BIOCHEMICAL CHARACTERISTICS OF MARAMA BEAN (*Tylosema esculentum*): QUALITY EVALUATION AND HEALTH POTENTIALS IN FUNCTIONAL CELL MODELS

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ABSTRACT

Marama bean (*Tylosema esculentum*) is a drought-tolerant legume native to Southern Africa. The potential of its seeds have been compared to soybeans. The studies of the potential of this plant are limited and at the moment there are various ongoing researches on the marama bean plant. The main objectives of this study were to evaluate the quality of the marama bean and determine its health potential. The following studies were carried out: roasting optimization, sensory analysis, texture profiling, proximate and mineral composition, microorganism profile, anticancer and lectin studies. The roasting process of marama bean was optimized using a standard roaster. Roasted marama beans were compared to roasted macadamia nuts by a sensory consumer panel. The marama beans had a satisfactory consumer ranking. This was due to the presence of the bitterness attribute which was more significant in marama beans then macadamia nuts. Sensory attributes obtained with a sensory descriptive panel were compared to the attributes obtained with instrumental TPA; a good relationship was found between the two techniques for hardness and crunchiness. Standard AOAC methods were used to determine proximate and mineral composition of marama beans. The proximate analysis showed marama beans to have high crude protein, carbohydrate and fat contents. The beans were found to be rich in various minerals with high values occurring for potassium, magnesium, phosphorus and calcium. Trace iron was also detected in the bean samples. No harmful microorganisms were detected in the roasted marama bean samples. The marama bean plant extracts were found to have promising health potential activities, as well as the ability to enhance glycan expression of the epithelial cells in the gut.

TABLE OF CONTENTS

DEDICATION

This thesis is dedicated to my parents, Josef and Aino Kapewangolo. You are the reason for my success. Thank you so much for the encouragements, prayers and for making sure that failure was never an option for me. You are the best.

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DECLARATIONS

- I, Petrina Taatsu Kapewangolo, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Petrina Taatsu Kapewangolo

ACRONYMS

AEC-DMF	3-Amino-9-Ethylcarbazole – N,N-Dimethylformamide
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
CO_2	Carbon dioxide
CON A	Concanavalin Agglutinin
DMEM	Dulbecco's Modified Eagle's Medium
E. coli	*Escherichia coli*
H_2O_2	Hydrogen peroxide
HPA	*Helix pomatia* Agglutinin
LDL	Low-Density Lipoproteins
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N	Nitrogen
OD	Optical Density
PBS	Phosphate Buffered Saline
PNA	Peanut Agglutinin
QDA	Quantitative Descriptive Analysis
RDA	Recommended Daily Allowance
TPA	Texture Profile Analysis

UEA I	*Ulex europaeus* – I Agglutinin
UNAM	University of Namibia
USDA	United States Department of Agriculture
WGA	Wheatgerm Agglutinin

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background

Marama beans, *Tylosema esculentum* is a long-lived perennial species endemic to arid areas of Southern Africa (Botswana, Namibia and South Africa). Its potential as a nutritional crop results from the high oil and protein content of its seeds. *Tylosema esculentum* is classified in the Leguminosae family (Hartley, Tshamekeng & Thomas, 2002) and the Caesalpinioideae subfamily (Johnston, 1977, p. 61). In Botswana and South Africa it is referred to as "morama bean". The species produces a nutritious and edible food called the marama bean. According to Menninger (1970), the word "*esculentum*" means edible and was given to the plant because not only does it produce seeds and tubers which are eaten, browsing stock and game also consume the tuberous stems.

Above the ground, *Tylosema esculentum* produces seeds that rival peanut and soybean in composition and nutritive value. The ripe seeds are chestnut brown and spherical about 15 mm in diameter (Fig. 1). The inner flesh comprising the cotyledon is firm, cream coloured, oily and without fibers (Mmonatau, 2005, p. 9). Below the ground, it produces a high-protein tuber much bigger than sugar beet and more nutritious than potato or yam (Vietmeyer, 1978, p. 9). The base of the leaves and the base of the petioles contain flexible tissue, which enables the leaves to fold close under stress and to orientate the closed leaves so that the radiating surface area is

minimized. In winter, parts of the plant above the ground die off and the tuber remains dormant until the following rain season (Vietmeyer, 1978, p. 21).

The seeds of the marama plant are collected in its native area from the wild and by hand. The tubers are harvested by hand digging. The raw seeds of the marama bean plant can be stored and they remain edible for years. Dry storage is preferable (Brink & Belay, 2006, p. 189).

The plant yields top-quality vegetable oil (Vietmeyer, 1978, p. 16). Oil can be extracted from the seeds by conventional pressing or solvent extraction (Brink and Belay, 2006, p. 189). In addition, it thrives in poor-quality soil and under the harshest climates. Indeed, in its native habitat droughts often last years on end, a feature ruinous to mainstream crops and most living creatures but not to marama beans (Vietmeyer, 1978, p. 9).

The marama plant is presently known only in the wild state and it is native to the Kalahari Desert and the neighbouring sandy regions of Namibia, Botswana and South Africa. In some areas of Botswana and Namibia, the plant occurs in areas several kilometers across. It is found to a lesser extent in South Africa (Northern Cape Province and Gauteng). The typical habitat is an undulating grassveld (savanna), with marama sprouting among the native grass and acacia-thorn scrub on sandy vlies (Bower, Hertel, Oh & Storey, 1988).

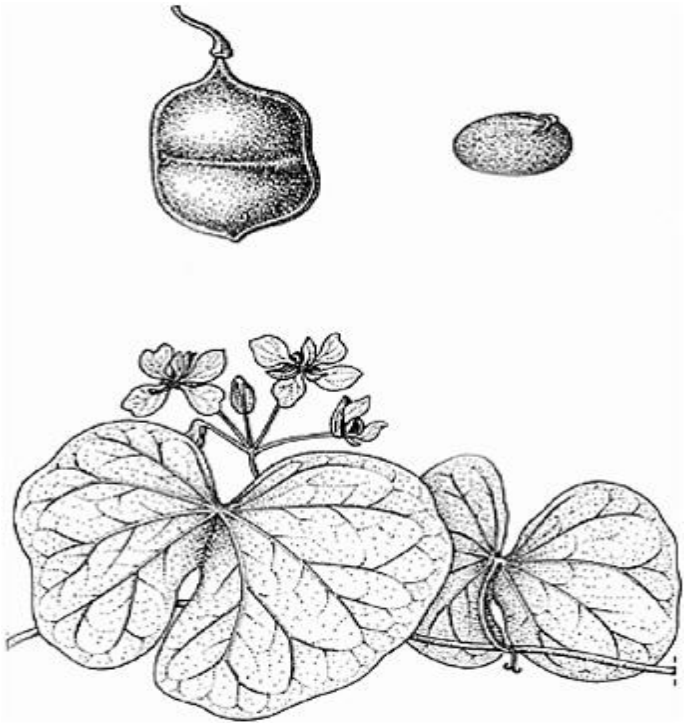


Figure 1. A marama bean pod, bean and the bilobed marama leaves (Vietmeyer, 1978).

The marama bean plant is classified as an underutilized crop. An underutilized crop is by explanation the one that has not been fully exploited in terms of its potential use as a food and/or non-food product (Azam-Ali, 1996, as cited in Naomab, 2004, p. 2). In particular, from nutritional and health perspective, there has been very little research efforts that have targeted to increase its commercial value in the countries where it is found. An earlier EU funded project (MARAMA I) "Improvement of marama bean *T esculentum* an underutilized grain and tuber producing legume from southern Africa" has provided preliminary scientific information on the marama bean. It looked at understanding the factors involved in production and domestication of the marama plant.

This research primarily focused on the quality evaluation of processed and raw marama beans and chemical physiological modulation with human and pig cell lines. Quality evaluation included biochemical tests such as *In-vitro* protein digestibility, proximate analysis and the microbiological profile. The physiologic and immunomodulatory activity was measured using crystal violet, mitochondrial activity, nitric oxide release and lectin assays. The outcome of this study will hopefully be used to create awareness on the nutritional and health potential of the marama bean.

1.2 Statement of the research problem

According to Watt and Breyer-Brandwijk (1962), the marama plant has the potential of becoming a nutritional crop because of the high oil and protein content of its seed. It also has potential for use in human health, as reported from the areas where marama beans are found (Lima de Faria & Saraiva, 2008, p. 11).

In Namibia, the marama bean still remains an underutilized plant with little information available about its quality and health benefits. This research on the nutritional value and health potential of the marama plant is important therefore inorder to make the local people that consume this plant and the whole world at large understand the full potential of the marama plant.

1.3 Objectives

The overall objective of this study was to determine the quality of roasted marama beans based on consumer preferences and provide information that could be used to target niche markets and therefore improve nutrition and increase economic opportunity.

The specific objectives of this study were:

a) To optimize the roasting process of marama beans

b) To determine the sensory attributes and consumer acceptance of roasted marama beans

c) To determine the texture profile of roasted marama beans

d) To determine the nutritional content of roasted marama beans

e) To develop a microbial food safety profile of roasted marama beans

f) To determine the effect of consuming marama bean products on health potential activities in functional cell models

1.4 Hypotheses

It was hypothesized that marama beans will have importance as follows:

a) A standard roaster will be able to optimize the roasting process of marama beans.

b) The roasted marama beans will have a high consumer preference compared with other snack products such as macadamia nuts.

c) The roasted marama beans will have high nutritional value in comparison to other beans or nuts to increase economic opportunity within the local communities.

d) The texture profile analysis attributes of roasted marama beans generated with the texture analyzer will be closely related to those generated by a sensory evaluation panel.

e) Marama beans roasted at the optimal roasting condition will have a relatively low content of potentially harmful microorganisms.

f) Marama bean products have good health potential effects especially in the gut.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of chapter content

In this chapter the processing of marama beans, its sensory evaluation, texture profile analyses, nutritional value, microorganism profile and its health benefits will be discussed.

2.2 Processing of Marama beans

The marama bean compares well in protein and oil content with both soya and peanut and is usually collected and eaten by local people (Hartley et al., 2002, p. 67). In the case of Namibia the local people that consume marama beans are found in Okakarara and Gobabis. Marama beans have an unpleasant taste when raw (Hartley, 1997, as cited in Mmonatau, 2005, p. 10).

The uncooked seeds are never eaten raw because they are tasteless and have an unpleasant slimy texture. The most common way that local people prepare marama beans is by roasting the bean in hot sand. Menninger (1970) indicated that, after roasting, the seeds take on a nutty flavor that has been compared with roasted cashew nuts.

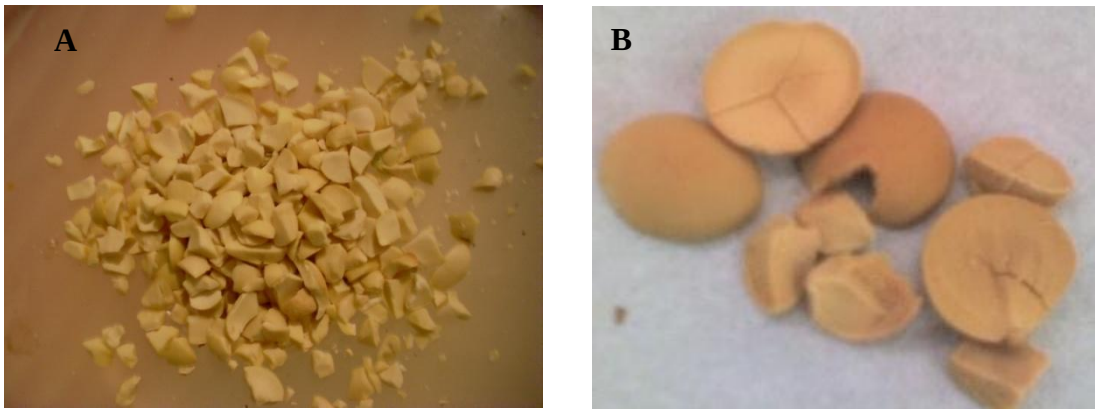


Figure 2. Unroasted (A) and roasted (B) marama beans

The fire is prepared either directly on the sand and beans are roasted on the hot sand or a container of sand is placed over a fire and the beans are placed inside the container with sand (Lima de Faria & Saraiva, 2008, p. 17). They are ready when they pop. When they pop they can be dangerous as they spring when they are very hot that is why they are stirred with caution. One or two beans are cracked to see if they are ready (Lima de Faria & Saraiva, 2008, p. 17). The sand is always used to distribute the heat evenly and prevent the seed coat from breaking. Young tubers are also roasted and eaten (Van Wyk & Gericke, 2000). The San people use a shallow hole in the ground to cook the marama beans. They make a fire inside the hole and wait until it is very hot before they drop the beans inside (Lima de Faria & Saraiva, 2008, p. 17).

Europeans in Southern Africa have grounded the roasted seeds and used them as a culinary substitute for almonds. Local people also boil them with cornmeal, grind or pound them into a powder that is boiled in water to produce either a cocoa-like beverage or porridge (Ripperger-Suhler, 1983, p. 18). The immature seeds and stems

may be eaten cooked as a vegetable or in soups. Roasted marama bean are sometimes sold locally but only at a small scale (Brink & Belay, 2006, p. 188).

Legumes are known to contain protease inhibitors (more commonly referred to as trypsin inhibitors). These inhibitors are one of the most studied antinutritional factors in legumes such as soybeans. The term protease inhibitor is preferred to trypsin inhibitor because this family of proteins inhibits a wide variety of proteinases in addition to trypsin (Liener, 1981, p. 921).

Trypsin inhibitor activity in marama can be destroyed by heat. Baking the defatted seed meal at 140ºC for 30 minutes decrease the activity in aqueous protein-extracts, giving a decrease of 70% in total trypsin inhibitor of the meal (Bower et al., 1988, p. 538).

2.3 Sensory attributes and consumer preference

Consumer attitudes to foods are mainly influenced by quality attributes and consumer preference of foods is mainly determined by their sensory perception (Brennan & Kuri, 2002).

Sensory science is a scientific method used to measure, analyze, and interpret human responses to products as perceived through their senses of touch, taste, sight, smell, or sound (Meilgaard, Civille & Carr, 1999, p. 71). Sensory science is often used to improve existing products or to test people's views on new products, such as the softness of tissues, the crunchiness of an apple variety, or the aroma of air fresheners.

It is also used to test the taste and color acceptance of new products, such as ketchup, or the sound characteristics of products, as in the crunch of snack foods (Meilgaard et al., 1999, p. 71).

The sensory analysis environment is extremely important as the environment can interfere or confound sensory perceptions. Removal of as many environmental factors as possible or minimizing and standardizing those factors that cannot be removed across treatments is important (Miller, 1998, p. 2). This assures that the sensory response of a panelist is the result of the product characteristics, not a response confounded by the environment in which the response was evoked (Miller, 1998, p. 2).

"A quantitative descriptive analysis (QDA) approach has gained acceptance for sensory evaluation of various food and dairy products. In a QDA approach, panelists recruited from the general public work together in a focus group to identify product attributes and appropriate intensity scales specific to a product" (Stone & Sidel, 1998, as cited in Chapman, Lawless & Boor, 2001, p. 12). This group is then trained to reliably identify and score product attributes. As panelists generate the attribute terms, the resulting descriptors are meaningful to consumers, and thus, analyses provide information amenable to modeling predictions of consumer acceptability (Chapman et al., 2001, p. 13).

Marama beans have been reported to have a pleasant sweet flavor when boiled or roasted, comparable to roasted cashew nuts or almonds, although bitter types of

marama beans are known (Brink & Belay, 2006, p. 188). In analysis of such studies where sensory and consumer-related data are collected, it is important to extract the relevant information such as the right attributes that describes the product under study (Westad, Hersleth, Lea & Martens, 2003, p. 1).

The product quality drives consumer acceptance and demand. The ability to measure sensory attributes characteristic of high quality products is necessary for the development and production of products that meet consumer expectations (Chapman *et al*., 2001, p. 12).

Results obtained during sensory evaluation can provide valid and reliable information to product manufacturers and entrepreneurs with regard to the product's sensory properties and eventually insight into positioning the products in relation to other similar products (Museler, 2005, p. 83).

2.4 Texture profile analysis

Texture is a quality of food that we can feel either with the fingers, the tongue, the palate or the teeth. The range of textures in foods is very large and the departure from an expected texture is a quality defect (Gupta, A. Sharma & R. Sharma, 2007, p. 455). Instrumental texture analysis studies have been carried out on legumes in the past using various probes (Revilla & Vivar-Quintana, 2008, p. 312).

Peleg (1983), as cited in the Exponent *stable micro systems texture analysis software* states that characterization of food texture commonly falls into two main groups,

based on sensory and instrumental methods of analysis. Sensory analysis includes use of the senses of smell, taste, sound and touch. Evaluation of food texture by touch includes the use of the fingers, as well as the lips, tongue, palate and teeth in the mouth. As would be expected, sensory methods of analysis are subject to wide variability, though this variability can be reduced by using trained assessors. It is sometimes preferable to use instrumental methods of assessing food texture rather than sensory analysis because they can be carried out under more strictly defined and controlled conditions. Furthermore, problems of experimental variability are more likely to be caused by sample heterogeneity than by instrumental imprecision. Another reason for instrumental analysis may be that often changes in ingredient levels cause several simultaneous changes in product characteristics. Some of these changes are difficult to mask and thus tend to make sensory analysis difficult, e.g. variation in cake firmness due to sugar content. Therefore a main goal of many texture studies is to devise one or more mechanical tests with the capacity to replace human sensory evaluation as a tool to evaluate food texture.

According to Gupta *et al.* (2007), instrumental texture profile analysis (TPA) was pioneered in 1963 by Szczesniak who defined the textural parameters first used in this method of analysis. Computer assisted texturometers were further developed and this helped obtain directly all the TPA parameters directly by means of a software. TPA is based on the recognition of texture as a multi-parameter attribute. For research purposes, a texture profile in terms of several parameters determined on a small homogeneous sample may be desirable (Gupta *et al*., 2007, p. 455).

The test consists of compressing a bite-size piece of food two times in a reciprocating motion that imitates the action of the jaw and extracting from the resulting force-time curve a number of textural parameters that correlate well with sensory evaluation of those parameters. Most of the published reports on food texture have dealt with the evaluation of a single characteristic in a specific food product. This single characteristic has not always adequately explained changes in textural properties of different foods. Single compression or penetration tests are mostly used but provide little information about the texture of a food product (Gupta *et al.*, 2007, p. 456).

2.5 Nutritional value of roasted marama beans

Several plants exist with very high nutritive value and yet remain unexploited for human and animal benefits (Oladele & Oshodi, 2007, p. 345). Legumes are rich sources of food protein and have provided a protein source for man since the earliest civilizations (Sandberg, 2002, p. 281).

According to Kanamori *et al* (1982) and Vietmeyer (1986) as cited in Bower *et al*. (1988), the protein and fat in marama bean are higher than in several types of bean and many other potential crops. Marama bean is an excellent source of good quality protein (30-39%); which is roughly equal to that of soya beans at 38% protein (Powell, 1987, p. 217). A study carried out by Bower *et al*. (1988), showed that marama bean protein appears to be comparable to soybean in essential amino acid content, with cystine and methionine as the limiting amino acids. Compared to soya

bean, the albumin content of marama was found to be higher while that of globulin was lower (Bower *et al*., 1988, p. 537).

Although legumes are recognized as being high in protein, the quality of bean protein is often underestimated. This is because the protein-efficiency ratio, which is based on the growth of laboratory animals (most commonly rats), was the standard method of evaluating protein quality until recently (Messina, 1999, p. 440). According to Sarwar *et al*. (1989) as cited in Messina (1999), rats have a methionine requirement that is approximately 50% higher than that of humans.

A study carried out by Amarteifio and Moholo (1998) showed that marama bean had the highest fat, protein, phosphorus and calcium contents compared to bambara groundnut, mung bean and tepary bean.

The content of minerals is generally high in legumes. However, the legumes also contain antinutritional factors, such as proteinase inhibitors, lectin, raffinose oligosaccharides, saponins, polyphenols and phytate (Sandberg, 2002, p. 281). Anti-nutritional factors lower the nutritional value of a food by lowering the digestibility or bioavailability of nutrients. Phytate, and some of the degradation products of phytate, are well-known inhibitors of absorption of essential dietary minerals, in particular non-haem iron and zinc (Sandberg, 2002, p. 281).

Marama bean oil (35-43%) is rich in mono- and di-unsaturated fatty acids and contains no cholesterol (Powell, 1987, p. 217). Although not commercially available,

the marama oil has a pleasant odour and taste and can be used in the food and cosmetic industries (Müseler, 2005, p. 28). The high oil and protein content of marama bean as indicated in table 1 would provide a good source of protein and energy in the rural areas.

Table 1. Nutrients of marama beans (g/100g - raw) on dry basis (Bower *et al. 1988*, p. 534)

Nutrient	**Marama bean ± SD**
Protein (g) (N x 6.25)	31.8 ±1.1
Oil (g)	42.2 ± 1.6
Fatty acids	34.0 ± 1.6
Waxes	8.2 ± 1.9
Carbohydrate (g)	18.9 ± 2.2
Ash (g)	3.2 ± 0.1
Moisture (g)	3.9 ± 1.0
Energy (MJ)	2.66 ± 0.08

2.6 Microorganism profile of roasted marama beans

The composition and chemistry of a food dictates the microorganisms that will grow well on it. The chief compositional factors that influence microbial activity are pH, moisture, available oxygen, nutrients, and the presence of natural inhibitors (Shurtleff & Aoyagi, 2000, p. 108).

Many foods serve equally well to nourish microorganisms and human beings. To ensure that food is safe for human consumption it must be carefully processed, stored, and prepared. Foods that are not safe can transmit diseases that are either infectious or intoxicating. Food infections are caused by a number of different organisms (Pelczar, Reid & Chan, 1977).

Many microorganisms are also beneficial to human beings because their fermentations of raw materials produce beverage alcohol, cheese, leavening of bread, and other important products (Pelczar *et al*., 1977). Bacteria, yeasts, molds, and viruses are important in food for three main reasons: namely, their ability to cause foodborne diseases and food spoilage and to produce food and food ingredients (Bibek, 1996, p. 471).

The plate count is one of the most accurate means of enumeration of viable microbes because a visual indicator for every cell in the specimen is obtained. The technique stems from Robert Koch's insight gained from viewing colonies growing on the surface of a spoiling slice of potato. In practice, a small aliquot of a liquid suspension of microbes is spread on the surface of solidified nutrient medium, which when incubated, leads to each cell 'developing' into a visible colony through repeated fission. Many samples may have so many microbes that even a small aliquot would have more than the 30-300 colony forming units per aliquot. In that case, the sample must be diluted to roughly 300-3,000 CFU per mL of which 0.1 mL can then be plated out. This technique is applicable to total microbial count, coliform, *E. coli*, yeast and mold count (Fankhauser, 2001).

