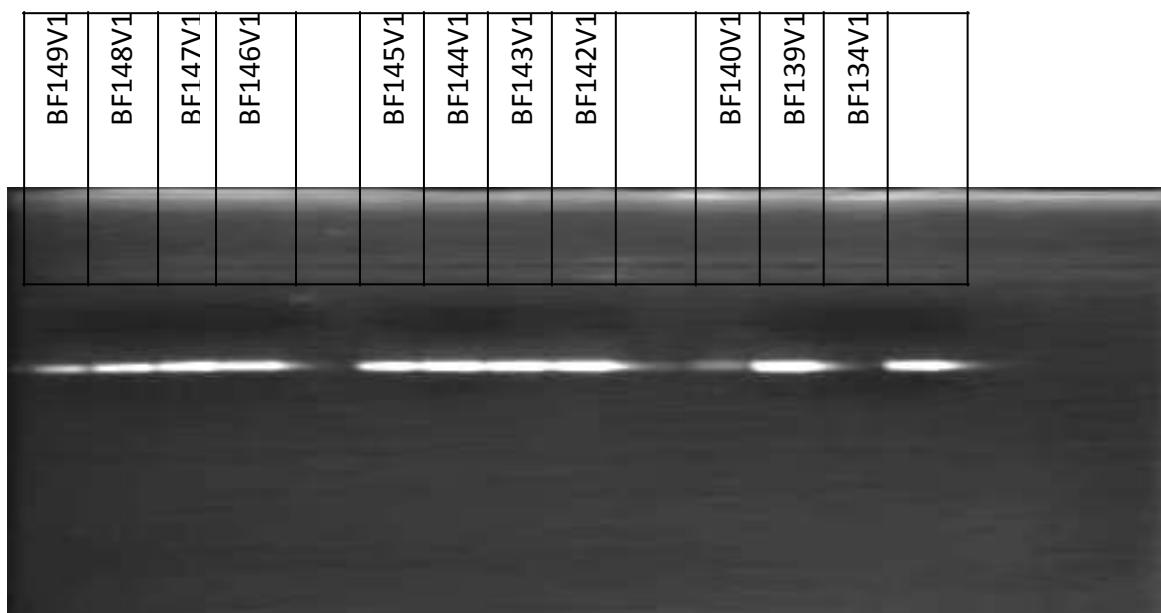
A master mix containing 1 $\mu$ l random primers, 1 $\mu$ l annealing buffer, 10 $\mu$ l RNase free water and 3 $\mu$ l of the sample RNA was incubated in a thermocycler machine (BioRad, UK) at 65 $^{\circ}$ C for 5 minutes then immediately placed on ice for 1 minute. The contents of the tube were then centrifuged briefly. The 2X first strand reaction mixture containing 10 $\mu$ l superscript III were then added. 2 $\mu$ l RNase out enzyme was then added. The sample mixture was then incubated at 50 $^{\circ}$ C for 50 minutes, then 25 $^{\circ}$ C for 10 minutes and finally 50 $^{\circ}$ C for 50 minutes. The reaction was terminated by incubating the mixture at 85 $^{\circ}$ C for 5 minutes. Amplification reaction mixes consisted of 1–1000 ng of template DNA, 0.5 IU of thermostable DNA polymerase, 0.2 mmol/l dNTPs, 1.5–3.0 mmol/l magnesium chloride, 10–30 pmoles of primers and PCR reaction buffer (20 mmol/l Tris-HCl, pH 8.4 and 50 mM KCl), in a final volume of 20  $\mu$ l. All samples were initially denatured at 94 $^{\circ}$ C for 5 min followed by 30 cycles of 94 $^{\circ}$ C for 1 min, 53 $^{\circ}$ C for 2 min and 65 $^{\circ}$ C for 3 min. A final extension step of 65 $^{\circ}$ C for 6 min was included. All PCR reactions were performed on a pre-programmed automated DNA thermal cycler (BioRad, UK). Using primers with FT1 5'ATGAGCTCCCAGATTCGT 3' as the forward primer and FT2

5'TTAGTCGTGTTGAGAGTGAG 3' a 431 bp fragment of the ferritin gene from human serum RNA was amplified (18).

