BIOACTIVITY OF FAMINE FOOD PLANTS FROM THE FAMILY: AMARANTHACEAE

Alveera Singh

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Supervisor: **Prof. B. Odhav**

Co-Supervisor: **Dr. L. Reddy**

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REFERENCE DECLARATION IN RESPECT OF A MASTER'S DISSERTATION

I, Alveera Singh (Student Number: 20201901), Prof. Bharti Odhav and Dr. L. Reddy do hereby declare that in respect of the following dissertation:

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A. Singh _____ March 2009

Student

B. Odhav _____ March 2009

Supervisor

L. Reddy _____ March 2009

Co-Supervisor

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

2,4-D	**:**	**2,4-Dichlorophenoxyacetic acid**
2iP	**:**	**2-isopentyl adenine**
A	**:**	**larvae that reached the adult stage**
ABA	**:**	**Abscisic acid**
AOAC	**:**	**Association of Official Analytical Chemists**
BAP	**:**	**Benzylaminopurine**
BSL	**:**	**Brine Shrimp lethality**
COX	**:**	**Cyclooxygenase**
DBT	**:**	**Durban University of Technology Culture Collection**
GA_3	**:**	**Giberrilic Acid**
IAA	**:**	**indole-3-acetic acid**
IBA	**:**	**Indole butyric acid**
IC_{50}	**:**	**concentration of inhibitor necessary for 50% inhibition**
ICP	**:**	**inductively coupled plasma**
K562	**:**	**human eythroleukemia cell line**
L	**:**	**Larvae**
LOX	**:**	**Lipoxygenase**
MIC	**:**	**minimum inhibitory concentration**
MRC	**:**	**Medical Research Council**
MS	**:**	**Murashige and Skoog**
NAA	**:**	**1- Naphthaleneacetic acid**
NaN_3	**:**	**Sodium azide**
NRC	**:**	**National Research Council**
P	**:**	**larvae that reached the pupal stage**
PGR's	**:**	**Plant growth regulators**
RDA	**:**	**Recommended Daily Allowances**
WHO	**:**	**World Health Organization**

ABSTRACT

Information regarding the nutritional value of wild food plants in Africa and current information varies from source to source. Prior to commercialization of wild foods the nutritional, ethnobotanical, medical, chemical, anthropological and toxicity requires investigation. Plants from the Amaranthaceae family were chosen because the family is characterized by several species which are used by indigenous communities as a source of nutrition in different plants of the world. The focus of this study was to investigate the nutritional and biological activities of three plants from the Amaranthaceae family viz. *Achyranthes aspera*, *Alternanthera sessilis* and *Guilleminea densa* that are considered famine plants.

This study aimed to determine the nutritional value (proximate, minerals and vitamins), biological activity, toxicity and potential of a tissue culture system for three species from the family Amaranthaceae. Nutritional analysis comprised of determining moisture, ash, protein, fat, carbohydrate, dietary fibre and energy. Mineral analysis of calcium, copper, iron, magnesium, manganese, phosphorus, sodium and zinc was performed by microwave digestion and then analyzed by ICP Spectrophotometry. Vitamin A, Vitamin B_1, Vitamin B_2, Vitamin B_3 and Vitamin C were also analyzed. For biological and safety analyses aqueous and methanolic extracts were prepared. Anti-oxidative and anti-inflammatory properties of the extracts were tested; antimicrobial activity was tested by evaluating the bactericidal, fungal effect and minimum inhibitory concentration on selected bacteria and fungi using the agar disk diffusion method. Anti mosquito potential was determined by setting up repellency, larvacidal assay and insecticidal assay. The safety and toxicity analysis was carried out by measuring cytotoxicity, toxicity and mutagenicity. The potential of an *in vitro* tissue culture system of *A. aspera, A. sessilis* and *G. densa* was determined using micropropagation.

A. aspera indicated significant amounts moisture, ash, dietary fibre, protein, vitamin B_1, vitamin B_2, magnesium and manganese. Plant extracts of *A. aspera* had antibacterial activity against the Gram negative bacteria *Esherichia coli, Pseudomas aeroginosa* and *Salmonella typhi*; Gram positive bacteria *Staphylococcus epidermis* and *Staphylococcus aureus*. The methanolic extract had antifungal activity against *Sacchromyces* cerevisiae and exhibited significant free radical scavenging activity as well as 85% repellency against *Anopheles arabiensis*. The aqueous extract stimulated the growth of the K562 (Chronic Myclogenous Leukaemia) cell line and the plant

extracts showed no mutagenicity or toxicity. ***A. sessilis*** indicated significant levels of ash, dietary fibre, protein, energy, vitamin A, vitamin B_1, vitamin B_2, vitamin B_3, iron, magnesium and manganese present. Plant extracts of *A. sessilis* had antibacterial activity against Gram negative bacteria *P. aeroginosa* and Gram positive bacteria *S. epidermis*. The plant also showed antifungal activity against the yeasts *S. cerevisiae* and *Candida albicans*. The methanolic plant extract showed excellent antioxidant activity. The aqueous plant extract stimulated the growth of the K562 cell line and the plant extracts possessed no mutagenicity or toxicity. This plant grew well in a tissue culture system where it was propagated from callus to a fully grown plant able to survive in environmental conditions. ***G. densa*** has ash and dietary fibre, vitamin B_2, vitamin B_3 and iron. The plant extracts had antibacterial activity against Gram negative bacteria *E. coli, P. aeroginosa* and *Klebsiella. oxytoca;* Gram positive bacteria *Baccilus stereathermophilus* and *S. aureus*. The plant also has antifungal activity against *C. albicans* and significant repellency activity against *A. arabiensis* where it showed 100% repellency. This plant was not found to be mutagenic or toxic.

The results obtained from this study show promising potential for the plants to be exploited as famine food plants. The nutritional value, biological activity and ability to micropropagate *A. aspera*, *A. sessilis* and *G. densa* indicates a good potential for purposes of harnessing biotechnological products.

CHAPTER ONE: STUDY RATIONALE AND OBJECTIVES

During times of natural disasters, populations suffering from severe food shortages become heavily reliant on wild food plants for survival (Leborgne *et al.*, 2002). This gives rise to the concept of famine plants (Chopak, 2000). Rodale and McGrath (1991) stated that famine plants have been eaten and utilized for centuries and they can be reintroduced as standard crops to stabilize the land and mitigate the cycles of famine. Improved strains of native "famine plants," edible plants native to Africa that thrive through adverse weather cycles of drought and excess rainfall, should be reintroduced and cultivated in place of foreign cash crops as these crops are more nutritious than foods introduced from abroad, are more highly adapted to the climatic adversity of Africa, are more resistant to local pests and disease, and require relatively little management (Rodale and McGrath, 1991). Additionally, imported cash crops that have been introduced into African agricultural systems are also detrimental to the sustainability of African farming.

Publications (Maundu *et al.*, 1999, Freedman, 2006) showed that wild plants are essential components of many Africans' diets, especially in periods of seasonal food shortage and ethnobotanic studies deal with their medicinal properties. The nutritional, antibacterial, antifungal, anthelmintic, anti-amoebic, antischistosomal, antimalarial, anti-inflammatory and antioxidant activity, as well as psychotropic and neurotropic activity using appropriate *in vitro* tests forms basis for validating the usefulness of famine plants. Final commercialization potential is then often provided through the isolation of active compounds. It is also important to investigate how safe, these plants are for consumption, since some are potentially toxic and can produce toxic constituents during stressful environmental conditions.

In Africa, one of the plants frequently consumed as leafy vegetable belongs to the family Amaranthaceae. As these are not the usually commercially cultivated vegetables data pertaining to amaranths is limited to *Amaranthus dubius*, *A. spinosus* and *A. hybridus*. Other members in this family serve as examples of famine plants that may be superior to crop plants introduced such as maize. Although, their seeds are smaller than maize, they yield higher nutritional properties. The average protein content of amaranth seeds is 16% whereas maize yields an average of only 12%. Additionally, amaranth is considered better nutritionally balanced; having

lysine content three times greater than maize, and its leaves and young stems can be eaten as greens which have high mineral and vitamin content.

Plants from the Amaranthaceae family are used in indigenous system of medicine for their antiarthritic, antifertility, laxative, ecbolic, abortifacient, anthelmintic, aphrodisiac, antiviral, antispasmodic, antihypertensive, anticoagulant, diuretic and antitumour activities. They are used to treat cough, renal dropsy, fistula, scrofula, skin rash, nasal infection, chronic malaria, impotence, fever, asthma, amennorrhoea, piles, abdominal cramps and snake bites. Furthermore, some of the members from this family have important phytochemicals such as rutin which is a strong antioxidant compound and saponins.

A review of literature showed that many of the medicinal properties are attributed to the common amaranths i.e. *A. dubius*, *A. spinosus* and *A. hybridus* and through the literature and other sources, in this study we selected *Achyranthes aspera*, *Alternanthera sessilis* and *Guilleminea densa* because there is little information available, thus our aim was to evaluate the nutritional and biological activities of *Achyranthes aspera*, *Alternanthera sessilis* and *Guilleminea densa* and develop a protocol for cultivating them in a tissue culture. To achieve this we embarked on: (i) determining the nutritional value i.e. moisture, ash, protein, fat, carbohydrate, dietary fibre, energy, vitamins and mineral content and compared it to other species from the Amaranthaceae family; (ii) evaluating the anti microbial activity, anti-oxidant activity, anti-inflammatory activity and activity against the malaria vector. *Anopheles arabiensis*; (iii) determining toxicity and mutagenicity *in vitro*; and (iv) establishing a protocol for the micropropagation of the plants.

CHAPTER TWO: LITERATURE REVIEW

2.1 Wild plants and Famine Foods

2.1.1 Introduction

Useful wild plants are found all over the world. Some people call wild plants "weeds" although many have important uses. The term 'wild-food' often implies that there is an absence of human influence and management, however according to Bell (1995) people have indirectly shaped many of the plants and some have been largely domesticated in home gardens and in the fields together with farmers' cultivated food and cash crops. The term 'wild-food' is used to describe all plant resources outside of agricultural areas that are harvested or collected for the purpose of human consumption in forests, savannah and other bush land areas. Wild-foods are incorporated into the normal livelihood strategies of many rural people (Bell, 1995) and is usually considered as an additional diet to farmers' daily food consumption pattern, generally based on their crop harvest, domestic livestock products and food purchased from local markets. Not all wild plants are safe for food or medicine. Plants classified as typical 'famine-food' plants are normally not consumed due to their limited seasonal availability, local taboos, offensive nature of the plants such as abundance of thorns and tiny spines, certain unpleasant characteristics and side-effects such as bad taste, complicated and prolonged preparation, and association with stomach complaints, constipation, diarrhea and even intoxication. Certain 'wild-foods' are enjoyed and therefore collected and consumed every time when ripe and these are important 'famine-foods' during periods of food shortage (Guinard and Lemessa, 2000). Typical 'famine-food' consists of a variety of plants which contain leafy and tender parts of stalks, pseudo-stems, fruits, berries, seeds, husks and roots, i.e. tubers and corms.

Many of the wild plants also contribute to herbal medicines which form an important part of the culture and traditions of African people. Today, most of the populations in urban South Africa, as well as smaller rural communities, are reliant on herbal medicines for their health care needs. Apart from their cultural significance, herbal medicines are generally more accessible and affordable (Mander *et al.*, 1996). As a consequence, there is an increasing trend, worldwide, to integrate traditional medicine with primary health care.

In the Hausa-speaking regions of the Republic of Niger the majority of people rely on millet as the staple foodstuff of their diet (Kim *et al.*, 1997). However, during times when crop yields are low or nonexistent the population must seek other available foods. These foods, referred to as ‘‘famine foods,’’ are consumed during periods of food shortage because they are edible and available.

In some regions of the Sahel, farmers’ crop yields are often insufficient to last until the following harvest season. In many villages in the Republic of Niger, for example, granaries often become exhausted months before the next planting season begins (Glew *et al.*, 2004). This results in a ‘‘hungry season’’ during which people are forced by circumstances to purchase grain in local markets at an elevated price. Consequently, during times when staple cereals are scarce or costly, the inhabitants of rural Niger are compelled to increase their reliance on wild edible plants for food. In the Sahel region of West Africa wild plant foods are widely consumed to supplement cultivated cereals such as millet and sorghum (Glew *et al.*, 2004).

In Tanzania, wild vegetables accounted for over 80% of all the leafy vegetables used. Wild vegetables served as major side dishes or condiments to the meals. Wild vegetables played central dietary roles where 49% of vegetables consumed were from wild sources and they supplied significant sources of micronutrients. In Swaziland, wild foods were used throughout the year. Burkina Faso documented that 20% of all food plants consumed were of the wild species (Grivetti and Ogle, 2000). A study conducted in Zimbabwe revealed that some poor households rely on wild fruits as an alternative to cultivated food for a quarter of all dry season's meals (Balemie and Kebebew, 2006).

Since wild plant foods play an essential role in the survival and health of human populations, it would be beneficial to understand how these plants contribute to human nutrition and to recognize their potential for sustaining populations during future food shortages.

2.1.2 Categories of Wild Food Plants

Each registered wild-food plant species has been categorized and listed in one of four proposed plant categories, depending on their availability and usage (Guinard and Lemessa, 2000);

- typical 'famine-food' plants

- 'wild-food' plants with 'famine-food' components
- wild-food' plants attracting additional consumer categories during food shortage periods
- on-farm food crops with 'famine-food' components

Typical 'famine-food' plants

A typical famine-food plant has leaves, stalks, inflorescence, roots (tubers, corms and rhizomes) or barks (mainly of *Acacia* sp) that are edible. Many of the root-type famine-food plants are drought tolerant and can stay in the soil intact for a long time. Most of the leafy-type famine-food plants are locally referred to and classified as 'weeds', sprouting and flourishing after rains. They generally mature within a short period of time. There are two main periods of maximum consumption of the leaves and tender parts of such "famine-food" plants. The first period is while farmers are waiting for the upcoming crop harvest and the second is when they run out of food stocks from the previous harvest. People try to add "famine-food" to local staple foods or to mix it with other foodstuff to mask the often offensive nature of the food and to reduce any characteristic and unpleasant side effects.

'Wild-food' plants with 'famine-food' components

Within this category multi-purpose wild-food plants are represented. Fruits plus one or more additional food products such as leaves and tender parts of stalks or root parts can be used at different times of the year and at different stages of food shortage.

'Wild-food' plants attracting additional consumer categories during food shortage periods
For most species classified in this category people's consumption behaviour is the same, that is, only the fruits or the berries are eaten or considered edible. Children consume the fruits in normal times, but when food is short, adults, as an additional consumer category, will collect and consume fruits from wild trees and bushes.

On-farm food crops with 'famine-food' components

On-farm crops with famine-food components are few and are likely to be perennial plants. The famine-food components are normally not consumed because it may imply the total destruction

of the plant. Examples of on-farm crops bearing famine-food components are banana *(Musa paradisica)*, false banana *(Ensete ventricosum)*, grass pea *(Lathyrus sativus)*, cotton *(Gossypium spp.), cabbage tree (Moringa oleifera)*, sorghum, beans and tomato plants.

2.1.3 Usefulness of wild foods

Wild food plants represent inexpensive, locally available and versatile food sources capable of improving nutrition and health quality. Furthermore, they often represent food sources with low labor requirements, to the extreme that children, aged people and some sick people can participate in their harvesting, provided adequate indigenous knowledge on their localization and identification has been adequately transferred. Thus, wild food plants can also represent an easy income source, especially if specific policies and programs were deployed to expand market niches and support the integration of poor rural households in their sustainable harvesting, processing, and commercialization (FAO, 2003). The wide indigenous knowledge on the preservation and processing of wild food plants further enhances their roles in household food security and nutrition.

Wild foods have both agricultural and medicinal importance.

2.1.3.1 Agricultural Importance

Agricultural development and cultivation in developing countries are primarily based on subsistence crops and edible wild plant species, and only secondarily, on the cultivation or utilization of a wide diversity of food crops (Martin, 1984). Dietary utilization of non domesticated plants has received little attention and a dramatic narrowing of the food base in many traditional societies has occurred. For example, of the thousands of edible wild and domesticated plants documented globally (Tanaka, 1976), as few as 150 enter the commercial market. Out of these 150 species only 15 constitute main sources of human food energy (Wilkes, 1977). The narrowing of domesticated species used as crops creates a vulnerable position in which the crops could be destroyed by drought, diseases and pests (Turton, 1977). The diversification of wild crops can ensure dietary balance and the intake of essential micronutrients. Wild food plants become a critical food security resource in times when main crops cannot be harvested especially in dry areas. Wild food plants represent untapped resources with potential to

improve nutrition in arid and semi-arid lands. They play an analogous role to that of vegetable crops in humid and sub-humid areas (FAO, 2003).

For many years the importance of wild plants in subsistence agriculture in the developing world as a food supplement and as a means of survival during times of drought and famine has been overlooked. Generally, the consumption of such so-called 'wild-food' has been under-estimated (Bell, 1995). Wild food plants represent a versatile agro biodiversity resource, providing different benefits and opportunities depending on agro-ecological conditions, food security dynamics, nutritional needs, cultural dimensions, and other circumstances. The most relevant and distinctive value of wild food plants are that they are:

- A supplementary food source, adding nutritional quality and food variety to local diets, excellent supply of micronutrients (vitamins and minerals), critical food source during seasonal food shortage periods, which are recurrent among the rural poor dwellings in arid lands (FAO, 2003).
- Inexpensive and easy food source, often requiring low labour inputs.
- Vital ingredients in food habits and culinary practices in some areas of Sub-Saharan Africa, especially for the so-called relishes that accompany main meals.
- Emergency food source, for instance in cases of food shortage, during drought, crop failure, and civil conflict.
- Source of income, as some wild food plants may access specific market niches (FAO, 2003).

2.1.3.2 Medicinal importance

Infectious diseases caused by bacteria, fungi, viruses and parasites are a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance (Okeke *et al.*, 2005). Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Clardy and Walsh, 2004). Only a minute portion of the available diversity among fungi, marine fauna and flora, bacteria and plants has yet been explored and ample opportunities lie ahead. Current research on natural molecules and products

primarily focuses on plants since they can be sourced more easily and can be selected on the basis of their ethno-medicinal use (Verpoorte *et al.*, 2005).

Renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. Instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Cox and Balick, 1994). Already an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001).

Wild food plants have a potential in the mitigation of AIDS impact, especially among the rural poor. In particular, they are relevant in three main concerns by households affected by AIDS; they improve food access, improve nutrition and health quality, and alleviate labor constrains related to food production (FAO, 2003). Wild food plants represent a relevant food security and nutrition option for households suffering labor shortages, such as typically those affected by HIV/AIDS. In fact, the recognized role of wild food plants in emergency situations is well extensible to the context of AIDS, which in fact constitutes an emergency scenario (FAO, 2003). Preserved wild food plants can help AIDS affected households and families to cope with labor shortages confront sudden food scarcity, or build food reserves for times of acute production constrains.

2.2 Amaranthaceae Family

The amaranth (or pigweed) family is a large group of dicotyledonous flowering plants known as the Amaranthaceae. Amaranthaceae is divided into two subfamilies: the Amaranthoideae and the Gomphrenoideae, based on certain morphological characteristics of their flowers. It is a relatively large family, having about 65 genera and 900 species. Most of the 900 species of Amaranthaceae are native to tropical and subtropical regions of Africa, Central America, and South America. The number of Amaranthaceae species declines as one approaches the northern and southern temperate zones.

The species in this family are mostly annual or perennial herbs, although a few species are shrubs or small trees. Many species of Amaranthaceae are considered weeds, since they invade disturbed

areas, such as agricultural fields and roadsides. *A. aspera*, *A. sessilis* and *G. densa* fall under the category typical 'Famine-Food' plants.