2.6.1 Microbial Total count

Microbial population in foods, food ingredients, and the food contact environment normally constitute many different species of microbes coming from different sources. The total microbial population in food varies greatly depending upon the level of sanitation used at all phases, the degree of abuse that leads to microbial growth, and the processing and preservation methods used to kill and prevent growth of microorganisms (Bibek, 1996, p. 471).

The methods used for the microbiological evaluation or detection of foods, food ingredients, and environments are broadly grouped as quantitative and qualitative methods (Bibek, 1996, 471).

The quantitative methods are designed to enumerate or estimate directly or indirectly the microbial load in a test material (Bibek, 1996, 472). Qualitative methods are designed to determine if a representative amount (a sample) of food or a certain number of samples in a batch of food contain a specific microbial species among the total microbial population or not. These methods are used to detect the possible presence of certain foodborne pathogens, especially those capable of inflicting high fatality rates among consumers (Bibek, 1996, p. 472).

2.6.2 Coliforms count

The coliform group of bacteria includes all the aerobic and facultatively anaerobic, gram-negative, nonsporulating bacilli that produce acid and gas from the

fermentation of lactose. The classical species of this group are *Escherichia coli* and *Enterobacter aerogenes* (Pelczar *et al*., 1977).

Coliform organisms are constantly present in the human intestine in large numbers. It is estimated that billions of these organisms are excreted by an average person in one day. A healthy person would not, of course, normally excrete pathogenic organisms, but should an intestinal-tract infection develop, the pathogen is likely to appear in the feces (Pelczar *et al*., 1977).

The presence of coliforms in a food usually indicates that it has been processed under unsanitary conditions (Pelczar *et al*., 1977).

2.6.3 *Escherichia coli*

This gram-negative facultative anaerobe is found in the intestinal contents of humans, warm-blooded animals, and birds. Many strains are nonpathogenic, but some strains are pathogenic to humans and animals. *Escherichia coli (E. coli)* is involved in foodborne diseases. It is used as an indicator of sanitation (theoretically nonpathogenic strains) in the coliform and fecal coliform groups (Bibek, 1996, p. 473).

2.6.4 Mold count

Molds are important in food because they can grow under conditions in which many bacteria cannot grow, such as low pH, low water activity (A_w), and high osmotic pressure. They are important spoilage microorganisms. Many strains also produce

mycotoxins and have been implicated in foodborne intoxication. Many are used in food bioprocessing, producing food additives and enzymes (Bibek, 1996, p. 473).

2.6.5 Yeast count

Yeasts as well as molds are fungi, but they are distinguished from the molds because their usual and dominant form is unicellular. Their vegetative reproduction is usually by budding. As single cells they grow faster and reproduce more rapidly than the filamentous molds. Yeasts do not form a well-defined group of microorganisms. They are not a natural taxonomic entity, even though they exhibit a uniformity of morphology among them (Pelczar *et al*., 1977).

Yeasts have served people for many centuries by fermenting fruit juices, by leavening bread, and by making certain foods palatable and nutritious (Pelczar *et al*., 1977). Yeasts are widely distributed in nature and are disseminated by insect carriers and by wind and air currents. Most are saprophytes, living on dead organic matter, but some are parasites, depending on a living host for nutrition (Pelczar *et al*., 1977). A few obligate or facultative parasites among the yeasts can cause disease in people, other animals, and plants. Many different species of yeasts are found in soil. The kinds of yeasts found in soil are therefore related to the sources of the inoculums, their ability to survive in a specific environment, the composition of the soil, and the temperature, sunlight, moisture, and other factors. They are found in bare soils as well as in soils that natural vegetation (forests, jungles, bush, and grasslands) (Pelczar *et al*., 1977).

2.7 Health potential of marama beans

It is noteworthy that there have been no reported toxic substances detected in any of the seed components (Mmonatau, 2005, p. 9). Marama bean is a good source of micronutrients such as calcium, iron, zinc, phosphate, magnesium, B vitamins including folate. It has also been reported to be a potential source of phytonutrients including isoflavones, tannins, trypsin inhibitors, phytates and oligosaccharides, components which have been shown in other foods to contribute to health in particular prevention of non-communicable diseases such as cardiovascular diseases, diabetes and some cancers (Powell, 1987, p. 217).

In general, beans are known to have cholesterol lowering effects in humans. This is mainly due to the phytoestrogens content of beans. The cholesterol lowering effect of beans especially soya is not only caused by the presence of phytoestrogens alone, but rather a combination of the components in beans (Jenkins *et al*., 2006, p. 582-591) and the overall composition of diets with a high soya content have been suggested to affect cholesterol metabolism. Phytoestrogens are plant-derived chemicals that have oestrogenic activity (Rosell, Appleby, Spencer & Key, 2004).

Fibres in beans help the body get rid of bile acids in the liver, which can be turned into cholesterol (Meng, Lewis, Wahala, Adlercreutz & Tikkanen, 1999). Laboratory experiments have previously demonstrated that low-density lipoproteins (LDL) cholesterol isolated from individuals after soya intake was less susceptible to oxidation compared with LDL cholesterol isolated from individuals on a soy-free diet (Meng *et al*., 1999).

Beans like soya have been found to contain several components that traditionally have been considered to be anti-nutritional for example phytates and trypsin inhibitors (Dixon, 2001, p. 844). Phytates are said to act as anti-nutrients because they have the ability to bind to minerals and possibly reduce the assimilation of calcium, copper, iron, magnesium and zinc (Dixon, 2001, p. 844).

Trypsin is an enzyme needed to break down proteins to make them available, therefore inhibitors prevent this from happening. However, research suggests that this may be an oversimplification. For example, boiling beans removes most of the trypsin inhibitors and these are also inactivated by sprouting and fermentation (Dixon, 2001, p. 845).

There is a suggestion that the human digestive system is stimulated to reject anti-nutrients and the body can adequately deal with these 'non-nutritive' components without any detriment to nutritional states. There are many factors affecting the absorption of nutrients, but few will alter the body's normal mineral status if a person is physically well and following a balanced diet (Dixon, 2001, p. 846).

According to Daly *et al*. (1995) as cited in Messina (1999), beans are an excellent source of folate, which in addition to being an essential nutrient is thought to reduce the risk of neural tube defects.

The glycemic index is a measure of the potential for carbohydrates in different foods to raise blood glucose levels. Consuming foods with high-glycemic index values causes blood glucose levels to rise more rapidly, which results in greater insulin

secretion by the pancreas, than after consuming foods with low-glycemic index values (Higdon, 2006, p. 13). Legumes are generally known to have low-glycemic index values; hence, substituting legumes for high-glycemic index foods lowers the glycemic load of a person's diet (Higdon, 2006, p. 13).

There are various assay methods for evaluating cytotoxic effects of chemicals or food products on cultured cells. One of these techniques is the mitochondrial activity (MTT) assay (Chiba, Kawakami & Tohyama, 1998). The MTT assay is based on the uptake and the reduction by the mitochondrial succinic dehydrogenase of the soluble yellow MTT tetrazolium salt to an insoluble blue MTT formazan product (Chiba, Kawakami & Tohyama, 1998, p. 251).

According to Itagaki *et al*., (1991) and Saotome *et al*., (1989) as cited in Chiba *et al*. (1998), the crystal violet staining (CVS) assay is another simple and reproducible assay of cytotoxicity. This assay is based on the growth rate reduction reflected by the colorimetric determination of the stained cells (Chiba *et al*., 1998, p. 251).

2.7.1 Lectin histochemistry

Lectins are natural occurring proteins and glycoproteins which selectively bind non-covalently to carbohydrate residues. The term 'lectin' was first proposed by Boyd and Shapleigh in 1954. It is derived from the Latin verb *'legere'*, which means to pick out, select or choose, and refers to the remarkable selectivity and specificity with which lectins recognize and bind to carbohydrates (Brooks, Leathem & Schumacher, 1997, p. 1).

The most important property of a lectin, as stated in its definition, is the ability to bind to specific carbohydrates and hence agglutinate cells. The agglutination is due to the fact that the lectins have more than one binding site and it is therefore able to cross-link cells through interaction with carbohydrates on the cell membrane (Brooks *et al*., 1997, p. 13). This makes lectins powerful tools for detecting changes in the carbohydrate structure of glycoproteins (Ching, Black, Helliwell, Savage, Barr & Rhodes, 1988, p. 324-328).

Lectins are commonly used as tools in biomedical diagnostics, for observing glycosylation changes associated with cell behavior, development and disease (Mislovičova, Gemeiner, Kozarova & Kozar, 2009; Brooks *et al*., 1997). Glycosylation is the enzymatic process that links saccharides to produce glycans, attached to proteins, lipids, or other organic molecules. Glycans refers to a polysaccharide or oligosaccharide. They can be found attached to proteins as in glycoproteins and proteoglycans. Glycans are generally found on the exterior surface of cells (Brooks *et al*., 1997; Slifkin & Doyle, 1990, p. 197). Lectin assay is used to study surface glycan signatures mostly in mammalian cell lines (Tao *et al*., 2008, p. 1-31).

CHAPTER 3

METHODOLOGY

3.1 Optimization of roasted marama beans

Roasting is one of the common form of processing marama beans. Purpose of roasting is to increase overall palatability of the product. Roasting alters and significantly enhances the flavor, texture and appearance of bean and nut products (Nikzadeh & Sedaghat, 2008, p. 478). Marama bean has an unpleasant taste when raw, therefore heat treatments of the raw bean with has an effect of reducing parameters such as moisture content of the bean and changes in the taste of the bean (Garrow & James, 1993, Hartley, 1997, as cited in Mmonatau, 2005, p. 10).

Marama beans were collected in May 2007 and also April and May 2008, in Gobabis and Okakarara, Namibia. The beans were first roasted at different temperatures of 135°C, 152°C and 160°C for 20min and 40min respectively in an oven in 2007 and at different speed settings (200-1000xg) at a fixed temperature in a standard roaster in 2009. A consumer preference t54est was used to determine the optimal roasting conditions. Based on the optimal roasting conditions, the quality of the optimally roasted marama bean and its health effect on humans was then further investigated. The shells/husks and tuber were also evaluated for their health potentials.

3.1.1 Traditional roasting of marama beans

Traditional roasting of marama beans was closely observed and carried out. It was this traditional method from which the lab-scale roasting was based on. Traditionally,

roasting of marama beans was carried out in hot sand while the beans were still in shells (see appendix 1). Dehulling of the bean before roasting proved impossible as the shell was extremely intact and only cracks partially. The shell becomes easy to remove after treating it with heat (personal observation).

During the months of February and March 2007, trips were undertaken to the areas where marama beans grow in Namibia. These are Gobabis area in Omaheke region and Okakarara in Otjozondjupa region. This was done to find out the suitable harvesting period in order to carry out the traditional roasting method in those specific areas.

3.1.2 Lab-scale roasting of marama beans

The roasting of marama beans in the laboratory was carried out using a forced convection continuous tumble roaster (Bastion Industries CC, (www.roastech.com) South Africa). The roaster (Fig. 3) consists of a rotor that carries the beans through the machine. The rotor speed is adjustable, meaning the marama beans were either roasted fast or slow at varying temperatures. The product is tumbled about 45 times before it leaves the rotor. Hot air is forced right through the product during the roasting process, creating a very even roasted product (F. Teseling, Bastion Industries, personal communication, 21 February 2009).

The temperature is adjustable between 0 to 400ºC, while the speed controller can be adjusted between 0 and 1200xg. At a speed setting of 1000xg, the residence time is

350 seconds. Residence time is the time that a product stays in the roaster (F. Teseling, Bastion Industries, personal communication, 21 February 2009).

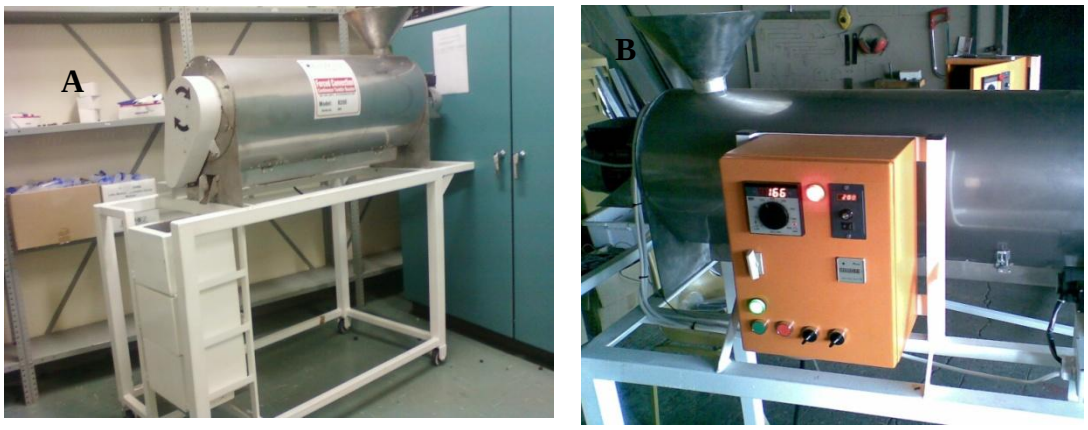


Figure 3. The convection continuous tumble roaster that was used to roast marama beans. (A) The backside of the roaster. (B) The front part that holds the box. The box contains the temperature and speed control knobs, and the on and stop push buttons.

Optimal roasting condition was achieved by adjusting the speed settings to 1000xg, 600xg, 550xg and 200xg at a fixed temperature of 153ºC.

3.2 Sensory analyses

3.2.1 Panelist screening

Sensitivity is an important factor when selecting assessors. A threshold test was carried out to determine an individual's sensitivity to taste i.e. sweetness, sourness, saltiness and bitterness. Thus 20 individuals were screened by tasting solutions of 8% sucrose, 0.15% citric acid, 0.5% sodium chloride and 0.5% caffeine.

The solutions were then poured in the Styrofoam cups which were labeled with 3 digits random numbers, whereby each panelist was served with about 20 mL of each solution. Panelists evaluated the solutions by tasting and recorded the perceived taste in the threshold score sheet. Panelists were reminded to rinse their mouths before and after tasting each sample. The threshold test took place in a laboratory and 10 of 20 panelists qualified for descriptive testing, i.e. those who discriminated the samples correctly.

3.2.2 Descriptive sensory evaluation test

Panelists who qualified from the threshold test were trained for descriptive testing. Apart from generating the descriptive words provided in Table 2, panelists were trained on how to perceive different sample attributes using roasted marama beans and macadamia nuts. They were familiarized with the descriptive sensory form and made comments and queries on areas that were not clear to them. Panelists were also reminded about the important conditions that one needs to adhere to during the evaluation; these included no re-tasting of the sample, since it may affect the perceived attributes from the other sample and no communication and noise during the evaluation since these may affect the concentration of other panelists.

3.2.2.1 Descriptive sensory sample preparation and presentation

The descriptive sensory evaluation test was conducted in the laboratory at Neudamm campus. It was done in triplicates. Sample preparation occurred in the laboratory, and was served in Styrofoam cups. Roasted marama beans were evaluated together with roasted macadamia nuts. Each panelist received about 20 g of roasted marama beans

and macadamia nuts. Samples were then served in Styrofoam cups that were covered with lids.

Panelists were presented with evaluation forms on every seat. The panelists were then presented with the two labeled sample of the marama beans and macadamia nuts in Styrofoam cups that were labeled with letter A or B and another Styrofoam cup containing water for rinsing their mouth. Panelists were urged to read the instructions carefully before starting the evaluation. Samples were evaluated for different sensory attributes that included, flavor, sweetness, oiliness, crunchiness, aroma and the after taste as indicated in Table 2.

3.2.3 Consumer evaluation

Consumer sensory evaluation does not require trained panelists. No screening was required. Consumers were recruited from students, faculty, and staff. Two trials of consumer evaluation were carried out. The first trial was carried out at the University of Namibia (UNAM) main campus in order to determine the optimal roasting conditions of marama beans from the different speed settings and it consisted of 20 panelists. The second consumer evaluation trial was carried out to determine the consumer preference between roasted marama beans and roasted macadamia nuts. The second trial took place at Neudamm campus, UNAM and it consisted of 50 consumer panelists, with an equal balance of males and females between the ages of 19-50 years. The two groups were made up of people from different ethnic groups; Herero, Coloured, Rwandese and Wambo.

Table 2. Lexicon of the sensory attributes of roasted marama beans developed by the 10 panelists

Attribute	**Definition of attributes**
Aroma	
Nutty aroma	The aroma associated with nuts
Burnt aroma	The aroma associated with burnt nuts
Flavour	
Sweetness	The taste on the tongue associated with sugars
Bitterness	A taste associated with bitter agent such as caffeine in coffee
Nutty flavor	The flavour associated with nuts
Burnt flavor	The flavour associated with burnt nuts
Oily flavor	The flavour associated with vegetable oil / cooking oil
Texture	
Crunchiness	The force needed and amount of sound generated from chewing roasted nuts with molar
Hardness	The force associated with compressing food between molar teeth
Tooth picking	This attribute is associated with peanuts when they remain stuck in the teeth after chewing
After taste (residual)	
Oiliness	The after taste experienced after drinking oil
Particles	The attribute associated with the residues that remain in the mouth after eating peanuts

3.2.3.1 Consumer evaluation sample preparation and presentation

For the first trial at UNAM, each panelist was presented with marama beans roasted at 153ºC at the four different speed settings; 1000xg, 600xg, 550xg and 200xg. For the second trial, each panelist received about 20g of roasted marama beans and macadamia nuts. The samples were presented in styrofoam cups that were labeled with letter A or B. For all trials, panelists were provided with water to rinse their mouths in-between tasting.

Panelists were urged to read the instructions carefully before starting the evaluation. The consumers carried out the evaluation by tasting the samples they were presented with, and filled their perceptions on the evaluation forms. The 9-points hedonic scale was used for rating the products (Like extremely-9 and dislike extremely-1) (Young *et al*., 2005, p. 39).

3.3 Texture profile analysis of marama beans

Texture measurements were carried out using a TA.XTplus Texture analyser (Stable Micro Systems, UK) connected to a computer with the Exponent stable micro systems texture analysis software.

A 75 mm diameter platen (compression plate) was attached to a 50 kg load cell. The following samples were subjected to texture profile analysis (TPA); roasted macadamia nuts, roasted marama beans and raw marama beans. The samples were approximately 15 mm in length, 6 mm width and 7 mm in height. The condition-set up in the Texture Analyser for measuring textural properties was as follows:

Pre-test speed: 1.00 mm/s; test speed: 5.00 mm/s; post-test speed: 5.00 mm/s; target mode: strain; strain: 75%; time: 1 sec; trigger type: auto; trigger force: 5.0 g.

The TPA test was carried out at a strain setting of 75%, which had a two-bite time difference of 1s. The test was replicated a minimum of eight times and mean values for each parameter were calculated. Hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience were determined from the texture profile. The parameters were measured from force to time curves and the results were expressed in grams.

Hardness is defined as the maximum peak force during the first compression cycle (first bite) and has often been substituted by the term firmness. Fracturability (originally called brittleness) is defined as the force at the first significant break in the TPA curve. Adhesiveness is defined as the negative force area for the first bite and represents the work required to overcome the attractive forces between the surface of a food and the surface of other materials with which the food comes into contact, i.e. the total force necessary to pull the compression plunger away from the sample. For materials with a high adhesiveness and low cohesiveness, when tested, part of the sample is likely to adhere to the probe on the upward stroke. Springiness (originally called elasticity) is related to the height that the food recovers during the time that elapses between the end of the first bite and the start of the second bite. Chewiness is defined as the product of gumminess x springiness (which equals hardness x cohesiveness x springiness) and is therefore influenced by the change of any one of these parameters. Gumminess is defined as the product of hardness x

cohesiveness. Gumminess is a characteristic of semisolid foods with a low degree of hardness and a high degree of cohesiveness. Cohesiveness is defined as the ratio of the positive force area during the second compression to that during the first compression. Cohesiveness may be measured as the rate at which the material disintegrates under mechanical action. Resilience is a measurement of how the sample recovers from deformation both in terms of speed and forces derived. Stringiness is the distance the product is extended during decompression before separating from the compression probe (adapted from *exponent stable micro systems texture software*).

3.4 Nutritional evaluation of roasted marama beans

Proximate analysis of roasted and unroasted marama beans was determined in this study. Trace mineral, iron was also determined in roasted marama beans. The analyses were carried out in triplicates on the nut itself, after separating it from the shell. These analyses were done in triplicates and they were conducted at Analytical Laboratory Services, Windhoek, Namibia. Standard AOAC methods were used for proximate analysis (Horwitz, 1970).

3.4.1 Determination of moisture

The moisture was assayed on 4-5g of the sample in a covered, flat, aluminium dish and dried to a constant weight at 100ºC (James, 1995). The following formula was used to determine percentage moisture in a sample of marama bean:

$$\text{Moisture content (\%)} = \frac{\text{Weight fresh sample} - \text{Weight dry sample}}{\text{Weight fresh sample}} \times 100$$

3.4.2 Determination of nitrogen and protein content

Crude protein is based on a laboratory nitrogen (N) analysis, from which the protein content in a feedstuff can be calculated by multiplying by 6.25. This is from the assumption that nitrogen is derived from protein containing 16% nitrogen. However, some portion of the N in most feeds is found as non-protein nitrogen and, therefore, the value calculated by multiplying N x 6.25 is referred to as crude rather than true protein (James, 1995).

Total nitrogen was determined by the Kjeldahl method. This method involved digestion of the sample with concentrated sulfuric acid in the presence of catalysts (selenium tablets) for 1.5 hours. The mixture was then distilled and the distillate was titrated with 0.100M HCl (James, 1995).

The following formulas were used to determine the nitrogen and protein content in the marama bean sample:

$$\text{Nitrogen content of the sample (\%)} = \frac{\text{millilitre acid x Normality of standard acid}}{\text{Weight of sample}} \times 1.4$$

Crude protein content (%) = nitrogen content x 6.25

3.4.3 Determination of extractable fat

A soxhlet apparatus was used to extract fat from the samples. The samples (weight dried sample between 2-3 grams) were placed in a thimble inside the soxhlet apparatus. Petroleum ether (boiling point, 40-60ºC) was used to extract the fat for 4

hours. The solvent was removed using a rotavapor. The dry sample was then cooled in a dessicator and weighed (James, 1995).

The following formula was used to determine crude fat in the marama bean sample:

$$\text{Crude fat (\% of dry matter)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

3.4.4 Determination of fiber

Crude fibre is included in the carbohydrate content and listed separately. It includes, theoretically, materials that are indigestible in the human and animal organism. Crude fibre determinations are greatly affected by manipulations and procedures. Particle size is very important; the finer the material is ground, the lower the determined crude fibre content (developed by Analytical laboratory services, 2009).

The residue from a crude fibre determination contains about 97% cellulose and lignin. It does not represent, however, all the cellulose and lignin present initially. In addition, the crude fibre is a mixture of cellulosic materials and does not represent any specific compound or groups of compounds. Crude fibre levels are stated on feed labels but total dietary fibre is a more useful measure of dietary fibre content (James, 1995).