#### 6.2.4.2 Gel Electrophoresis of PCR results of Ferritin expression

Five microlitres of nested PCR product was mixed with a 1-2  $\mu$ l of 10 $\times$  loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with Ethidium bromide (0.5  $\mu$ g/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 30 minutes. DNA was visualized by ultraviolet transillumination.

The **Figure 6.4** is an example of a typical gel after development and photography of the results from electrophoresis of the ferritin gene enhanced through PCR.



**Figure 6 4:** Gel of amplified Ferritin genes from human blood using a transilluminator (an ultraviolet light box). Ethidium bromide-stained DNA in gels seen as bright glowing bands.

### 6.2.4.3 Analysis of Ferritin Gene

After 28 days of using *Chinsaga* in the diet, the Ferritin gene was generally weakly expressed with the mean being 1.91 at 95% confidence level and confidence interval of between 3.62 and 0.2011. Out of the 112 patients whose serum ferritin levels were analyzed, 44 (39.3%) showed no gene expression, 36 (32.1%) had weak gene expression, 30 (26.8%) had normal gene expression while 2 (1.8%) showed over expression of the gene (**Table 6.4**).

**Table 6 4:** *Percentage change in the average expression of ferritin gene as determined by PCR cDNA template amplification at baseline.*

<b>Level of ferritin gene expressed at baseline</b>	<b>Frequency</b>	<b>Percentage</b>	<b>Cumulative Percentage</b>
None (1)	44	39.3	39.3
Weakly Expressed (2)	36	32.1	71.4
Normally Expressed (3)	30	26.8	98.2
Over Expressed (4)	2	1.8	100
TOTAL	112	100	

On Day 14, that is two weeks of using *Chinsaga* in the diet, 45 samples were analyzed for ferritin levels. There was a slight increase in the level of ferritin gene expression at a mean of 2.16 and 95% confidence level. The confidence interval ranges from 3.91 to 0.400. Out of the 45 samples analyzed, 13 (28.9%) showed no expression, 13 (28.9%) had weak expression of the ferritin gene, 18 (40.0%) had normal gene expression while 1 (2.2%) showed over expression of the gene as summarized in the **Table 6.5**. Most of the patients did not turn up for the follow up even after making every effort to pursue them.

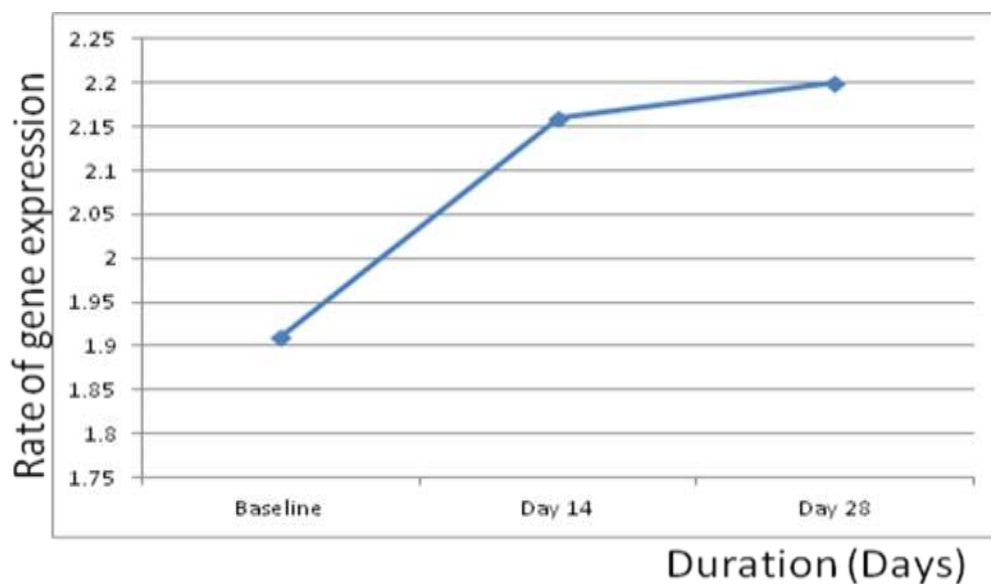
**Table 6 5:** Percentage change in the average expression of ferritin gene as determined by PCR cDNA template amplification on Day 14.