2.2.1 *Achyranthes aspera*

Description

The flowers are hermaphrodite (have both male and female organs) (Fern, 1996). They are perennial or annual plants. The stems are 0.4-2 m, pilose or puberulent. They have elliptic, ovate, or broadly ovate leaf blades. They inflorescences to 30 cm; bracts membranous; bracteoles long-aristate, spinose; wings attached at sides and base. The flowers occur as tepals 4 or 5, length 3-7 mm; pseudostaminodes with margins fimbriate at apex, often with dorsal scale. The utricles are cylindric, 2-4 mm, apex truncate or depressed. Below is an illustration of the *A. aspera* plant (Fig.1).

Nomenclature

Common Name: Prickly Chaff-flower
Hindi Name: Chirchita, Latjira, Onga
Chemical Constituent: Achyranthine
Useful Parts: All parts of the plants are used

Fig. 1. ***Achyranthes aspera***

Habitat

The plant prefers light sandy, medium loamy, heavy clay soils, can grow in semi-shade (light woodland) or no shade and requires moist soil (Fern, 1996). It grows as wasteland herb everywhere. It has been used as folk medicine. It is in flower from July to September, and the seeds ripen in October.

Nutritional properties and Biological properties

The seeds are rich in protein.

According to Ayurveda, it is bitter, pungent, heating, laxative, stomachic, carminative and useful in treatment of vomiting, bronchitis, heart disease, piles, itching abdominal pains, ascites, dyspepsia, dysentery, blood diseases etc. The juice of the plant is used in the treatment of boils, diarrhea, dysentery, hemorrhoids, rheumatic pains, itches and skin eruptions. The leaf is emetic and a decoction is used in the treatment of diarrhea and dysentery. A paste of the leaves is applied

in the treatment of rabies, nervous disorders, hysteria, insect and snake bites (Manandhar, 2002). The ash from the burnt plant, often mixed with mustard oil and a pinch of salt, is used as a tooth powder for cleaning teeth. The dried twigs are used as toothbrushes.

2.2.2 *Alternanthera sessilis*

Description

A. sessilis is an annual or perennial herb, grows up to 1 m tall. It sometimes become woody at the base, prostrate or erect to floating or scrambling. The leaves are opposite and entire. It has inflorescences heads or short spikes, axillary, sessile or pedunculate, solitary or fasciculate, bracteate: bracts persistent. The flowers are bisexual, solitary in axils of bracts; 2 bracteoles, usually shorter than perianth, persistent or not, perianth falling with fruit. There are 4 or 5 tepals, free or concrescent at the base, glabrous of furnished with smooth or denticulate hairs. There are 2-5 stamens, some occasionally anantherous, hypogynous; filaments monadelphous at the base into a cup or tube, alternating with large and laciniate to small pseudostaminodes or these rarely obsolete; anthers thecous. The ovaries are suborbiculare or obovate in outline, usually compressed; ovule solitary, pendulous, basal; style short or absent; stigma capitellate. The capsule is thin walled or sometimes corky, in dehiscent. The seeds are lenticular (Glen, 2000). *A. sessilis* is unique in the genus in that its tepals are generally shorter than the utricle (Fig. 2). The utricles of other *Alternanthera* species sit well below the tops of the tepals (Scher, 2004). Below is an illustration of the *A. sessilis* plant (Fig.2).

Nomenclature

Family: Amaranthaceae

Sub Family: Gomphrenoideae,

Common Name: sessile joyweed

Disseminule: Fruit

Fig.2. ***Alternanthera sessilis***

Habitat

It prefers wet conditions, but occurs in both wetlands and uplands and can grow on a variety of soil types. The plant is propagated by seeds, which are wind and water-dispersed, and by rooting at stem nodes. Widespread throughout the tropics and subtropics: tropical Africa, southern Asia to Japan, Southeast Asia, Australia, and the Pacific Islands (Scher, 2004). It is a pantropical; found in damp shady areas, swamps, pond margins, shallow ditches, roadsides, low-lying waste places, damp pastures, cultivated areas (Scher, 2004).

Nutritional properties and Biological properties

It is a weed of rice throughout tropical lands, and of other cereal crops, sugarcane, and bananas. The young tips are eaten as a vegetable. It is a very famous leafy vegetable in tribal and rural areas. The leaf is very rich in iron (17 mg/100 mg), Vitamin A (192 mg/100 mg) and dietary fiber (12 g/100 g). The plant contains about 5% protein and soups made with the leaf are given to anaemic patients in rural areas. It contains abundant carotene, therefore it is used for curing night blindness. The plant has a virtue of galactogogue and enhances the secretion of milk in new mothers (Naples, 2005). Chemical constituents are Sitosterol, stigmasterol, campesterol, cc-

spinasterol, oleanolic acid rhamnoside, 24-methylene cycloartenol, cycloeucalenol, lupeol, 5-cc-stigmasta-7-enol and palmitate.

An infusion of the entire plant is used in Indonesia as a remedy against intestinal cramps, diarrhoea and dysentery (intestinal disorder), and externally as a cooling agent to treat fever. In Malaysia, it is used internally against intestinal inflammation and fever, and externally to treat wounds. In Taiwan, this plant is used in local medicine, often in mixtures with other medicinal plants, to treat hepatitis, congestion, bronchitis and asthma, ailments of the lung, to stop bleeding and as a hair tonic. It is used in India against dysentery (intestinal disorder), as a cholagogue (increases bile flow in liver), abortifacient (causes abortion) and febrifuge (reduces fever) and to treat snake bites, inflamed wounds and boils (Naples, 2005).

2.2.3 *Guilleminea densa*

Description

It is a woolly, prostrate mat-forming perennial herb. The leaves are narrowly elliptic to broadly ovate; glabrous on the upper surface and with long matted white hairs on the lower surface. Inflates to 6 mm, dense, ovoid, and whitish (Fig 3) (Hyde and Wursten, 2002).

Nomenclature

Family: *Amaranthaceae*

Synonyms: *Brayulinea densa* (Willd.) Small

Fig.3. ***Guilleminea densa***

Habitat

It is found on roadsides, in grassland and open woodland, usually in well-trodden disturbed places (Hyde and Wursten, 2002). It is naturalized in Australia and South Africa. It is native of the warmer parts of North and South America, from the southern USA to northern Argentina, and now a widespread tropical weed (Hyde and Wursten, 2002).

Nutritional properties and Biological properties

There is no information available of nutritional or biological properties.

2.3 Nutritional and Biological Properties

2.3.1 Nutritional Requirements

The basic nutritional needs for humans are to supply energy and raw materials for all the various activities and processes that occur in the body. In addition to the need for water, humans require five types of nutrients from their food supply: three of which are required in large amounts and are called macronutrients, consisting of carbohydrates, protein and fats. The other two types of nutrients, vitamins and minerals, are required in small amounts are known as micronutrients.

Although several reports have been published on the nutritional value of famine food plants, the database of their chemical and nutrient composition is far from exhaustive (Cook *et al.*, 2000).

Diets of various types can be devised to meet recognized nutritional needs based on Recommended Daily Allowances (RDA). The National Research Council defines the levels of intake of essential nutrients that, on the basis of scientific knowledge, are judged by the Food and Nutrition Board to be adequate to meet the known nutrient needs of practically all healthy persons (NRC, 1989). In principle the RDAs are based on various kinds of evidence but in practice, there is only limited data on which estimates of nutrient requirements can be used. According to the Food and Nutrition Board, RDAs should be provided from a selection of foods that are acceptable and palatable to ensure consumption. In a few cases where deficiency is commonly observed, food fortification and individual supplementation are appropriate. Attention and public interest have also been focused on the possible effects of nutrients, often at high intakes, on conditions other than those associated with specific deficiencies. At higher levels of intake, both the toxicity and the pharmacological action of specific nutrients must be considered (NRC, 1989).

2.3.1.1 Macronutrients

Human energy requirements (1200-3200 calories per day) vary with age, sex, and activity level of the individual. A calorie is the measure of energy (amount of energy needed to raise the temperature of one gram of water by one degree Celsius). Although all the macronutrients can be used as a source of energy, normally only carbohydrates and fats do so, while proteins provide the raw materials or building blocks, required for the synthesis of essential metabolites, growth and tissue maintenance (Arntzen and Ritter, 1984). Previous studies on leafy vegetables (Odhav *et al.*, 2007) showed that other species from the Amaranthaceae family namely *A. dubius*, *A. hybridus* and *A. spinosus* had significant moisture and carbohydrate values. A comparison was later performed between the famine food plants and the leafy vegetables from the Amaranthaceae family in this study.

2.3.1.2 Micronutrients

Micronutrients are essential for proper nutrition but are required in much smaller amounts. There are two categories of micronutrients, the organic compounds known as vitamins and the inorganic compounds known as minerals (Arntzen and Ritter, 1984). Odhav *et al.* (2007) reported significant calcium values for *A. spinosus* and *A. hybridus*; significant sodium and manganese values for *A. dubius* and *A. hybridus. A. spinosus, A. dubius* and *A. hybridus* possessed substantial quantities of magnesium.

2.3.2 Biological Screening

2.3.2.1 Antimicrobial activity

Many countries ravaged by war and poverty do not have the sanitation and hygiene levels comparable to those of First World countries which exposes the people to a wider array of microbial pathogens. These communities have to depend on local and indigenous plants as these are often the only available means of treating such infections (Taylor *et al.*, 2001).

Many plants have been screened to rationalize their use in traditional medicines (Rabe and van Staden, 1997; Lin *et al.*, 1999; Luyt *et al.*, 1999; Shale *et al.*, 1999; Kelmanson *et al.*, 2000; Tetyana *et al.*, 2002). Antibacterial compounds were isolated from *Warburgia salutaris* which is traditionally used as a topical application for sores and inflammation (Rabe and van Staden. 2000). Reid and coworkers in 2001 showed vernodalin was extracted from the leaf extract of *Vernonia colorata* (Willd.) Drake (Asteraceae). Rabe *et al.* (2002) isolated sesquiterpene lactones. Pillay *et al.* (2001) showed that bark extracts of *Erythrina lysistemon* Hutch. (Fabaceae) had good antibacterial activity and was attributed to the compound wighteone. In studies conducted by Grace *et al.* (2002) on the fruits of *Kigelia africana* (Lam.) Benth. (Bignoniaceae) they found activity against *Bacillus subtilis* which was due to the presence of the antibacterial compound palmitic acid.

Plants may also offer protection against some opportunistic fungal infections such as *Candida albicans* where Western medicines are unavailable in HIV patients (Fan-Harvard *et al.*, 1991). Motsei *et al.* (2003) reported that the aqueous extracts of *Tulbaghia violacea* Harv., *Allium*

sativum L. (both Alliaceae), *Polygala myrtifolia L.* (Polygalaceae) and *Glycyrrhiza glabra L.* (Fabaceae) exhibited anti-candidial activity.

2.3.2.2 Antimalarial activity

Malaria is the world's leading killer among the infectious diseases. It is estimated that more than two million people die from the disease each year (Prozesky *et al*., 2001).

One of the approaches for control of mosquito-borne diseases is the interruption of disease transmission by killing or preventing mosquitoes from biting humans. Herbal products with proven potential as an insecticide or repellent can play an important role in the interruption of the transmission of mosquito-borne diseases at the individual as well as at the community level. Some herbal products such as nicotine obtained from tobacco leaves, *Nicotiana tabacum*, anabasine and lupinine, the alkaloids extracted from Russian weed *Anabasis aphylla* (Campbell *et al*., 1993), rotenone from *Derris elliptica* and pyrethrums from *Chrysanthemum cinererifolium* flowers have been used as natural insecticides even before the discovery of synthetic organic insecticides (Jacobson and Crosby, 1971). The discovery, development and use of synthetic organic chemicals with persistent residual action not only overshadowed the use of herbal products against mosquitoes but also became the major weapon for mosquito control. Since the discovery of DDT (Dichloro-Diphenyl-Trichloroethane), mosquito control approach has been almost completely based on synthetic organic insecticides. But the extensive use of synthetic organic insecticides during the last five decades has resulted in environmental hazards and also in the development of physiological resistance in major vector species. This has necessitated the need for search and development of environmentally safe, biodegradable, low cost, indigenous methods for vector control, which can be used with minimum care by individuals and communities in specific situations.

Prozesky *et al*., (2001) showed plants to have antiplasmodial activity against a chloroquine-resistant strain of *Plasmodium falciparum*. *Ajuga remota* Benth. (Lamiaceae) and *Caesalpinia volkensii* Harms (Fabaceae) are two medicinal plants commonly used by traditional healers in Kenya to treat malaria (Kuria *et al*., 2001). Petroleum ether extracts of *Morinda lucida* Benth. (Morindaceae) inhibited the maturation of *Plasmodium falciparum in vitro* (Awe and Makinde,

1998). A recent report by Fennel *et al.* (2004) revealed that 134 South African plant species were tested for *in vitro* antiplasmodial activity against a *Plasmodium falciparum* strain D10 using the parasite lactate dehydrogenase assay. The species selected were based on their traditional use. Of the 134 species 49% showed promising antiplasmodial activity ($IC_{50} \leq 10$ µg/ml). A further 23 species were found to be highly active with IC_{50} values of ≤5 µg/ml and warrant further investigation as possible plant-based antimalarial drugs. At present many of the African medicinal plants tested for antiplasmodial activity have shown potential to be developed as new antimalarial drugs.

2.3.2.3 Anti-inflammatory activity

To evaluate the efficacy of a plant in reducing pain and inflammation, extracts can be tested for prostaglandin synthesis, these are involved in the complex process of inflammation and are responsible for the sensation of pain inhibitory activity in an *in vitro* assay (White and Glassman, 1974). Plants used in the treatment of pain and inflammation have been screened for COX-1 and COX-2 inhibition in an attempt to rationalize their traditional usage (Jäger *et al.*, 1995, McGaw *et al.*, 1997, Lindsey *et al.*, 1999, Shale *et al.*, 1999). To classify a plant as active, the minimum inhibition by aqueous extracts tested at a final concentration of 250 µg per test solution must be 59% and for ethanolic extracts, 70%, when tested at a final concentration of 250 µg per test solution.

Attention has recently focused on developing safer anti-inflammatory drugs following new evidence for the metabolism of arachidonic acid. Arachidonic acid metabolism through cyclooxygenase (COX) and lipoxygenase (LOX) pathways generates various biologically active intermediates that play an important role in inflammation, thrombosis and tumor progression. In addition to arachidonic acid's conversion to prostaglandins by the COX enzymes, it may also be converted to leukotrienes by the action of 5-lipoxygenase. Drugs that are able to block both COX and 5-LO metabolic pathways (dual inhibitors) equally well, seem to be the best option in terms of NSAIDs. (Fiorucci *et al.*, 2001)

2.3.2.4 Antioxidant activity

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage caused by free radicals. These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists (Choi *et al.,* 2002). There are increasing suggestions by considerable evidence that the free radicals induce oxidative damage to biomolecules (lipids, proteins and nucleic acids), the damage which eventually causes atherosclerosis, ageing, cancer, diabetes mellitus, inflammation, AIDS and several degenerative diseases in humans (Choi *et al.,* 2002). Examples of antioxidants include β-carotene, lycopene, vitamins A, C, E, and other substances. Plants contain a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous metabolites and such natural products are rich in antioxidant activities (Choi *et al.,* 2002).

In addition to the antioxidant vitamins, edible wild plants are rich in phenols and other compounds that increase their antioxidant capacity. According to Simopolous (2004), it is important to systematically analyze the total antioxidant capacity of wild plants and promote their commercialization in both developed and developing countries. Studies by Lindsey *et al.* (2002) reveal that the plants that are eaten as foods in Southern Africa were shown to provide important health benefits in the form of antioxidant activity. .

Extracts with inhibition values greater than 70%, and thus with high antioxidant activity, were found in the potherbs *Amaranthus* sp. (Amaranthaceae), *Sisymbrium thellungii* O.E. Schulz (Brassicaceae) and *Urtica dioica* L. (Urticaceae). Traditionally, these plants are used in the preparation of ‘imfino’ which forms an important part of the diet. High activity (greater than 70%) was also shown by the tuberous *Colocasia esculanta* Schott (Araceae) and the teas *Galium aparine* L. (Rubiaceae) and *Aspalathus linearis* (Burm.f.) R. Dahlgren (Fabaceae) (rooibos) (Fennell *et al.*, 2004).

2. 4 Safety Analaysis

Significant risks are associated with the inappropriate use of medicines in all healthcare systems. In South African traditional healthcare, these risks are of greater consequence due to the form in which plant medicines are sold. Medicinal plants, most commonly bark bulbs and roots (Mander *et al*., 1996) are dried and sold as semi and processed products, ranging from plant organs to crude powders. Products are seldom labeled but bulk stock may be identified by signage indicating a local vernacular name, and packaging is rudimentary. Botanical identification of medicinal plant products using vernacular nomenclature is notably difficult. Furthermore, taxonomically reliable characters are usually lost through desiccation during drying; rendering medicinal plant products extremely difficult to identify using morphological characters (Fennell *et al.*, 2004). Frequently used plants in traditional medicine are assumed safe, due to their long-term use (Elgorashi *et al*., 2002) and are considered to have no side effects because they are 'natural' (Popat *et al.*, 2001). However, scientific research has shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic *(Schimmer et al.*, 1988, Higashimoto *et al.*, 1993, Kassie *et al.*, 1996, De Sã Ferrira and Ferrão Vargas, 1999).

2. 4.1 Toxicity

The eggs of the brine shrimp, *Artemia salina*, have been used in the Brine Shrimp lethality (BSL) assay, which is a simple bench top bioassay used to test toxicity of compounds. *A. salina*, with same purine metabolism as that of mammalian cells and the DNA-dependent RNA polymerases of *A. salina* are also similar to the mammalian type (McLaughlin, 1991, Solis *et al.*, 1993).

2.4.2 Mutagenicity

The Ames test also called the *Salmonella*/microsome assay (Maron and Ames, 1983) is used as it is widely used in the determination of possible gene mutations caused by extracts. A positive response in any single bacterial strain either with or without metabolic activation is sufficient to designate a substance as a mutagen (Zeiger, 2001). The assay acts by detecting back mutations in the His operon to (→His+) when growing *Salmonella typhimurium* bacteria in a His-poor

medium. Elgorashi *et al.* (2003) reported that *Crinum macowanii*, *Chaetacme aristata* Planch. (Celastraceae), *Plumbago auriculata* Lam. (Plumbaginaceae), *Catharanthus roseus* (L.) G.Don. (Apocynaceae) and *Ziziphus mucronata* Willd. (Rhamnaceae) had mutagenic effects in the *Salmonella*/microsome assay

2.4.3 Cytotoxicity

Scientific strategies for the *in vitro* evaluation of natural products with biological activity have changed in the past few years. Interest in a large number of traditional natural products has increased (Cordell, 1995, Kurokawa *et al.,* 1993, Vlietinck *et al.,* 1995, Taylor *et al.,* 1996). MTT (Tetrazolium blue) colorimetric assays are used to evaluate the reduction of viability of cell cultures in presence and absence of the extracts (Betancur-Galvis et al., 1999). It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral and antitumor agents (Chung *et al.*, 1995, Vlietinck *et al.*, 1995). These techniques which considered quick and inexpensive for the evaluation of antitumor (Carmichael *et al.*, 1987, Rubinstein *et al.*, 1990) and antiviral activity (Weislow *et al.*, 1989) of a large number of natural product extracts. They can be used to guide the isolation and purification of their biologically active principles (Cordell, 1995).

2.5 Micropropagation of Wild Food Plants

For many years the importance of wild plants in subsistence agriculture in the developing world as a food supplement and as a means of survival during times of drought and famine has been overlooked. Native plants are disappearing from their habitat in an alarming rate. In addition to their contribution to the integrity of the environment, plants are invaluable sources of useful genes for genetic improvement of crop plants. Conservation of the components of biological diversity is vital in the arid zone, where renewable natural resources are scarce. While the contribution of native wild plants to the preservation of the environment has been well recognized, development of methods for tissue culturing of many native wild plants is lagging behind. Lack of well defined economic or commercial interests may be the cause for little interest in these plants. Cell and tissue culture of native plants is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large scale revegetation with perennial plants and for genetic manipulation studies. Among the

requirements for an applicable large-scale propagation system for native plants are cost effectiveness, reproducibility and simplicity.