Crude fibre was determined as that fraction remaining after digestion with standard solutions of sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH) under carefully controlled conditions. The samples were defatted prior fibre analysis (James, 1995).

The following formula was used to determine the crude fibre content in the marama bean sample:

Crude fibre (% of fat-free dry matter)

$$= \frac{\text{(weight crucible + dried residue)-(weight crucible + ashed residue)}}{\text{(weight of sample)}} \times 100$$

3.4.5 Determination of energy

Classically, the total energy content of a sample was determined by burning the marama bean sample in a bomb calorimeter and measuring the amount of energy released. Energy was calculated in kJ/100g (James, 1995).

3.4.6 Determination of ash

The marama bean samples were ashed at 550 °C to a constant weight for 6 hours.

The formula below was used to determine the ash content of marama bean samples.

$$\text{Ash (\%)} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100$$

3.4.7 Determination of carbohydrates

The total carbohydrate content of the marama bean samples was calculated by difference after determining all the other nutritional components.

% Carbohydrate = 100 – (% moisture + % fat + % ash + % crude fat & oil + % protein + % fiber)

3.4.8 Determination of phosphorus

A spectrophotometer was used for phosphorus (P) determination. An aliquot of the sample was pipette into a 100 mL flask and 20 mL of the molybdovanadate reagent was added. Aliquots of the working standards containing 0.5, 0.8, 1.0 and 1.5 mg P were transferred to 100 mL flasks and treated as above. Samples were read at 400 mμ setting the 0.5 mg standard at 100% transmission. The mg of P in each sample aliquot was determined from a standard curve (James, 1995).

3.4.9 Determination of Sodium (Na), Potassium (K), Magnesium (Mg), Calcium (Ca) and Iron (Fe)

Ash was dissolved in HCl and the elements (Na, K, Mg, Ca and Fe) were determined by inductively coupled plasma – optical emission spectroscopy. The results were reported as mg/100g (James, 1995).

3.5 Microorganism profile of roasted marama beans

In this study, microorganisms including total count, coliforms count, *E. coli*, yeast and mould count of roasted marama beans was profiled. This was done to determine whether processing marama beans with a standard roaster and manual dehulling resulted in microbial contamination of the food sample.

3.5.1 Preparation of roasted marama bean samples for plating

Marama beans were roasted at 153ºC at a speed setting of 600xg. After the beans have cooled down, they were dehulled manually (by hand). The area where dehulling took place was sterilized with 70% ethanol (Fig. 4). The beans were then transferred

to a sterilized pestle and mortar and grinding was carried out in the biosafety hood (Fig. 5). One gram of the milled bean sample was diluted at 1:10 (sample: peptone diluent, w/v) ratio and homogenized with a vortex (HPA standard method, 2008). The homogenate was 10^{-1} from which a series of dilutions at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared in peptone diluents (see appendix 2).

Every dilution was plated in duplicates into various media specific for certain microorganisms as described below. The pour plate method was used in this study (HPA standard method, 2008). All agars used in this study were prepared according to the manufacturer's instructions (see appendix 3).

Figure 4. Dehulling of marama beans was carried out manually at a sterilized bench.

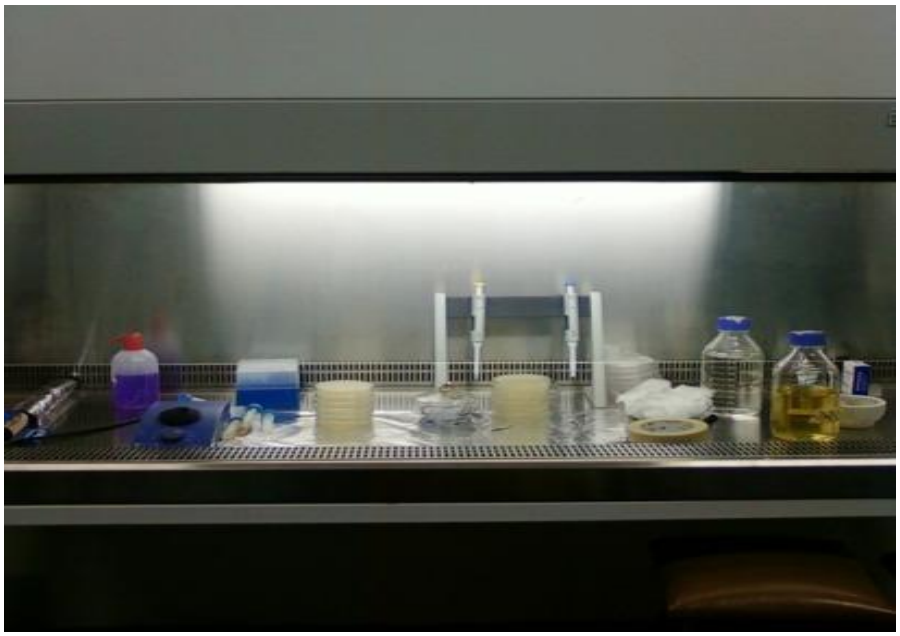


Figure 5. Sample grinding, dilution and plating took place in the biosafety hood.

3.5.2 Microbial total count

The plate count agar (Biolab) was used for the total count of organisms in roasted marama beans. Incubation was carried out at 22ºC for 68 hours and also at 37ºC for 44 hours (HPA standard methods, 2008).

Total count on plate count agar was repeated with marama bean samples that were roasted and stored for 2 days. Incubation was done at the same conditions as above, 22ºC for 68 hours and 37ºC for 44 hours (HPA standard methods, 2008).

3.5.3 Coliforms count

MacConkey agar (Biolab) was used for testing and counting of coliforms in roasted marama beans. Incubation of the plates was carried out at 30ºC for 48 hours (National standard methods, 2005).

3.5.4 *Escherichia coli*

E. coli Direct Agar (Scharlau) was used as the solid culture medium for the detection of *E. coli* in roasted marama beans. Incubation of the plates was carried out at 30ºC for 48 hours (National standard method, 2005).

3.5.5 Yeast and mould count

The Rose Bengal Chloramphenicol Agar (Biolab) was used as a selective medium for the enumeration of yeasts and moulds in roasted marama beans. Incubation was carried out at 28ºC for 72 hours (Fankhauser, 2001).

3.6 Chemical physiological modulation of human and pig cell lines with marama bean extracts

To study physiologic activity of marama bean, human and pig cells were exposed to marama water and ethanolic extracts and the response of the cells to the extracts was observed. Cell growth, proliferation, cell vitality and potential cytotoxic activity was measured by crystal violet assay (Chiba *et al*., 1998). The effects of the marama bean extracts on surface glycan expression were determined by lectin assay (Brooks *et al*., 1997). This study was carried out in triplicates.

3.6.1 Marama bean extracts

Aqueous water extracts were prepared from dehulled marama beans using the O'Dell and De Boland (1976) protocol. The crude ethanolic marama bean extract was prepared following Robinson (1963) as cited in A.V Khan and A.A Khan (2002); the protocol is described in appendix 4. A serial dilution of extracts in advanced

Dulbecco's Modified Eagle's Media (DMEM) was performed (10^{-1} – 10^{-8}). The media was pre-treated with a double dose of antibiotics, penicillin and streptomycin.

Marama extracts were introduced on the cell monolayers in 96-well plates and incubated for 24 hours at 37ºC in a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% air. This was done in order to obtain a cell medium/supernatant and a cell monolayer on which the assays were carried out.

3.6.2 Cell lines

The following cell lines were used in this study; Human intestinal epithelial cell line (H4), Normal pig liver cells (HEP20), pig intestinal epithelial cells (CLAB & PSI), porcine macrophages (POM), gastric adenocarcinoma (AGS), skin cancer cells (melanoma), human carcinoma of large intestine (Caco2) and breast cancer cells (MCF7).

All cell lines were grown in advanced DMEM media supplemented with 10% foetal bovine serum (Sigma-Aldrich), L-glutamine (Sigma), penicillin (Sigma) and streptomycin (Fluka). The cell lines were maintained by routinely passaging in 75 cm^2 culture flasks (Nunc) at 37ºC in a humidified atmosphere of 5% CO_2 (as established by University of Maribor, Slovenia).

3.6.3 Crystal violet assay

The cell monolayer with the extracts was washed with advanced DMEM media supplemented with only L-glutamine. A 100µL of 0.1% crystal violet was added into

each well with monolayer. The plate was then incubated for 2 min at room temperature. After incubation, the plate was rinsed with tap water. Acetic acid was introduced into each well after drying to extract the dye from the cells. The plate was placed on a shaker for 60 min to mix well. Absorbance was then measured at 595nm using a Labsystems multiscan spectrophotometer (as modified from Chiba *et al*., 1998).

3.6.4 Lectin assay

Lectins are simply defined as proteins which specifically bind (or crosslink) carbohydrates. The lectins used in this study to determine the effect of the marama bean water and ethanolic extracts on glycan expression with the different cell lines are presented in Table 3.

Table 3. Lectins used in the study; their full names, origin and their sugar specificity (Brooks *et al*., 1997; Schumacher, Adam, Brooks & Leathem, 1995).

Lectin	**Full name**	**Source of lectin**	**Sugar specificity**
Con A	Concanavalin agglutinin	*Canavalia ensiformis* (Jack bean)	Mannose
HPA	*Helix pomatia* agglutinin	*Helix pomatia* (Roman snail)	*N*-Acetylgalactosamine
PNA	Peanut agglutinin	*Arachis hypogaea* (peanut)	Galactose
UEA I	*Ulex europaeus*- I agglutinin	*Ulex europaeus* (gorse)	Fucose
WGA	Wheatgerm agglutinin	*Triticum vulgaris* (wheatgerm)	*N*-Acetylglucosamine

The lectin assay was performed according to the modified method of Brooks et al. (1997). After incubation of the cell lines with extracts, the cells were fixed with 3% formaldehyde in PBS at room temperature. The cells were washed with 0.2% BSA in lectin buffer (see appendix 5). Incubation was carried out for 20 minutes with H_2O_2 in methanol and further incubation was done for 1 hour at 37ºC with lectin solution.

After the incubation period, the cells were washed with 0.2% BSA in lectin buffer. AEC-DMF- H_2O_2 (see appendix 5) was added for 40 minutes at room temperature. The colour development was checked under a microscope and 96% ethanol was added for 1 hour. Optical density was measured at 540 nm using a Labsystems multiscan spectrophotometer (Multiscan, Finland).

3.7 Data analysis

3.7.1 Sensory analysis

The data from sensory evaluation was analysed using analysis of variance (ANOVA) and multivariate statistics (Young, Sanders, Drake, Osborne & Civille, 2005). For ANOVA, the means for the different attributes were evaluated at the 5% significant level using the least significant test. Test for normality of data was carried out using Kolmogorov-Smirnov test. For multivariate statistics, principal component analysis (PCA) was performed using the XLSTAT software to model the relationship in the data of sensory evaluation. Pie charts were also used to illustrate the consumer results.

3.7.2 Texture profile analysis

Data was analysed using SPSS 17 statistical package. The significance of the parameters were calculated at $\alpha = 0.05$ level using the Kruskal-Wallis test, where there was significant difference among the samples Mann-Whitney test was used to determine the samples that differed (see appendix 8). Test for normality of the data was done using Kolmogorov-Smirnov test.

3.7.3 Nutritional evaluation

The first trial of proximate analysis was done on roasted and unroasted marama beans. It was carried out in duplicates and the data were analysed by one-way ANOVA. The second trial was on proximate analysis of roasted marama beans; it also included mineral analysis of roasted marama beans. These data were also evaluated by one-way ANOVA. Mean differences were evaluated at the 5% significance level ($p \leq 0.05$) using the least significant test (see appendix 7). Test for normality of the data was carried out using Kolmogorov-Smirnov test.

3.7.4 Microorganisms profile

Plating was carried out in duplicates for each analyses. The colonies in each plate were counted with the naked eye. The colony forming units (CFU) were calculated using the following formula:

CFU/mL = Number of colonies x dilution factor x volume factor.

3.7.5 Chemical physiological modulation of human and pig cell lines

The optical density (OD) results from the chemical physiological modulation analysis of human and pig cells with the marama extracts were obtained using a Labsystems Multiscan Mass Spectroscopy Spectrophotometer. The absorbance for crystal violet assay was done at 595 nm (standard wavelength), while that of lectin assay was done at 540 nm (standard wavelength). The OD measurements were then analysed using basic statistical tools incorporated in the Microsoft excel software. Results were shown in the form of column graphs using Microsoft excel.

CHAPTER 4

RESULTS

4.1 Roasting of marama beans

The results of roasting marama beans in a forced convection continuous tumble roaster are illustrated in Table 4. The beans were roasted at 153ºC at the following speed settings; 200xg, 550xg, 600xg and 1000xg.

Table 4. Results of marama beans roasted at the four different speed settings

Trial	**Temperature (*ºC*)**	**Speed setting (xg)**	**Time (min)**	**Description(after roasting)**
1	153	1000	06:17	Still raw
2	153	600	10:20	Very well cooked
3	153	550	10:27	well cooked
4	153	200	25:37	Burned

The roasting temperature was kept fixed at 153ºC. The different speed settings resulted in differently roasted beans. With the help of a consumer panel, a speed setting of 600xg at 153°C was selected as the optimal roasting condition and roasting time under these conditions was 10 min and 20 sec.

4.2 Sensory analyses

4.2.1 Descriptive analysis

Descriptive analysis results of the attributes of roasted marama beans and macadamia nuts are presented in Table 5 in the following categories; aroma, texture, flavour and after taste. The mean values for the two samples as well as the significant differences ($p \leq 0.05$) are also shown in the table (see appendix 7 for Anova tables).

Table 5. Descriptive attribute means (*n=10*) for roasted marama beans and macadamia nuts

Sensory attribute	**Marama**	**Macadamia**	**p-value**
Aroma			
Nutty aroma	7.2[a]	4.1[b]	0.004181
Burnt aroma	5.4[a]	2.2[b]	0.000627
Flavour			
Nutty flavour	7.5[a]	4[b]	0.000123
Burnt flavour	5.9[a]	2.2[b]	0.000228
Oily flavour	4.5[a]	7.7[b]	0.000382
Bitterness	4.8[a]	2[b]	0.000402
Sweetness	3.6[a]	4.5[a]	0.324017
Texture			
Hardness	5.6[a]	3.9[b]	0.009909
Crunchiness	5.2[a]	5.2[a]	1.000000
Tooth picking	5.4[a]	3.3[b]	0.000114
After taste (residual)			
Oiliness	3.4[a]	5.5[b]	0.010656
Particles	5.6[a]	3.9[b]	0.002588

Means in a row followed by different letters represent significant differences ($p \leq 0.05$).

4.2.1.1 Aroma

There was a significant difference ($p \leq 0.05$) between roasted marama beans and macadamia nuts for the nutty aroma attribute. The panel also found a significant difference ($p \leq 0.05$) between roasted marama beans and macadamia nuts for the burnt aroma.

4.2.1.2 Flavour

A significant difference was found ($p \leq 0.05$) between roasted marama beans and macadamia nuts for the nutty flavour attribute. The panel also found a significant difference ($p \leq 0.05$) between the two samples for the following attributes burnt flavour and bitterness. There was no significant difference ($p \geq 0.05$) between roasted marama beans and macadamia nuts with regard to the sweetness attribute.

4.2.1.3 Texture

There was a significant difference ($p \leq 0.05$) in hardness and tooth picking attribute between roasted marama beans and macadamia nuts. There was however no significant difference found ($p \geq 0.05$) in the crunchiness of roasted marama beans and macadamia nuts.

4.2.1.4 After taste (residual)

A significant difference was found ($p \leq 0.05$) between roasted marama beans and macadamia nuts for the oiliness and particles after taste residual attribute.

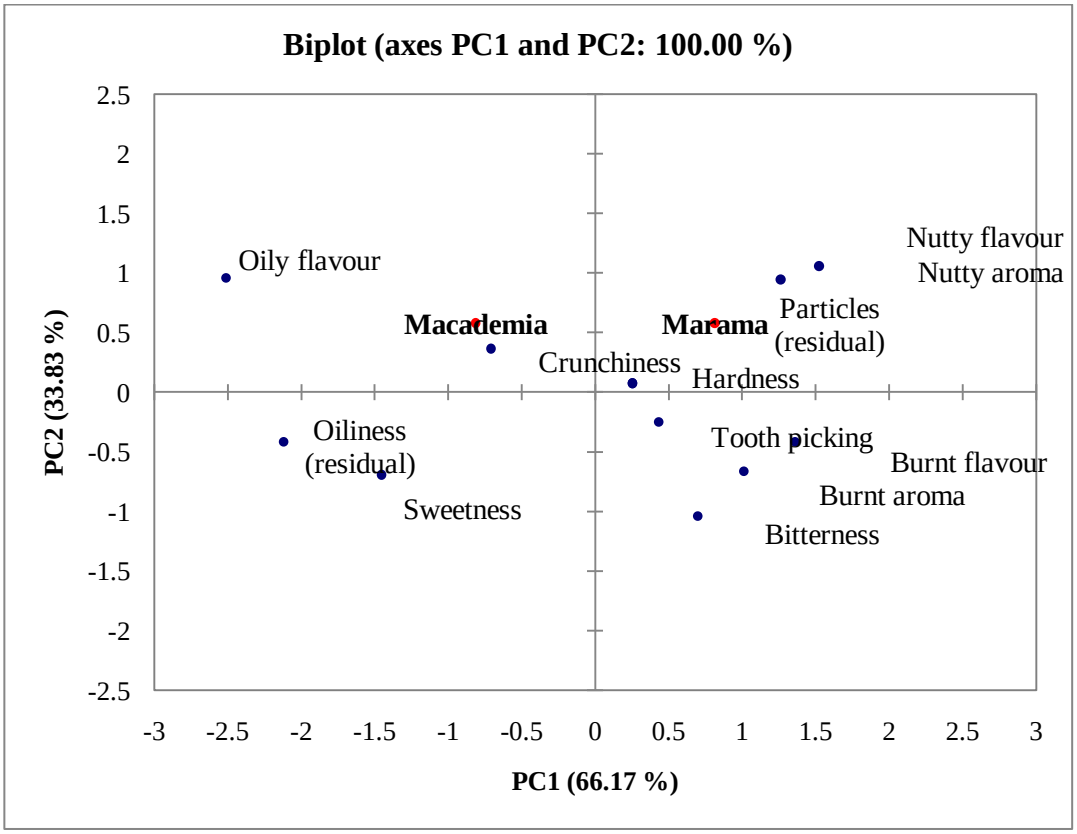


Figure 6. Principle component analysis biplot of roasted marama beans and macadamia nuts by descriptive analysis

According to the PCA biplot (Fig. 6), roasted macadamia nuts were more associated with oily flavour, oily residue or after taste, and sweetness. Roasted marama beans were associated more with nutty flavour, nutty aroma, particles residual or after taste, hardness, burnt flavour, burnt aroma, bitterness and tooth picking. Both samples were found to have equal crunchiness.

4.2.2 Consumer evaluation

For the first trial of consumer analysis, the panel preferred ($p \leq 0.05$) the marama beans roasted at a speed setting of 600xg at a temperature of 153ºC and 10 min; 20 sec.

The second trial of consumer analysis involved comparing roasted marama beans to roasted macadamia nuts. The consumer mean scores are presented in table 6. The results were further illustrated in figures 5, 6 and 7.

Table 6. The means of answer percentage for each point in hedonic scale for the consumer preference (n = 50) of roasted marama beans and macadamia nuts.

Consumer preference	**Marama**	**Macadamia**
9- Like extremely	8^a	18^b
8- Like very much	14^a	34^b
7- Like moderately	28^a	12^b
6- Like slightly	26^a	18^b
5- Neither like nor dislike	2^a	4^a
4- Dislike slightly	10^a	6^a
3- Dislike moderately	0^a	0^a
2- Dislike very much	6^a	8^a
1- Dislike extremely	6^a	0^b

Means in a row followed by different letters represent significant differences ($p \leq 0.05$).

The answer percentage for consumer scores 1 to 5 was higher for roasted marama beans. That is 24% for roasted marama beans and 18% for roasted macadamia nuts.

The answer percentage for consumer scores 6 – 9 was higher for roasted macadamia nuts. That is 82% for roasted macadamia nuts and 76% for roasted marama beans.

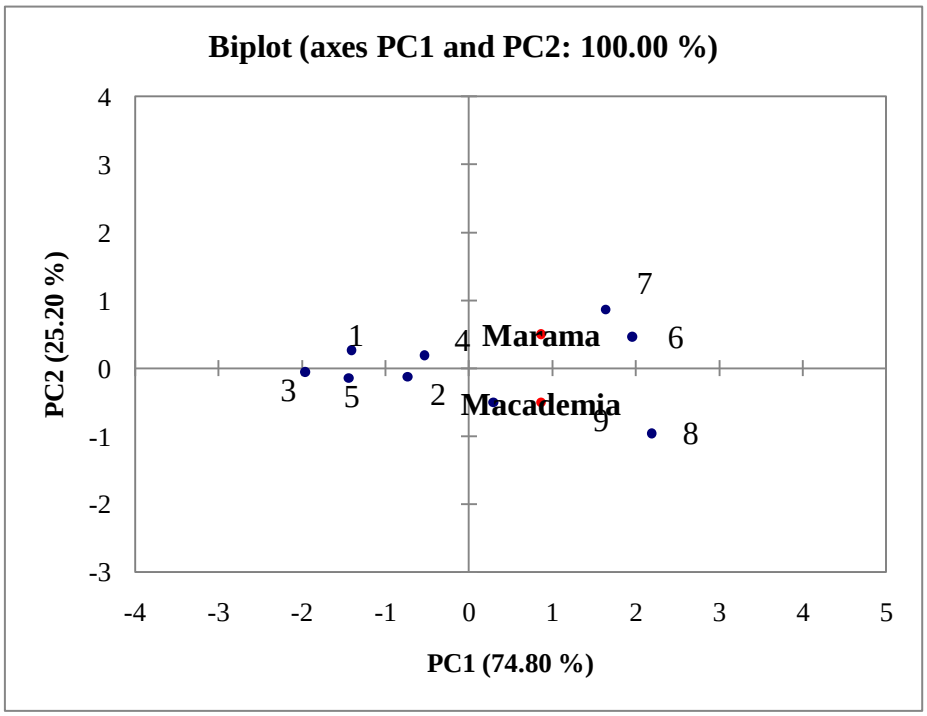


Figure 7. Principle component analysis biplot of roasted marama and macadamia nuts by consumer analysis.

The consumer scores represented the following; 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

The PCA biplot (Fig. 7) indicates that roasted marama bean samples received a high percentage score for points 1, 4, 7 and 6. The roasted macadamia nuts received a high percentage score for points 2, 5, 8 and 9. None of the panelists chose score 3

(dislike moderately) for either of the samples. These results are also illustrated in Figures 8 and 9.