Level of ferritin gene expressed at Day 14	Frequency	Percentage	Cumulative Percentage
None (1)	13	28.9	28.9
Weakly Expressed (2)	13	28.9	57.8
Normally Expressed (3)	18	40.0	97.8
Over Expressed (4)	2	2.2	100
TOTAL	45	100	

On Day 28, some patients did not turn up for the follow up. Samples for 33 participants were analyzed for ferritin levels. Again a very slight increase in the level of ferritin gene expression was observed at a mean of 2.2 and 95% confidence level. The confidence interval ranges from 4.1 to 0.3. Out of the 33 samples analyzed, 10 (30.3%) showed no gene expression, 8 (24.2%) had weak gene expression, 13 (39.4%) had normal gene expression while 2 (6.1%) showed over expression of the gene. Ferritin gene was generally weakly expressed with the highest mean observed on Day 28 at 2.2. The increase is statistically significant  $p= 0.00751$ ,  $F_{41}$  test value of 0.71, higher than the tabulated value at  $F_{41}$  of 0.49 ( **Table 6.6**).

**Table 6 6:** Percentage change in the average expression of ferritin gene as determined by PCR cDNA template amplification in visit three

Level of ferritin gene expressed at Day 28	Frequency	Percentage	Cumulative Percentage
None (1)	10	30.3	30.3
Weakly Expressed (2)	8	24.2	54.5
Normally Expressed (3)	13	39.4	93.9
Over Expressed (4)	2	6.1	100
TOTAL	33	100	



**Figure 6 5:** Change in the average Ferritin gene expression days; 0,14, and 28 as determined by PCR cDNA template amplification..

### 6.2.5 Profiling for Lactoferrin expression in breast milk

Milk samples were obtained from 10 participants in the control group (Kale) and 10 from the *Chinsaga* group selected randomly after delivery. As long as the mother was willing to participate and was breast-feeding, 10 ml milk samples were taken from each mother at midmorning feeding (9: 00 to 11:00 AM) during the clinic visits (day 0, 14 and 28). This sampling technique has been proved to be adequate for water-soluble components such as total protein, non-protein nitrogen, individual proteins, and lactose (48). Hand expression was used for collection of milk specimens. The samples were pooled and stored at -20 C until the time of analysis. All analyses were performed on defatted milk collected on days; 0, 14 and 28 after delivery as previously reported (49) (50).

#### 6.2.5.1 Extraction of lactoferrin mRNA

Human breast milk (100µl), was drawn into a 1.5ml eppendorf tube. Lysis buffer, 200µl, was then added and incubated at 37°C for 30 minutes in a

thermomixer shaking at 1000rpm. The lysates were homogenized by vortexing briefly. Flow was applied through the RNeasy column, it was then subjected to two washes with the first wash using 200µl of wash buffer I and the second wash using 400µl of wash buffer II. RNA, 100µl, was eluted in RNA free water. Of the RNA, 20µl, was subjected to DNaseI digestion by adding 1µl of DNaseI then 1µl of DNase inhibitor.