Tissue culture is basically defined as *in vitro* growth of plantlets from any part of the plants in suitable nutritive culture medium. It is also known as `micropropagation' in scientific technology (Ghatnekar and Kavian, 2000). The application of tissue culture for large-scale plant production meant for commercial purposes is well demonstrated in the case of several crops and horticulture species. Many varieties have grown remarkably well *in vitro*, allowing various kinds of experimental manipulations. In many instances, the objectives of the investigations have been more than fulfilled and many solutions have been found to problems of plant growth, differentiation, morphogenesis and in the application of different growth substances (Ghatnekar and Kavian, 2000).

There are currently no published literatures available on micropropagation of *A. aspera, A. sessilis* and *G. densa*. Literature related to tissue culture on *Amaranthus* spp. belonging to the family Amaranthaceae is available.

Flores *et al.* (1982) developed *in vitro* culture system for some species and varieties of *Amaranthus*. Leaf disks and hypocotyls segments from 2-3 week old *A. hypochondriacus*, *A. cruentus* and *A. tricolor* seedlings were cultured in B5 (Gamborg) and MS media supplemented with 2,4-D, NAA, BA and zeatin in various combinations. 2,4-D induced callus and abnormal roots in the first two species. Flores and Teutonico (1986) reported that *in vitro* technology may provide very useful methods for the genetic improvement of grain *Amaranthus* species. In particular the micropropagation and regeneration of plants from primary explants offers the possibility to multiply superior genotypes as biotic and abiotic stress resistant plants, selected plants for increased production of proteins or specific amino acids or male sterile plants.

Bagga *et al.* (1987) found that hypocotyl segments of *A. paniculatus* callused on B_5 medium supplemented with Kn (0.5 ppm) and NAA (0.1 ppm) and even without transfer, shoots were formed in such cultures. About 20% of the cultures produced multiple shoots. In medium with 1 ppm each of Kn and NAA direct shoots were formed at one end of the hypocotyl segment and callusing was initiated at the other end. The plants obtained in either medium formed roots and could be transferred to soil for further growth.

Callus induction, callus growth and organogenetic processes were investigated in hypocotyl and stem cultures of four species of *Amaranthus* each of which comprised several varieties by Bennici *et al.* (1992). The combinations of NAA plus BAP or 2,4-D plus Kinetin were very effective in causing callus formation. As far organogenesis-based on a few varieties - only *A. caudatus* and *A. hypochondriacus* responded well forming shoots from callus when cultured in presence of IAA plus Kinetin and/or IAA plus BAP. Root regeneration was also observed in several varieties (Bennici *et al.*, 1992).

Bennici *et al.* (1997) studied *in vitro* behaviour of *A. cruentus*, *A. hybridus* and *A. hypochondriacus* and found that callus formation occurred from explants from almost all the lines tested. The results obtained demonstrate that in *Amaranthus* the genotype, growth regulator dose and combination, the type and the physiological stage of explants are factors of great importance for *in vitro* plant regeneration.

In general, the genus *Amaranthus* shows the great potential with regard to dedifferentiation and morphogenetic processes and the possibility to micropropagate selected genotypes via direct or indirect regeneration or via. somatic embryogenesis. The data obtained from different experiments provide unequivocal evidence that plant regeneration from primary explants varies with the genotype. Optimum conditions for shoot induction are high cytokinin/auxin ratio. Strong cytokinin such as BAP or 2-iP seem to be effective agents for shoot regeneration. The endogenous auxin / cytokinin balance and age dependant competence of explants tissues may play an important role in regeneration (Bennici and Schiff, 1997).

CHAPTER THREE: METHODOLOGY

3.1 Nutritional Analysis

All analyses were conducted in duplicate and the reagents used were that of analytical grade. Results are based on fresh weight per 100 g of sample.

3.1.1 Sample Collection and Preparation

A. aspera, A. sessilis and *G. densa* were collected from the greater Durban area, Kwa-Zulu Natal in 2006. The plants were collected and identified by Prof. Baijnath using taxonomic keys.

Upon receipt, the leaves were separated and washed several times with distilled water until no foreign material remained and air dried for 24 h. Thereafter, they were dried in an oven (Memmert, South Africa) at 25°C for 7 days. The dried leaves were powdered using an industrial grinder (Retsch Gmbh, West Germany) and stored in Schott bottles until use.

3.1.2 Determination of Moisture Content

Moisture analysis was carried out using the drying oven method (AOAC, 1990). Porcelain crucibles were weighed and their masses recorded. In this study 4 g of each fresh plant sample was weighed into pre-weighed crucibles and dried in the drying oven (Memmert, South Africa) at 105°C for 3 h. These were cooled in desiccators for 1 h and reweighed.

The percentage moisture content was determined according to the formula:

$$\text{Percentage moisture} = \frac{\text{initial mass (g)} - \text{mass after 3 h (g)}}{\text{mass of sample (g)}} \times \frac{100}{1}$$

3.1.3 Determination of Ash Content

Ash content was analysed as per method outlined in the AOAC (1990). Dried porcelain crucibles were weighed and their masses recorded. In this study 3 g of each plant sample was weighed into

the crucibles. An aliquot of 7 ml glycerol: methanol (1:1 v/v) was added to the crucibles. The crucibles were ignited and burnt until all organic material volatilised. The samples in the crucibles were ashed by placing them in a muffle furnace (Labcon, Laboratory Consumables and Chemical Supplies) at 600°C for 6 h. The crucibles were placed in desiccators and allowed to cool. The resulting crucibles were weighed and the percentage ash content was determined as follows:

$$\text{percentage ash} = \frac{\text{mass of ash (g)}}{\text{mass of sample (g)}} \times 100$$

3.1.4 Determination of Protein Content

The Kjeldahl method (AOAC, 1990) was used for protein analysis using a Buchi 430 Digestor (Switzerland). This method is based on the assumption that a mixture of pure proteins will contain 16% nitrogen. The nitrogen concentration is determined by converting the nitrogen present in the sample to ammonium sulphate by digesting it in concentrated sulphuric acid (H_2SO_4). The digested sample is then made alkaline with 32% sodium hydroxide (NaOH) (m/v). The ammonia is distilled into excess 2% boric acid solution and is determined by titration with standardized 0.1N H_2SO_4. Protein content was obtained by multiplying the percentage determined nitrogen by the appropriate factor, which was 6.25.

In this study 0.5 g of sample, 4 g of catalyst mixture (192 g anhydrous sodium sulphate, 7 g copper sulphate crystals and 0.71 g selenium powder were crushed using a mortar and pestle) and 20 ml of concentrated H_2SO_4 were weighed into clean dry digestion tubes (Buchi, Switzerland). The digestion tubes were connected to a NaOH trap for absorbing the noxious fumes and a vacuum was used to draw the fumes into the NaOH trap. The tubes containing the sample mixture were heated and the heating power was maintained such that the samples were always boiling. Digestion occurred for 45 min and was completed when the solution turned light green. The digested samples were distilled by inserting the digestion tubes into the preheated Buchi 321 Distillation Unit (Switzerland) and were diluted with distilled water (1:3 v/v) followed by the addition of 70 ml of a 32% NaOH (w/v) solution. An aliquot of 60 ml of 4% boric acid and 6 drops of methyl red indicator was added to Erlenmeyer flasks that were placed in the distillation unit. Samples were distilled for 3 min. The distillate was titrated with 0.1N H_2SO_4 and the end

point was reached when the light blue solution turned colourless to grey. The percentage nitrogen content was calculated as follows:

$$\% N = \frac{(\text{sample titre in ml} - \text{blank in ml}) \times 1.4 \times \text{normality of acid} \times 100}{\text{mass of sample} \times 10}$$

The percentage protein content was calculated as follows:

$$\% \text{ Protein} = 6.25 \times \%N$$

3.1.5 Determination of Fat Content

The Soxhlet method was used to determine total fat (AOAC, 1990). This method is based on acid hydrolysis that liberates the bound fat followed by solvent extraction of the fat.

Acid hydrolysis was achieved by adding 3 g of sample, 5 g of Celite 545 (Capital Lab Suppliers, New Germany) and 100 ml of 4N hydrochloric acid (HCl) to clean, dry digestion flasks (Buchi, Switzerland) and swirled to mix contents. 10 g of sea sand (Merck, Germany) and 5 g of celite 545 were added into filter crucibles (Buchi hydrolysis unit B-425 Switzerland). The raise/lower device was placed in the top boil position and the filter crucibles were placed in the preheated digestion block. Sample aspiration tubes as well as the water jet pump for the vacuum extraction system was connected and samples were filtered into filter crucibles. After filtration was complete, the heater was switched off. Empty digestion tubes were rinsed out with aliquots of warm distilled water (50°C) into filter crucibles until no sample remained. Filter crucibles were dried in a drying oven at 80°C overnight.

The fat was extracted using a Buchi 810 Soxhlet system (Switzerland). The system was switched on, water tap opened and glass steam generator tap closed. Pre-dried beakers together with 2 boiling chips in each were weighed and their masses recorded. Beakers were filled with petroleum ether and inserted into the system. Filter crucibles containing samples were sealed with cotton wool and inserted into the extraction chamber. Beakers were constantly topped up with

petroleum ether (40-60°C) using a plastic hypodermic sponge during the 6 hour extraction period. At the end of the extraction period, the side lever was opened which allowed the solvent to drain out of the beakers. Heating continued until all the solvent evaporated from the beakers. These were placed in a drying oven overnight at 105°C, cooled in desiccators and weighed. Results were analyzed according to the formula:

$$\text{Percentage fat} = \frac{\text{mass of flask after drying - initial mass of flask}}{\text{mass of sample}} \times 100$$

3.1.6 Carbohydrate Analysis

Carbohydrate analysis was performed using the Phenol Sulphuric Acid Carbohydrate Assay Method (Dubois, 1956)

Plant samples were dissolved in distilled water and filtered using a C_{18} Sep-Pak Plus cartridge (Waters Corp. Wilford, Mass. USA), previously conditioned with 10 ml distilled water (MillQ. R.G. Millipore, USA) and 45 µl nylon filter (Scheicher & Schuell, Germany).

100 µl of plant sample or glucose standards were added to 50 µl of 80 % phenol followed by 2 ml of concentrated sulphuric acid. Dilutions were made from the standard solution in the range of 10 µg/ml to 100 µg/ml to construct a standard curve. These solutions were kept at room temperature for 10 min and the resultant absorbance was measured at 490 nm using a spectrophotometer (Ultraspec 2). The concentration of the sugars was determined from the standard curve obtained.

3.1.7 Dietary Fibre Analysis

Procedure was carried out according to Scheizer and Wuersch (1979) with some modifications.

The moisture of the samples was determined as described in 3.1.2. In this study 1 g sample was weighed into a 250 ml beaker and the fat was extracted with 3 × 25 ml portions of hot petroleum ether. The defatted sample was extracted with 3 × 25 ml portions of 80% ethanol. 50 ml distilled water was added to sample and autoclaved for 1 h at 121°C to gelatinize the starch. The sample was cooled and the pH was adjusted to 1.5 using dilute HCl. 50 mg Pepsin and 2 ml of chloroform was added and this mixture was incubated for 20 h at 37°C. After 16 h the pH was

readjusted to 1.5 and a further 25 mg Pepsin was added. After pepsin digestion the pH was changed to 6.0 with dilute NaOH solution. 25 ml phosphate buffer (pH 6) containing 100 mg pancreatin and 50 mg glucoamylase was added to the mixture. This mixture was incubated for 18 h at 37°C. A few drops of thymol solution prevented bacterial growth during incubation.

The digested sample was centrifuged at 3000 rpm for 30 min and the supernatant was transferred to a 600 ml beaker. The residue was transferred to a glass sintered crucible with distilled water, acetone and diethyl ether. This was Fraction A. Four volumes of ethanol were added to the beaker containing the supernatant (Fraction B) to precipitate the soluble fibres. The supernatant was drained off and the precipitate was transferred to a glass sintered crucible and filtered (Fraction C). Fraction C was washed with 80% ethanol, acetone and diethyl ether. The crucibles containing fraction A and C were dried for 16 h in an oven at 105°C. The crucibles were weighed (W1) and placed in a furnace at 45°C for 2 h. These were cooled in desiccators and reweighed (W2). The difference between the weights W1 and W2 of fractions A and C represent the insoluble and soluble fibres respectively. The sum of the two represents the total dietary fibre. A reagent blank was used during the procedure.

The calculations were as follows:

$$\% \text{ Insoluble Fibre} = \frac{(W1 - W2) \times 10}{\text{mass sample}}$$

$$\% \text{ Soluble Fibre} = \frac{((W1 - W2) - \text{Blank}) \times 100}{\text{mass sample}}$$

Calculation for sample with < 2% fat:

$$\% \text{ Dietary fibre} = \frac{\text{Total dietary fibre}}{\text{sample mass}} \times 100$$

Calculation of % dietary fibre on dried, defatted samples expressed on:

$$\% \text{ Dietary Fibre} = \frac{\text{(Total dietary fibre x 100)}}{\text{mass of sample}} \times \frac{\text{(100 - \% moisture)}}{100} \times \frac{\text{(100 - \% fat)}}{100}$$

3.1.8 Energy Determination

The Atwater system was used to determine the energy values. This system uses factors to estimate available energy from the protein, fat, carbohydrate, and alcohol components of food items. Energy was calculated using the general Atwater's factors of 4 kilocalorie (kcal) per g protein, 9 kcal per g fat and 4 kcal per g carbohydrate. These conversion factors were multiplied by 4.186 in order to obtain energy values in kilojoules (kJ) (WHO, 1985):

$$\text{Energy (kJ)} = (4 \text{ kcal/g} \times \text{g protein} \times 4.186) + (4 \text{ kcal/g} \times \text{g carbohydrate} \times 4.186) + (9 \text{ kcal/g} \times \text{g fat} \times 4.186)$$

3.1.9 Mineral Analysis

The mineral metallic elements calcium, copper, iron, magnesium, manganese, zinc, sodium and phosphorus were determined on dried samples that were digested in a microwave digester using the method of Milestone Microwave Lab Systems (MMLS, 1999). The concentrations of the minerals were determined with an Inductively Coupled Plasma (ICP) spectrometer (Perkin-Elmer). Sample solutions were quantified against standard solutions of known concentrations that were analyzed concurrently according to Perkin Elmer (Perkin, 1996). All assays were carried out in duplicate. Mean values and standard deviations are based on these results.

Sample Digestion

Duplicate aliquots (0.5 g) from each of the dried plant specimens were weighed into teflon vessels to which 5 ml concentrated nitric acid and 2 ml hydrogen peroxide were added. Each vessel was closed with its teflon cover and adapter and tightened with a spring disc. Vessels were

positioned on the rotor and were secured by placing a circular safety band around them. The rotor was placed onto its base and each vessel was tightened using a torque wrench. The microwave oven and the fume extractor were switched on and the rotor was transferred to the microwave oven. The appropriate program from the instrument user manual was selected and the following parameters were entered:

	Step 1	Step 2	Step 3	Step 4	Step 5
Power(W)	250	0	250	400	600
Time (Min)	1	2	5	5	5

Once the operation was complete, the oven was switched off and the rotor was taken out of the oven. The vessels were allowed to cool and the contents were transferred into 50 ml volumetric flasks and made up to 50 ml using double deionised water (MMLS, 1999).

Mineral Analysis

Mineral analysis by Inductively Coupled Plasma Spectrophotometry was carried out according to protocols obtained from Perkin (1996) at ALEX STEWART TES BRETBY (KZN) (Pty) Ltd.

Stock solutions of the mineral elements were prepared from standard solutions of 1 mg/ml. The ICP spectrometer was ignited and the ICP 400 software program (Perkin, 1996) was loaded. The extractor fan, argon gas and the spectrometer were switched on. The peristaltic pump was turned on and deionised water was aspirated for 1 min. The torch was ignited and the nebulizer argon flow was set by performing the bullet test. This was achieved by aspirating a 1000 mg/ml solution of sodium. The plasma was examined through the viewing window of the torch compartment door and a yellow-orange bullet, extending from the base of the discharge to a point about 2-3 mm above the top of the RF coil, was visible in the central channel of the discharge. A satisfactory bullet height was achieved by adjusting the nebulizer argon flow incrementally using the nebulizer adjustment knob. After setting the nebulizer argon flow, the system was allowed to stabilize for 1 hour before running samples.

A method was developed by entering the element parameters into the element mode and the samples, standards and blanks were aspirated and read. The calibrated wavelengths as well as the element parameters were stored in the element mode whilst the element parameters and element

file names were entered and stored in the method mode. The method file name was accessed and the standards, blank and samples were aspirated and read. A quality control standard of known concentration of each element analyzed was determined after every five samples in order to verify accuracy of the procedure. The concentrations of minerals were calculated using the concentration from the ICP analysis reports using the formula:

$$\text{Concentration (mg/100 g)} = \frac{\text{concentration (ppm)} \times \text{volume (ml)}}{\text{mass of sample (g)}} \text{ X } 100$$

3.1.10 Vitamin Analysis

3.1.10.1 Vitamin A

Vitamin A analysis was carried out using the Carr Price Method (Hoffman, 1969).

In this study 5 g of the plant sample and 5 g of the retinol standard were weighed individually into a 100 ml flask containing 63 ml of the alkali alcohol solution (7.5 g potassium hydroxide in 63 ml ethanol). The solution was heated for 25 mins under nitrogen. Then the contents of the flask were transferred into a separating funnel and allowed to cool. 100 ml of petroleum ether was added to the funnel. Following which the air was displaced using nitrogen. Shook the funnel vigorously and the cap was removed to allow the layers to separate. The contents were centrifuged for 5 min at 3000 rpm to separate the layers. Pipetted out 20 ml of the petroleum ether layer and filtered through a dried funnel containing anhydrous sodium sulphate. The filtrate was evaporated using a rotary evaporator (Buchi RE Rotoevaporator including a Buchi 461 water bath) and 5 ml of chloroform was added to the flask. From this 1 ml of the sample and standard was taken and added to 2 ml of antimony trichloride solution. The absorbance was measured at 620 nm. Each test was carried out in duplicate.

The amount of Vitamin A present in the samples was calculated based on the following formula:

$$\text{Vitamin A (I.U.)} = \frac{E_A \text{ x C x 5x 100 x100}}{E_B \text{ x 20 x sample weight}}$$

E_A = average extinction of the sample preparation

E_B =average extinction of the standard preparation

C =I.U. of vitamin A contained in 1 ml of the vitamin A standard solution

3.1.10.2 Vitamin B_1

Vitamin B_1 analysis was carried out using a fluorescence method described as the Thiochrome Method (Hoffman, 1969).

2 ml of each plant sample and standard was taken separately into a 100 ml flask containing 50 ml distilled water and 20 ml of 10% HCl. This solution was heated for 10 min in a water bath at 60°C. The solution was cooled and then volume was made up to 100 ml using distilled water. The solution was centrifuged at 5000 rpm for 10 min. After centrifugation, 5 ml of the supernatant was added to 100 ml of distilled water and then 5 ml of Solution A (30 g of potassium hydroxide in 100 ml distilled water) and Solution B (300 mg of potassium ferricyanide in 6 ml distilled water) was also added. Then, after 90 s, 10 ml of isobutanol was added and the samples were centrifuged for 5 min at 5000 rpm to separate the layers. 5 ml of the upper layer was pipetted out into a test tube and 2 ml of alcohol was added and mixed. The fluorescence of the sample and standard were measured with a fluorimeter using a primary filter (maximum transmission at 360-365 mol) and a secondary filter (maximum transmission at 460-480 mol).