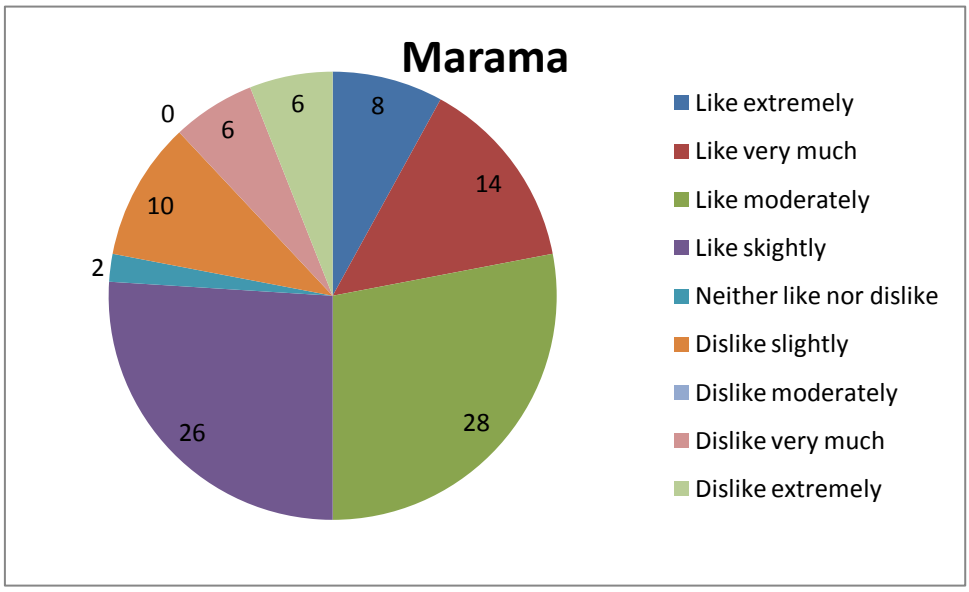


Figure 8. Results of the consumer preference (n = 50) of roasted marama beans.

The numbers in the chart represent the percentage of the panelists that selected a certain category.

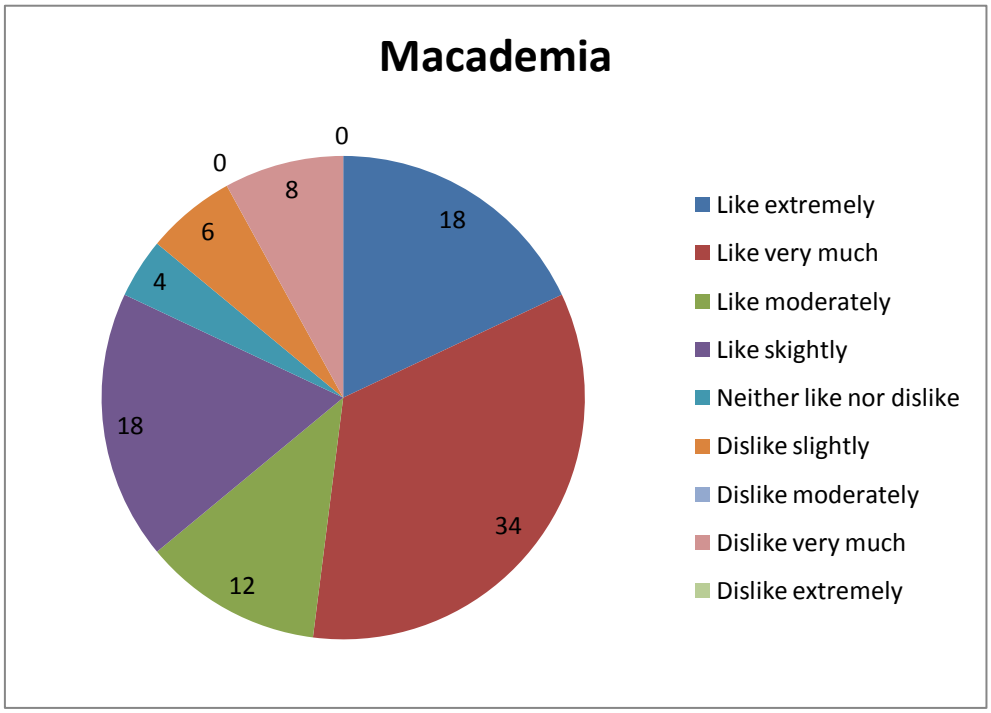


Figure 9. Results of the consumer preference (*n* = 50) of roasted macadamia nuts.

The numbers in the chart represent the percentage of the panelists that selected a certain category.

4.3 Texture profile analysis

The TPA results are presented in Table 7. Adhesiveness of roasted and raw marama beans was significantly low compared to that of roasted macadamia nuts. A similar observation occurred with chewiness. Fracturability and hardness of roasted and raw marama beans were significantly higher than that of roasted macadamia nuts. Raw marama beans were found to have significantly high cohesiveness, gumminess, springiness and resilience compared to roasted marama bean and macadamia nuts. TPA on raw marama beans was carried out just to compare its textural properties to roasted marama beans.

Table 7. Texture profile properties of roasted marama beans, raw marama beans and roasted macadamia nuts.

Sample tested	**A**	**C**	**Co**	**F**	**G**	**H**	**S**	**R**
Roasted marama	0.5^{a}	1.2^{a}	0.4^{a}	10.3^{a}	3.5^{a}	8.8^{a}	0.3^{a}	0.3^{a}
Raw marama	0.6^{a}	1.7^{a}	0.5^{b}	10.1^{a}	4.2^{b}	8.6^{a}	0.4^{b}	0.4^{b}
Roasted macadamia	6.2^{b}	4.5^{b}	0.2^{c}	7.8^{b}	1.4^{c}	5.5^{b}	0.3^{a}	0.1^{c}

Data with different letters in a column are statistically different at $p<0.05$

[A – adhesiveness; C – chewiness; Co – cohesiveness; F – fracturability; G – gumminess; H – hardness; S – springiness; and R – resilience]

4.4 Nutritional value of roasted marama beans

4.4.1 Comparing the proximate analyses of roasted and unroasted marama beans

The study conducted in 2007 compared the nutritional value of roasted and unroasted marama beans. The results are presented in Table 8. The roasted sample was prepared in an oven at 153ºC for 40 minutes. Analyses were done in duplicates.

Table 8. Proximate analysis results (%) of roasted and unroasted marama beans (±SD).

Test / Identification	**Moisture**	**Crude fats & Oils Extractable**	**Ash**	**Nitrogen**	**Crude Protein**	**Carbohydrates**
Roasted marama bean	$3.0^{a} \pm 0.14$	$32.2^{a} \pm 3.82$	$3.2^{a} \pm 0.42$	$5.6^{a} \pm 0.28$	$35.0^{a} \pm 0.57$	$26.7^{a} \pm 0.71$
Unroasted marama bean	$4.4^{b} \pm 0.28$	$26.5^{b} \pm 0.71$	$3.5^{a} \pm 0.42$	$5.7^{a} \pm 0.57$	$33.9^{a} \pm 1.56$	$31.9^{a} \pm 2.0$

Means in a column followed by different letters represent significant differences ($p \leq 0.05$).

A significant difference ($p \leq 0.05$) was found in the moisture and crude fats content of roasted and unroasted marama beans.

There was no significant difference ($p \geq 0.05$) in the ash, nitrogen, protein and carbohydrate contents of roasted and unroasted marama beans.

4.4.2 Proximate and mineral evaluation of marama beans prepared in a standard roaster

The marama beans prepared in the standard roaster were analysed for proximate and mineral value. The results are presented in Table 9 and 10. The samples that were analysed were prepared at the following speed settings; 550xg (sample 1) and 600xg (sample 2). The temperature of the roaster was kept fixed at 153ºC. Sample 2 was preferred by the consumer panel (4.2.2).

Table 9. Proximate analysis (%) results of roasted marama beans (±SD)

	Dry matter	**Crude protein**	**Crude fats & oil extractable**	**Carbohydrates**	**Ash**	**Crude fiber**
Sample 1	$97.2^{a} \pm 0.17$	$33.8^{a} \pm 0.15$	$35.2^{a} \pm 0.93$	$25.3^{a} \pm 0.79$	$2.9^{a} \pm 0.0$	$13.1^{a} \pm 2.10$
Sample 2	$97.0^{a} \pm 0.12$	$32.4^{a} \pm 0.60$	$31.5^{a} \pm 4.93$	$30.0^{a} \pm 4.85$	$3.0^{a} \pm 0.0$	$14.7^{a} \pm 0.87$

Means in a column followed by different letters represent significant differences ($p \leq 0.05$).

The results for proximate analysis studies of marama beans roasted at the speed settings of 550xg (sample 1) and 600xg (sample 2) at a fixed temperature of 153 ºC are shown in Table 9.

There was no significant difference ($p \geq 0.05$) in the nutritional value of the two roasted marama bean samples. The energy value was found to be 2222 kJ/100g for sample 1 and 2219 kJ/100g for sample 2. No significant difference ($p \geq 0.05$) was found between the two values either.

Table 10. Mineral analysis (mg/100g) results of roasted marama beans (±SD)

	Sodium	**Potassium**	**Magnesium**	**Calcium**	**Iron**	**Phosphorus**
Sample 1	45.3^{a} ±9.61	790.3^{a} ±33.7	284.7^{a} ±6.03	107.3^{a} ± 6.66	1.1^{a} ± 0.64	384.7^{a} ± 5.51
Sample 2	64.0^{a} ±21.7	741.7^{a} ±50.2	299.7^{a} ±13.3	122.7^{b} ± 5.51	1.4^{a} ± 0.15	427.7^{b} ± 5.13

Means in a column followed by different letters represent significant differences ($p \leq 0.05$).

The results of mineral analysis of marama beans roasted at a speed setting of 550xg (sample 1) and 600xg (sample 2) at 153ºC. The significant difference was only found ($p \leq 0.05$) in the calcium content of the two roasted marama bean samples.

The results for mineral analysis were compared to the mineral values of the recommended daily allowance (RDA) in Table 11.

Table 11. Daily recommended mineral allowance for all population groups (Kretser, 2006).

Mineral	**Recommended daily allowance (RDA) (mg)**
Calcium	1000
Phosphorus	700
Potassium	3500
Sodium	1600
Iron	18
Magnesium	400

4.5 Microorganism profile of roasted marama beans

There was no colony forming units on all the plates inoculated with 1 mL of the roasted marama sample dilutions 10^{-1} to 10^{-6}. The results for total count, coliforms count, *E. coli*, yeast and mould count were all 0/1mL of dilutions (0 = not detected). The plates that were used in this study are presented in figures 10, 11, 12 and 13.

No colony forming units were detected with the dilutions of 10^{-1} to 10^{-6} of roasted marama beans as shown in Figures 10, 11, 12 and 13.

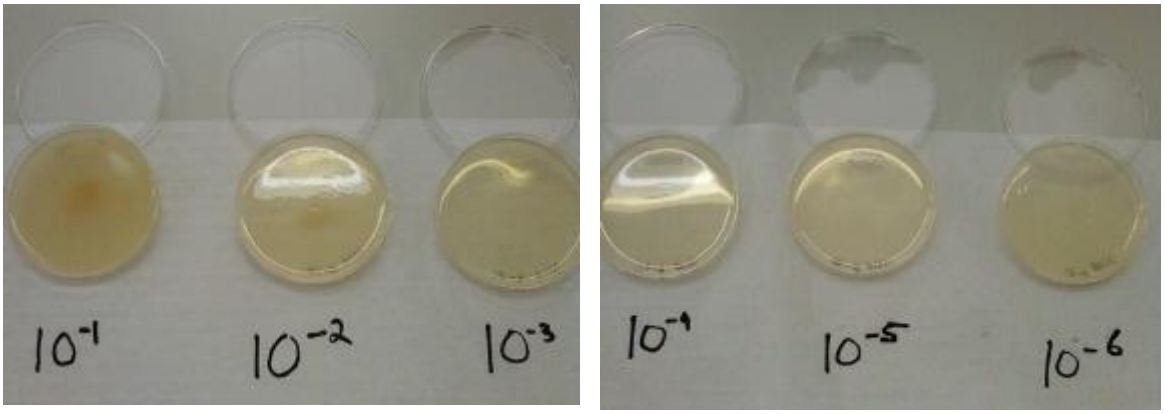


Figure 10. Total count with plate count agar.

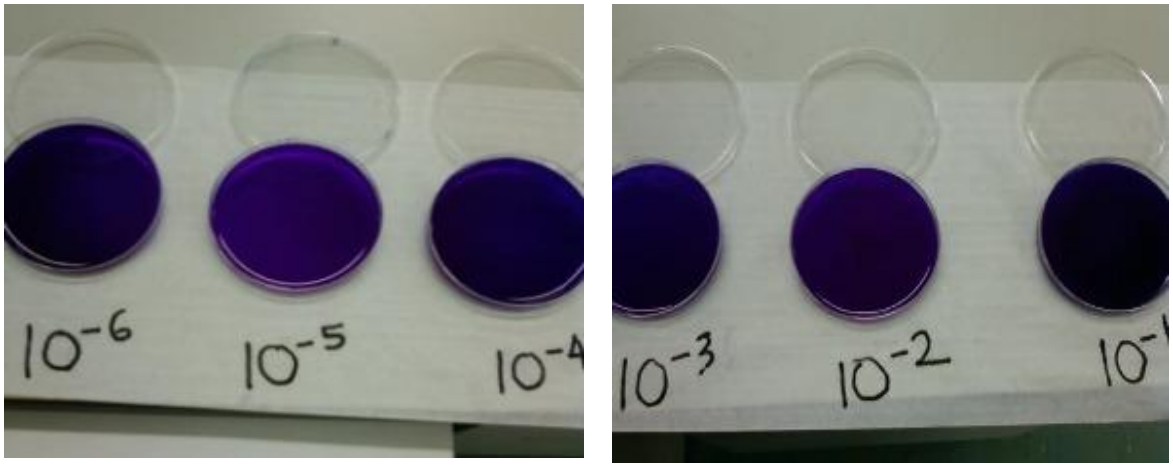


Figure 11. Coliforms count with MacConkey agar.

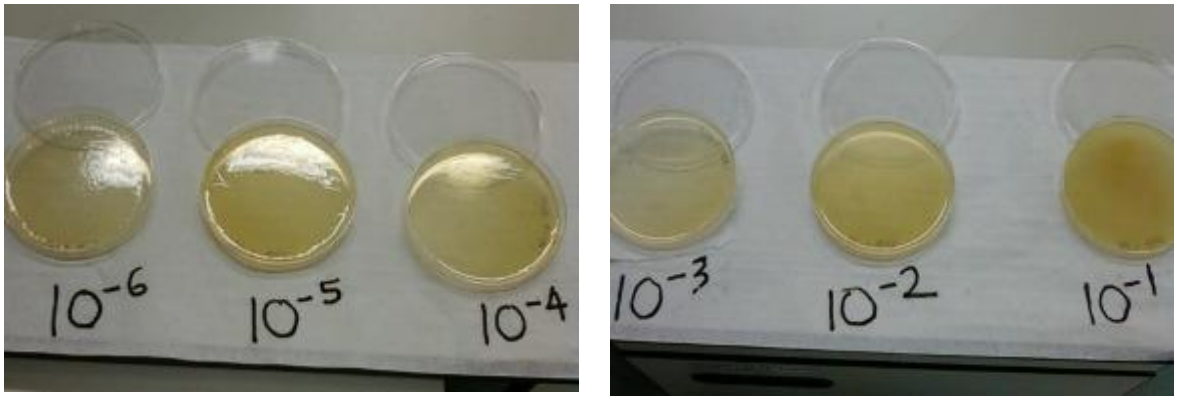


Figure 12. *E. coli* detection with *E. coli* Direct Agar.

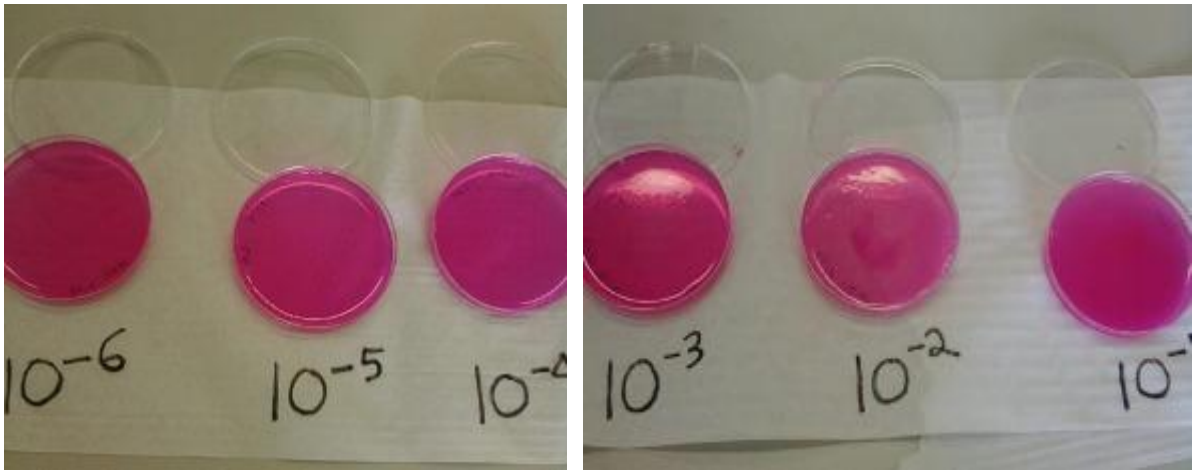


Figure 13. Yeast and mould count with the rose bengal chloramphenicol agar.

4.6 Chemical physiological modulation of human and pig cell lines with marama beans extracts

4.6.1 Cell viability with crystal violet assay

The cell lines were exposed to the following extracts; marama bean cotyledon water extract (MP7), marama husk water extract (HP7), marama tuber water extract (TDP7 & MTD7), marama cotyledon ethanolic extract (ME5) and marama husk ethanolic extract (HE5). The results for the extracts used showed ability to enhance and reduce viability in the normal and cancer cells. The results were obtained after 24 h exposure of the cells to the extracts. The results of the marama extracts with the normal cells are shown in Figures 14-18, while those with cancer cells are shown in Figures 19-23.

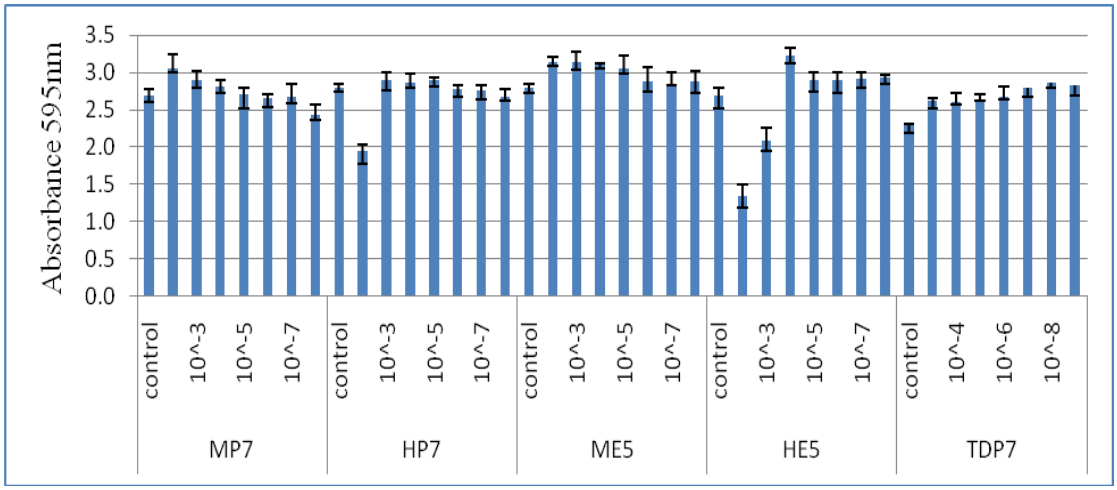


Figure 14. Effect of marama water and ethanolic extracts on normal human cells (H4) after 24 h exposure.

Concentration of 10^{-2} of the marama husk extracts (HP7 & HE5) led to the lowest viability of H4 cells (Fig. 14). The concentration, 10^{-2} of the water tuber extracts completely destroyed H4 cells. However for the cotyledon extracts (ME5 & MP7), best viability was obtained at the concentration of 10^{-2} and viability was increasing with increasing extract concentration. For the husk and tuber (HP7, HE5 & TDP7) viability increased inversely with the concentration of the extracts.

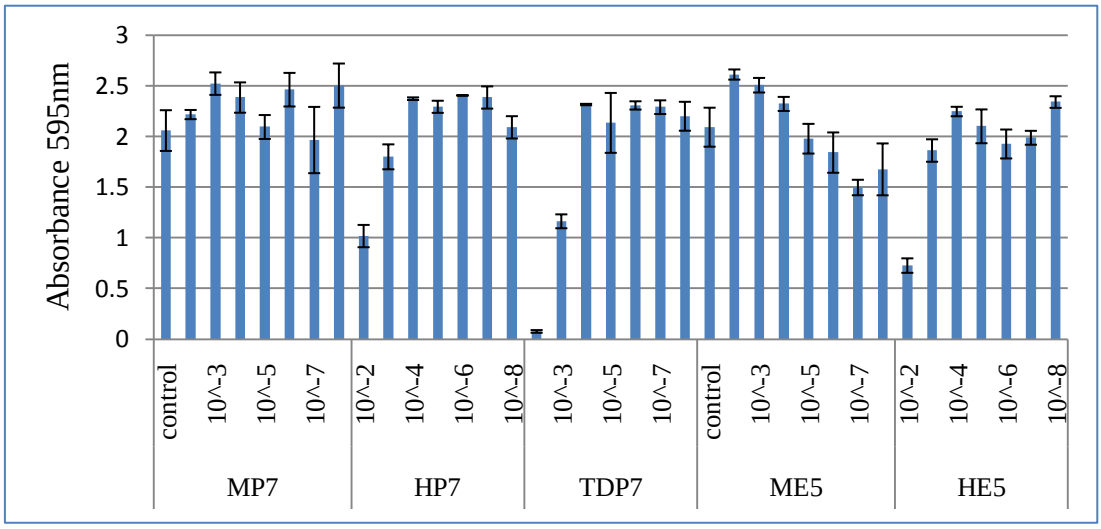


Figure 15. Effect of marama water and ethanolic extracts on viability of normal pig liver cells (Hep20) after 24 h exposure.

From the work carried out, it was observed that HEP20 cells were quite sensitive compared to other cells used. Hep20 cells were partially destroyed at 10^{-2} of the husk extracts (HP7 & HE5) and completely destroyed at 10^{-2} by the tuber water extract (TDP7). With the cotyledon water extract (MP7) excellent viability was observed between the concentrations of 10^{-3} to 10^{-7}. This observations was similar to that of the husk water extract (HP7). Growth at 10^{-2} for cotyledon ethanolic extract (ME5) was much more compared to that of cotyledon water extract (MP7).