#### **6.2.5.2 Synthesis of lactoferrin cDNA**

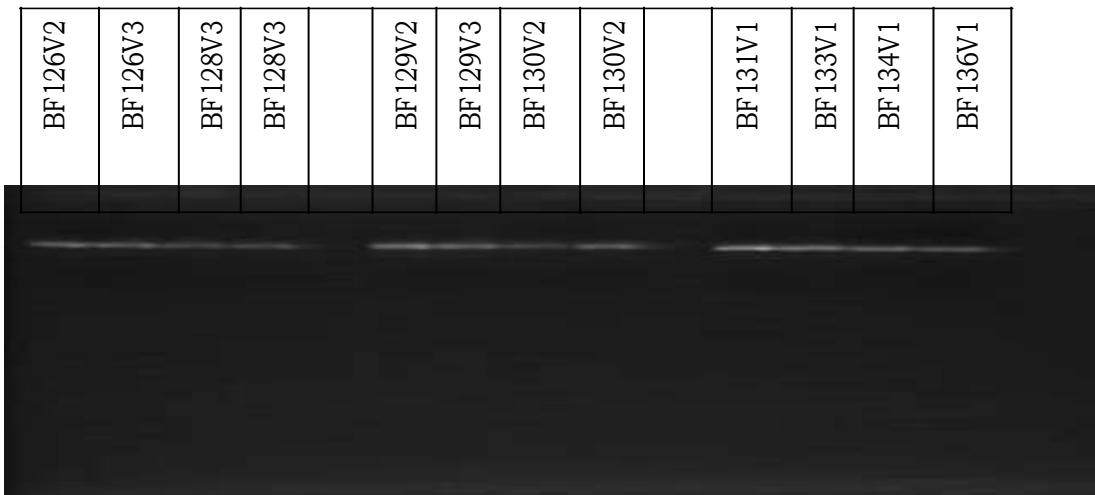
A master mix containing 1µl random primers, 1µl annealing buffer, 10µl RNase free water and 3µl of the sample RNA was incubated in a thermocycler machine (BioRad, UK) at 65°C for 5 minutes then immediately placed on ice for 1 minute. The contents of the tube were then centrifuged briefly. The 2X first strand reaction mixture containing 10µl superscript III was then added. RNase out enzyme, 2µl, was then added. The sample mixture was then incubated at 50°C for 50 minutes, then 25°C for 10 minutes and finally 50°C for 50 minutes. The reaction was terminated by incubating the mixture at 85°C for 5 minutes. Amplification reaction mixes consisted of 1–1000 ng of template DNA, 0.5 IU of thermostable DNA polymerase, 0.2 mmol/l dNTPs, 1.5–3.0 mmol/l magnesium chloride, 10–30 pmoles of primers and PCR reaction buffer (20 mmol/l Tris-HCl, pH 8.4 and 50 mM KCl), in a final volume of 20 µl. All samples were initially denatured at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 2 min and 65°C for 3 min. A final extension step of 65°C for 3 min was included. All PCR reactions were performed on a pre-programmed automated DNA thermal cycler (BioRad, UK). Using primers with LF1 5'AGGAGGATGCCATCTGGAAT 3' as the forward primer and LF2 5'AACCCAATGGCAGAGTCCTT 3' a 398 bp fragment of the lactoferrin gene from human breast milk RNA was amplified (45).

### 6.2.5.3 Gel Electrophoresis

Five microlitres of nested PCR product was mixed with a 1-2  $\mu$ l of 10 $\times$  loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with Ethidium bromide (0.5  $\mu$ g/ml) in Tris-acetate-EDTA buffer. The electrophoresis was run at 80 volts for 30 minutes. DNA was visualized by ultraviolet transilluminator.

### 6.2.5.4 Results of lactoferrin PCR in serum

**Figure 6.6** is a typical gel after development and photography of the results from electrophoresis of the transferrin gene enhanced through PCR.



**Figure 6 6:** Gel of amplified Transferrin genes from human milk using a transilluminator (an ultraviolet light box), showing Ethidium bromide-stained DNA in gels as bright glowing bands.

### 6.2.5.5 Analysis of Lactoferrin Gene.

Lactoferrin gene during baseline, at the beginning of a period of one month of using *Chinsaga* in the diet, was generally weakly expressed with the mean being 2.3 at 95% confidence interval and a confidence level of between 4.15 and 0.391. Out of the 48 patients whose serum lactoferrin levels were analyzed

14 (29.2 %) showed no expression, 9 (18.8 %) had weak expression of the lactoferrin gene, 23 (47.9 %) had normal gene expression while 2 (4.2 %) showed over expression of the gene as shown in the **Table 6.7**.

**Table 6 7:** *Percentage change in the average gene expression of lactoferrin as determined by PCR cDNA template amplification at baseline*

<b>Level of lactoferrin gene expressed at baseline</b>	<b>Frequency</b>	<b>Percentage</b>	<b>Cumulative Percentage</b>
None (1)	14	29.2	29.2
Weakly Expressed (2)	9	18.7	47.9
Normally Expressed (3)	23	47.9	95.8
Over Expressed (4)	2	4.1	100
TOTAL	48	100	

On Day 14 of using *Chinsaga* in the diet, 51 samples were analyzed for lactoferrin levels. There was a slight decrease in the level of lactoferrin gene expression at a mean of 2.2 and 95% confidence level. The confidence interval ranges from 4.0 to 0.5. Out of the 51 samples analyzed, 13 (25.5%) showed no expression, 14 (27.4%) had weak expression of the lactoferrin gene, 22 (43.1%) had normal gene expression while 2 (3.9%) showed over expression of the gene, **Table 6.8**.