Vitamin B_1 was calculated according to the following formula:

$$\text{Vitamin } B_1 \ (\mu g/ml) = \frac{F_A \times 0.8}{F_B}$$

F_A = average fluorescence of the sample preparations

F_B = average fluorescence of the standard preparations

0.8 = µg/ml of thiamine hydrochloride contained in 1 ml of the thiamine standard solution

3.1.10.3 Vitamin B_2

Vitamin B_2 analysis was carried out using the Fluorimetric method (Hoffman, 1969)

In this study 0.8 mg of plant sample and standard solution was added to a 100 ml volumetric flask containing 80 ml of solvent mixture (pyridine: glacial acetic acid : distilled water, 10:1:40 v/v) The solution was heated on a water bath at 60°C for 10 min. The flask was cooled and diluted to 100 ml with the solvent mixture. Thereafter, 20 ml of the solution was transferred to a centrifuge tube and 1 g of purified kieselguhr was added. This was centrifuged at 5000 rpm for 10 min. 5 ml of the supernatant solution was diluted with 100 ml of the solvent mixture (pyridine: glacial acetic acid: distilled water, 10:1:40 v/v). The fluorescence of the sample and the riboflavin standard solution was measured with a fluorimeter, using a primary filter (maximum transmission at 400-420 mu.) and a secondary filter (maximum transmission at 550-700 mu).

Vitamin B_2 was calculated according to the following formulae:

$$\text{Vitamin } B_2\ (\mu g/ml) = \frac{FA \times 0.4}{F_B}$$

F_A = average fluorescence of the sample solution

F_B = average fluorescence of the riboflavine standard solution

0.4= µg of riboflavine contained in 1 ml of the riboflavine standard solution

3.1.10.4 Vitamin B_3

Vitamin B_3 analysis was carried out using a method described by (Horowitz, 2000).

Pipetted out 0, 5, 10, 15 and 20 ml of standard solution of Niacin in a series of flasks and 5 g of the sample was weighed in a separate flask. 1.5 g of calcium hydroxide and 60 ml of distilled water was added to each of the flasks and autoclaved at 121°C for 15 min. The contents were made up to 100 ml and centrifuged at 5000 rpm for 10 min followed by placing it in an ice bath for 15 min. From this, 20 ml of the supernatant was transferred into separate centrifuge tubes

containing 8 g ammonium sulphate $(NH_4)_2S0_4$ and 2 ml phosphate buffer solution. The tubes were placed in a water bath at 55°C for 15 min and then centrifuged at 5000 rpm for 15 min and filtered through Whatman No. 1 filter paper. To the supernatant 10 ml distilled water was added and placed in an ice bath for 30 min. 1 ml sulphanilic acid was added and placed into the ice bath for 15 min. For the reagent blank, 10 ml cyanogen bromide and 1 ml sulphanilic acid was added and placed in an ice bath for 15 min. The absorbance was measured at 470 nm.

Vitamin B_3 was calculated as follows:

$$\text{mg Vitamin } B_3/\ 100\text{g sample} = \frac{\text{Concentration}}{10 \times \text{weight of sample}}$$

3.1.10.5 Vitamin C

Vitamin C analysis was carried out using a method described by Hoffman (1969).

Pipetted out 2 ml of Vitamin C solution (Ascorbic Acid) and 5ml of 1% oxalic acid was added to a conical flask. This solution was titrated against the indicator (0.050 g Phenol- indo- 2:6 - dichloro - phenol was dissolved in 200 ml of hot water containing 0.042 g Sodium bicarbonate) until a light pink end point was obtained. This is A, the average of the duplicate standard titres.

Pipetted out 7 ml of 1% Oxalic acid and 16ml distilled water was added into a conical flask. This solution was titrated against the indicator until a light pink end point was obtained. This is B, the average of the duplicate blank titres.

For the sample, 10 g of plant sample was dissolved in 1% Oxalic acid. Then it was diluted in 100 ml distilled water and filtered. Pipetted out 10 ml of the filtered sample and titrated against the indicator until a light pink end point. This is C, the average of the duplicate sample titres.

Vitamin C was calculated as follows:

$$\text{Weight of Standard (W)} = \frac{\text{Weight of Vitamin C (g)}}{100\text{ ml of Standard Vitamin C Solution}}$$

$$\text{mg Vitamin C/100g} = \frac{100 \times W \times 2 \times 100 \times C}{(A - B) \times \text{Weight of sample}}$$

3.2 Biological Screening

3.2.1 Sample Collection and Preparation

The plants were collected and stored as described in Section 3.1.1.

3.2.2 Preparation of Aqueous and Methanolic Extracts

Preparation of the Aqueous and Methanolic Extracts

Aqueous extraction of the dried plant material was carried out according to the procedure outlined by (Jeremy and Whiteman, 2003) with minor modifications. 20 g of the dried plant material was stirred for 24 h in 200 ml of distilled water. The slurry was filtered using Whatman No. 1 filter paper and the supernatant was collected. This was then concentrated by placing the supernatant in a biofreezer (Snijders Scientific, Holland) at -70°C and then freeze dried (Virtis Benchtop Freeze Dryer). The freeze dried material was used as a stock and working solutions were prepared for appropriate applications.

Methanolic extracts of the dried plant material were carried as above but the sample was extracted in 80% methanol. The supernatant was concentrated using a Buchi RE Rotoevaporator including a Buchi 461 water bath set at a temperature of 50°C. The concentrate was placed in a biofreezer and freeze dried using a Virtis Benchtop Freeze Dryer. Aliquots were prepared from the dried crude extract and dissolved in methanol, acetone or dimethyl sulfoxide (DMSO) depending on the experimental protocol.

The yield per 100 g of plant material was calculated using the following equation:

Amount of dry extract per 100 g = Amount of Final Product X 100

Amount of Sample Added

3.2.3 Determination of Antioxidant Activity

The anti-oxidative properties of the crude extracts were tested using the DPPH (1.1-diphenyl-2-picrylhydrazyl radical) photometric assay (Choi *et al.,* 2002).

Preparation of plant material

The freeze dried aqueous and methanolic plant material (1000 μg/ml) were diluted to final concentrations of 500 μg/ml, 250 μg/ml, 100 μg/ml, 50 μg/ml, 10 μg/ml and 1 μg/ml in ethanol.

Preparation of Standard

Rutin (Sigma) found in the buckwheat plant *Fagopyrum esculentum*, was used as a comparative standard.

DPPH Photometric Assay

1 ml of 0.3 mM DPPH in ethanol, was added to 2.5 ml of plant sample solution of different concentrations and were allowed to react at room temperature for 30 min. 1.0 ml ethanol plus plant extract solution (2.5 ml) was used as a blank, while DPPH solution and 2.5 ml ethanol was used as a negative control. The positive control was DPPH solution (1 ml) plus 2.5 ml 1mM Rutin. Each test was carried out in triplicate and results are expressed as the mean and standard deviation of the mean. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm and the average absorbance values was converted into the percentage antioxidant activity, using the following equation:

Scavenging capacity %=100- (Abs of sample-Abs of blank) × 100

Abs of negative control

3.2.4 Anti-inflammatory properties

Determination of the 5- lipoxygenase activity.

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm on a UV/Visible spectrophotometer (Varion Cary 1E UV- Visible spectrophotometer). Nordihydroguaiaretic acid (NDGA) and Rutin known inhibitors of soybean lipoxygenase, were used as controls. The reaction was initiated by the addition of aliquots (50 µl) of a soybean lipoxygenase solution (prepared daily in potassium phosphate buffer 1M pH 9.0) in a sufficient concentration to give an easily measurable initial rate of reaction to 2.0 ml of sodium linoleate (100 µM) in phosphate buffer. The enzymatic reactions were performed in absence or in presence of inhibitor and their kinetics were compared. The inhibitors were dissolved in DMSO in such a manner that an aliquot of each (30 µl) yielded a final concentration of maximum 100 ppm in each assay. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 30 µl of phosphate buffer (pH 9.0) instead of 30 µl of the inhibitor solution). Each inhibitor concentration was tested in triplicate and the results averaged; the concentration that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration (Njenga and Viljoen, 2006). Aqueous extracts ($IC_{50} \geq 100$ µg/ml) were not taken in this study. All the analysis were carried out in triplicate and the results were expressed the mean ±SD. Regression analysis was used to calculate IC_{50}, defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction.

3.2.5 Antimicrobial Activity

The antimicrobial activity of methanolic and aqueous plant extracts were carried out on selected bacteria and fungi by evaluating the bactericidal and anti fungal effect and the minimum inhibitory concentration on selected bacteria and fungi in a petri dish using the agar disk diffusion method (Vlotman, 2003).

Determination of Antibacterial Activity

The ten bacteria used as test organisms were as follows: *Bacillus cereus* (DBT*_F), *B. stearothermophilus* (DBT*_Q), *Escherichia coli* (DBT*_L), *Klebsiella oxytoca* (DBT*_AM), *Micrococcus sp.* (DBT*_AR), *Pseudomonas aeruginosa* (DBT*_D), *Proteus mirabilis* (DBT*_O), *Salmonella typhimurium* (DBT*_AF), *Staphylococcus aureus* (DBT*_E) and *S. epidermis* (DBT*_ Q). * DBT is a reference for the Durban University of Technolgy Culture Collection which is based at the Department of Biotechnology and Food Technology.

Stock cultures were prepared from the Culture Collection and stored in micro bank vials using 50% glycerol. When required the cultures were plated out on Tryptone Soya Agar (Biolab) plates and were subsequently grown in Tryptone Soya Broth (Biolab) for 24 h at 37°C. The absorbance of bacterial cells was adjusted to MacFarland Standard of 0.5 which corresponded to 10^8 CFU/ml.

Molten (45°C) sterile tryptone agar (10 ml) in a flask was inoculated with a 0.1 ml of 10^8 cfu/ml of each of the respective bacterial strains. This was poured over the base plates containing 10 ml tryptone agar in sterile 9 cm Petri dishes. 50 µl of plant extracts at different concentrations (1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml) were pipetted on 5 mm sterile filter paper disks (Whatman No 1); and air dried in a biological safety cabinet laminar. Sample containing discs were placed on the surface of the bacterial plates inoculated and incubated at 37°C for 24 h except for *B. stearothermophilus*, which was incubated at 50°C. Control disks with ethanol (5 µl) served as the negative control, whilst Ciprofloxacin (5 mg per disk) was the positive control. All tests were carried out in triplicate. The minimum inhibitory concentration (MIC) was taken as the lowest concentration of plant extract that inhibited growth after incubation.

Determination of Antifungal Activity

The two yeasts and seven fungi used as test organisms were: *Candida albicans* (DBT*_AB) and *Saccharomyces cerevisiae* (DBT*_R), *Aspergillus flavus* (DBT*_AR), *Cladosporium* sp (DBT*_AS), *Fusarium verticilloides* (DBT*_AT), *Geotrichum* sp (DBT*_AA), *Penicillium* sp (DBT*_AC), *Rhizopus* sp (DBT*_Y) and *Trichoderma* sp (DBT*_AU).

The yeast cultures were recovered by growing from stock cultures in Sabourand Dextrose Broth for 24 h at 37°C. The moulds were grown on Sabourand Dextrose Agar at 28°C for 4 to 7 days until sporulation. The spores were collected in 10 ml sterile distilled water, counted in a counting chamber (Neubauer) and the concentration adjusted to 10^6 spores/ml. Sterile distilled water containing the fungal spores (10^6 spores/ml) were poured over the Sabourand Dextrose Agar (SDA) base plates (Biolab). 50 µl of each extract (1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml) was transferred onto each of three sterile 9 mm discs (Whatman No. 1). 50 µl of ethanol served as the negative control, and 5 µg/ml Amphotericin B (Fluka, Biochemika), was used as a positive control. Each plant extract and control was tested in triplicate. The plant extracts and ethanol impregnated discs were dried in sterile Petri dishes and incubated at 30°C. Antifungal activities were recorded as the width (mm). The minimum inhibitory concentration (MIC) was taken as the lowest concentration that inhibited growth.

3.2.6 Anti Mosquito Activity

Mosquito repellency, larvicidal and insecticidal activity against *Anopheles arabiensis* were carried out at the Medical Research Council, South Africa under the guidance of Ms R. Gayaram.

Determination of Mosquito Repellency

Repellent activity was assessed by topical application of 1000 µg/ml of plant extract to the skin and subsequent exposure of the treated areas of skin to unfed female mosquitoes. The rodent *Mastomys coucha* was the test animal used for the screening of extracts for repellency activity.

The repellency analysis followed the South African Medical Research Council standard protocols and is depicted by figures. 4a-4d below. Ethical approval for the use of Mastomys in these trials was obtained from the MRC's Ethics Committee for Research on Animals.

Animal preparation

For each plant extract four adult Mastomys were weighed individually and injected intraperitoneally with sodium pentobarbital (Fig. 4b). The anesthetized rodents were shaved on the ventral surface and 1000 µg/ml of plant extract was applied to each of two rodent's abdomens

(Fig. 4c). The third animal served as a negative control and the fourth animal was a positive control (N, N-Diethyl-meta-Toluamide (DEET)).

Repellency assay

Paper cups (500ml) were modified by replacing the base of the cup with mosquito netting held in place with a rubber band and covering the mouth of the cup with transparent plastic film. Thirty unfed 4-day old *A. arabiensis* females (Fig. 4a) were placed in the cup and held in contact with the treated ventral surface of each rodent. Mosquito activity was observed through the transparent plastic film. After a period of two mins the numbers of mosquitoes probing were recorded (Fig. 4d). The rodent was then returned to the animal facility and allowed to recover from anesthetic. Each rodent was monitored for 7 days for adverse reactions to the applied plant extracts.

Repellency of the extracts was calculated using the formula:

$$\% \text{ mosquito repelled} = \frac{\text{number repelled}}{\text{number introduced}} \times 100$$

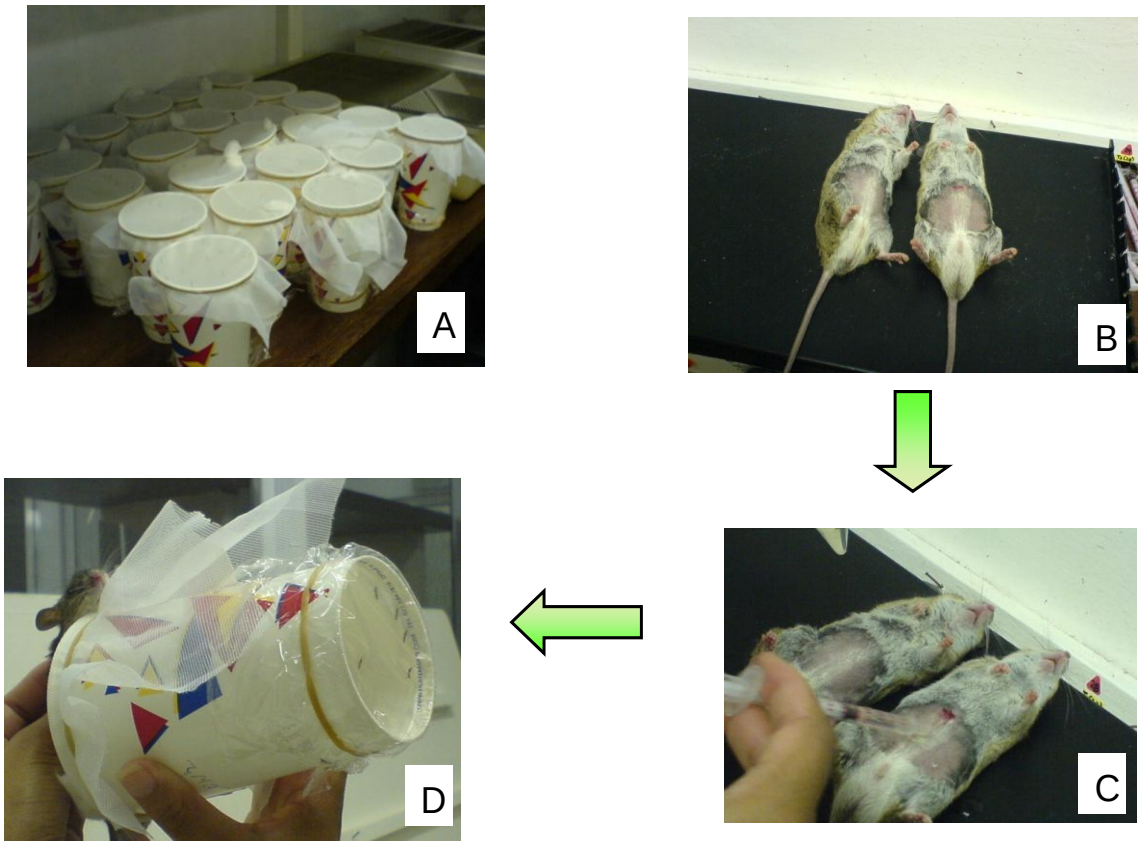


Fig. 4: Repellency (a) Unfed *A. arabiensis* females introduced into cup. (b) Rodent anaesthetized using sodium pentobarbital. (c) Plant extract/Positive control (DEET) applied to rodent's abdomens. (d) Unfed *A. arabiensis* females held in contact with treated ventral surface of rodent

Larvicidal assay

The larvicidal bioassay followed the WHO standard protocols (WHO, 1981b) with slight modifications. 1 ml of the extract solution was added to polypropylene containers (10 cm x10 cm) containing 0.25 liters of distilled water. Thirty 3rd instar larvae of *A. arabiensis* were placed in the container. A negative control was set up in which solvent was only added instead of extract. A positive control was set up using Mostop, an organo-phosphate (used by the malaria

control program as a larvicide). Each container was monitored for larval mortality over a 7 day period.

Insecticidal Assay

The adulticidal effect was assayed following a slightly modified version of the WHO standard method (WHO, 1981a) depicted by figures 5a-5e below.

1 ml extract solution or 1 ml of the positive control (K-Orithrine) or 1 ml negative control (acetone or distilled water) was sprayed onto a clean dry non-porous ceramic tile using the pre-calibrated Potter's Tower. The negative controls in this experiment were acetone and distilled water. The Potter's Tower (Fig. 5a) was cleaned with acetone between each different extract application. The sprayed tiles (Fig. 5b) were air dried and assayed within 24 h of spraying. A standard bioassay cone was fixed in place over the sprayed tile and thirty blood-fed *A. arabiensis* females 3-5 days old were introduced into the cone (Fig. 5c). The mosquitoes were then observed for knockdowns after 30 and 60 min of exposure (Fig. 5d). The test species were thereafter removed from the bioassay cone and transferred to a holding cage containing a nutrient solution (Fig. 5e). After 24 h the number of dead mosquitoes was recorded and percentage mortality calculated.

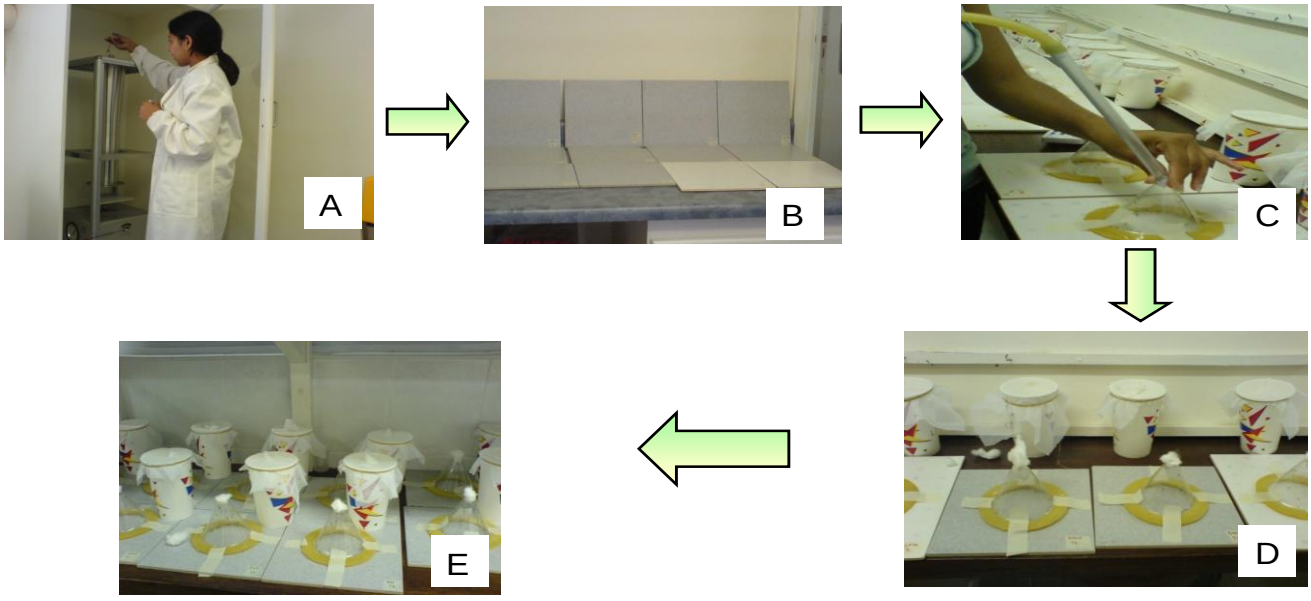


Fig.5: Insecticidal Assay (a) Potter's Tower. (b) Plant extract/ Positive control (K-orithine) sprayed on ceramic non porous tiles. (c) *A. arabiensis* (30) females introduced into bioassay cone. (d) Observed for knockdown after 30 & 60 min of exposure. (e) Transferred to holding cage containing nutrient solution overnight to check for mortality

3.3 Safety Evalution of Plants

3.3.1 Cytotoxicity

Cell proliferation inhibition was assessed by the MTT assay (Hanelt *et al.*, 1994).