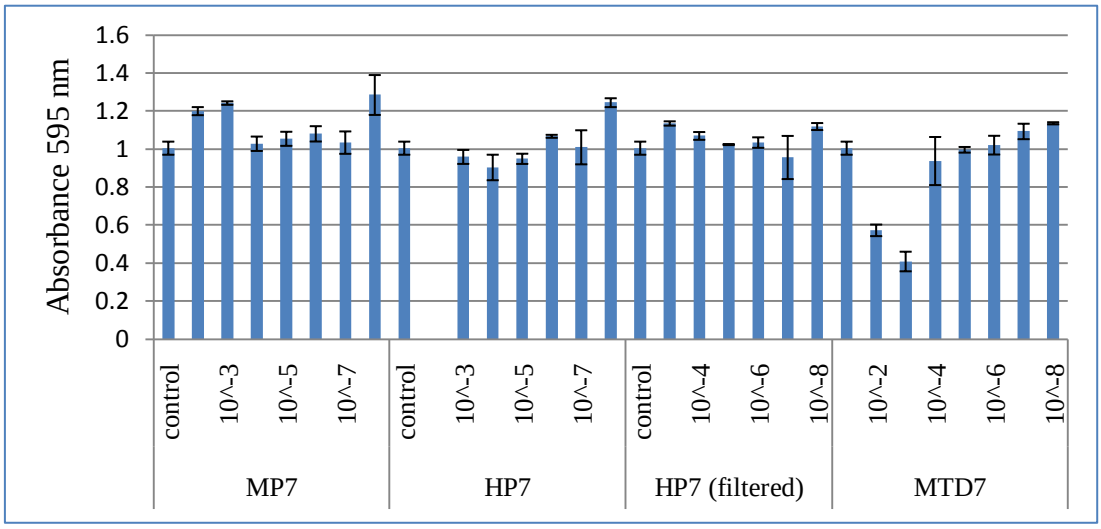


Figure 16. Effect of marama water extracts on the viability of pig intestinal epithelial (CLAB) cells after 24 h exposure.

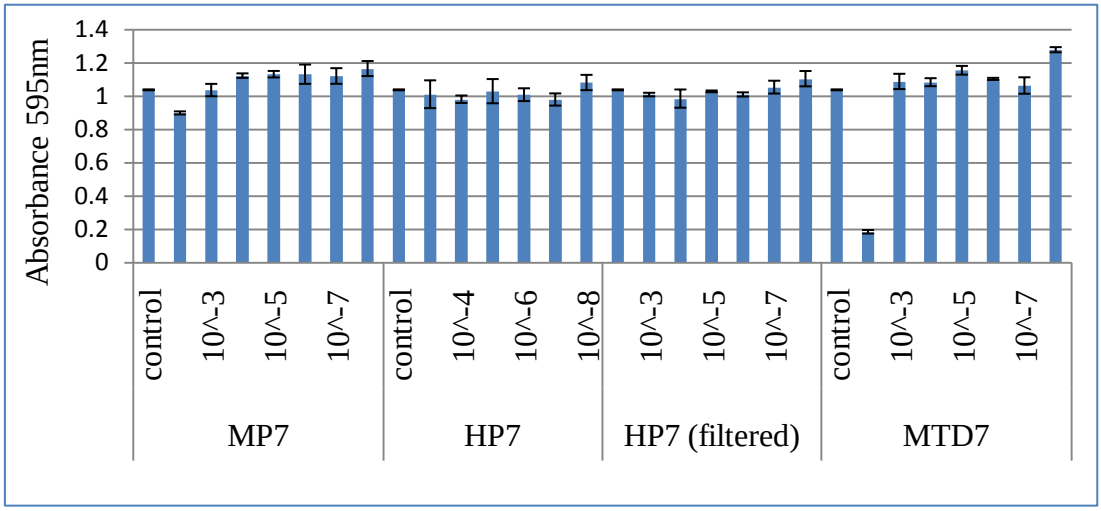


Figure 17. Effect of marama water extracts on the viability of pig intestinal epithelial (PSI) cells after 24 h exposure.

Figures 16 and 17 show that the concentration of 10^{-2} of the husk water extract (HP7) completely destroyed the CLAB and PSI cells; viability increased at between dilutions of 10^{-5} and 10^{-8}. Concentration of 10^{-2} of MP7 decreased the viability of PSI

cells; overall, the MP7 extract increased the survival of CLAB and PSI cells. The tuber water extract (MTD7) also led to high viability of the cells with decreasing concentration from 10^{-3}.

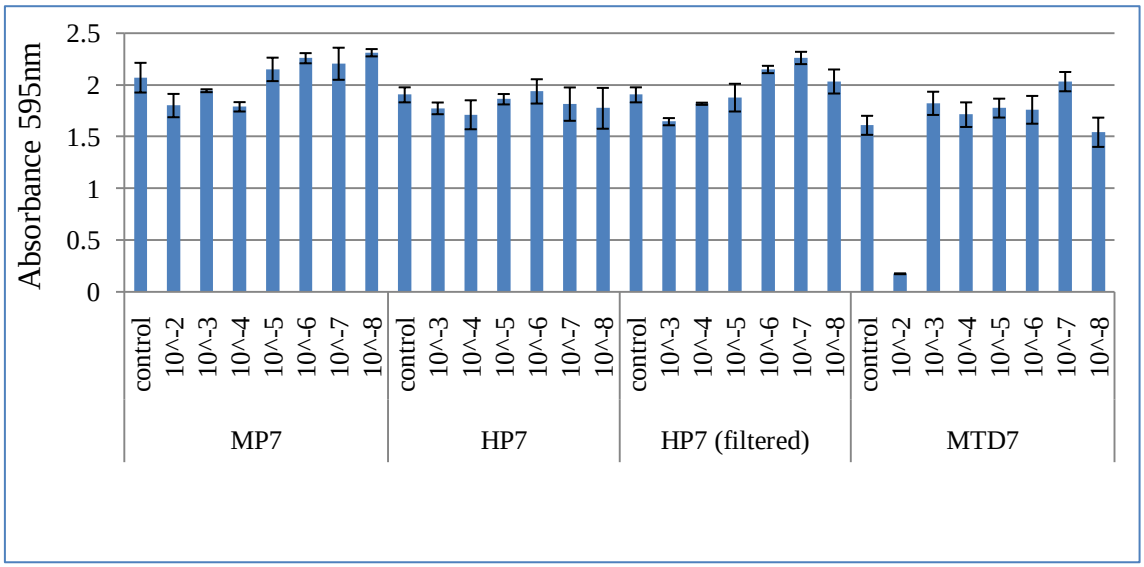


Figure 18. Effect of marama water extracts on the viability of porcine macrophages (POM) cells after 24 h exposure.

In this experiment, a filtered sample of the husk protein extract was used in order to compare its effect to that of the unfiltered husk water extract. The lower dilutions of all the water extracts increased the viability of the porcine macrophages except for the unfiltered husk water extract. Positive activation of macrophages like what was observed with these results means better immune response.

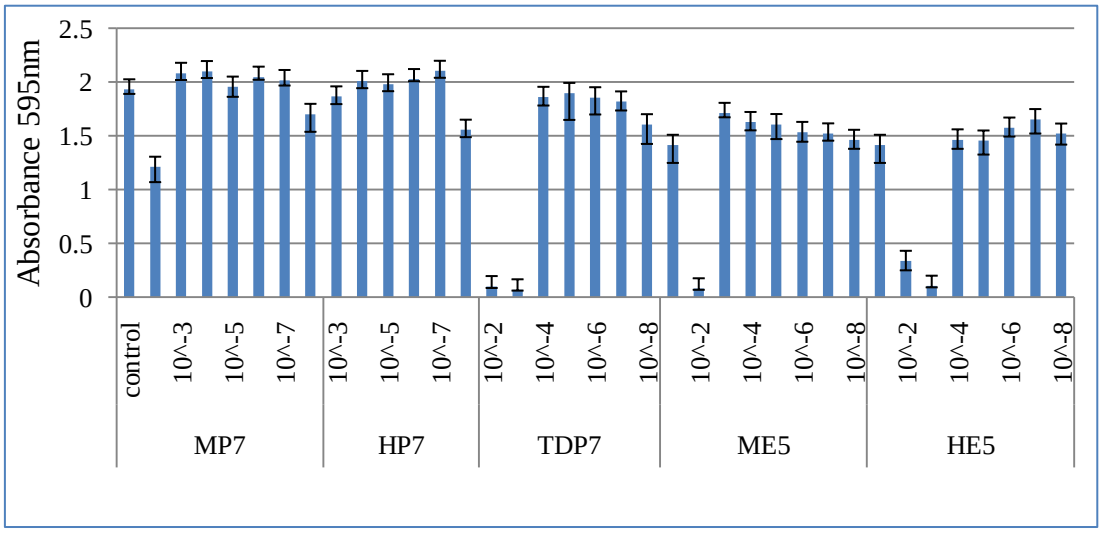


Figure 19. Effect of marama water and ethanolic extracts on viability of gastric adenocarcinoma (AGS) cells after 24 h exposure.

Figure 19 indicates that the concentration of 10^{-2} for all the extracts used decreased the survival of AGS cells, in some cases cells were completely destroyed (HP7, TDP7 & ME5). The concentration of 10^{-3} of the water and ethanolic extracts also decreased the viability of the cells except for the cotyledon protein. Overall, the ethanolic cotyledon & tuber extracts (ME5, HE5) and aqueous tuber water (TDP7) decreased the viability of the cancer cells as compared to the control and only the lower dilution of the cotyledon and husk water extracts (MP7 & HP7) had an effect of decreasing the viability of AGS cells.

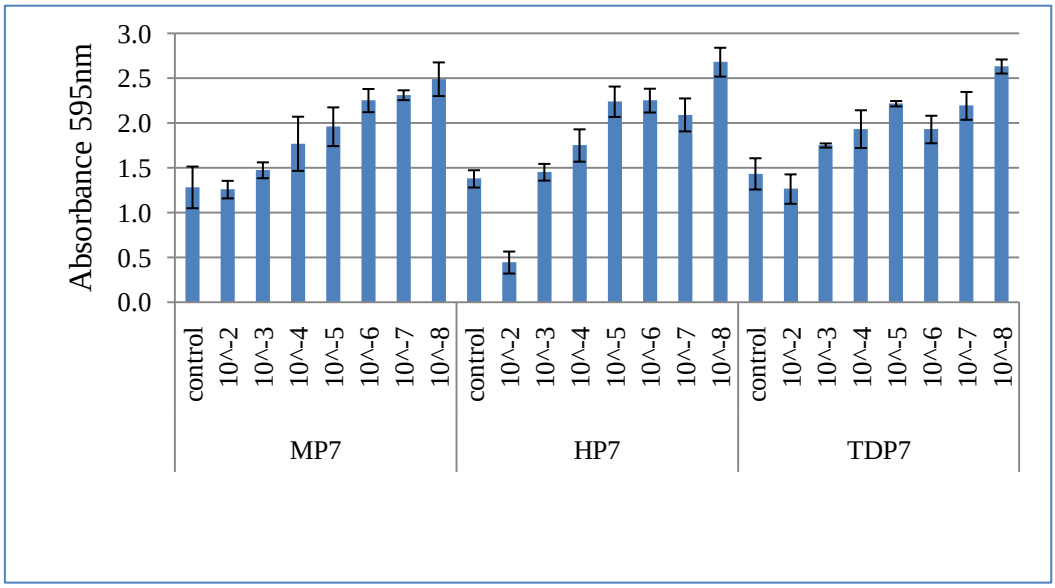


Figure 20. Effect of marama water extracts on the viability of human intestinal cancer cells (Caco2) after 24 h exposure.

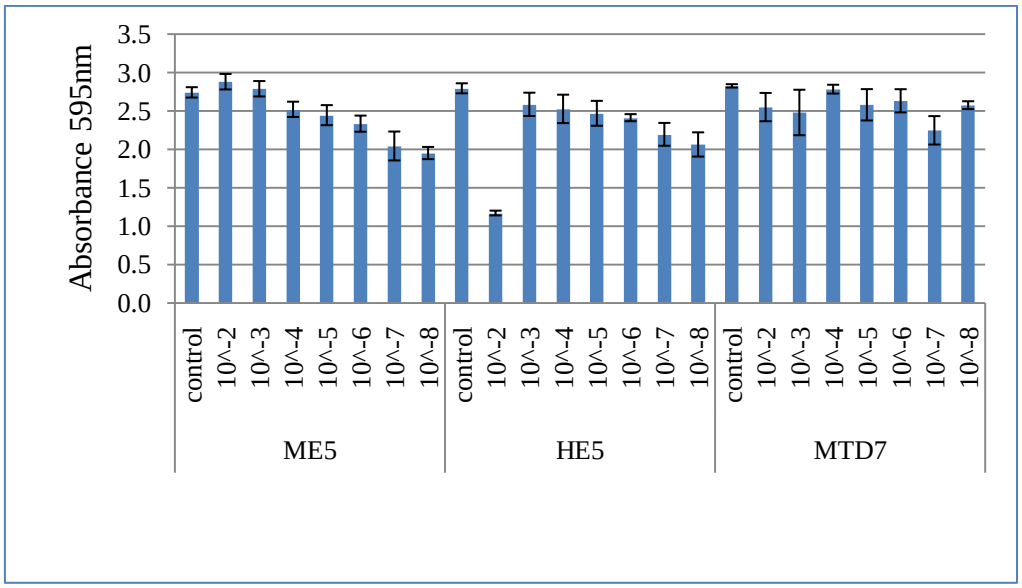


Figure 21. Effect of marama ethanolic extracts on the viability of Caco2 cells after 24 h exposure.

The results obtained in Figures 20 and 21 show that the viability/survival of Caco2 cells increased inversely proportional to the concentrations of the water extracts (Fig. 20). With the ethanolic extracts, the viability of the Caco2 cells decreased as the concentrations of the ethanolic extracts was decreased (Fig. 21). Caco2 cells had low viability in all dilutions of HE5 (husk ethanolic) and MTD7 (tuber water).

The results for the effect of water and ethanolic extracts on breast cancer cells (MCF7) are shown in Figures 22 and 23. The two graphs showed that the cotyledon, husk and tuber water extracts were both decreasing viability of cells at 10^{-2}. The same effect was also observed for the husk ethanolic extract. MTD7 (tuber solution), completely destroyed the cancer cells at 10^{-2}. Significant growth suppression of the breast cancer cells was observed with all the extracts at their lower concentrations. While the cotyledon water extract was suppressing growth at 10^{-2}, the cotyledon ethanolic extract was enhancing growth of the cancer cells at 10^{-2}.

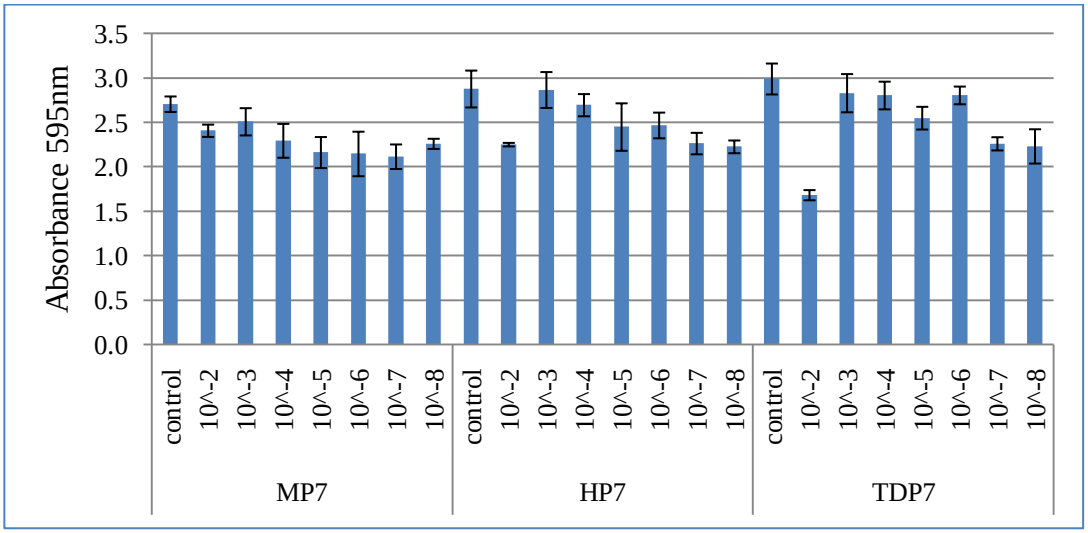


Figure 22. Effect of marama water extracts on the viability of breast cancer cells (MCF7) after 24 h exposure.

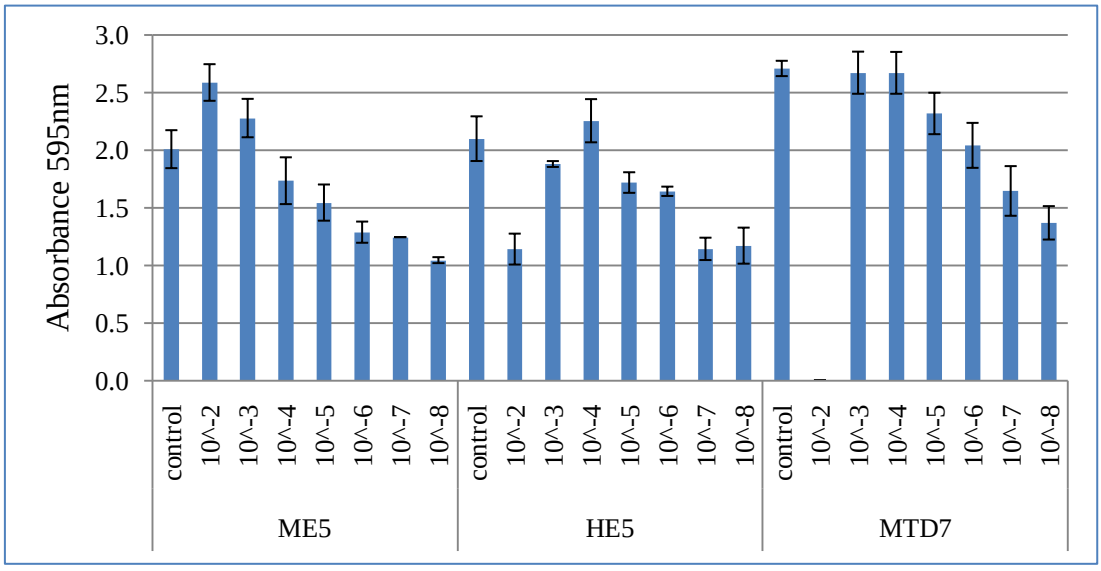


Figure 23. Effect of marama ethanolic extracts on the viability of MCF7 cells after 24 h exposure.

4.6.2 Lectin assay

The following lectins were tested in normal cells; WGA, CON A, HPA and UEA I. They all binded to the cell line (Fig. 24). Increased glycan production was observed with all concentrations of the extracts used except for MP7 and HP7 with WGA. Note that only dilutions that previously showed increase in H4 viability were used (Fig. 14). This means that, the dilutions of the water and ethanolic extracts used increased surface glycan production except for HP7 which did not induce sugar residues specific for WGA.

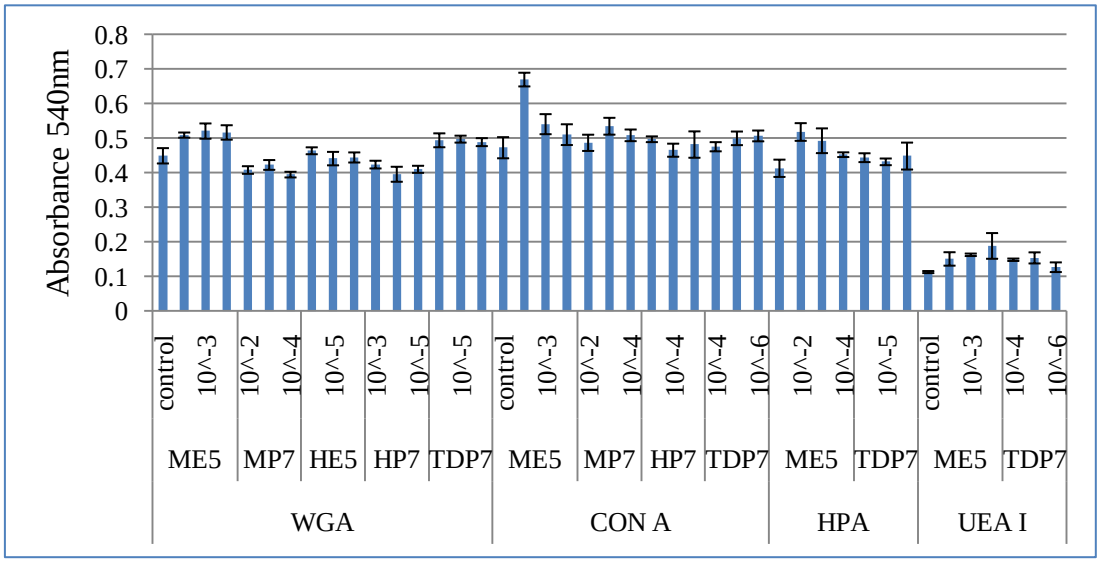


Figure 24. Effect of marama water and ethanolic extracts on glycan production with H4 cells.

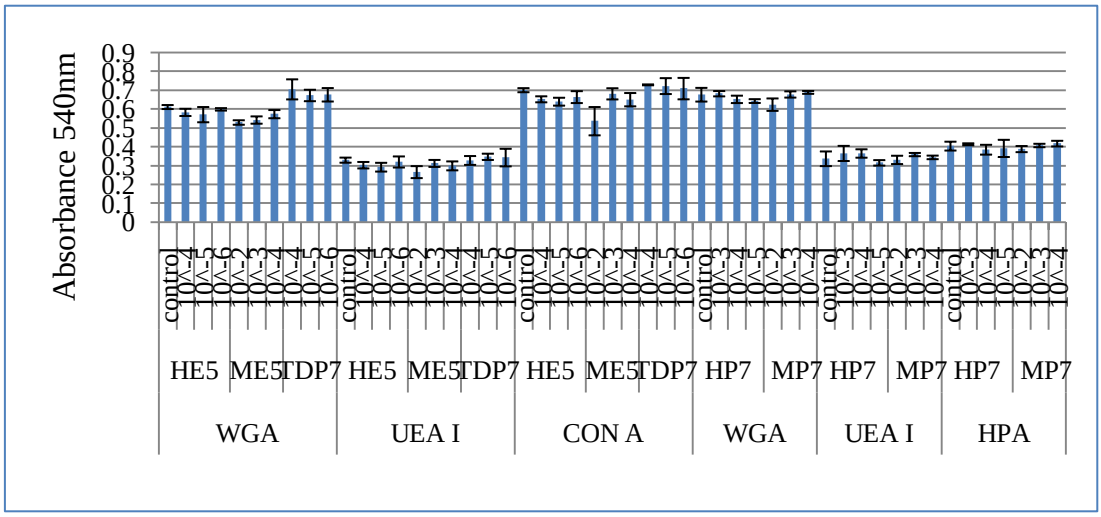


Figure 25. Effect of marama water and ethanolic extracts on glycan production in CLAB cells.

