PHENOTYPIC AND MOLECULAR CHARACTERISATION OF SOME *AMARANTHUS* ACCESSIONS AND HEPATOPROTECTIVE ACTIVITY OF THEIR ETHANOL EXTRACT ON SODIUM ARSENITE-INDUCED TOXICITY IN MALE RATS

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ABSTRACT

Amaranth is an underutilised crop with great potential as a source of essential nutrients. It also contains bioactive compounds with potential health benefits. However, its characterisation for agronomic and nutritional traits is limited. Likewise, information on its hepatoprotective potential is scarce. In this study *Amaranthus* accessions were characterised using phenotypic and molecular markers. The quality of its seed protein and hepatoprotective activity of its Ethanol Seed Extract (ESE) on sodium arsenite (NaAS)-induced toxicity in male rats were also investigated.

Twenty-nine accessions (27 from the United States Agricultural Research Station and 2 from National Horticultural Research Institute, Ibadan) were characterised using 27 phenotypic traits and 16 Random Amplified Polymorphic DNA (RAPD) primers. Protein quality was assessed using Kjedahl method, amino acid analyzer and one-dimensional electrophoresis. The ESE of all accessions were analysed for phytochemical contents and antioxidant activities. Two out of the 29 accessions with the highest nutritional contents were used for hepatoprotective study. Experimental design consisted of two main groups (representing the two accessions), each consisting of eight treatment groups of 5 rats each. Treatment groups comprised of control, NaAS (2.5mg/kg body weight), amaranth seed extracts (100, 200, 300 mg/kg body weight) and NaAS plus amaranth seed extracts (100, 200, 300 mg/kg). After 14 days of treatment, serum Alanine Aminotransferase (ALT) and Gamma Glutamyl Transferase (GGT) activities were assayed spectrophotometrically. Hepatic Superoxide Dismutase (SOD), Catalase and Glutathione Peroxidase (GPx) activities were assayed likewise. Data were analysed using ANOVA and Cluster at $p=0.05$.

For phenotypic traits, 57.5% variability was observed and accessions were grouped into five clusters. The RAPD analysis yielded 193 loci. Genetic similarity coefficient ranged from 0.6 to 0.9 while dendogram grouped accessions into nine clusters. Total protein contents ranged from 11.8 to 19.0%. Total essential amino acids ranged from 31.2 to 44.9% and were limited in tryptophan and leucine. Albumin, globulin and glutelin were the major protein fractions. Phytate, total flavonoid, total polyphenol, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), total antioxidant and 2,

2-azino-bis-3-ethylbenz-thiazoline-6-sulfonate (ABTS) values ranged from 0.8-1.9%; 7.8-11.0mg/100g; 23.9-35.4mg/100g; 82.8-95.4%; 111.3-271.6%; 157.6-208.8mM Trolox Equivalent (TE), respectively. Accessions A23 (*Amaranthus hypochondriacus*, AHC) and A28 (*Amaranthus hybridus*, AHB) had higher protein and phytochemical contents than the other accessions. The activities of ALT (16.7±1.0U/L) and GGT (3.5±0.9U/L) in NaAS-treated group were significantly higher than control (ALT - 9.4±1.3U/L, GGT - 1.7±0.7U/L) and lower in groups treated with AHC plus NaAS (100mg/kg - 14.1±0.8U/L, 3.2±0.6U/L), (200mg/kg - 12.6±0.3U/L, 2.6±1.1U/L) and (300mg/kg - 9.2±0.2U/L, 1.7±0.7U/L). Activities of ALT and GGT were also lower in AHB plus NaAS treated groups (100mg/kg - 12.5±1.4U/L, 2.9±0.7U/L), (200mg/kg - 11.8±0.8U/L, 2.3±0.9U/L), and (300mg/kg - 8.6±2.7U/L, 1.8±0.6U/L) when compared with NaAS-treated group. Hepatic SOD, Catalase and GPx activities were significantly lower in NaAS-treated group when compared with control and groups administered different doses of the amaranth extracts.

Amaranth accessions A23 (*Amaranthus hypochondriacus*) and A28 (*Amaranthus hybridus*) contained a good balance of essential amino acids and the ethanol extract showed dose-dependent hepatoprotective activity. The diverse clusters can be used as parents in hybridization programmes.