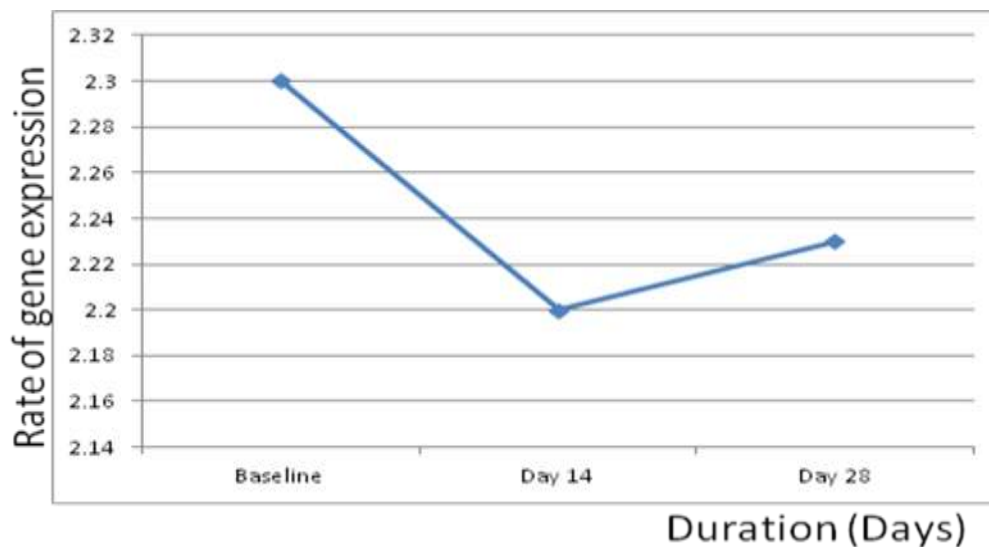
**Table 6 8:** *Percentage change in the average gene expression of lactoferrin as determined by PCR cDNA template amplification on Day 14.*

<b>Level of lactoferrin gene expressed at Day 14</b>	<b>Frequency</b>	<b>Percentage</b>	<b>Cumulative Percentage</b>
None (1)	13	28.9	28.9
Weakly Expressed (2)	13	28.9	57.8
Normally Expressed (3)	18	40.0	97.8
Over Expressed (4)	2	2.2	100
TOTAL	51	100	

On Day 28, four weeks into the period of using *Chinsaga* in the diet, 35 samples were analyzed for lactoferrin levels; some patients did not turn up for the follow up even after pursuing them. Again a slight decrease in the level of lactoferrin gene expression was observed at a mean of 2.23 and 95% confidence level. The confidence interval ranges from 4.23 to 0.223, **Table 6.9** and **Figure 6.7**. Out of the 35 samples analyzed, 10 (28.6%) showed no expression, 11 (31.4%) had weak expression of the lactoferrin gene, 10 (28.6%) had normal gene expression while 4 (11.4%) showed over expression of the gene.

**Table 6 9:** Percentage change in the average gene expression of ferritin as determined by PCR cDNA template amplification in visit three.

Level of lactoferrin gene expressed at Day 28	Frequency	Percentage	Cumulative Percentage
None (1)	10	28.6	28.6
Weakly Expressed (2)	11	31.5	60.0
Normally Expressed (3)	10	28.6	88.6
Over Expressed (4)	4	11.4	100
TOTAL	35	100	



**Figure 6 7:** Change in the average Lactoferrin gene expression days; 0,14 and 28 as determined by PCR cDNA template amplification

Generally lactoferrin gene in breast milk has been weakly expressed with the greatest expression being during the baseline with a mean of 2.3. However most of the samples in the baseline are either normally expressed or over expressed in 52.1% of the samples. The expression of lactoferrin gene with every subsequent visit was decreasing significantly. The decrease is statistically significant at  $p = 0.05$ , an  $F_{25}$  test was done that gave a value 1.06 higher than the tabulated value at  $F_{25}$  which is 0.72 and a  $p$  value of 0.001

### **6.3 Discussion**

This study was a randomized clinical trial to establish the effect of including *Chinsaga* in the diet on iron balance in the body of lactating mothers. Ferritin, Transferritin and Lactoferrin were selected as surrogate biomarkers to evaluate the relationship.