In CLAB cells, the lectins that showed significant binding were WGA and CON A with the tuber water extract (TDP7) as compared to their controls. This means that TDP7 induces the production of glycans on cell surface of CLAB cells. The marama water cotyledon (MP7) also showed weak binding of WGA to cell surface at 10^{-4}. With the lectin UEA I, binding was also observed with cells incubated with TDP7 even though there was reduced expression of UEA I and HPA with the cells.

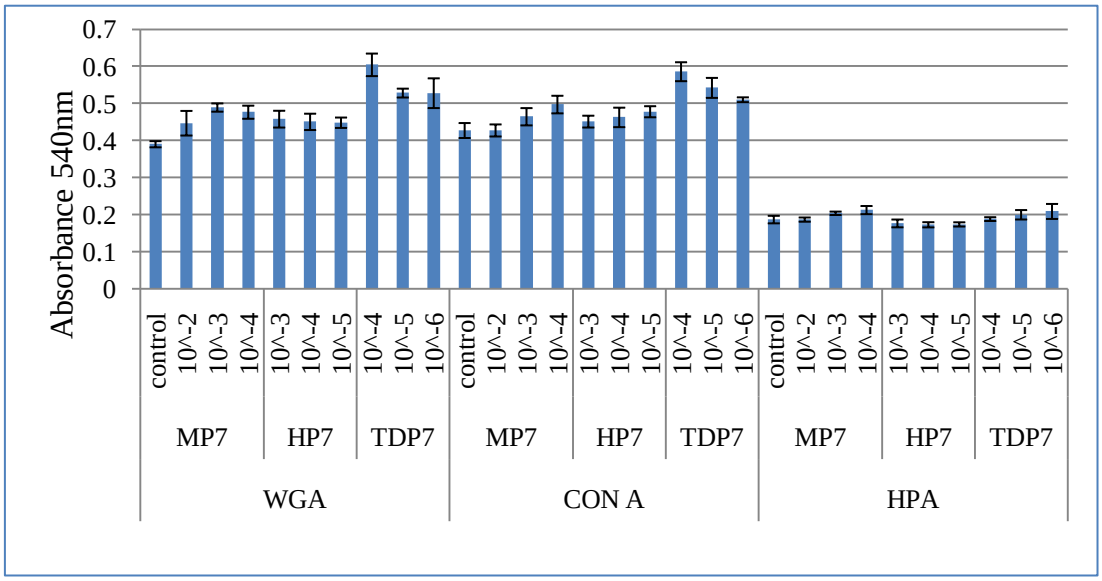


Figure 26. Effect of marama water extracts on glycan production in IPEC cells.

WGA and CON A strongly binded to IPEC cells incubated with tuber water extract (TDP7). Other strong binding of the same lectins was observed with the cells incubated with cotyledon water extract (MP7). This shows the presence of glycans specific for WGA and CON A present on the surface of the IPEC cells. HPA showed weak binding with these cells.

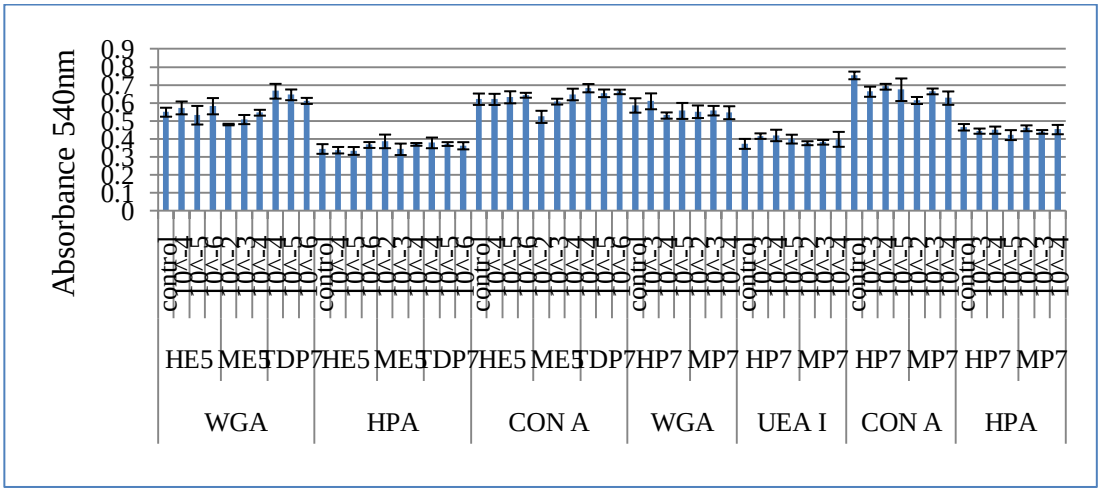


Figure 27. Effect of marama ethanolic and water extracts on glycan production with PSI cells.

As previously observed with CLAB and IPEC cells (Figs. 25 and 26), WGA and CON A showed significant binding with PSI cells (Fig. 27) incubated with tuber water extract (TDP7). Even though there was reduced glycan expression with HPA & UEA I, HPA showed strong binding with cells incubated with TDP7 and UEA I showed some strong binding with cells incubated with Husk water extract (HP7).

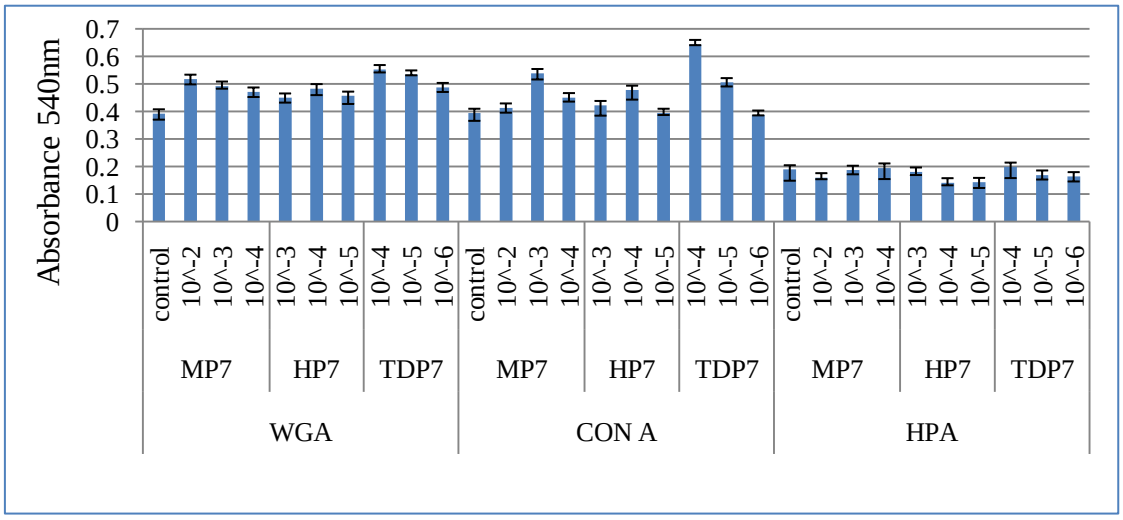


Figure 28. Effect of marama water extracts on glycan production in TLT cells.

The lectins that strongly binded to the cell surface of TLT incubated with water extracts were WGA and CON A (Fig. 28). This shows the presence of sugar residues specific for those two lectins on the cell surface of human macrophage cells.

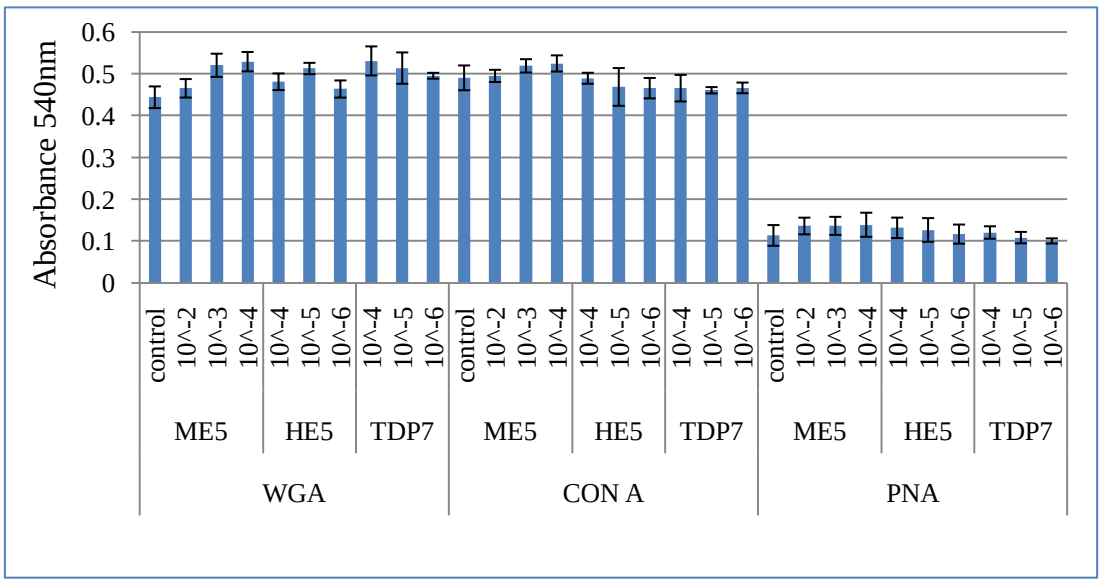


Figure 29. Effect of marama ethanolic and water extracts on glycan production in melanoma cells.

Melanoma is a type of skin cancer, originating in the melanocytes, the cells containing skin color. Figure 29 showed strong binding of lectins WGA and CON A in melanoma cells incubated with the cotyledon ME5 ethanolic extracts. WGA also binded strongly to melanoma cells incubated with the husk ethanolic and tuber water extract (TDP7). PNA binded weakly to the melanoma cells.

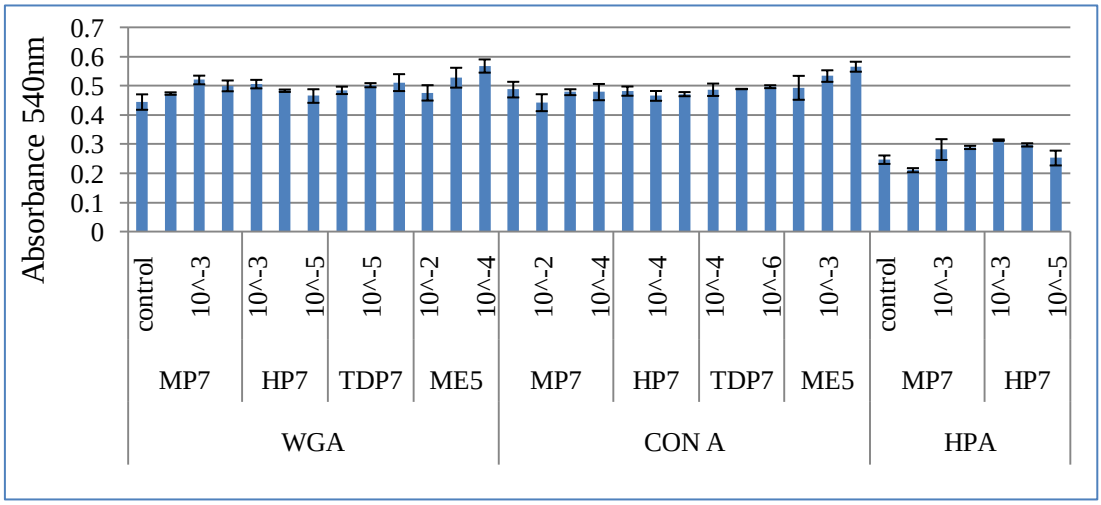


Figure 30. Effect of marama water and ethanol extracts on glycan production in Hep20 cells.

Strong lectin binding was observed with the liver cells incubated with the cotyledon ethanolic extract (ME5). HPA and WGA showed strong binding with cells incubated with cotyledon and husk water extracts (MP7 and HP7).

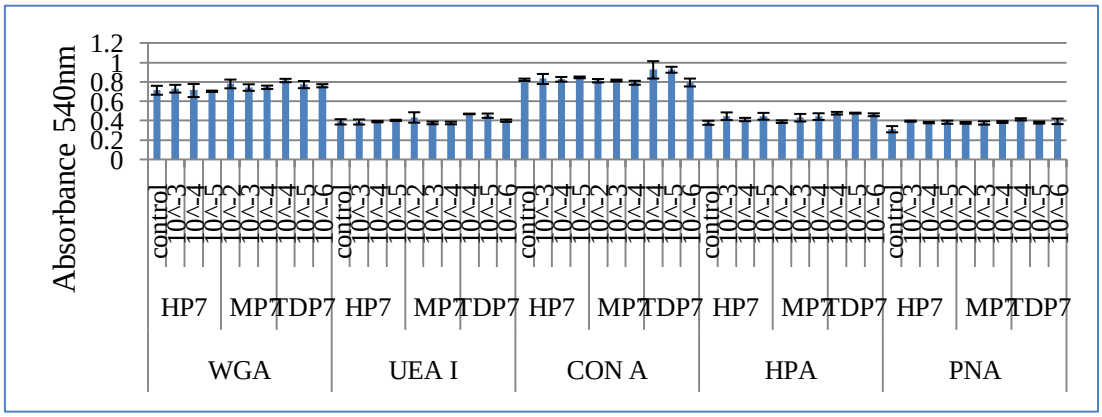


Figure 31. Effect of marama water extracts on glycan production in MCF7 cells.

Strong binding with all the lectins was mainly observed with the breast cancer cells incubated with the tuber water extract (TDP7).

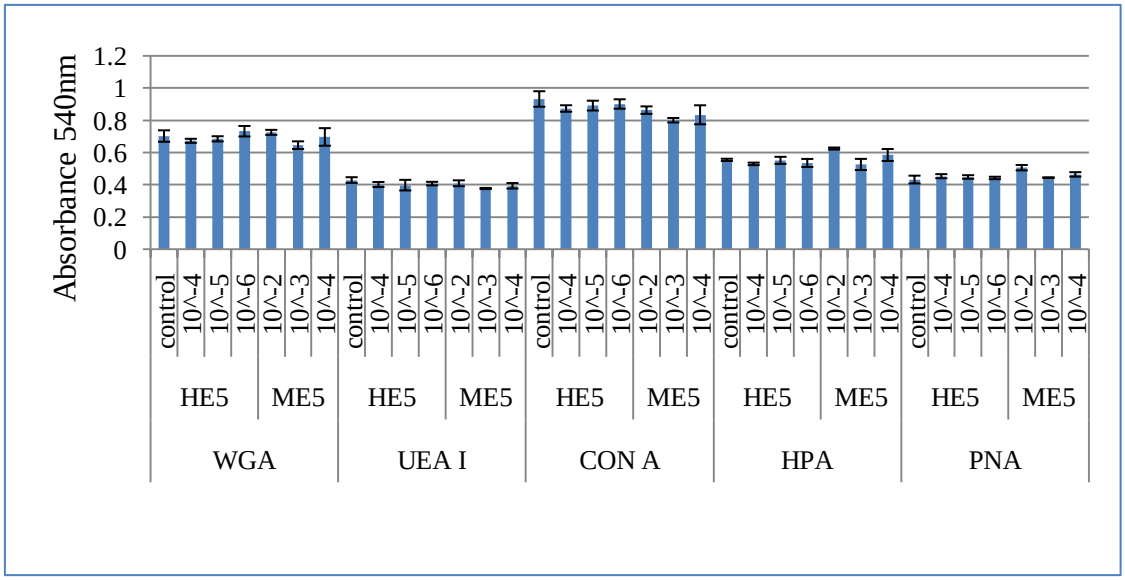


Figure 32. Effect of marama ethanolic extracts on glycan production in MCF7 cells.

With the breast cancer cells incubated with cotyledon and husk ethanolic extracts (ME5 & HE5), strong lectin to cell binding was observed with HPA and PNA lectins only. Weak lectin to cell binding was observed with WGA lectin.

CHAPTER 5

DISCUSSION

In this chapter, the results obtained on roasting of marama beans, sensory evaluation, texture profile analyses, nutritional evaluation, microorganism profile and its health benefits will be discussed.

5.1 Optimisation of roasted marama beans

The temperature that was found to be optimal to prepare marama bean using the standard roaster was 153ºC at a speed setting of 600xg for 10.33 min (Table 4). This temperature/speed roasting condition did not have any significant effect ($p < 0.05$) on the protein, carbohydrate and fat contents of the marama bean. According to the consumer evaluation study (Table 6), roasted marama beans have been found to have a slight bitter taste. This bitter taste is largely attributed to the presence of tannins and cholines in marama beans (Mmonatau, 2005). It is altered after roasting, making the marama beans to be consumable by humans (Mmonatau, 2005, p. 10). Too raw and too burned marama beans were found to be too bitter for human consumption. The consumer results produced acceptability of marama beans roasted at optimal temperature and speed setting.

A study carried out by Maruatona in 2008 at the University of Pretoria showed, dry heating marama beans at 150ºC for 20 minutes practically decreased the trypsin inhibitor activity by almost 100%. Trypsin inhibitors are commonly found in

legumes and they are known to lower digestibility of proteins in the intestine (Liener, 1986, p. 921).

5.2 Descriptive evaluation of roasted marama beans

A total of twelve attributes were described by the panelists of the trained sensory panel in the roasted marama beans and macadamia nuts analyzed. These included for aroma: nutty and burnt; for flavour: nutty, burnt, oily, bitterness and sweetness; for texture: hardness, crunchiness and tooth picking; for after taste (residual): oiliness and particles (Table 5).

The two products showed significant differences ($p \leq 0.05$) in almost all attributes except for sweetness and crunchiness. Roasted marama beans showed higher intensity ratings in the following attributes: nutty aroma, burnt aroma, nutty flavour, burnt flavour, bitterness, hardness, tooth picking and particles residual after taste. Roasted macadamia nuts had higher intensity ratings in oily flavour, sweetness and oiliness residual after taste. The discrimination in the attributes was also well illustrated in the PCA biplot (Figure 6).

Marama beans were found to take on a strong flavour and aroma that was nutty and burnt after roasting. Mmonatau (2005) reported that, flavour formation in foods is primarily the result of *inter alia*, the Maillard reaction, caramelisation, thermal degradation, oxidation and lipid-Maillard interactions, which, in the case of marama beans, needs to be researched. The end-products of Maillard reactions are flavor compounds and brown high molecular weight pigments called melanoids. These

reaction products change the flavor of roasted foods. However, up to now the knowledge about structural, functional and physiological properties of melanoids is rather limited. An example can be given with roasted peanut. According to Crippen *et al*. (1992); Buckholz and Duan (1981) as cited in Nepote *et al*. (2008) the roasted peanut flavour, can be attributed to the presence of pyrazines. Pyrazines can be formed as possible flavor compounds through the Maillard reaction.

5.3 Consumer evaluation of roasted marama beans

There was an equal balance of males and females (50% and 50% respectively). All consumers were between the ages of 19-50 years. The ethnicity of the consumer panel included persons from various tribes such as Coloured, Rwandese, Herero and Oshiwambo. Most of the panelists indicated that they consumed nuts and beans on a regular basis.

In this study, consumer testing was conducted to detect differences between marama beans roasted at different temperatures and also to compare the two products, roasted marama beans and macadamia nuts. The difference in the overall liking of roasted marama beans and macadamia nuts was not significant ($p < 0.05$). However, the roasted macadamia nuts received the highest consumer scores for overall liking. This high score can be attributed to the minimal bitterness taste in macadamia nuts ($p \leq 0.05$) compared to marama beans. The other attribute could have contributed to the consumer's preference of macadamia nuts over marama beans is sweetness; even though no significant difference was found in sweetness between the two products,

more than 50% of the descriptive panelists gave a high rating of sweetness in roasted macadamia nuts compared to roasted marama beans.

Consumers from the Herero ethnic group were found to have a high preference for marama beans; this was expected since they consume it on a daily basis during the marama bean season in their homesteads. People from other regions in Namibia where marama beans do not grow were found to have a moderate preference of the roasted marama beans. Therefore, the preferences reported may also likely reflect cultural preferences.

5.4 Texture profile analysis of marama beans

The TPA results obtained with the TA.XTplus Texture analyser (Stable Micro Systems) were compared to the texture evaluation results obtained from the descriptive sensory panel (Table 5). According to the TPA results (Table 7), roasted marama beans was found to be significantly harder ($p<0.05$) than roasted macadamia nuts. This compares well with the descriptive sensory panels results which also found roasted marama bean to be harder than roasted macadamia nuts (Table 5).

Fracturability of roasted and raw marama beans was found to be significantly ($p<0.05$) higher than that of roasted macadamia nuts. Fracturability is the force at the significant break in the TPA curve (see Appendix 6). The fracturability parameter is comparable to the crunchiness attribute and it was significant in the curves of roasted macadamia nuts (see Appendix 6: C). Results obtained from the descriptive sensory panel (Table 5) indicated that there was no significant difference ($p>0.05$) in

crunchiness of roasted marama beans and macadamia nuts. The fracturability values obtained with the TPA instrument were in the range of 7-11, this means that the instrument differentiated more clearly the difference in crunchiness between the two samples.

Adhesiveness is defined as the total force necessary to pull the compression plunger away from the sample. Food products with high adhesiveness and low cohesiveness will likely adhere to the probe on the upward stroke (Sadar, 2004, p. 62). This was observed during analysis of roasted macadamia nuts; these nuts were adhering to the probe 95% of the times.

Roasted macadamia nuts were found to have a significantly ($p<0.05$) high adhesiveness compared to roasted and raw marama beans (Table 7). Adhesiveness could be closely related to the tooth picking attribute that was developed by the descriptive sensory panel (Table 5). However, the panel found roasted marama beans to have a significantly ($p<0.05$) high tooth picking attribute compared to roasted macadamia nuts (Table 5). A study carried out by Kealy (2006) indicated that the complexity of movement in the mouth and the sophisticated interactions of the food products undoubtedly influence measurements of some attributes. For instrumental TPA the samples were measured dry, while with the panel, samples are mixed with saliva in the mouth which influences adhesiveness of the samples to the teeth.

Macadamia nuts have an oil content of 65-75% which is higher than that of marama bean, which ranges between 35-43% (Duke, 1983, p. 2 and Powel, 1987, p. 217).

The high oiliness of the macadamia may have contributed to its adhesiveness to the plunger/probe, whilst the stickiness of roasted marama beans in the mouth was likely influenced by the saliva.

Gumminess of raw marama beans was expected to be higher because of its slimy texture as described by Menninger (1970). According to the instrumental TPA, gumminess for raw marama beans was significantly higher ($p<0.05$) compared to roasted marama beans and macadamia nuts.