Cell Line

Human chronic myelogenous leukaemia (K562) was used in this study. The K562 cell line was purchased from Highveld Biological, Modderfontein, South Africa. The cells were received in an active state and immediately incubated at 37ºC in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town South Africa) with 5% CO_2. When the cells were 80% confluent, they were sub-cultured, and stock cultures were stored at -70ºC until required.

Cell maintenance

Cell maintenance was performed according by protocols obtained from Freshney (1987).

All cell culture procedures were carried out in a laminar flow cabinet containing a UV light, (Scientific Engineering INC). The unit was swabbed /sterilized with 70% ethanol (Merck, South Africa) before each use.

The cells were grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Greiner, Germany) using filter sterilized (0.22 mm) 10% Complete Culture Medium (CCM) which comprised of Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum and supplemented with antibiotics (penicillin: 10 000 U/ml, streptomycin sulphate 10 000 U/ml) and 1 mM sodium pyruvate). Cells were incubated in a humidified incubator under 5% CO2 at 37°C and passaged weekly. All the above media and chemicals were obtained from Highveld Biological, South Africa. The cultures were incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town South Africa) with a 5% CO_2 atmosphere. The culture flasks were examined for colour changes and turbidity of the media on a daily basis. This determined the frequency of media changes. The culture was examined under an inverted microscope (Nikon) for cell growth

The cells were harvested when the culture was 80% confluent and divided into two separate flasks, then more medium added to each culture flask and incubated at 37°C humidified incubator (Snjiders Hepa, United Scientific group, Cape Town South Africa) with a 5% CO_2 atmosphere.

The cells were enumerated using a haemocytometer. The cell suspension was mixed with equal aliquot of 0.2% Trypan Blue [Biowhittaker, Wakersville (USA)] (v/v 1:1). This mixture was drawn across the grid by capillary action. The volume of cell suspension that occupied one

primary square is 0.1mm^3 (1.0mm^2 ´ 0.1mm/ 1.0 ´ 10^4 mL). Only the viable (translucent) cells that lay within or that touched, the left or top boundary was counted. The number of viable cells per ml in the original sample was calculated as follows:

Cells/ml = Average number of cells per primary square × 10^4 dilution factor

Storage of cells

Storage of cells was performed according by protocols obtained from Freshney (1987).

The cells were pelleted and washed twice with pre-warmed Phosphate Buffered Saline, pH 7.2 (PBS). Resuspended in 0.5 ml FCS and cooled on ice. A 20% dimethylsulphoxide (DMSO) in DMEM (V/V 1:4) was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5ml) of the cell suspension and the cryoprotective agent were added to a cryotube (Corning , South Africa). The tubes were transferred to the thermos flask and kept overnight at -20°C. The cells were subsequently transferred to a –70°C bio-freezer and stored until required.

Regeneration of cells

Regeneration of cells was performed according to protocols obtained from Freshney (1987).

Cells were removed from the –70°C biofreezer, swabbed with 70% ethanol and rapidly thawed. The cells were then transferred to 20 ml of pre-warmed 10% CCM in 75 cm^2 tissue culture flasks and incubated at 37°C humidified incubator with a 5% CO_2 atmosphere.

MTT assay

The MTT cytotoxity assay was conducted according to Mosmann, (1983) with minor modifications. The assay was carried out in 96 well, flat bottomed microtitre plates (Cellstar, Greiner, Germany). 200 µl of ±1.2 x10^3 of cells was added into each well, 20 µl of the plant (1000 µg/ ml, 100 µg/ ml 10 µg/ ml) extracts were added to the respective wells. In the control wells 20 µl DMSO and 20 µl media only respectively were added. The plate was incubated in a 37°C humidified incubator with a 5% CO_2 atmosphere for 20 h. Then 20 µl of MTT reagent (Sigma, St Louis, USA) was added, the plates were then incubated for a further 4 hours at 37ºC in a humidified incubator with 5% CO_2 atmosphere. Subsequently 100 µl of 100% DMSO was

added to each well and the plate was incubated for an additional 1 h. The absorbance was read at 578 nm on an ELISA plate reader (Digital Analogue Systems, Italy).

The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.3.2 Toxicity

The brine shrimp lethality assay was used with minor modifications (Meyer *et al*., 1982).

Sample Preparation

10, 100, and 1000 µg/ml of plant extract were dissolved in DMSO and 50 µl was impregnated on filter paper disks. The disks were allowed to dry in an open sterile Petri dish in a biological safety cabinet with a vertical laminar flow for 1 h. Control disks were prepared using only DMSO. Three replicates of each dose and the control were tested.

Hatching the shrimp

25 mg of Class C *Artemia salina* eggs (Natures Petland, Durban, South Africa) was added to artificial salt water (23 g NaCl, 11g $MgCl_2 \cdot 6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl in 1000 ml distilled water) and kept at room temperature. The pH was adjusted to 9.0 using Na_2CO_3 to avoid risk of death to the *Artemia* larvae by decrease of pH during incubation. This was incubated in a hatching chamber at room temperature. After 24 h, 15 ml of yeast solution was added to the chamber for every litre of salt water in order to feed the larvae, 48 h after the eggs were incubated, the larvae were extracted by picking up the moving larvae and visibly counted.

Bioassay

Every vial with 100 µl of plant sample at different concentration (10, 100, 1000 µl) contained 10 larvae of brine shrimp, including the control group, and was filled to 5 ml total volume with artificial salt water. A drop of yeast suspension (3 mg in 5 ml sea water) was added to each vial. The vials were then incubated at 27°C for 24 h. After 24 h, dead larvae were counted and percentage death determined.

3.3.3 Ames Mutagenicity Test

The *Salmonella* mutagenicity assay was conducted according to the method described by Maron and Ames, 1983 with minor modifications. The tester strains TA 98 and TA 100 were obtained on disc cultures from MOLTOX^{TM}. The disc cultures described were prepared from master cultures obtained from Dr. B.N. Ames (Berkeley, California, USA)

From the frozen disc culture of the *S. typhimurium* TA 98 and TA 100 tester strain, broth cultures were made. Using a flamed bacteriological needle, one of the culture disks was aseptically removed and inoculated into a sterile 250 ml Erlenmeyer flask containing 25 ml of nutrient broth (Oxoid) and 78 µl of 8 mg/ml Ampicillin (to maintain the stability of the plasmid). The flask was incubated on a shaker (150 rpm) at 37ºC for 16 h to obtain an optical density at 660 nm of between 1.2 and 1.4.

In a sterile test tube, 100 µl of culture was added to 2 ml of 0.05 mM histidine/0.05 mM biotin top agar (Appendix A), vortexed and plated onto a minimal glucose agar plate. The plate was incubated at 37ºC for 48 h. Well separated colonies were used from this plate for initial broth cultures.

Broth cultures of *S. typhimurium* were made by inoculating nutrient broth with master plate colonies. These cultures were incubated on a shaker (150 rpm) at 37 ºC for 24 h. The plant extracts were dissolved in DMSO to obtain concentrations of 100 µg/ml, 1000 µg/ml and 10 000 µg/ml. Sodium azide (NaN_3) is a highly mutagenic compound and was used as a positive control. NaN_3 was dissolved in DMSO to obtain concentrations of 5 µg/ml, 10 µg/ml and 20 µg/ml. Sterile distilled water was used as a negative control.

Three plates were prepared for each concentration of test compound. In a sterile test tube, 100 µl of bacterial culture, 100 µl of test compound and 2.9 ml of soft agar (Appendix A) held at 45 ºC, were added. This was briefly mixed with a vortex mixer and poured onto glucose minimal agar plates (Appendix A). Once the agar overlay solidified, the plates were inverted and incubated at 37ºC for 48 h, after which the number of revertant colonies (i.e. histidine dependant) colonies were counted and the mutant frequency determined. The mutant frequency was expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control.

This can be expressed in the formula:

$$\text{Mutant Frequency} = \frac{\text{Revertant number of colonies}}{\text{Negative control}}$$

3.4 Microprogation of *A.aspera, A.sessilis* and *G.densa*

Plant Material

A. aspera, A. sessilis and *G. densa* were used in this study. The plant material was collected and the leaves were harvested and washed thoroughly in tap water to remove the impurities and subsequently washed three times in sterile distilled water.

Surface sterilization of explants

The leaves were disinfected either for 5 min in 0.1% Mercury Chloride and for 20 min in 40% Sodium Hypochlorite or for 5 min in 0.1% Mercury Chloride and for 15 min in 30% Sodium Hypochlorite or for 20 min in 30% Sodium Hypochlorite only. It was then thoroughly rinsed three times with sterile distilled water under a laminar flow hood.

Callus induction

Preparation of media for callus induction

The MS medium (Murashige and Skoog, 1962) purchased from Sigma was used in this study. For callus initiation the basal MS media was manipulated with auxin (2, 4-D) and cytokinin (BAP) in different concentrations. The different combinations from 0.5 mg/L to 1 mg/L were used. Fresh stock solutions were made twice a month. The pH of the media was always verified to be 5.8. Cefotaxamine 25 mg/L and Fungizone 25 mg/L were the antibiotics used to prevent microbial contamination.

After sterilization the leaves were cut into square pieces using sterile scalpel blades. Five leaf squares were placed on each Petri plate containing the basal salt MS medium (42.2 g/L MS) and plant growth regulators. All inoculations were performed under the laminar flow to maintain aseptic conditions. All cultures were maintained at 25 °C in a 16 h photoperiod (16 h light/16 h dark) in a growth chamber.

Shoot Regeneration using Callus

Callus obtained from MS medium supplemented with 1 mg/L 2, 4-D and 1 mg/ L BAP were transferred on shoot regeneration medium having different concentrations of hormones. This was kept in a growth chamber (Polychem Supplies, South Africa) with a 16 h photoperiod and observed for shoot formation. All cultures were transferred every four weeks unless contamination was noted. After shoot formation had occurred the individual shoots were transferred into tissue culture grade bottles and the shoots were allowed to further multiply.

Root induction

Regenerated shoots are separated from the cultures individually and used for root induction. The media used for root induction was half strength MS (21.1 g/L) supplemented with different concentrations of NAA and IBA respectively to determine the best root induction medium.

Hardening of regenerated plantlets

When the plantlets attained adequate growth by producing 4-6 leaves with sufficient root system, they were removed from the culture medium and washed in running tap water carefully to remove the media adhering to roots. They were subsequently transplanted in bottles containing

sterilized soil. The plants were covered with polyethylene bags and kept in the culture room. After 15 days, the polyethylene bags were removed and well established plants were transferred to greenhouse.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Nutritional profile

Proximate composition

The carbohydrate, protein, moisture, energy, fat, dietary fibre and ash levels of the famine food plants and those of other edible species from Amaranthaceae family are shown in Table 1. *A. sessilis* had the highest energy value (636.11±8.56 g/100 g), followed closely by *A. aspera* (513.99±9.88 g/100 g) and *G. densa* (341.37±2.63 g/100 g).

The moisture levels in *A. aspera, A. sessilis* and *G. densa* were 83.24±2.5 g/100g, 65.5±5.0 g/100g and 54±1.4 g/100g respectively. The high moisture content of *A. aspera* is keeping with its succulent nature and the low moisture content in *G. densa* is due its woody nature.

The carbohydrate values for *A. aspera. A. sessilis* and *G. densa* were 4.29±0.03 g/100 g, 4.01±0.25 g/100 g and 3.07±0.03 g/100 g respectively. The highest protein levels were found in *A. sessilis* (27.70±0.12 g/100 g) when compared to *A. aspera* (21.87±0.10g/100g) and *G. densa* (12.95±0.08g/100g). The fat content of *A. aspera* was marginally lower than *A. sessilis* followed by *G. densa (*2.06±0.16 g/100 g, 2.8±0.06 g/100 g and 1.95±0.05 g/100 g respectively). *G. densa* (48.84±1.43) contained the highest amount of dietary fibre in comparison to the other famine food plants *A. aspera* and *A. sessilis*. Odhav *et al.* (2007) reported low dietary fibre contents for *A. spinosus*, *A. hybridus* and *A. dubius*. The famine food plants analyzed in this study contained higher dietary fibre contents than the leafy vegetables from the Amaranthaceae family.

A large proportion of our daily energy and nutritional needs is obtained from plants. The proportion of energy from plant foods varies from about 96% in poor countries to as low as 50% in urban societies. During famine conditions or food shortages, non commercialized wild plants may be the only source of energy. The nutrients in the plants that provide energy are carbohydrates, fats and proteins. Vitamins and minerals are present in small amounts and are important in maintaining specific functions in the body. The energy value depends on the water content and a combination of carbohydrates, fats and proteins. Adults require about 1 500 to 3 000 kilocalories per day. Carbohydrates, fats and proteins and provide about 400 kcal per 100 g carbohydrates, 900 kcal per 100 g fat and 400 kcal per 100 g protein. The amount of water

(moisture) in the food has an important influence on the calorific value. Nuts and grains have a low water content (5 to 10%) and therefore high energy value, while starch tubers and fruits have high water content (80 to 90%) and thus lower energy value.

These results correlate well with the recently published results of Freedman (2006) for *A. sessilis* where the values for moisture, fat and protein are similar. For *A. aspera* and *G.densa* we did not find any published data, these results in Table 1 below are the first. A comparison of the energy values with cultivated species in the Amaranthaceae family viz. *A. spinosus*, *A. hybridus*, *A. dubius* and *A. hypochondriacus* indicate that the famine species *A. aspera* and *A. sessilis* in this study have twice as much energy and also higher values than Maundu *et al.* (1999) on indigenous leafy vegetables as well as those reported by Odhav *et al.* (2007). Furthermore, in *A. sessilis* the protein quantity is sufficient to make up 50% of the RDA.

Table 1. The proximate values of famine foods *A. aspera*, *A. sessilis* and *G. densa* and cultivated species *A. spinosus*, *A. hybridus*, *A. dubius* and *A. hypochondriacus*

Plants	Moisture (g)	Ash (g)	Dietary Fibre (g)	CHO (g)	Fat (g)	Protein (g)	Energy (kJ)
A. aspera	83.24±2.5	13.50±1.03	40.85	4.29±0.03	2.06±0.16	21.87±0.10	513.99±9.88
A. sessilis	65.5±4.9	15.6±1.81	38.44±0.47	4.01±0.25	2.8±0.06	27.70±0.12	636.11±8.56
G. densa	54±1.4g	12.51±1.05	48.84±1.43	3.07±0.03	1.95±0.05	12.95±0.08	341.37±2.63
COMPARATIVE STUDY							
A. spinosus[a]	91.36±0.25	2.76±0.34	2.48±0.08	1.16±0.16	0.60±0.01	4.12±0.06	111.02±1.12
A. hybridus[a]	82.55±0.07	4.91±0.11	2.81±0.03	6.09±0.31	0.53±0.08	5.92±0.04	221.07±1.29
A. dubius[a]	84.59±0.38	3.42±0.03	2.87±0.04	7.86±0.55	0.24±0.06	3.89±0.08	205.79±5.68
A. sessilis[b]	70.40	20.54	11.47	47.99	3.65		
A. hypochondriacus[c]					8.81	15.8	365

Data are mean ± SD(n=2), [a](Odhav *et al*, 2007), [b](Freedman, 2006), [c]**(van Wyk, 2005)**

Mineral content

A. aspera and *G. densa* had similar levels of calcium present (142.97±10 mg/100 g and 140.84±2.5 mg/100 g respectively) and *A. sessilis* had a lower value (83.97±0.5 mg/100 g) (Table 3). The RDA of calcium for adults is 800 mg per day (NRC, 1989). Thus 100g/day of the famine plants in this study do not meet the RDA. These values reported in this study are low. Calcium is an important mineral involved in the building of rigid structures to support the body (Aletor *et al.*, 2002). Reports of calcium levels in other famine plants *Ximenia americana*, *A. viridus*, and

the leaves of the baobab tree (*Adansonia digitata)* contained higher quantities of calcium (Glew *et al*., 2005).

A. aspera and *A. sessilis* had similar amounts of copper present with values of 0.22 mg/100 g and 0.20 mg/100 g respectively (Table 3). *G. densa* had a slightly lower amount of copper present than the other two famine food plants with a concentration of 0.17 mg/100 g (Table 3). According to the National Research Council, the daily requirement for copper is 2 mg per day (NRC, 1989). *A. aspera* and *A. sessilis* meet about 10 % of the RDA for adults. In comparison to the leafy vegetables (*A. hybridus*, *A. dubius* and *A. spinosus*) in the Amaranthaceae family reported by Odhav *et al.* (2007), the famine food plants in this study had low copper concentrations. Plants which have high copper concentrations could be useful in preventing a deficiency of copper which normally results in anemia and bone problems (Arntzen and Ritter, 1984).

A. sessilis and *G. densa* had iron concentrations of 8.57±3 mg/100 g and 8.71±0.5 mg/100 g respectively and *A. aspera* had 4.70±1.6 mg/100 g (Table 3). The RDA for iron is 10 mg per day adults (NRC, 1989). *A. sessilis* and *G. densa* satisfied more than 85% and 87% respectively of the iron requirement for the RDA for adults. Iron is necessary for the optimal immune function (Glew *et al.*, 2005). There is a relatively high prevalence of iron deficiency, anaemia among all age groups in the rural populations (Glew *et al*., 2005). Odhav *et al.* (2007) reported very high levels of iron in the leafy vegetables in the Amaranthaceae family.

In *G. densa* we found low levels of magnesium (45.27±2.9 mg/100 g), however in *A. aspera* and *A. sessilis* the magnesium concentration were 73.03±2.6 mg/100 g and 53.23±0.2 mg/100 g respectively (Table 3). The daily consumption of magnesium should amount to 120 mg (NRC, 1989). The famine food plants *A. aspera* satisfies 60% and *A. sessilis* satisfies 53% of the RDA for magnesium. Magnesium is needed for healthy bones and teeth, proper nervous system functioning, and energy metabolism.

In *G. densa* we found very low levels of manganese (0.31 mg/100 g) and *A. aspera* and *A. sessilis* had amounts of 5.00±0.2 mg/100 g and 4.47±0.1 mg/100 g respectively (Table 3). The RDA for manganese is 7 mg (NRC, 1989). *A. aspera* and *A. sessilis* meet more than 50% of the daily

recommended and have a greater manganese concentration than the food plant *A. spinosus* (Table 3). Manganese is needed for enzyme structure.