The study has showed that there was a statistically significant increase in transferrin gene expression during the days 0,14 and 28, with every subsequent visit (  $F_{40}$  -test = 2.6,  $p$  value = 0.007). Transferrin gene has been shown to be under-expressed in cases of an iron overload (51). The highest mean was observed on Day 28 at 2.25, this value tells us that the average transferrin gene expression is weak. This could mean that generally Iron levels in the study population are high and as a result transferrin gene has been under expressed.

The dietary intake of *Chinsaga* could be raising the iron levels in blood and the brain and thus leading to low expression of the transferrin gene as a compensatory reaction. This finding also corroborates that by Barton *et al* who in 1998 observed an inverse kind of relationship between transferrin saturation and iron stores in African-american as well as in Caucasian populations in

United States (52). It is also possible that other nutritional factors as well as hormones regulated the expression of the gene coding the transferrin protein.

Ferritin gene was generally weakly expressed with the highest mean observed on Day 28 at 2.2. The increase is statistically significant at  $p = 0.05$ , an  $F_{41}$  test was done that gave a value 0.71 higher than the tabulated value at  $F_{41}$  which is 0.49,  $p$  value of 0.007. It has been suggested that iron overload induces ferritin synthesis by removing a repressor from ferritin mRNA, thereby increasing its translational efficiency (53). Evidence for an increase in the cellular level of ferritin mRNA has also been reported and it is possible that both of these effects contribute to the overall increase in ferritin subunit synthesis (23).

The results above suggest that there is no iron overload in most of the samples so as to trigger over expression of the ferritin gene. However, 6.1% of the samples showed that this gene over expressed in the last visit with a majority of the samples 39.4% showing normal gene expression. This is indicative of high iron levels in most of the patients. It is possible that *Chinsaga* being consumed by the patients may have raised iron blood levels and hence increased ferritin expression since it plays a major role in storage of iron.

Generally lactoferrin gene in breast milk has been weakly expressed with the greatest expression during the baseline with a mean of 2.3. However most of the samples in the baseline are either normally expressed or over expressed that is 52.1% of the samples. The expression of lactoferrin gene with every subsequent visit was decreasing significantly. The decrease is significant at  $p = 0.05$ , an  $F_{25}$  test was done that gave a value 1.06 higher than the tabulated value at  $F_{25}$  which is 0.72 and a  $p$  value of 0.001.

Milk protein synthesis is under multiple hormonal controls. Prolactin, corticosterone and insulin are known to affect gene expression of several milk proteins (54). While iron status is known to affect transferrin synthesis, it is

not known whether iron status has any effect on lactoferrin synthesis. However, if iron entry into the mammary gland is closely regulated, cellular iron levels may be similar in both iron deficiency and during adequate iron status, and an effect would not be expected.

The factors regulating concentration of iron and iron binding proteins in milk are incompletely understood. It has recently been suggested that the number of transferrin receptors in the mammary gland will be up- or down regulated during iron deficiency or sufficiency and that this is a mechanism for homeostatic control of milk iron (55). Support for this hypothesis has been obtained in lactating rats with different iron status; mammary gland transferrin receptors were inversely correlated to iron status (56). Thus, an anemic woman would be expected to have a high number of transferrin receptors in her mammary glands, thereby more efficiently 'extracting' the low levels of serum iron. On the other hand, the iron depleted woman would have a lower number of receptors in her gland, which aid the accumulation of iron from relatively low serum iron levels.

In conclusion, this study has established that *Chinsaga* supplementation increases transferrin and ferritin gene expression. However, the Lactoferrin gene expression was noted to decrease over the 28 days. Overall, the results suggest *Chinsaga* consumption has a positive effect on iron status in lactating women through its effects on iron metabolism as believed by the *Abagusii*.

Additional studies need to be carried out to elucidate *Chinsaga* interactions with dietary and supplementary sources of iron as a possible adjunct in the enhancement of iron status among breastfeeding women.