Cohesiveness of roasted and raw marama bean was higher than that of roasted macadamia nuts. Cohesiveness is defined as the strength of the internal bonds within the body of the product (Mazaheri-Tehrani, Yeganehzad, Razmkhah-sharabiani & Amjadi, 2009). This suggests that the internal bonds of marama beans are stronger than those of macadamia nuts.

According to Larmond (1976) cited in Anzaldua-Morales (1999), chewiness is the energy required to reduce the food to a suitable consistency for swallowing. Lower chewiness was recorded for roasted and raw marama beans, while roasted macadamia nuts was found have a significantly high ($p<0.05$) chewiness.

Both roasted marama beans and macadamia nuts gave the same values of springiness. Raw marama beans had a significantly high ($p<0.05$) value for springiness compared to roasted marama beans and macadamia nuts. The value of resilience of macadamia nuts was lower than those of marama beans. Springiness decreased in roasted marama beans, hence one can infer that roasting decrease

springiness of marama beans. Resilience is how well a product regain its original position (Gupta *et al.*, 2007, p. 456), this parameter also decreased in roasted marama beans, hence one can also infer that heat treatment of marama beans lowers its resilience.

5.5 Proximate composition of marama beans

5.5.1 Roasted and unroasted marama beans

There was a difference ($p \leq 0.05$) in the moisture content of roasted and unroasted marama beans (Table 8). These results are in agreement with that of Mmonatau (2005), who found a significant difference in the moisture content of marama bean flour that was treated at 120ºC and 150ºC, respectively.

The content of crude fats of roasted marama beans was significantly higher ($p \leq 0.05$) compared to that of the unroasted sample (Table 8). The high content of oil in roasted marama beans can be attributed to the fact that roasting or heat treatment disrupts lipid bodies (Vance, 2002, p. 86).

The carbohydrate content of roasted marama beans was a bit lower than that of unroasted marama bean sample. However, the value of the carbohydrate content of the roasted sample was close to that of the marama beans roasted at 153ºC at a speed setting of 550xg (Table 9).

The protein content of the two samples was not significantly different ($p \leq 0.05$) (Table 8). This means that, heat treatment does not significantly alter the protein

content of marama beans. The results obtained for protein for roasted and unroasted marama beans were 35% and 34%, respectively (Table 8). These values compares well with that obtained by Bower *et al*. (1988), Museler (2005), Amarteifio and Moholo (1998).

Even though statistically the values of the protein content of roasted and unroasted marama beans did not differ, the value of that protein for the roasted sample was a bit high with 1% compared to that of the unroasted sample. Increase in protein content can be attributed to a decrease in moisture content. A similar observation was reported by Obatolu, Fasoyiro and Ogunsumi (2001) on effects of processing on functional properties of yam bean.

5.5.2 Marama beans prepared in a convection continuous tumble roaster

According to the proximate analyses of the two samples roasted at the different speed settings, no significant difference was found in the nutritional values between the two samples (Table 9).

The proximate content analysed were dry matter, crude protein, crude fats and oil, carbohydrates, ash, crude fiber and energy. The similarity in the results obtained was expected since the roasting conditions of the samples analysed were close (Table 4). The moisture, protein, fats and oil, carbohydrates and ash compares well with the results previously discussed in section 5.4.1.

The fat content of roasted marama beans compared well with that of soya beans. Messina (1999), described soya beans to have a fat content of 47%, while the USDA Nutrient Database indicated soya beans to have a fat content of 19.9 %. This range is comparable to the one obtained in this study 32 – 35% (Table 8 and 9).

5.6 Mineral content

The two samples of marama beans roasted at the two different speed settings were analysed for the presence of the following minerals; sodium, potassium, magnesium, calcium, iron and phosphorus. The results for the mineral analysis (Table 10) on sample 1 and 2 indicated that there was no significant difference ($p \leq 0.05$) in the sodium, potassium, magnesium and iron between the two samples.

Marama beans roasted at a speed setting of 600xg at 153ºC (sample 2), which were highly preferred by the consumer panel, had a high mineral content of sodium, magnesium, calcium and phosphorus compared to sample 1 (speed 550xg at 153ºC). A high content of potassium was also observed in both two samples. Trace amount of iron was also found in the roasted samples.

Recommended mineral intake for all age groups reported by Kretser (2006) are given in Table 11. The mineral values obtained in this study (Table 10) were compared to the RDA and it was observed that the roasted marama beans contain sufficient minerals to prevent mineral deficiency in humans.

The results obtained compares well with those obtained by Shikongo (2008) on her study of chemical composition of marama bean leaves and seeds. The marama bean seeds used in her study were not roasted. The mineral content of potassium for raw marama beans was however low compared to the value of potassium for roasted marama beans. The sodium, calcium and trace iron content of raw marama beans was found to be significantly higher compared to the values obtained in roasted marama beans.

These minerals are essential for osmotic relations, acid base balance, bones and teeth formation, and activation of many enzymes concerned with carbohydrate metabolism. The trace iron found in the roasted marama beans is necessary for hemoglobin formation. Phosphorus plays a vital role in the formation of adenosine triphosphate (ATP) in energy synthesizing reactions (Sandberg, 2002, p. 281-283).

According to Weaver and Plawecki (1994), the amount of protein and sodium in food influences the amount of calcium lost in the urine. Individuals who consume consistently lower-protein or sodium diets may require lower calcium intakes than those on higher protein or sodium intakes.

5.7 Microorganism profile of roasted marama beans

The results obtained in this study showed that no microorganisms were detected in marama beans roasted at 153ºC at a speed setting of 600xg. This can only mean that the high temperature used completely destroyed any form of microorganism that could have been present in marama beans.

According to Shurtleff and Aoyagi (2000), some bacteria are able to form spores and others are not. Bacterial spores remain dormant due to unfavorable growth conditions. When introduced to a favorable growth media, spores tend to grow. Bacterial spores are known to survive heat treatment of up to 100ºC. However, the most heat resistant spores are commonly destroyed at temperatures of 121ºC. The temperature used in this study was high enough to eliminate any form of microorganism that could have been present in marama beans.

The main source of microorganism contamination in food products is the processing methods (Bibek, 1996, p. 471). Proper care and handling of roasted marama beans should always be followed to avoid contamination. The results obtained also indicated that the manual method (Fig. 4) used in this study did not pose any threat as a source of microbial contamination in roasted marama beans.

5.8 Health evaluation of marama beans

5.8.1 Crystal violet assay

The marama water and ethanolic extracts were found to increase the viability of normal/healthy human and animal cells. This was observed in lower dilutions for the cotyledon extracts and in higher dilutions of the shell/husk and tuber extracts (Figures 14-18).

The lower dilutions of the husk/shell and tuber extracts destroyed most normal/healthy cells (Figures 15-18). In other words, they decreased the viability of cells. A similar effect was observed in the cancer cells as shown in Figures 19-23.

The lower dilutions of the husk ethanolic and water extracts as well as that of the tuber extracts was found to reduce the viability of cancer cell lines. In some cases (Fig. 20), the lower diluted extracts completely destroyed the cancer cell lines.

The reduction in cell viability is largely attributed to the presence of a class of phyto-oestrogen called isoflavones. These isoflavones are genistein, daidzein, formononetic and biochanin which are found in leguminous plants and have been found to have a defense against a variety of cancers too (US patent 6004558).

Kelly (1998) indicated that administering a human with sufficient amount of phyto-oestrogen on a daily basis of a sufficient period such as at least a month may prevent health conditions or ameliorate cancers of the uterus, cancer of the bowel, benign (or cystic) breast disease, pre-menstrual syndrome (also known as pre-menstrual tension). The phyto-oestrogen supplement also improves the health of a human having elevated levels of blood cholesterol.

Activation of macrophages was observed with the extracts (Fig. 18). Positive activation of macrophage like what was observed in this study mean consumption of marama bean will result in better immune response, due to the presence of specific compounds in marama that are able to activate macrophages (Hume & Denkins, 1989, p. 248).

5.8.2 Lectin assay

Lectins can distinguish glycan expression in all cell lines; they can also identify cancer cell behavior due to their ability to serve as prognostics tools. It is for this reason that normal cells were used to observe lectin binding in them and compare the results obtained to lectin binding to cancer cell lines. From the results obtained, lectin binding and uptake into the normal and cancer cell lines incubated with the marama extracts varied considerably. The lectins, WGA and Con A had bound and were subsequently internalized to a large extent in all cell lines investigated, whereas weak binding and internalization was observed with UEA-I, HPA and PNA (Fig. 24-32).

Con A was found to have a high affinity with normal human and pig cell lines (Figures 24-27). Strong binding was observed in CLAB cells, this means that the marama water and ethanolic extracts were able to increase synthesis of glycans with mannose residues on the cell surface membrane of CLAB cells.

Con A is a mitogenic lectin and it was also found to have chondrogenic and osteogenic activity (Izbicka et al. 1996). This explains the results obtained with melanoma and MCF7 cells (Fig. 29 & 31). The binding strength in the two cell lines for Con A was high even for the control; this means that the marama extracts did not play any role in the binding strength. One of the reasons can be Con A induced activation of MMP-2 in the cancer cell lines which are critical for cancer cell metastasis (Yu, Sato, Seiki & Thompson, 1995, p. 3272).

Some lectins such as ricin and abrin are known to be cytotoxic, they are potent toxins which kill cells by arresting protein synthesis they kill cells by arresting (Lord, 1987, p. 1). However, no toxic lectins were used in this study and the weak binding of HPA, UEA-I and PNA to the cell lines used can only be attributed to the fact that the marama water and ethanolic extracts did not induce sufficient glycans with the following sugar residues N-acetylgalactosamine, fucose and galactose respectively for HPA, UEA-I and PNA. In a study carried out by Schumacher (1995), HPA was used as a marker of metastatic competence and poor prognosis in human breast cancer. Prognosis is a prediction of the probable course and outcome of a disease.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The development of a standard roaster made it possible to commercially roast marama beans in the laboratory and assess its nutritional and biochemical characterization. With the help of the consumer panel this made it easier for the roasted marama beans that were analysed to be closely linked to the actual product consumed in the areas were marama beans are found. The consumer panel that was used consisted of panelists that consume marama beans on a regular basis. They described the laboratory-roasted marama beans as tasting similar to those prepared traditionally. This study therefore accepts hypothesis (a), concluding that a standard roaster was developed that was able to optimize the roasting process of marama beans.

The initial objective of this study was to do analysis on marama beans and compare the results with similar soya bean products. However, soya beans could not be obtained due to unavailability of them in the markets. Roasted macadamia nuts were instead used to compare with roasted marama beans. From the results of the consumer analysis, the consumer panel preferred roasted macadamia nuts to roasted marama beans. This was attributed to the high level of bitterness in roasted marama beans compared to roasted macadamia nuts. It should be noted that, the consumer ranking of roasted marama beans was also high although not significantly as high as the ones of roasted macadamia nuts. Therefore, this study could not accept or reject hypothesis (b).

The nutritional evaluation of roasted and unroasted marama beans yielded richness in the nutritive value of marama beans. Marama beans were found to have significantly high protein, carbohydrate and fat contents (Tables 8 and 9). The mineral content of both roasted and unroasted marama beans was also found to be significantly high. The presence of potassium, sodium, calcium, phosphorus, magnesium and trace iron indicates that not only will marama bean provide a high protein and carbohydrate diet but it will also serve as a mineral source for important metabolic reactions in the body such as ATP production. This study therefore accepts hypothesis (c), concluding that the developed roasted marama beans had a high nutritional value in comparison to other bean products such as soya beans and this will help increase economic opportunities within the local communities were marama beans grow.

The instrumental texture profile analysis indicated marama beans to be harder then macadamia nuts. Fracturability was found to be higher in marama beans compared to macadamia nuts. All these results were expected as hardness in marama beans is due to its high protein content and macadamia nuts have a waxy like texture that makes it less crunchy. Macadamia nuts had a higher adhesiveness due to its high oil content; it was sticking to the compression plunger during TPA testing. Chewiness was found to be higher in macadamia nuts; more force is required to reduce it to a suitable swallowing consistency compared to marama beans. Other parameters that were found to be high in marama beans compared to macadamia nuts including cohesiveness and springiness. The parameters obtained with instrumental TPA testing compared well those obtained with a sensory panel. The study could not deduce a concrete relationship between the sensory panel's tooth picking attribute

with the instrumental TPA parameters. Descriptive sensory analysis indicated marama beans to be harder then macadamia nuts and also a high value of crunchiness was ranked for both marama beans and macadamia nuts. Crunchiness is related to fracturability. This led to the acceptance of hypothesis (d), concluding that TPA attributes generated with a texture analyzer are closely related to those generated by a sensory evaluation panel.

The microbial safety evaluation found out that the roasting condition that was selected as the optimal roasting condition by the consumer panel resulted in no harmful microorganism being detected in the roasted marama bean samples. Plating was done on serial dilutions and no microorganisms were detected in any of the dilution of the marama bean roasted samples. This indicated that all possible microbes that might have been present in marama beans before roasting were completely destroyed during the roasting process. Hence, it can be said that it all depends on the specific roaster that is being used for roasting and the cracking method, whether they will provide a sterile environment for marama beans. Contamination of marama beans during roasting and cracking can be eliminated by providing a clean preparatory environment. It should also be noted that no cases have been reported among the local people of health complications after consuming roasted marama beans. This study therefore accepts hypothesis (e), concluding that roasting marama beans at the optimal roasting conditions led to the elimination of possible microorganisms.

The marama bean plant was found to have physiological, immunomodulatory and potential anticancerogenic effects when tested in functional cell models of human and pig. From the results obtained, it can be concluded that all the marama bean extracts used in this study played an important role in the viability of the cells; at high concentrations marama extracts especially husk ethanolic and tuber water extracts reduced viability of most cells. The most prominent extract was however the tuber water extract, not only did it increase the survival of normal cells at lower concentrations, it also completely destroyed cancer cells at higher concentrations. All the lectins used in this study binded to the cell lines used. The cell lines were pre-incubated with ethanolic and water marama extracts. This shows that marama bean has the ability to increase cell surface glycan production. Glycans serve a variety of structural and functional roles in membrane and secreted proteins. Increased glycan production on cell surface membranes is assumed to play a role in minimizing transmission of diseases. Hypothesis (f) is therefore accepted, suggesting that marama bean plant has functional components that could be used for health and therefore may be used as a food supplement or nutraceutical for improving health status.

6.2 Recommendations

a) The microbial food safety evaluation in this study was carried out on roasted marama beans. It will be ideal if a microorganism study is carried out on raw marama beans. The presence of bacterial spores should also be determined for raw marama beans using microscopy and cytological techniques.

b) This study found anticancer effects in marama ethanolic and water extracts. It is therefore recommended for the specific compounds present in the extracts to be isolated, purified and identified. These isolates can help in the production of purified marama bean supplements for cancer patients.

c) Further studies can be carried out on the effects of the marama bean extracts on HIV and leprosy cells as well as other cancer cell lines that were not looked at in this study. These cancer cell lines are such as those of the lungs, brain, cervical and leukemia.

d) The consumer preference study indicated that roasted macadamia nuts were more preferred to roasted marama beans. Other snack products such as roasted soya beans and cashew nuts should be used in future consumer evaluation studies to compare with roasted marama beans. At the time of this study, no soya beans were available on the market in Windhoek.

REFERENCES

Amarteifio, J.O. & Moholo, D. (1998). The chemical composition of four legumes consumed in Botswana. *Journal of Food Composition and Analysis, 11*, 329-*332*.

Anzaldua-Morales, A., Brusewitz, G.H. & Junus, S. (1999). Instrumental texture profile analysis of pecans of various oil and moisture contents after freezing treatments. *Journal of Food Quality, 22*, 573-581.

Bibek, R. (1996). *Fundamental Food Microbiology*. Florida: CRC Press Library of congress, pp. 471-473.

Bower, N., Hertel, K., OH, J. & Storey, R. (1988). Nutritional evaluation of Marama Bean (*Tylosema esculentum*, Fabaceae): Analysis of the Seed. *Economic Botany, 42*, 533-540.

Brennan, C.S. & Kuri, V. (2002). Relationship between sensory attributes, hidden attributes and price in influencing consumer perception of organic foods. *UK Organic Research: Proceedings of the COR Conference, 26-28 March 2002, 65-68.*

Brink, M. & Belay, G. (2006). *Plant resources of tropical Africa 1. Cereals and pulses*. Netherlands: Backhuys Publishers.

Brooks, S.A., Leathem, A.J.C. & Schumacher, U. (Eds.) (1997). *Lectin Histochemistry a concise practical handbook*. pp. 131 – 145. United Kingdom: BIOS Scientific Publishers Limited.

Chapman, K.W., Lawless, H.T. & Boor, K.J. (2001). Quantitative descriptive analysis and principal component analysis for sensory characterization of ultra-pasteurized milk. *Journal of Dairy Science, 84*, 12-20.

Chiba, K., Kawakami, K. & Tohyama, K. (1998). Simultaneous evaluation of cell viability by Neutral red, MTT and Crystal violet staining assays of the same cells. *Toxicology in vitro*, *12*, 251-258.

Ching, C.K., Black, R., Helliwell, T., Savage, A., Barr, H. & Rhodes, J.M. (1988). Use of lectin histochemistry in pancreatic cancer. *Journal of Clinical Pathology*, *41*, 324-328.

Dixon, R.A. (2001). Natural products and plant disease resistance. *Nature*, *411*, 843-847.

Duke, J.A. (1983). *Handbook of energy crops*. Unpublished book retrieved from http://hort.purdue.edu/newcrop/duke_ebergy/Macadamia.html

Fankhauser, D.B. (2001). Yeast plate count protocol. University of Cincinati Clermont College, Batavia. Unpublished paper retrieved from http://biology.clc.uc.edu/Fankhauser/Labs/Microbiology/Yeast_Plate_Count htm

Gupta, R.K., Sharma, A. & Sharma, R. (2007). Instrumental texture profile analysis (TPA) of shelled sunflower seed caramel snack using response surface methodology. *Food Science and Technology International*, *13*(7), 455-460.

Health Protection Agency Standard Methods. (2008). Wales: National public health service.

Hartley, M.L., Tshamekeng, E. & Thomas, S.M. (2002). Functional heterostyly in *Tylosema esculentum* (Caeslpinioideae). *Annals of Botany*, *89*, 67-76.

Higdon, J. (2006). *An evidence-based approach to dietary phytochemicals*. New York: Thieme Medical Publishers.

Horwitz, W. (1970). *Official methods of analysis for the AOAC* (11th ed.)

Hume, D.A. & Denkins, Y.M. (1989). Activation of macrophages to express cytocidal activity correlates with inhibition of their responsiveness to macrophage colony-stimulating factor (CSF-1): Involvement of a pertussis toxin-sensitive reaction. *Immunology and Cell Biology, 67*, 243-249.

James, C.S. (1995). *Analytical chemistry of foods*.

Jenkins, D.J., Kendall, C.W.C., Faulkner, D.A., Nguyen, T., Kemp, T., Marchie, A., *et al*. (2006). Assessment of the longer term effects of a dietary portfolio of cholesterol-lowering foods in hypercholesterolemia. *American Journal of Clinical Nutrition*, *83*(3), 582-91.

Johnston. (1977) The Flora of Southern Africa, *African Affairs, 16,* 61.

Kealy, T. (2006). Application of liquid and solid rheological technologies to the textural characterization of semi-solid foods. *Food Research International, 39*, 265-276.

Kelly, G. E. (1998). Health supplements containing phyto-oestrogens, analogues or metabolites thereof. *United States Patent: 5,830,887*. 1-16

Khan, A.V. & Khan, A.A. (2002). *Antibacterial Potential of Clerodendrum inerme Crude Extracts Against Some Human Pathogenic Bacteria*. Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, India.

Kretser, A.J. (2006). The new dietary reference intakes in food labeling: the food industry's perspective. *The American Journal of Clinical Nutrition, 83,* 1231-1234.

Liener, I.E. (1986). Trypsin inhibitors: Concern for human nutrition or not? *The Journal of Nutrition, 116*, 920-923.

Lima de Faria, M. & Saraiva, M.C. (2008). Report on consumer demand using consumer focus groups in Namibia urban and rural areas. Unpublished manuscript available at the Instituto de Investigação Científica Tropical, Portugal.

Lord, J.M. (1987). The use of cytotoxic plant lectins in cancer therapy. *Plant Physiology, 85*, 1-3.

Mazaheri-Tehrani, M., Yeganehzad, S., Razmkhah-sharabiani, S. & Amjadi, H. (2009). Physicochemical and sensory properties of peanut spreads fortified with soy flour. *World Applied Sciences Journal, 7*(2), 192-196.

Meilgaard, M., Civille, G.V. & Carr, B.T. (Eds.) (1999). *Sensory evaluation techniques* (3rd ed.). Boca Raton: CRC Press LLC.

Meng, Q.H., Lewis, P., Wähälä, K., Adlercreutz, H., Tikkanen, M.J. (1999). Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. *Biochimica et Biophysica Acta, 1438*, 369-376.

Menninger, E.A. (1970). *Flowering Vines of the World, An Encyclopedia of Climbing Plants*. New York: Hearthside Press, Inc.

Messina, M.J. (1999). Legumes and soybeans: overview of their nutritional profiles and health effects. *American Journal of Clinical Nutrition, 70*, 439-450.

Mmonatau, Y. (2005), *Flour from the morama bean: composition and sensory properties in a Botswana perspective*. Unpublished master's research report, Stellenbosch University, South Africa.

Mislovicova, D., Gemeiner, P., Kozarova, A. & Kozar, T. (2009). Lectinomics I. Relevance of exogenous plant lectins in biomedical diagnostics. *Section Cellular and Molecular Biology, 64*(1), 1-19.

Müseler, D.L. (2005), *Evaluation of the quality characteristics of the marama bean (Tylosema esculentum), an underutilized grain and tuber producing legume in Southern Africa*. Unpublished master's research report, University of Namibia, Windhoek.

Naomab, E. (2004), *Assessment of Genetic variation in natural populations of Marama bean (Tylosema esculentum) using molecular markers*. Unpublished master's research report, University of Namibia, Windhoek.

National Standard Methods. (2005). Wales: National public health service.

Nepote, V., Mestrallet, M.G., Olmedo, R.H., Ryan, L.C., Conci, S. & Grosso, N.R. (2008). Chemical composition and sensory analysis of roasted peanuts coated with prickly pear and algarrobo pod syrups. *Grasas Y Aceites, 59*(2), 174-181.

Nikzadeh, V. & Sedaghat, N. (2008). Physical and sensory changes in pistachio nuts as affected by roasting temperature and storage. *American-Eurasian journal of Agricultural and Environmental sciences, 4*(4), 478-483.

O'Dell, B.L. & De Boland, A. (1976). Complexation of phytate with proteins and cations in corn germ and oilseed meals. *Journal of Agricultural and Food Chemistry, 24*(4), 804-808.