A. sessilis had the highest amount (53.58±2.4 mg/100 g) of phosphorus present, followed by *G. densa* (35.08±1.0 mg/100 g) and thereafter *A. aspera* (26.41±0.6 mg/100 g) (Table 3). The RDA for phosphorus is 800 mg for adults. According to Odhav *et al.* (2007) the leafy vegetables analyzed from the Amaranthaceae family satisfied more than half the RDA for phosphorus, however the plants analyzed in this study had very low phosphorous concentrations (Table 3). Phosphorus is needed for healthy bones and teeth, energy metabolism, and acid-base balance in the body.

A. aspera (114.72±6.7 mg/100 g) had a very high concentration of sodium in comparison to *G. densa* which only had 12.76±0.7 mg/100 g (Table 3).The RDA for sodium is 300 mg. No deficiency of sodium is found in the human diet, however excessive intake of sodium can result in high blood pressure (Jaworska and Kmiecik, 1999).

A. sessilis had the highest concentration of zinc (2.65±1.9 mg/100 g) compared to the other two famine food plants which had values of 1.85±0.1 mg/100g and 1.01 mg/100g for *A. aspera* and *G. densa* respectively (Table 3). The RDA for zinc is 10 mg per day for adults (NRC, 1989). Thus, the famine plants from this study do not meet the RDA. Poor zinc status is widespread, especially amongst populations that consume cereal based diets (Brown and Wuehler, 2000).

Table 2. Comparison of mineral levels of the famine food plants (*A. aspera*, *A. sessilis* and *G. densa*) to the leafy vegetables (*A. spinosus*, *A. hybridus* and *A. dubius*)

Plants	Calcium	Copper	Iron	Magnesium	Manganese	Phosphorus	Sodium	Zinc
	mg/ml							
A. aspera	142.97±10	0.22	4.70±1.6	73.03±2.6	5.00±0.2	26.41±0.6	114.72±6.7	1.85±0.1
A. sessilis	83.97±0.5	0.20	8.57±3	53.23±0.2	4.47±0.1	53.58±2.4	72.94±2.1	2.65±1.9
G. densa	140.84±2.5	0.17	8.71±0.5	45.27±2.9	0.31	35.08±1.0	12.76±0.7	1.01
COMPARATIVE STUDIES								
A. spinosus[a]	3930.58±15.3	3.36±0.3	31.92±2.4	1165.64±2.	3.02	628.60±7.2	392.92±3	15.47±0.8
A. hybridus[a]	2363.26±0.4	2.38±0.3	21.2±0.5	1316.88±2	24.38±0.9	603.94±1	427.1±0.1	17.93±0.2
A. dubius[a]	1686.2±0.4	2.76±0.1	25.11±0.3	805.63±2.1	81.92±0.9	487.40±0.2	346.99±1	56.12±1.4

Data are mean± standard deviation (n=2) [a] (Odhav *et al.*, 2007)

Vitamin composition

The famine food plant *A. sessilis* had the highest amount of Vitamin A (982.32±33.07 mg/ml) followed by *G. densa* (929.46±11.60 mg/ml) and *A. aspera* (806.55±27.34 mg/ml). According to the Food and Nutrition Board, the recommended daily allowance is 800 micrograms for adult females and 1000 micrograms for adult males (NRC, 1989). Vitamin A has widespread physiological functions in the body. Apart from its effects on vision, the role of vitamin A in maintaining the structural and functional integrity of mucosal epithelial cells is important. Vitamin A controls cellular proliferation and differentiation, and thus has significant effects on the immune system (Bhaskaram, 2002). Deficiency of Vitamin A causes night blindness, xerophthalmia and keratinisation of skin.

A. aspera had the highest content of Vitamin B_1 compared to the other famine food plants tested, almost twice the amount of *A. sessilis* and *G. densa* had 11.08±0.12 mg (Table 2). The RDA for Vitamin B_1 is 1.1-1.5 mg. Vitamin B_1 (thiamine) is a coenzyme in pyruvate biosynthesis and for 2-oxo-glutarate dehydrogenases, and transketolase and it also regulates Cl^-channel in nerve conduction. Deficiencies in Vitamin B_1 result in peripheral nerve damage or central nervous system lesions (Bender, 2003).

The Vitamin B_2 concentrations in this study ranged from 9.7±0.4 mg in *A. sessilis* to 41.75±0.35 mg in *G. densa* (Table 2). This meets more than the recommended daily allowance for Vitamin B_2. The recommended daily allowance is 1.2-1.7 mg (Bender, 2003). Vitamin B_2 (riboflavin) which acts as a coenzyme in the oxidation and reduction reaction is also a prosthetic group of flavoproteins. Deficiencies of riboflavin result in lesions of the corner of the mouth, lips, and tongue and sebhorreic dermatitis.

A. sessilis and *G. densa* had similar Vitamin B_3 levels with values ranging between 8.96±0.10 mg and 9.05±0.08 mg respectively (Table 2). *A. aspera*'s Vitamin B_3 level was only 5.98±0.16 mg. *A. sessilis* and *G .densa* meet only 60 % of the RDA. According to the Food and Nutrition Board, the recommended daily allowance is 15-19 mg (NRC, 1989). Vitamin B_3 acts as a coenzyme in oxidation and reduction reactions, it is a functional part of NAD and NADP and plays a role in intracellular calcium regulation and cell signalling. Deficiencies lead to pellagra-photosensitive dermatitis and depressive psychosis (Bender, 2003).

Vitamin C levels range from 16.27 mg in *G. densa* to 43.57 mg in *A. aspera* (Table 2). According to the Food and Nutrition Board, the RDA for Vitamin C is 60 mg. The famine food plants studied do not meet the daily allowance standard set for Vitamin C by the Food and Nutrition Board (NRC, 1989). Vitamin C (ascorbic acid) functions as a coenzyme in hydroxylation of proline and lysine in collagen synthesis; as an anti-oxidant and enhances absorption of iron. Deficiencies result in scurvy, loss of dental cement and subcutaneous haemorrhage (Bender, 2003).

The vitamins of relevance to plant foods are the fat soluble vitamins (A, D, E and K) and the water soluble vitamins (B_1, B_2, B_6 and B_{12}, biotin, C, folic acid, niacin and pantothenic acid). The recommended daily allowance is the quantity which is required to prevent disease. Micronutrient deficiencies and infectious diseases often coexist and exhibit complex interactions leading to the vicious cycle of malnutrition and infections among underprivileged populations of the developing countries, particularly in preschool children. Several micronutrients such as vitamin A, β-carotene, folic acid, vitamin B_{12}, vitamin C, riboflavin, iron, zinc, and selenium, have immunomodulating functions and thus influence the susceptibility of a host to infectious diseases and the course and outcome of such diseases (Bhaskaram, 2002).

Table 3. Vitamin levels of *A. aspera*, *A. sessilis* and *G. densa*

Plants	Vitamin A	Vitamin B_1	Vitamin B_2	Vitamin B_3	Vitamin C
	mg/100 g of dry weight				
A. aspera	806.55±27.34	20.02±0.12	22.2±0.57	5.98±0.16	43.57
A. sessilis	982.32±33.07	10.98±0.24	9.7±0.42	9.05±0.08	27.19
G. densa	929.46±11.60	11.08±0.12	41.75±0.35	8.96±0.10	16.27

Data are mean± standard deviation (n = 2)

4.2 Biological Screening

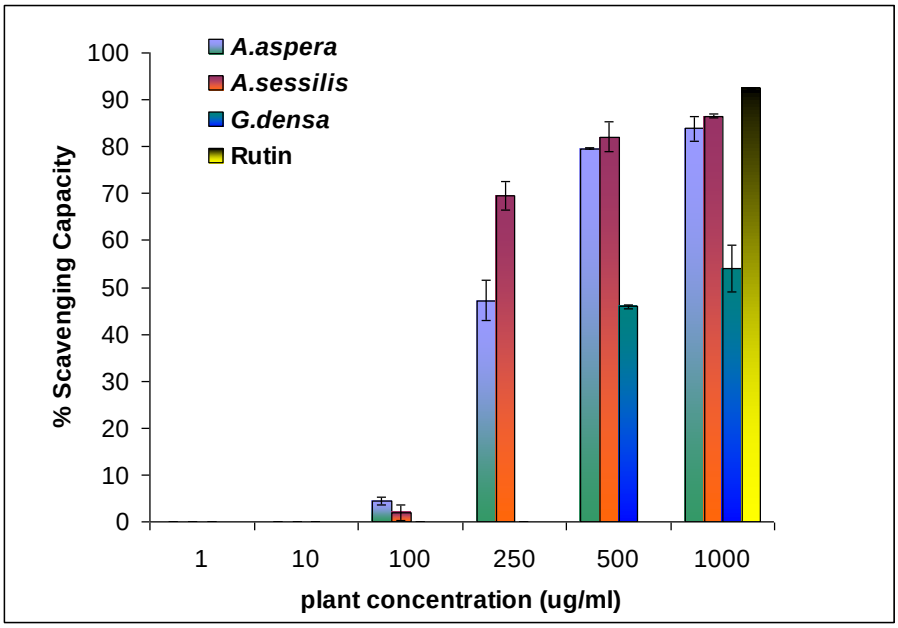
4.2.1 Antioxidant activity of plant extracts

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite.

An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage (Cheng *et al.*, 2003). Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's). Flavonoids may help provide protection against these diseases by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense.

In this study, we used the DPPH assay as it determines the activities of both hydrophilic and lipophilic chemicals. Rutin, the positive control used reduced 92.43 ±0.22 % whilst the famine food plants *A. aspera, A. sessilis* and *G. densa* reduced 83.86±2.63%, 86.51±0.37 % and 54±55 respectively (Fig. 6). The minimum concentration at which these two plants exhibited free radical scavenging activity was 100 µg/ml. The aqueous extracts of the three plants showed no antioxidant activity. Akula and Odhav (2008) reported high antioxidant activities from plants in the Amaranthaceae family. *A. hybridus* was reported to have 90.5±0.24%, *A. spinosus* had 88.2±0.22% and *A. dubius* had 78.4±0.22% (Akula and Odhav, 2008).

The recognized dietary antioxidants are vitamin C, vitamin E, selenium, and carotenoids. In our study *A. sessilis* and *A. aspera* had vitamin C present. This may explain the high level of anti-oxidative properties displayed. Furthermore, there is a connection between plant stress levels and the production of secondary metabolites, including many polyphenols and antioxidants. There is substantial agreement among plant pathologists, physiologists, and entomologists that relatively higher levels of antioxidant secondary plant metabolites are produced by plants in response to biotic and abiotic stress. Famine plants grow in stress conditions and hence it is plausible that they have better anti-oxidative capacity than commercially cultivated (amaranth species).



Data are mean ± standard deviation, n=3

Fig. 6. Free radical scavenging capacity of methanolic extracts of *A. aspera*, *A. sessilis*, *G. densa* and Rutin

4.2.2 Anti-Inflammatory Properties

The *in vitro* inhibition of soybean lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential (Abad *et al.*, 1995). In our study, we used NDGA as a standard for the comparison of anti-inflammatory potential of the three famine plants. The IC_{50} value of NDGA was 2.48 µg/ml and the famine food plants *A. aspera*, *A. sessilis* and *G. densa* were 246 µg/ml, 341.1 µg/ml and 582.1 µg/ml respectively (Table 4). These results indicate that the famine food plants analyzed had low anti-inflammatory activity. Reduced IC_{50} values suggest better inhibitory action on 5 LOX. A negative result in the lipoxygenase assay does not necessarily mean that the plant is without anti-inflammatory activity. The active compounds could work at other sites in the complex process of inflammation (Jäger *et al.*, 1995).

Previous work in our laboratory on anti-inflammatory activity with methanolic extracts among leafy vegetables from commonly consumed leafy Amaranthaceae species showed that *A. dubius* had IC_{50} value of 69.4 µg/ml and *A. spinosus* had 57.3 µg/ml (Akula and Odhav, 2008). These results suggest that the plants of Amarantheceae family do not have chemicals that can induce anti-inflammatory activity in this specific site. It has been reported by Gokhale *et al.* (2002) that

the ethanolic extract of *A. aspera* possessed anti-inflammatory and anti-arthritic activity and he also reported that *A. aspera* is used in the indigenous system of medicine for the treatment of inflammatory conditions; however, there is no reported literature on detailed investigation for rationality behind their use in inflammation.

Table 4: Anti-inflammatory properties of aqueous and methanolic extracts of *A.aspera*, *A.sessilis* and *G. Densa* using 5- lipooxygenases

	5-LOX IC50 (µg/ml)		
Plant	**Aqueous**	**Methanolic**	**Positive Control**
A . aspera	0	246	nd
A. sessilis	0	343.1	nd
G. densa	0	582.1	nd
NDGA	nd	nd	2.48

(n=2), nd: not determined

4.2.3 Antimicrobial Activity

Under many famine and food shortage circumstances, a wide array of microbial pathogens, and a low immunity increases susceptibility to bacterial and fungal infections. Wild plants are often the only available means of treating such infections (Taylor *et al.*, 2001). Fennel *et al.*, (2004) suggested that the use of the plants in treating fungal infections could be even more widely used as an alternative to the expensive and often unobtainable Western drugs.

The aqueous extract of ***A. aspera*** showed zones of inhibition against the Gram negative bacteria *E. coli*, *P. aeruginosa* and *S. typhi* (Table 5) with MIC values of 250 µg/ml, 100 µg/ml and 100 µg/ml respectively (Table 6) and against two Gram positive bacteria *S. epidermis* and *S. aureus* (Table 6) with MIC values of 1000 µg/ml and 1000 µg/ml respectively (Table 6). In the studies of Perumal Samy *et al.* (1998) the aqueous leaf extract of *A. aspera* did not show any activity against *E. coli*, *K. oxytoca* and *P. aeruginosa* at lower doses whereas, it exhibited activity against *P. bulgaricus* at higher doses of 4000 and 5000 ppm. Similar results related to Perumal Samy *et al.* (1998) were reported by Belachew Desta (1993). The extracts of *A. aspera* analyzed in our

study showed activity against *E. coli*, *P. aeruginosa*, *K. oxytoca* and *S. typhi* which is contradictory to Perumal Samy *et al.* (1998) and Belachew Desta (1993). Lamikarna (1999) reported that Gram negative bacteria have an impervious cell envelope which makes them resistant to many antibacterial agents. Although the *A. aspera* plant extracts did not inhibit fungal growth, the methanolic extract showed inhibition against the yeast *S. cerevisiae* (Table 7) at a minimum inhibitory concentration of 500 µg/ml (Table 8).

The aqueous extracts of ***A. sessilis*** showed activity against *P. aeruginosa* and *S. epidermis* (Table 5) and the methanolic extracts showed activity against *P. aeroginosa* and *S. aureus*. The aqueous extract of *A. sessilis* also showed the best inhibition against *C. albicans* (Table 7) at a minimum inhibitory concentration of 500 µg/ml (Table 8) in comparison to the rest of the plant extracts when determining the zone of inhibition. The aqueous and methanolic extract of *A. sessilis* showed antifungal activity against *S. cerevisiae* (Table 7) at a minimum inhibitory concentration of 500 µg/ml and 250 µg/ml respectively (Table 8).

G. densa extracts showed activity against the Gram negative *E. coli*, *P. aeroginosa* and *K. oxytoca* (Table 5) as well as the Gram positive *B. sterathermophilus* and *S. aureus* (Table 5). The methanolic and aqueous extracts of *G. densa* displayed antifungal activity against *C. albicans* (Table 7) at minimum inhibitory concentrations of 500 µg/ml and 250 µg/ml respectively (Table 8).

Table 5. Antibacterial activity of *A. aspera, A. sessilis* and *G. densa*

Plant Extracts (1mg/ml)	Zone of Inhibition (mm)									
	Ec	***Pa***	***Ko***	***Pm***	***St***	***Se***	***Bs***	***Bc***	***M***	***Sa***
***A .aspera* (aqueous)**	15	11.7±1.5	na	na	11.3±1.2	11.7±1.5	na	na	na	10
***A. aspera (*methanolic)**	13±2	12.7±0.6	12±1	na	11±1.7	Na	na	na	na	11±1.7
***A. sessilis* (aqueous)**	na	8.7±0.6	na	na	na	11±3.46	na	na	na	na
***A. sessilis* (methanolic)**	na	13	na	na	na	na	na	na	na	12
***G. densa* (aqueous)**	12±2	11.3±1.5	11.3±0.6	na	na	na	12.3±2.5	na	na	na
***G. densa* (methanolic)**	10	7.3±1.5	na	na	na	na	na	na	na	13
Ethanol	8.7±0.6	7±1	10.7±0.6	5	4	10	10	15	15	7
Ciprofloxacin	25	30.7±1.2	35 ±1	37±1	32±1	34±1	42.3±2.5	30±1	25±1	30±1

Data are mean± S D (n=3); na: no activity *Ec- Escherichia coli, Pa- Pseudomonas aeroginosus, Ko- Klebsiella oxytoca, Pm- Proteus mirabilis, St- Salmonella typhi, Se-Staphylococcus epidermis, Bs- Bacillus stereathermophilus, Bc- Bacillus cereus, M-Micrococcus, Sa- Staphylococcus aureus*

Table 6. Minimum Inhibitory Concentration of *A. aspera, A. sessilis* and *G. densa*

Plant Extracts	Minimum Inhibitory Concentration (µg/ml)									
	Ec	***Pa***	***Ko***	***Pm***	***St***	***Se***	***Bs***	***Bc***	***M***	***Sa***
***A. aspera* (aqueous)**	250	100	na	na	100	1000	na	na	na	1000
***A. aspera (*methanolic)**	250	100	1000	na	10	Na	na	na	na	500
***A. sessilis* (aqueous)**	na	500	na	na	na	1000	na	na	na	na
***A. sessilis* (methanolic)**	na	250	na	na	na	Na	na	na	na	1000
***G. densa* (aqueous)**	250	250	1000	na	na	Na	500	na	na	na
***G. densa* (methanolic)**	1000	1000	na	na	na	Na	na	na	na	500

Data are mean± S D (n=3); na: no activity *Ec- Escherichia coli, Pa- Pseudomonas aeroginosus, Ko- Klebsiella oxytoca, Pm- Proteus mirabilis, St- Salmonella typhi, Se-Staphylococcus epidermis, Bs- Bacillus stereathermophilus, Bc- Bacillus cereus, M-Micrococcus, Sa- Staphylococcus aureus*

Table 7. Antifungal activity of *A. aspera, A. sessilis* and *G. densa*

Plant Extracts (1 mg/ml)	Zone of inhibition (mm)								
	Sc	***F***	***P***	***Ca***	***R***	***T***	***C***	***G***	***Af***
***A. aspera* (aqueous)**	na	na	na	na	na	na	na	na	na
***A. aspera* (methanolic)**	18.33±2.8	na	na	na	na	na	na	na	na
***A. sessilis* (aqueous)**	15	na	na	na	na	na	na	na	na
***A. sessilis* (methanolic)**	17.33±2.5	na	na	15	na	na	na	na	na
***G. densa* (aqueous)**	na	na	na	15	na	na	na	na	na
***G. densa* (methanolic)**	na	na	na	16±1	na	na	na	na	na
Amphotericin B	25	25	25	26	20	20	20	20	20
Ethanol	10	10	10	10	0	0	0	0	0

Data are mean±SD (n=3); na: no activity; *F- Fusarium, P- Penicillium, Ca- Candida albicans, R- Rhizopus, T- Trichoderma, C- Cladosporium, G- Geotrichum, Af- Aspergillus flavus*

Table 8: Antifungal minimum inhibitory concentration of *A. aspera, A. sessilis* and *G. densa*