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## CHAPTER SEVEN

# 7.0 General Discussion Conclusion and Recommendations

## 7.1 General discussion, conclusion and recommendations

Despite well documented claims by the Kisii that *Chinsaga* has strong hematinic properties, there are no studies in scientific literature to support the claims. The Kisii community recommends the consumption of *Chinsaga* in the perinatal and postnatal periods to help the body restore the blood lost during the parturition, and improve lactational outcomes. This study set out to determine the effects of *Gynandropsis gynandra* (*Chinsaga*) consumption on the hematological profile and on iron metabolism biomarkers among Kenyan lactating women. The study also sought to document the socioeconomic value of *Chinsaga*, and to develop a chromatographic fingerprint of the vegetable. Data obtained from this study shows there is a scientific basis for the consumption of *Chinsaga* to improve the hematological profile and iron status of lactating Kenyan women in support of the recommendations by the *Abagusii*. *Chinsaga* raised the RBC count of the lactating women in the second and third visits compared to the control arm on Kales especially participants who had taken iron for at least 1 month. The mean Hb value for the *Chinsaga* group increased more steadily compared to the control group.

Since anemia in pregnancy contributes significantly to morbidities in this population, the Ministry of Health should consider adopting home grown affordable and accessible solutions to address this health challenge. Findings from this study confirm *Chinsaga* as a novel solution for iron deficiency anemia among lactating women. Similar studies should be extended to other populations to explore the possibility of wider application of *Chinsaga* in improving iron status.

At a molecular level, *Chinsaga* significantly increases transferrin gene expression with the highest mean observed in the fourth week of treatment. This could be due to the fact that *Chinsaga* is a rich source of beta carotene (a pro-vitamin A), with its content ranging between 6.7 -18.9 g per 100 g of

vegetable (4). Vitamin A supplementation enhances iron metabolism. Indicators of Vitamin A status including retinol and retinol-binding protein correlated with biomarkers of iron metabolism such as serum Iron and percentage saturation of transferrin. Though vitamin A has a beneficial effect, it has not been possible to co-formulate it with iron. *Chinsaga* is a natural hematinic agent with a unique rich in pro-vitamin A. It can therefore be used in nutrition programs as an alternative to synthetic formulations for vitamin A and iron.

Commercial production of *Chinsaga* as a nutraceutical should also be explored as a mechanism to alleviate anemia in lactating women and children. *Chinsaga* demand is increasing in the urban areas. This implies commercial agriculture of *Chinsaga* has good prospects; however declining farm sizes under traditional vegetables may constrain the farming of *Chinsaga*. There is need for review of agricultural policy to rationalize land use to support production of *Chinsaga* and other leafy African vegetables at the commercial levels using modern methods and good agricultural practices. Such a policy would readily get community support as there already exists a well structured supply chain with: producers; collectors; wholesalers; retailers and consumers in a variety of combinations.

To ensure consistency of quality of *Chinsaga* produced by the community, this study generated chromatographic fingerprint that can be applied for chemical identification and characterization of *Chinsaga*. TLC allowed quick and easy visualization of the components unique to the plant. This method can be used as a routine pharmacognostic assay. When higher resolution is desired, the HPLC method developed can be used. A combination of TLC and HPLC can be an effective way to differentiate *Chinsaga* by origin and from other herbs. The assay may be used to follow changes in quality during processing or storage and during pharmacokinetic studies.

Socio-demographic factors that affected RBC count included: marital status, age of husband, sharing the bed with the baby, drinking cocoa and eating *Ugali*. There was a significant correlation with duration of continuous stay in the current location ( $P < 0.01$ ). It may be possible that the long residence by the women helps overcome challenges including sourcing of affordable resources and the establishment of support networks beneficial to their health. Further well structured social studies are required to determine if there is a real association between the hematological profile and social factors.

In conclusion, this study confirms that the consumption of *Chinsaga* by lactating women leads to improvement in the hematological profile and markers of iron metabolism. The study therefore demonstrates a possible link between *Chinsaga* diet and the potential effect on blood restoration following delivery, as believed by the Kisii. The socioeconomic value of *Chinsaga* has been advanced.

This study therefore recommends the conduct of similar studies on other traditional vegetables consumed by various communities in Kenya as a way of preserving the knowledge and promoting their incorporation into the diet for the benefit of mankind. In addition, this study has demonstrated that it is possible to design and conduct similar studies with the currently available infrastructure in Kenya using the model developed in this work. The Ministries of Health and Agriculture should develop an integrated strategy to exploit the potential of *Chinsaga* for health and economic benefits. More studies are needed to examine the effect of *Chinsaga* diet on enhancing breast milk quantity.



# Appendix



## **Ethics Approval**

## **Consent form**

## **Questionnaire for visit 1**

## **Questionnaire for visit 2**

## **Questionnaire for visit 3**