Obatolu, V.A., Fasoyiro, S.B. & Ogunsumi, L. (2001). Effect of processing on functional properties of yam beans (Sphenostylis stenocarpa). *Food Science and Technology Research, 7*(4), 319-322.

Oladele, P.E-O. & Oshodi, A.A. (2007). Nutritional potential of Berlandier nettle spurge (*Jatropha cathartica*) seed. *Pakistan Journal of Nutrition, 6*(4), 345-348.

Pelczar, M.J., Reid, R.D. & Chan, E.C.S. (Eds.) (1977). *Microbiology*. (4th ed.). New York: McGraw-Hill Book Company.

Powell, M.A. (1987). Marama Bean (*Tylosema esculentum*, Fabaceae) Seed Crop in Texas. *Economic Botany, 41*(2**)**, 216-220.

Revilla, I. & Vivar-Quintana, A.M. (2008). Effect of canning process on texture of Faba beans (*Vicia Faba*). *Food Chemistry, 106*, 310-314.

Ripperger-Suhler, J.A. (1983), *Protein Quality of the African Morama Bean*. Unpublished master's research report, University of Texas, Austin.

Rosell, M.S., Appleby, P.N., Spencer, E.A. & Key, T.J. (2004). Soy intake and blood cholesterol concentrations: a cross-sectional study of 1033 pre- and postmenopausal women in the Oxford arm of the European Prospective Investigation into Cancer and Nutrition. *American Journal of Clinical Nutrition, 80*, 1391-1396.

Sader, L.N. (2004), *Rheological and textural characteristics of copolymerized hydrocolloidal solutions containing curdlan gum*. Unpublished master's research report, University of Maryland, Washington D.C.

Sandberg, A-S. (2002). Bioavailability of minerals in legumes. *British Journal of Nutrition, 88*(3), 281-285.

Schumacher, U., Adam, E., Brooks, S.A. & Leathem, A.J. (1995). Lectin-bonding properties of human breast cancer cell lines and human milk with particular

reference to *Helix pomatia* agglutinin. *The Journal of Histochemistry and Cytochemistry*, *43*(3), 275-281.

Shikongo, L. (2008). *Chemical composition of marama bean leaves and seeds.* Unpublished undergraduate dissertation, University of Namibia, Windhoek.

Shurtleff, T. & Aoyagi, A. (2000). T*ofu and soymilk production: a craft and technical manual*. (3rd ed.). California: Soyfoods Center.

Slifkin, M. & Doyle, R.J. (1990). Lectins and their application to clinical microbiology. *Clinical Microbiology Reviews*, *3*(3), 197-218.

United States patent 6004558 – Methods for treating cancer with legume plant extracts. (1999). Retrieved October 28, 2009 from http://www.patentstorm.us/patents/6004558/fulltext

Van Wyk, B-E. & Gericke, N. (2000). *People's Plants. A guide to useful plants of Southern Africa*. Pretoria: Briza Publications. Retrieved October 08, 2009 http://www.biodiversityexplorer.org/plants/fabaceae/tylosema_esculentum.htm

Vance, J.E. & Vance, D.E. (2002). *Biochemistry of lipids, lipoproteins and membranes, 36*. Amsterdam: Elsevier Science.

Vietmeyer, Noel. (1978). Lost Crops of Africa. *National Academy of Sciences, 2,* 1-57.

Watt J.M. & Breyer-Brandwijk M.G. (1962). *The medicinal and poisonous plants of southern and eastern Africa*. London: E & S Livingston.

Weaver, C.M. & Plawecki, K.L. (1994). Dietary calcium: adequacy of a vegetarian diet. *American Journal for Clinical Nutrition, 59,* 1238-1241.

Westad, F., Hersleth, M., Lea, P. & Martens, H. (2003). *Variable selection in PCA in sensory and consumer data*. Norwegian Food Research Institute, Norway.

Young, N. D., Sanders, T.H., Drake, M. A., Osborne, J. & Civille, G. V. (2005). Descriptive analysis and US consumer acceptability of peanuts from different origins. *Food quality and preference, 16,* 37-43.

Yu, M., Sato, H., Seiki, M. & Thompson, E.W. (1995). Complex regulation of membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation by concanavalin A in MDA-MB-231 human breast cancer cells. *Cancer Research, 55,* 3272-3277.

APPENDICES

Appendix 1. Traditional roasting of marama beans

Equipments required:

- Firewood
- Matches/Lighter
- Pot
- Sandy soil

1. A fire is lit and the flames are made sure to be enough to heat the pot.
2. Sandy-soil is placed in a pot and put it on the fire. The amount of sand placed in the pot depends on the amount of marama beans to be roasted.
3. Heat the sand until heat starts coming out of the sand.
4. Place the marama beans in the sand and immediately start stirring.
5. The marama beans will be stirred in the sand throughout the roasting process.
6. The time of roasting also depends on the amount of marama beans to be roasted and the intensity of the flame.
7. A total time of 5 minutes is required to completely roast 500g of marama beans.
8. When the shells of the marama beans start becoming dark brown, quickly remove the pot from the fire and spill the content on the ground.
9. The still hot marama beans are then spread on the ground.
10. Spreading enhance the cooling process of the roasted beans.
11. Roasting of the marama beans help partially crack the hard shell of the bean. To get to the inner part of the bean, the bean is placed on a hard surface such

as a brick and then hit with a hard object such as a piece of wood to completely remove the shell.

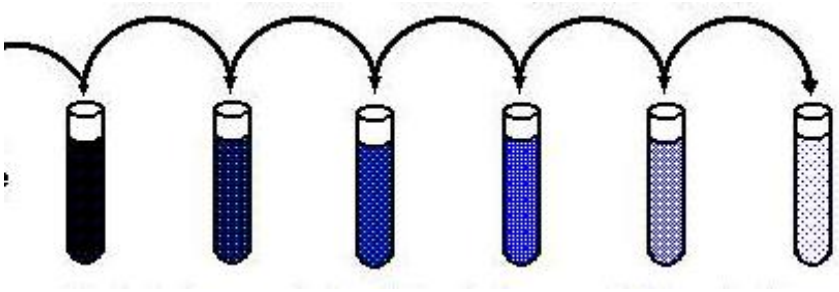
12. Spice the nut inside according to preference (chilly, salty etc.).

Images of traditional roasting of marama beans

Appendix 2. Serial dilution

Serial dilution was performed in the same medium (Peptone water)

Each step represents a 10-fold dilution. 1mL at each step. Each dilution was vortexed before transferring to the next tube. A different tip was used to transfer each dilution.



Each tube contained 9ml of peptone diluent

Appendix 3. Manufacturer's preparation instructions that appeared on each agar container

Plate count agar (Biolab)

Suspend 23g in 1L distilled water. Bring to the boil with frequent stirring. Mix and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

MacConkey agar (Biolab)

Suspend 58g in 1L distilled water. Heat to boiling point with continual agitation until it completely dissolves. Sterilize in the autoclave for 15 minutes at 121°C.

***E. coli* direct agar (Scharlau)**

Suspend 53g of powder in 1L of distilled water and add 5g/L of carbohydrate (lactose). Heat to the boiling. Distribute into suitable containers and sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and add MUG supplement.

Rose Bengal Chloramphenicol agar (Biolab)

Suspend 32g in 1L demineralised water boil whilst stirring until completely dissolved. Autoclave at 121°C for 15 minutes. Cool agar to 45-50°C. Mix well and pour plates.

Appendix 4. Protocol used to obtain the ethanolic marama extracts

1. Freshly dried and healthy plant material is ground into fine powder in an electric grinder. Powder so obtained is stored in a dessicator.

2. Five hundred grams of plant powder is refluxed with 95% ethyl alcohol (EtOH) in a round bottom flask on a water bath for 10 hours. Mother liquor (Crude EtOH extract) is filtered out and residual plant material is again refluxed with 95% ethyl alcohol for 10 hours. The process is repeated four times to obtain maximum yield of EtOH extract. The extract is evaporated to dryness at 50°C under reduced pressure.

3. Dried ethanol extract is refluxed with light petrol (60-80°C) for five hours. After filtration, the residual ethanol extract is again refluxed with petrol for five hours and filtered. This process is repeated five times. Petrol is evaporated under reduced pressure to obtain petrol soluble extract.

4. Petrol insoluble fraction of ethanol extract obtained in step (3) is refluxed with benzene for five hours. The process was repeated five times. Benzene is evaporated under reduced pressure to obtain benzene soluble extract.

5. Benzene insoluble fraction obtained in step (4) is refluxed with ethyl acetate for five hours. Thereafter, it is filtered and refluxed again with ethyl acetate for five hours and filtered. The process is repeated five times. Ethyl acetate is evaporated under reduced pressure to obtain ethyl acetate soluble extract.

6. Ethyl acetate insoluble fraction obtained in step (5) is refluxed with ethyl alcohol (95%) for five hours, filtered and is repeatedly refluxed for five times with ethyl alcohol. The ethanolic soluble fraction is evaporated under reduced pressure to obtain ethanolic extract, while ethanol insoluble residue is discarded.

Appendix 5. Protocols for lectin buffer and AEC-DMF- H_2O_2

Lectin buffer (10x)

60.57g Tris base

78.0g Sodium chloride

2.03g Magnesium chloride

1.11g Calcium chloride

Dissolve in 1L distilled water and adjust pH to 7.6 with hydrochloric acid

AEC-DMF- H_2O_2

Disslove 20mg AEC in 5mL DMF, add 75mL of 0,1M sodium acetate buffer (pH 5.2), filter the solution. (Prepare in advance and store at room temperature). Before use add 0.03% (v/v) hydrogen peroxide. Prepare 0.2M sodium acetate buffer (pH 5.2) by putting together the following:

Stock solution A: 0.2M sodium acetate in distilled water

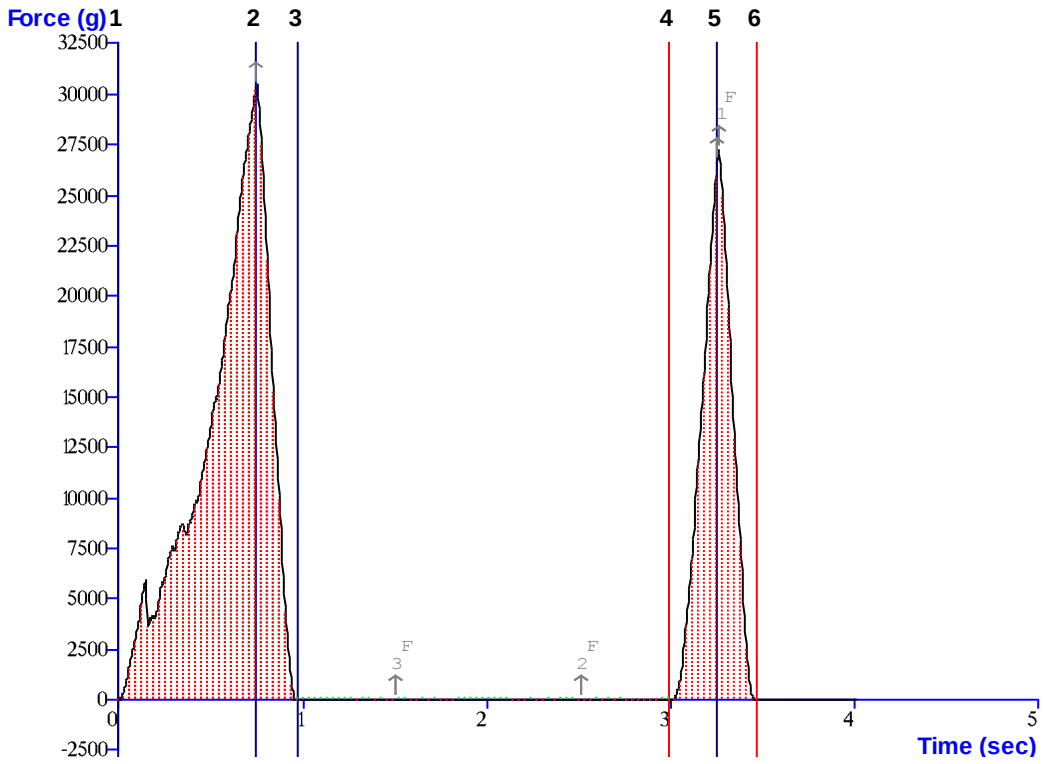
Stock solution B: 0.2M acetic acid in distilled water

Mix 80mL of stock solution A and 20mL of stock solution B.

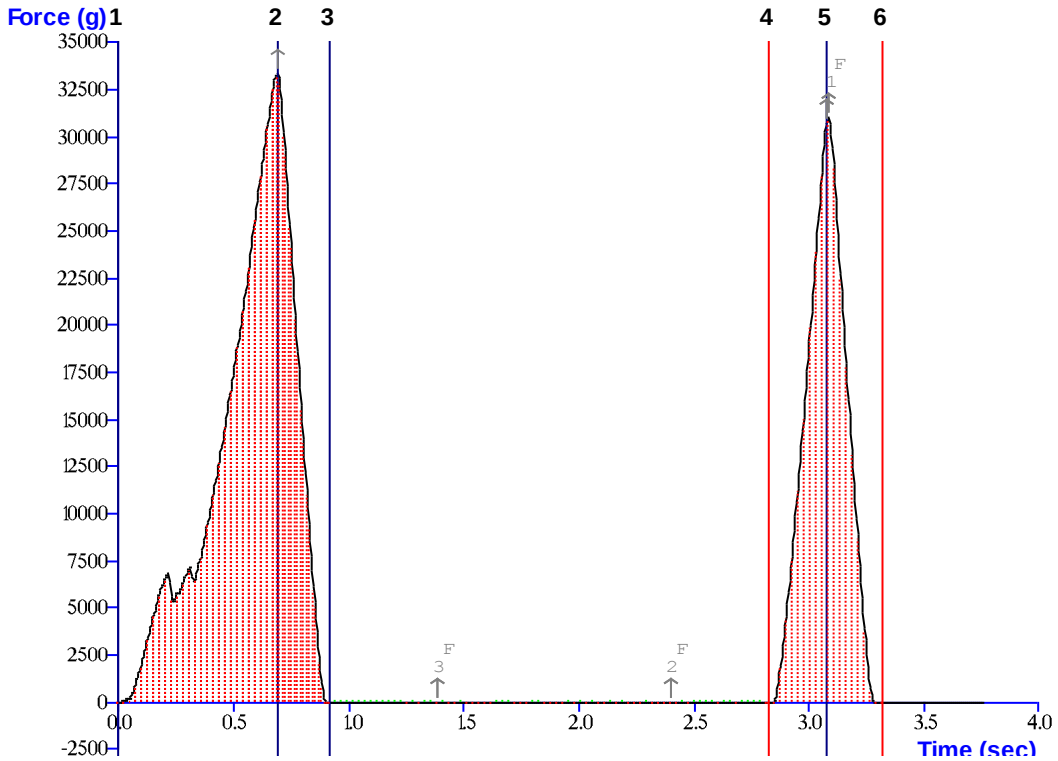
For 0.1M sodium acetate buffer, add 100mL of distilled water to the mixture of A and B.

Appendix 6. TPA curves for roasted marama beans, raw marama beans and roasted macadamia nuts

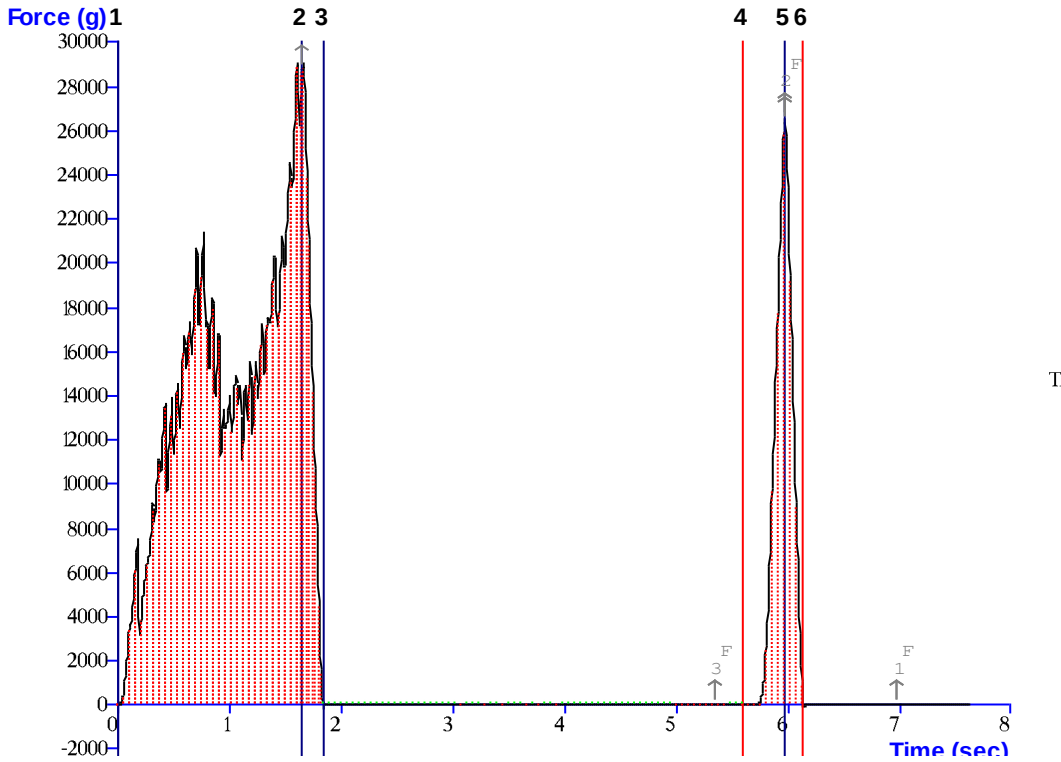
A: Roasted marama beans



B: Raw marama beans



C: Roasted macadamia nuts



Appendix 7. Anova tables for selected data

A: Descriptive sensory evaluation

Nutty aroma

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	72	7.2	2.4
Macadamia	10	41	4.1	6.544444

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	48.05	1	48.05	10.7441	0.004181	4.413873
Within Groups	80.5	18	4.472222			
Total	128.55	19				

Burnt aroma

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	54	5.4	4.488889
Macadamia	10	22	2.2	1.511111

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	51.2	1	51.2	17.06667	0.000627	4.413873
Within Groups	54	18	3			
Total	105.2	19				

Nutty flavor

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	75	7.5	1.166667
Macadamia	10	40	4	4

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	61.25	1	61.25	23.70968	0.000123	4.413873
Within Groups	46.5	18	2.583333			
Total	107.75	19				

Burnt flavor

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	59	5.9	4.544444
Macadamia	10	22	2.2	1.955556

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	68.45	1	68.45	21.06154	0.000228	4.413873
Within Groups	58.5	18	3.25			
Total	126.95	19				

Oily flavor

Oil flavour
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	45	4.5	4.277778
Macadamia	10	77	7.7	1.122222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	51.2	1	51.2	18.96296	0.000382	4.413873
Within Groups	48.6	18	2.7			
Total	99.8	19				

Bitterness

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	48	4.8	2.844444
Macadamia	10	20	2	1.333333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	39.2	1	39.2	18.76596	0.000402	4.413873
Within Groups	37.6	18	2.088889			
Total	76.8	19				

Sweetness

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	36	3.6	3.155556
Macadamia	10	45	4.5	4.722222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.05	1	4.05	1.028209	0.324017	4.413873
Within Groups	70.9	18	3.938889			
Total	74.95	19				

Hardness

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	56	5.6	2.266667
Macadamia	10	39	3.9	1.211111

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.45	1	14.45	8.309904	0.009909	4.413873
Within Groups	31.3	18	1.738889			
Total	45.75	19				

Crunchiness

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	52	5.2	2.844444
Macadamia	10	52	5.2	2.622222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.11E-15	1	7.11E-15	2.6E-15	1	4.413873
Within Groups	49.2	18	2.733333			
Total	49.2	19				

Tooth picking

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	54	5.4	1.155556
Macadamia	10	33	3.3	0.677778

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	22.05	1	22.05	24.05455	0.000114	4.413873
Within Groups	16.5	18	0.916667			
Total	38.55	19				

Oiliness

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	34	3.4	2.933333
Macadamia	10	55	5.5	2.5

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	22.05	1	22.05	8.116564	0.010656	4.413873
Within Groups	48.9	18	2.716667			
Total	70.95	19				

Particles

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Marama	10	56	5.6	1.377778
Macadamia	10	39	3.9	0.988889

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.45	1	14.45	12.21127	0.002588	4.413873
Within Groups	21.3	18	1.183333			
Total	35.75	19				

Appendix 8. SPSS 17 statistical evaluation of the instrumental texture profile properties of roasted marama beans, raw marama beans and roasted macadamia nuts

Roasted marama, raw marama and roasted macadamia (Kruskal-Wallis test)

Test Statistics[a,b]

	hardness	fracturability	adhesiveness	springiness	cohesiveness	gumminess	chewiness	resilience
Chi-Square	2.193	6.966	15.860	8.971	20.480	2.795	3.605	16.205
df	2	2	2	2	2	2	2	2
Asymp. Sig.	.334	.031	.000	.011	.000	.247	.165	.000

a. Kruskal Wallis Test

b. Grouping Variable: samples

Roasted and raw marama bean (Mann-Whitney test)

Test Statistics[b]

	fracturability	adhesiveness	springiness	cohesiveness	resilience
Mann-Whitney U	29.000	22.000	16.000	.000	19.000
Wilcoxon W	65.000	58.000	52.000	36.000	55.000
Z	-.316	-1.050	-1.685	-3.361	-1.365
Asymp. Sig. (2-tailed)	.752	.294	.092	.001	.172
Exact Sig. [2*(1-tailed Sig.)]	.798[a]	.328[a]	.105[a]	.000[a]	.195[a]

a. Not corrected for ties.

b. Grouping Variable: samples

Roasted marama bean and roasted macadamia (Mann-Whitney test)

Test Statistics[b]

	fracturability	adhesiveness	springiness	cohesiveness	resilience
Mann-Whitney U	12.000	.000	10.000	.000	.000
Wilcoxon W	48.000	36.000	46.000	36.000	36.000
Z	-2.104	-3.361	-2.312	-3.361	-3.361
Asymp. Sig. (2-tailed)	.035	.001	.021	.001	.001
Exact Sig. [2*(1-tailed Sig.)]	.038[a]	.000[a]	.021[a]	.000[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: samples

Raw marama and roasted macadamia (Mann-Whitney test)

Test Statistics[b]

	fracturability	adhesiveness	springiness	cohesiveness	resilience
Mann-Whitney U	9.000	.000	9.000	.000	.000
Wilcoxon W	45.000	36.000	45.000	36.000	36.000
Z	-2.424	-3.361	-2.415	-3.361	-3.361
Asymp. Sig. (2-tailed)	.015	.001	.016	.001	.001
Exact Sig. [2*(1-tailed Sig.)]	.015[a]	.000[a]	.015[a]	.000[a]	.000[a]

a. Not corrected for ties.

b. Grouping Variable: samples