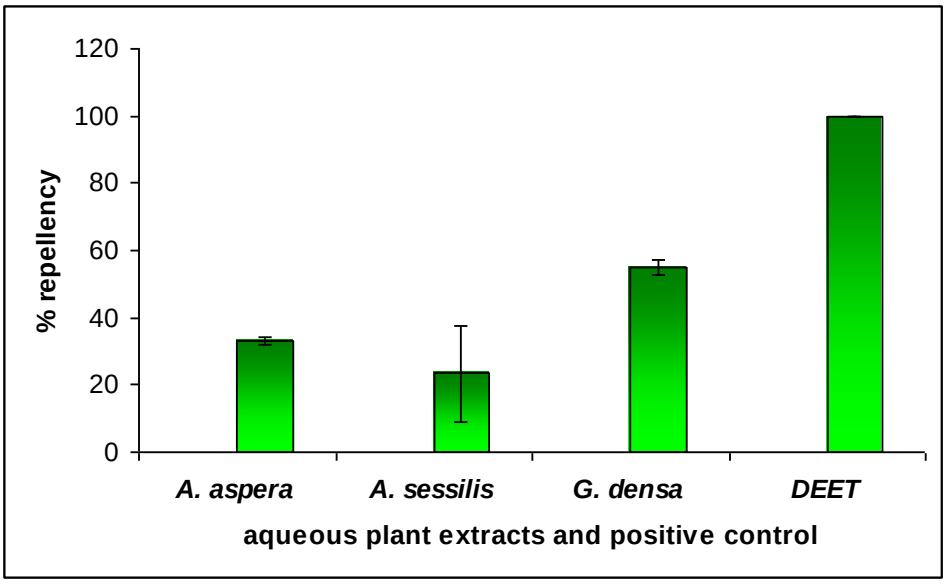
	Minimum Inhibitory Concentration (µg/ml)								
Plant Extract	***Sc***	***F***	***P***	***Ca***	***R***	***T***	***C***	***G***	***Af***
***A. aspera* (aqueous)**	na	na	na	na	na	na	Na	na	na
***A. aspera* (methanolic)**	500	na	na	na	na	na	Na	na	Na
***A. sesslis* (aqueous)**	500	na	na	na	na	na	Na	na	Na
***A. sessilis* (methanolic)**	250	na	na	500	na	na	Na	na	na
***G. densa* (aqueous)**	Na	na	na	250	na	na	Na	na	na
***G. densa* (methanolic)**	Na	na	na	500	na	na	Na	na	na

Data are mean±SD (n=3); na: no activity; *F- Fusarium, P- Penicillium, Ca- Candida albicans, R- Rhizopus, T- Trichoderma, C- Cladosporium, G- Geotrichum, Af- Aspergillus flavus*

4.2.4 Anti-Mosquito Properties

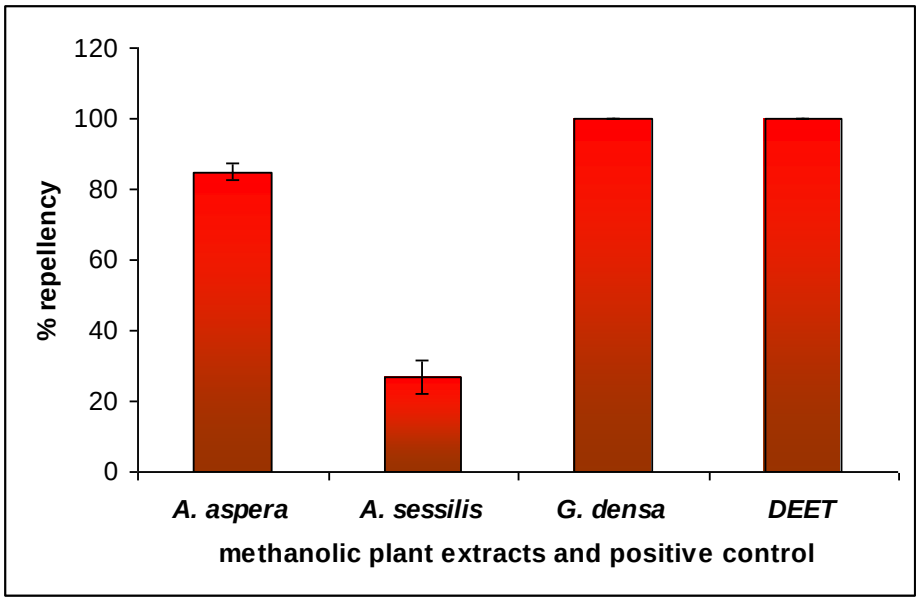
Repellent Activity

The repellency against *A. arabiensis* with the aqueous and methanolic extracts of the three famine plants is depicted in Fig. 7 and Fig. 8. The methanolic extract of *G. densa* and *A. aspera* has repellency effects against *A. arabiensis* and these can be used for personal protection against the mosquitoes by individuals. Further evaluation of *G. densa* and *A. aspera* is required before this could be used as an alternative mosquito repellent. When compared to the positive control which was DEET a well known synthetic organic insecticide, *G. densa* displayed 100% repellency and *A. aspera* displayed 85% repellency.



Data are mean± SD (n=2)

Fig. 7. Repellency activity from the aqueous extracts of *A. aspera*, *A. sessilis* and *G. densa* on *A. arabiensis* mosquito



Data are mean±SD: (n=2)

Fig. 8. Repellency activity from the methanolic extracts of *A. aspera*, *A. sessilis* and *G. densa* on *A. arabiensis* mosquito

Larvicidal Activity

For larvacidal trials none of the plant extracts have exhibited any significant effect on the immature stages of the *Anopheline* mosquito during the seven day exposure period, compared to

the positive control which in this case was Mostop, a commercial organophosphate. Transformation of larvae to pupae and subsequently to adult stage occurred as normal (Table 9). However recent studies by Bagavan *et al.* (2008) reported that the ethyl acetate extract of *A. aspera* showed larvicidal activity against the early fourth-instar larvae of *Aedes aegypti* L and *Culex quinquefasciatus* Say.

Table 9. Larvicidal trials of *A. aspera, A. sessilis* and *G. densa* at plant concentration of 1000 µg/ml on *A. arabiensis* mosquito

Plant Extracts	Larvicidal activity/ Survival							
	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7
***A. aspera* (Aqueous)**	L	L	L	L	P	P	A	A
***A. aspera* (Methanolic)**	L	L	L	L	P	P	A	A
***A. sessilis* (Aqueous)**	L	L	L	L	P	P	A	A
***A. sessilis* (Methanolic)**	L	L	L	L	P	P	A	A
***G. densa* (Aqueous)**	L	L	L	L	P	P	A	A
***G. densa (*Methanolic)**	L	L	L	L	P	P	A	A
Acetone	L	L	L	L	P	P	A	A
Distilled Water	L	L	L	L	P	P	A	A
Mostop (Organophosphate)	100	L	L	L	P	P	A	A

L- Larvae, P- Larvae that reached the pupal stage, A- Larvae that reached the adult stage

Insecticidal Activity

K orthrine, a commercial insecticide exhibited between 98.36±2.35 and 100% knockdown during the 60 min exposure time and 100% mortality (Table 10). The aqueous extract of *A. aspera* exhibited between 26 and 38.5% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h has shown a decrease in activity as only 21.67±7.07% (Table 10) of the test species had been affected by the extract. The methanolic extract of *A. aspera* exhibited between 30 and 32% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h showed a decrease in activity as only 16.67% (Table 10) of the test species was affected by the extract. Results showed that the aqueous extract of *A. sessilis* exhibited between 30 and 54% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h showed a

decrease in activity as only 21.67±7.07% (Table 10) of the test species was affected by the extract. The methanolic extract of *A. sessilis* exhibited between 15 and 37% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h showed a decrease in activity as only 5±2.36% (Table 10) of the test species was affected by the extract. The aqueous extract of *G. densa* has exhibited between 23 and 32% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h showed a decrease in activity as only 11.67±7.07% (Table 10) of the test species was affected by the extract. The methanolic extract of *G. densa* exhibited 18% (Table 10) knockdown activity against the *Anopheline* mosquitoes during the initial 60 min of exposure. Mortality measured after 24 h displayed that only 18% (Table 10) of the test species had been affected by the extract. None of the above plants can be used to kill the *Anopheline* vector as the level of knockdowns by the extracts are lower than the recommended standards set by the World Health Organization (WHO, 1981a).

Table 10: % Knockdown and Mortality of *A. arabienis* with aqueous and methanolic extracts of *A. aspera, A. sessilis* and *G. densa*

	Knockdown		Mortality
Plant Extracts	**30 mins**	**60 mins**	**24 hrs**
***A. aspera* (Aqueous)**	26.67±9.43	38.36±16.50	21.67±7.07
***A. aspera* (Methanolic)**	31.67±2.35	30±4.71	16.67
***A. sessilis* (Aqueous)**	30±14.14	53.33±28.28	21.67±7.07
***A. sessilis* (Methanolic)**	15±2.35	36.67±9.43	5±2.36
***G. densa* (Aqueous)**	23.33±14.14	31.67±7.07	11.67±7.07
***G. densa* Methanolic)**	18.36±2.35	18.36±2.35	18.36±11.79
Acetone	8.36±2.35	5±2.36	10±4.71
Distilled Water	1.67±2.35	10	11.67±2.35
K- Orithrine	98.36±2.35	100	100

Data are mean± S D (n=2)

Mosquitoes are the major vectors for the transmission of malaria, dengue fever, yellow fever, filariasis and several diseases (James, 1992). Effective use of controlling agents has disrupted natural biological control systems and led to the outbreaks of insect species showing resistance. According to Yang *et al.* (2002) it has provoked undesirable effects including toxicity to non-target organisms and fostered environmental and health concerns. These problems have highlighted the need for the development of alternate insecticides which will be effective, environmentally safe, biodegradable, safe and cheap. According to Wink (1993) extracts of plants may be alternate source of controlling agents since they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in integrated pest management programs. The initial repellency activity of *G. densa* and *A. aspera* was as effective as DEET (positive control), a synthetic organic insecticide, with the methanolic plant extracts showing 100% and 85% respectively. *A. aspera* is used as an antimalarial and antisplasmodic agent (Goyal *et al.*, 2007). For larvacidal trials none of the plant extracts exhibited any effect on the immature stages of the *Anopheline* mosquito during the seven day exposure period. The results for the insecticidal screening reflected in this study has not been very encouraging since the criteria set for determining if an extract is a potential insecticide according to the World Health Organization (WHO, 1981a) standards is 95% knockdown and greater than 80% mortality after 24 h.

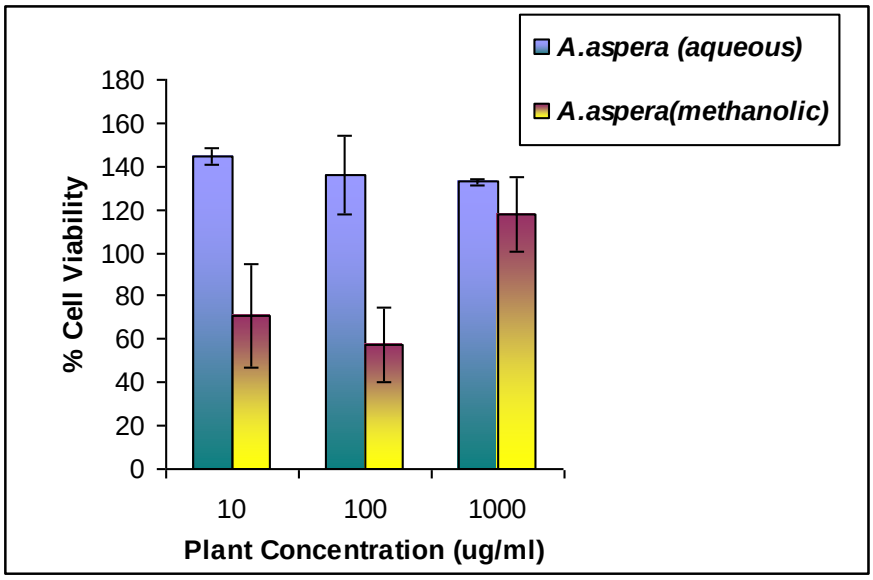
4.3 Safety Analysis

4.3.1 Cytotoxicity

The cytotoxic effect of aqueous and methanolic extracts of *A. aspera* on the K562 cell line is depicted in Fig. 9. The aqueous extract of *A. aspera* stimulated the growth of the K562 cell line. As the concentration of the extract increased, there was a slight decrease in cell viability. The methanolic extract of *A. aspera* showed some differentiation where at concentrations of 10 and 1000 µg/ml there was stimulation of cells but at a concentration of 100 µg/ml there was a decrease in cell viability. The same trend was observed for *A. sessilis*. The cytotoxic effect of aqueous and methanolic extracts of *A. sessilis* on the K562 cell line is illustrated in Fig. 10. The cytotoxic effect of aqueous and methanolic extracts of *G. densa* on the K562 cell line is shown in Fig. 11. The aqueous extract of *G. densa* stimulated the growth of the cell at low concentration but was toxic to the cells at a high concentration of 1000 µg/ml. The methanolic extracts

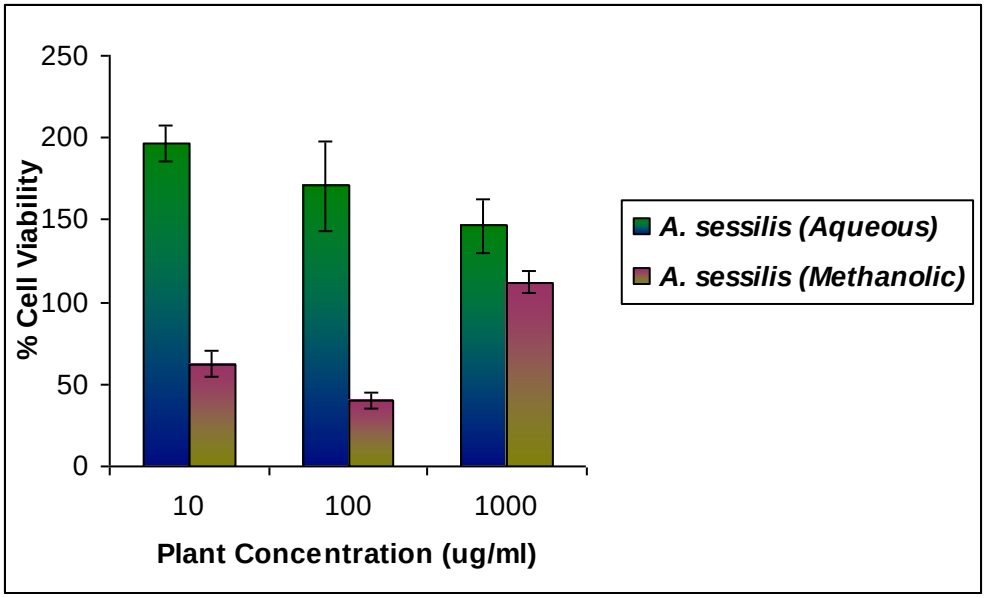
stimulated the growth of cells at low concentrations and as the concentration increased there was a decrease in the cell viability.

This particular cell line was chosen because K562 cells express the membrane complement regulatory proteins CD59, CD55 and CD46. Simpson and coworkers in 1997 reported that CD46 and CD55 inhibit the deposition of C3 fragments on the cell surface and thereby limit complement-dependent cellular cytotoxicity. CD59 prevents the formation of membrane attack complexes and the subsequent osmotic lysis of the target cell (Simpson *et al.*, 1997). Peer *et al.* (2005) reported that *A. spinosus* and *A. hybridus* from the Amaranthaceae family were cytotoxic to the HepG2 cell line.



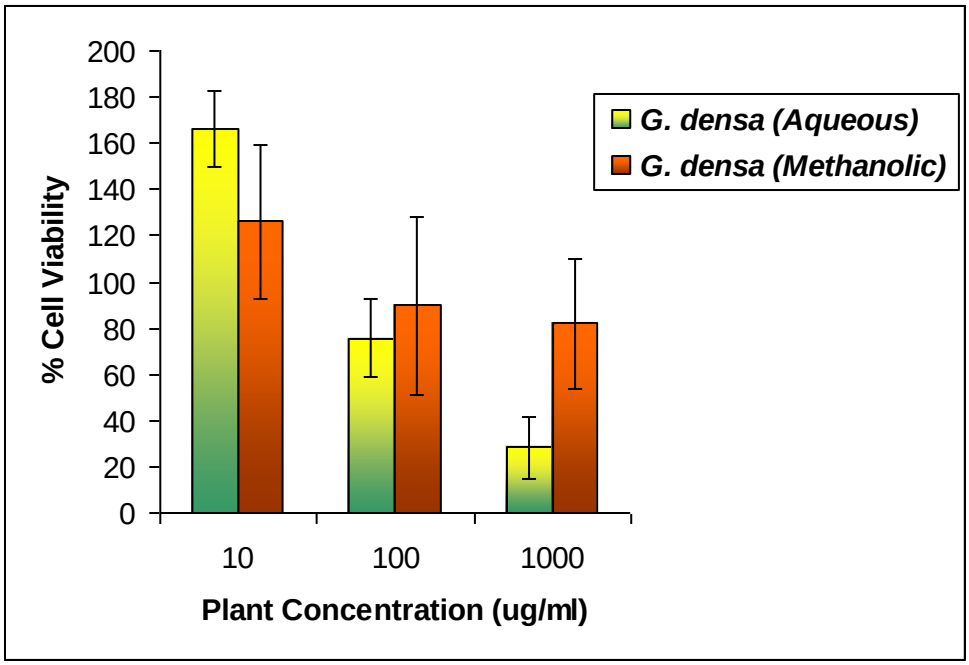
Data are mean± Standard Deviation (n=3)

Fig.9. Cytotoxic effect of aqueous and methanolic extracts of *A. aspera* on the K562 cell line



Data are mean± Standard Deviation (n=3)

Fig.10. Cytotoxic effect of aqueous and methanolic extracts of *A. sessilis* on the K562 cell line



Data are mean± Standard Deviation (n=3)

Fig.11. Cytotoxic effect of aqueous and methanolic extracts of *G. densa* on the K562 cell line

4.3.2 Toxicity to Brine Shrimp

None of the plants showed any level of toxicity with all the plants showing 100% of shrimp survival whereas the positive control which was an organophosphate showed 100 % mortality. A study performed by Peer *et al.,* (2005) showed that other plants from the Amaranthaceae family such as *A.dubius, A spinosus* and *A. hybridus* has some toxic effects. Gayathri *et al.* (2006) reported that histopathological testing revealed degenerative and necrotic changes in the liver and kidney in Swiss mice, caused by oral administration of water extract of *A. sessilis* in high doses. He suggested that this could be due to the effects of cytotoxic substances in *A. sessilis*, however, in our study no toxic activity was noted.

4.3.3 Mutagencity

The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. Table 11 shows the mutant frequency of the plant extract tested on the *S. typhimurium* TA 98 strain. Table 12 shows the mutant frequency of the plant extract tested on the *S. typhimurium* TA 100 strain. The greater the number of revertant colonies, the greater the mutant frequency. According to Maron and Ames (1983) a mutagenic potential is assumed if the mutant frequency is greater than 2; a possible mutagenic potential is assumed if the mutant frequency ranges between 1.7 and 1.9; and no mutagenic potential is assumed if the mutant frequency is lower than 1.6. None of the plant extracts up to concentrations of 1000 µg/ml showed any mutagenic potential. Sodium azide was the chosen mutagen used in this experiment and it showed a mutagenic potential; as the concentration increased so did the number of revertant colonies. Thus the extracts of *A. aspera, A. sessilis* and *G. densa* do not have compounds that may a potential to be a carcinogenic agent. Peer *et al.* (2005) reported that none of the leafy vegetables (*A. dubius, A. spinosus* and *A. hybridus*) had any mutagenic activity.

Table 11: Mutant frequency of the number of revertants in *S. typhimurium* strain TA 98 exposed to plant extracts

Plant Extract	Mutant frequency of revertants at different concentrations				
	5µg/ml	**10µg/ml**	**20µg/ml**	**100µg/ml**	**1000µg/ml**
***A. aspera*(aqueous)**	nd	0.5	nd	0.7	1.1
***A. aspera*(methanolic)**	nd	0	nd	0	0
***A. sessilis*(aqueous)**	nd	0.8	nd	1	1.4
***A. sessilis*(methanolic)**	nd	0	nd	0	0
***G. densa*(aqueous)**	nd	0.3	nd	0.4	0.8
***G. densa*(methanolic)**	nd	0	nd	0	0
Sodium Azide (positive control)	1.6	2.9	5.2	nd	nd

nd: not determined

Table 12: Mutant frequency of the number of revertants in *S. typhimurium* strain TA 100 exposed to plant extracts

Plant Extract	Mutant frequency of revertants at different concentrations				
	5µg/ml	10µg/ml	20µg/ml	100µg/ml	1000µg/ml
***A. aspera*(aqueous)**	nd	0.6	nd	0.7	0.9
***A. aspera*(methanolic)**	nd	0	nd	0	0
***A. sessilis*(aqueous)**	nd	0.6	nd	0.9	1
***A. sessilis*(methanolic)**	nd	0	nd	0	0
***G. densa*(aqueous)**	nd	0.5	nd	0.7	0.9
***G. densa*(methanolic)**	nd	0	nd	0	0
Sodium Azide (positive control)	1.7	2	2,5	nd	nd

nd: not determined

4.4 Micropropagation

4.4.1 Surface sterilization of explants

To identify the ideal surface sterilization conditions of the explants, different combinations of chemical sterilants viz., sodium hypochlorite and mercury chloride were used. Results are presented in Table 13 given below. Plants were exposed to sterilizing agents for varying durations. Maximum contamination (86±1.41%) was observed when explants are treated with 30% sodium hypochlorite (v/v) for 20 min, followed by combination of 0.1% mercury chloride (m/v) for 5 mins and 30% sodium hypochlorite (v/v) for 15 min. 0.1% mercury chloride for 5 min and 40% sodium hypochlorite for 20 min showed no contamination and were used to sterilize the leave explants for further use.

Table 13. Percentage of contamination using different surface sterilizing agents at different exposure times

Surface sterilizing agent	Duration of Exposure	% Contamination
30 % (v/v) Sodium hypochlorite	20 minutes	86±1.41
0.1 % (m/v) Mercury chloride + 30 % (v/v) Sodium hypochlorite	5 minutes + 15 minutes	52.5±3.53
0.1 % (m/v) Mercury chloride + 40 % (v/v) Sodium hypochlorite	5 minutes + 20 minutes	5.5±2.12

Data are mean±SD (n=2)

4.4.2 Callus induction

To determine the best callus induction response, various concentration and combinations of growth regulators were used. The leaves of the plants were used as explants. Callus initiation was observed on the surface or cut ends of the explants after 14 days of inoculation. The effect of different plant growth regulators and their concentration on callus induction is summarized in Table 14. The best callus induction response for leaf explants of *A. aspera* (Fig.12a-b) *A. sessilis* (Fig.14a-b) and *G. densa* (Fig.13a-b) were observed on MS medium supplemented with 1 mg/L– 2, 4-D and 1 mg/ L BAP. No callus induction was noted on 1 mg/L 2, 4-D or 1 mg/L BAP only, the explants just withered away. When equal concentrations of auxins and cytokinins were supplemented in the media high frequencies of callus induction were noted. When a high concentration of a strong auxin viz., 2,4-D was used in combination with a lower concentration of a cytokinin viz., BAP a low frequency of callus induction was noted. Similar reports of 2,4-D plus kinetin or NAA plus BAP induced callus development was observed in amaranthus by Flores *et al.* (1982) and Bennici *et al.* (1992). When a high concentration of cytokinin was used with a low concentration of auxin a low frequency of callus induction was noted. Hence the calli obtained from MS medium supplemented with 1 mg/L 2, 4-D and 1 mg/ L BAP were used for further analyses. The calli were subcultured onto fresh medium every two weeks.

Table 14: Effect of growth regulators on callus induction from leaves of *A. aspera*, *A. sessilis* and *G. densa* (After 2 weeks of culture)

MS medium plus	% of leaves forming callus		
	A.aspera	*A.sessilis*	*G.densa*
1 mg/L 2, 4-D + 1 mg/ L BAP	98.5±2.12	98±2.82	97.5±2.12
1 mg/L 2, 4-D	0	0	
1 mg/ L BAP	0	0	
0.5 mg/L 2, 4-D + 1 mg/ L BAP	37±2.82	49.5±6.36	27±2.82
0.5 mg/L 2, 4-D + 0.5 mg/ L BAP	74±1.41	77.5±3.53	79.5±6.36
1 mg/L 2, 4-D + 0.5 mg/ L BAP	54±5.65	53.5±2.12	40.5±0.70

Data are mean±SD (n=2)

4.4.3 Shoot regeneration from callus

Callus obtained from *A. sessilis* in MS medium supplemented with 1 mg/L 2, 4-D and 1 mg/ L BAP were transferred onto shooting media which consisted of half strength MS medium, 1 mg/L IAA and 1 mg/ L BAP. After 4 weeks, structures were observed with their basal ends embedded on the callus. These structures turned into green colored shoot buds (Fig. 14c). These structures were transferred into tissue culture bottles consisting of different combinations of growth hormones (Fig. 14d).

After 21 days the individual shoots were cut off and transferred into tissue culture bottles containing MS medium supplemented with 1 mg/L IAA and 1 mg/L BAP (Fig. 14e-f). This combination of growth regulators showed maximum shoot multiplication of 10 shoots per culture (Table 16). Bennici *et al.* (1997) found that high cytokinin:auxin ratio favours shoot regeneration in *Amaranthus*. The lowest number shoot regeneration was observed on half strength MS medium supplemented with 1 mg/L IAA only. No shoot multiplication was observed on half strength MS medium supplemented with 1 mg/ L BAP. Shoot multiplication was noted 14 days after the cultures were inoculated into the medium. *A. aspera* and *G. densa* did not successfully form shoots from callus.

Table 15: Effect of growth regulators on shoot differentiation from stem explants of *A. sessilis*

MS medium	Shoot multiplication of *A.sessilis*
1 mg/L IAA + 1 mg/ L BAP	10 shoots per culture
1 mg/L IAA	2 shoots per culture
1 mg/ L BAP	0

4.4.4 Rooting of regenerated shoots

For rooting, the shoots developed from the callus of *A. sessilis* were cultured on rooting medium consisting of half strength MS medium containing different concentrations of either NAA or IBA. Optimal rooting was observed on half strength MS medium supplemented with 1 mg/L IBA (Fig. 15a). Some rooting response was also observed on MS medium supplemented with 1mg/L NAA. Bagga *et al.* (1987) found that hypocotyls segments of *A. paniculatus* formed roots on B5 medium supplemented with NAA and Bennici *et al.* (1992) reported that *Amaranthus* responded well forming roots in IAA plus kinetin and/or IAA plus BAP.

4.4.5 Hardening of plantlets

The regenerated plantlets from the explant with healthy root and shoot system were transferred after washing with distilled water to remove the media traces from the roots. They were transferred into bottles containing sterilized soil. They were covered with sunbag vessels (Fig. 15b) to maintain a high humidity environment. These were maintained for one week. Thereafter the sunbags were removed and the plants were transferred into bottles containing normal soil and they were grown under full sunlight (Fig. 15d). To summarize, in all the three plants, callus formation was observed in most of the growth regulator combinations but the differences in callus growth were observed depending on the growth hormone combinations used. The best medium for callus induction was found to be MS medium supplemented with 1mg/L 2,4-D and 1 mg/L BAP. Shoot regeneration from the callus of *A. sessilis* was found in MS medium supplemented with 1mg/L IAA and 1 mg/L BAP. Optimal rooting was observed in MS medium with 1mg/L IBA. The results obtained from our study demonstrate that *A. sessilis* has great potential with regard to dedifferentiation and morphogenetic processes and the possibility to micropropagate these plants.

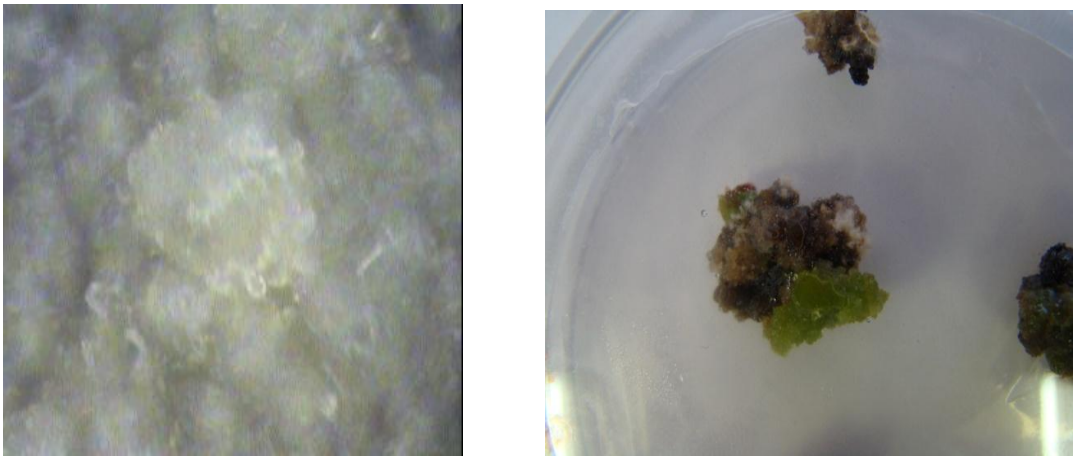


Fig.12. ***A.aspera*** **(a) callus derived from leaf explants (b) plate showing callus derived from leaf explants.**

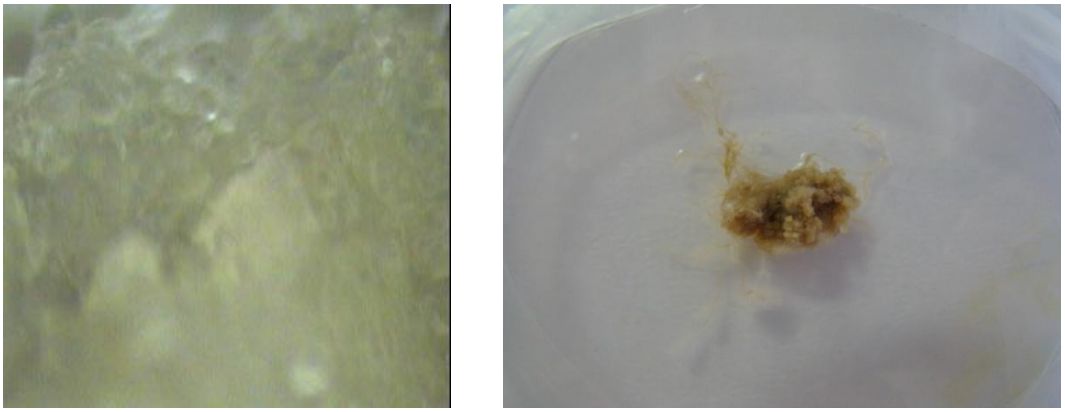


Fig.13. ***G.densa*** **(a) callus derived from leaf explants (b) plate showing callus derived from leaf explants.**

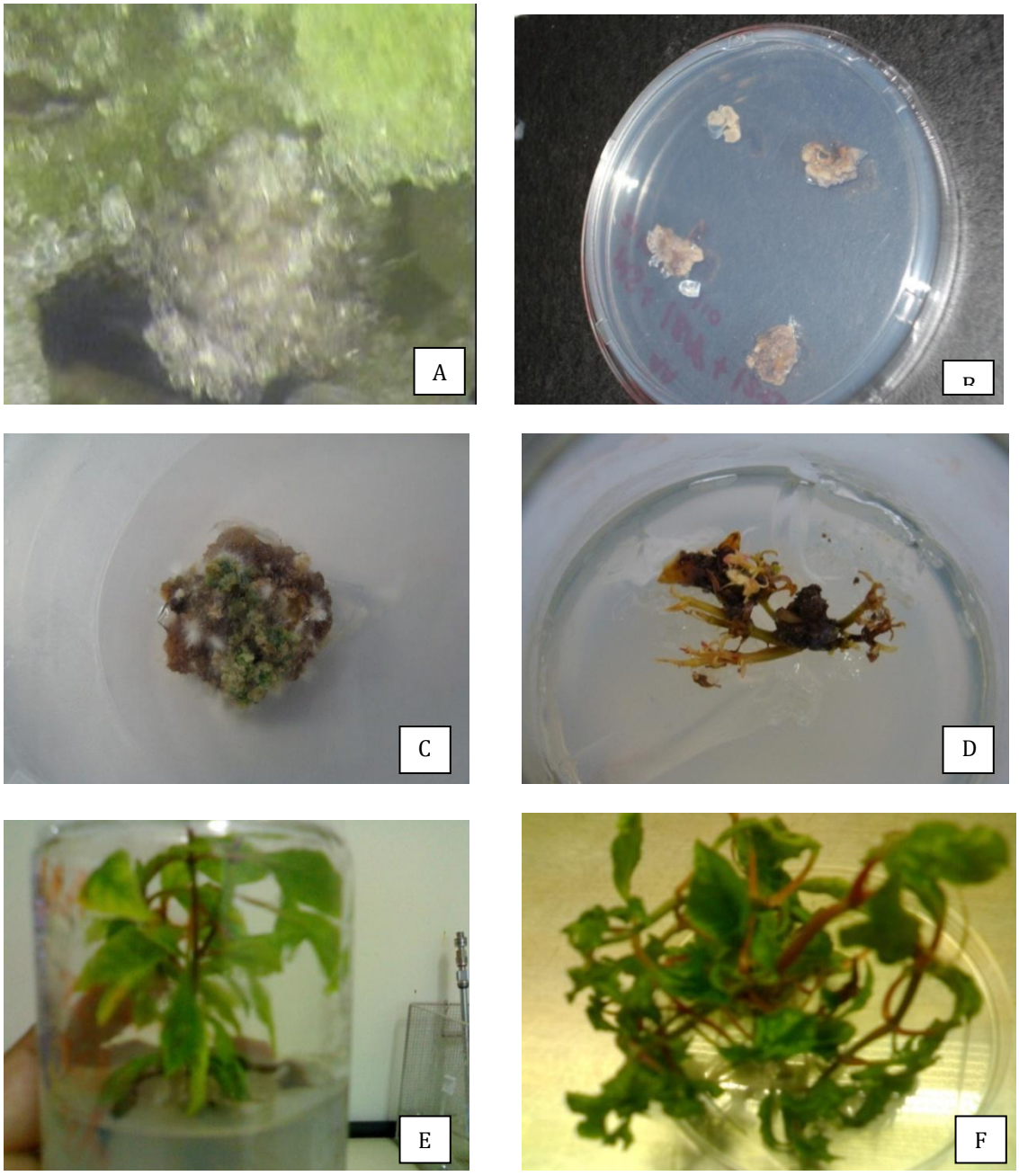


Fig.14. Micropropagation of *A. sessilis* (a) callus derived from leaf explants (b) plate showing callus derived from leaf explants (c) shoot formation from callus (d)shoot regeneration in tissue culture bottle (e) multiple shoot development in a bottle (f) multiple shoot development on a petri dish

Fig.15 . Root development in *A. sessilis* (a) Rooting in half MS medium supplemented with 1 mg/L IBA (b) *A. sessilis* plant in a sunbag vessel (c) hardened *A. sessilis* plant (d) Acclimatized plant

CHAPTER FIVE: CONCLUSION

The focus of this study was to investigate the nutritional, biological and safety of three plants from the Amaranthaceae family viz. *A. aspera*, *A. sessilis* and *G. densa* that are considered as famine plants.

The nutritional profile of *A. aspera* indicated that moisture, ash, carbohydrates, protein, fat, fibre and energy meet with the RDA levels. The plant extracts had high levels of Vitamin B_1, Vitamin B_2 and Vitamin C. The nutritional composition indicates that this neglected plant can be a valuable source of nutrients under famine conditions and the high levels of some vitamins and minerals can be used to prevent diseases. This plant can be cultivated on a large scale due to the fact that the extracts have high antioxidant activity. This activity can be used to prevent diseases of unknown aetiology such as cancer, ageing, atherosclerosis, ischemic injury and neurodegenerative diseases. The extracts can also provide an alternative source for current infectious diseases caused by multi drug resistant bacteria and can act as an agent for malarial control.

A. sessils has high level of ash, carbohydrates, protein, dietary, fat, fibre and energy needed to nourish individuals suffering from malnutrition. This plant can help eradicate malnutrition in areas rife where people are starving. The plant had high levels of Vitamin A and Vitamin B_3 and also high levels of magnesium, manganese and iron present, therefore, this plant can aid in proper nervous system functioning, energy metabolism, enzyme structuring and optimal immune functioning especially in rural populations where anemia is prevalent. The plant extracts also show high anti-oxidative activity. As far as the antibacterial activity results are concerned, it can be used to prevent diseases caused by *P. aeruginosa* and *S. aureus* and due to its fungicidal effect on *C. albicans*, this plant can be used against Candidiasis. A further advantage of cultivating this plant on a large scale like the other members from the family is that it is conducive for micropropagion by callus culture to a fully grown plantlet able to survive environmental conditions.

G. densa had sufficient amounts of ash, fibre and fat. This plant has excessive amounts of Vitamin B_1 and Vitamin B_2 therefore they could prevent deficiencies that cause peripheral nerve damage or central nerve system lesions, lesions of the corner of the mouth, lip and tongue and seborrheic dermatitis. Due to the sufficient amounts of Vitamin A and Vitamin B_3 this plant can fight illnesses such as night blindness, xerophthalmia, keratinisation of skin, pellagra- photosensitive dermatitis and depressive psychosis. The high iron levels found can be used to treat/prevent diarrhea. The plant has antibacterial effects against a range of bacteria so it can prevent diarrhea, vomiting and bronchitis and repellent properties against the mosquito whish could be used for personal protection especially by rural communities where chemical protection is very expensive.

A. aspera, *A. sessilis* and *G. densa* are also considered safe as they showed no toxicity or mutagenicity.

In conclusion the three plants from the Amaranthaceae family viz. *A. aspera*, *A. sessilis* and *G. densa* that are considered as famine plants should be cultivated on a large scale due to their nutritional and biological value.

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APPENDIX A

1. 0.5mM histidine/0.5mM biotin solution for the top agar in mutagenicity test

Ingredient	**per 250 ml**
D-Biotin	30.5 mg
L-histidine	26.2 mg
Dd H20	250 ml

Dissolved biotin in hot water first, filter sterilized and stored at 4°C in glass bottle.

2. Vogel –Bonner medium E (50 x strength stock) for minimal agar base

Ingredient	**per 1000 ml**
Warm ddH20	670 ml
MgSO4.7H2O	10 g
Citric acid monohydrate	100 g
K2HPO4	500 g
NaHNH4PO4.4H2O	175 g

Added salts in the order indicated to warm water in a 2 liter flask placed on a magnetic stirring hot plate. Allowed each salt to dissolve completely before adding the next. Adjusted the volume to 1 litre. Distributed into two 1 liter glass bottles. Autoclaved for 20 min at 121°C.

3. 40 % Glucose (autoclave sterile)

Ingredient	**per 1000 ml**
Glucose	400 g
ddH2O	1000 ml

4. Minimal Glucose agar plates for mutagencity test

Ingredient	**per 1000 ml**
Agar	15 g
Ddh20	930 ml
VB medium E stock (autoclaved sterile)	20 ml
40 % glucose (autoclaved sterile)	50 ml

Added agar to dd h20 in a 2 liter flask. Autoclaved at 121°C for 20 min using slow exhaust. When the solution cooled slightly, added sterile VB medium E stock and sterile 40% glucose.

5. Top Agar for mutagenicity test (soft agar)

Ingredient	**per 1000ml**
Agar	6 g
NaCl	5 g
Dd H2O	to 1000 ml

Microwaved to dissolve the agar. Mixed thoroughly and made 100 ml aliquots to 250 ml glass bottles with screw caps. Autoclaved at 121°C for 20 min with loosed caps.

* added 1/10 volume (10 ml) of the 0.5 mM histidine/0.5 mM biotin solution to the molten top agar.