Keywords: Amaranthus, Phenotypic and molecular characterisation, Sodium arsenite-hepatotoxicity, Word count: 494

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CERTIFICATION

I certify that this research work was carried out by Pamela Akin-Idowu under my supervision in the Department of Biochemistry, University of Ibadan.

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DEDICATION

This work is dedicated to God Almighty, my God who is ever faithful and makes everything beautiful in his own time.

And to my darling husband Akinlolu Idowu and my lovely Children, OreofeOluwa, Oluwatobiloba and Oyinkansola.

LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviations	Acronyms
AAS	Amino Acid Score
ABTS	2, 2-azinobis (3-ethylbenz-thiazoline-6-sulfonic acid)
AFLP	Amplified Fragment Length Polymorphism
ALP	Alanine Phosphatase
ALT	Alanine Amino Transferase
ANOVA	Analysis of Variance
AOAC	Association of Analytical Chemist
APC	Adenomatous Polyposis Coli
ARS	Agricultural Research Station
AST	Aspartate Amino Transferase
BSA	Bovine Serum Albumin
CVD	Cardiovascular Disease
DMA	Dimethylarsonic acid
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EAA	Essential Amino Acid
ECRP	Environmental Control and Research Program
FAO	Food and Agricultural Organization

GGT	Gamma Glutamyl Transferase
GPx	Glutathione Peroxidase
GSH	Glutathione
HCC	Hepatocellular Carcinoma
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IBGPR	International Board for Plant Genetic Resources
ICPAES	Inductively Coupled Atomic Emission Spectrometry
MAS	Marker Assisted Selection
MDA	Malondialdehyde
MMA	Monomethylarsonic acid
MN	Micronucleus Assay
$NaAsO_2$	Sodium arsenite
NAS	National Academy of Science
NCRPIS	North Central Regional Plant Introduction Station
NF-1	Necrosis Factor- 1
NIHORT	National Horticultural Research Institute
NRC	National Research Council
NTP	National Toxicological Programme
NTSYS	Numeric Taxonomic System of Statistics
PAH	Polyaromatic Hydrocarbon

PCA	Principal Component Analysis
PCE	Polychromatic Erythrocyte
PCR	Polymerase Chain Reaction
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PER	Protein Efficiency Ratio
PGR	Plant Genetic Resource
PIC	Polymorphic Information Content
PUFA	Polyunsaturated Fatty Acid
RAPD	Random Amplified Polymorphic DNA
RDA	Recommended Dietary Allowance
RFLP	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAM	S-Adenosyl-Methionine
SAS	Statistical Analysis Software
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
TBARS	Thiobarbituric Acid Reactive Substances

TCA	Trichloroacetic acid
UNICEF	United Nations International Children's Educational Fund
UPGMA	Unweighted Pair Group Arithmetic Mean
USDA	United State Drug Agency
USFDA	United State Food and Drug Administration
USDA-ARS	United State Department of Agriculture-Agricultural Research Station
WHO	World Health Organization

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CHAPTER ONE

1.0. INTRODUCTION

Food insecurity still persists after 40 years of the Green Revolution (Hobbs, 2007), and the first Millennium Development Goal is to reduce hunger and poverty by 2015 (Dixon *et al*., 2006). The demand for food is increasing, not only to meet the growing population but to provide quality food with high nutrient content and nutraceutical compounds. Food crops rich in protein with a good balance of essential amino acids appear to be the most potential solution to overcome the crisis of protein-calorie malnutrition in the less developed countries.

In sub-Saharan Africa, about 200 million people are hungry while 40% of the total populations suffer from "hidden hunger" i.e. the deficiencies in proteins, iron, vitamins, zinc and other nutrients (FAO, 2009). Thus, the burden of hunger occurs at two levels in rural Africa; energy deficiencies, and specific nutrient deficiencies. Stunting and underweight in children is a key indicator of these deficiencies (UNICEF, 2009; Black *et al*., 2008). The economic implication of this malnutrition burden has been estimated to stand at 5 and 17% decline in productivity for each of the two deficiencies respectively (Martorell, 1999). In addition, both the World Health Organization (WHO) and the World Bank have estimated that between 18-25% of the developing countries burden of child disease is caused by under nutrition (Black *et al*., 2008). The relative risk of infection has been shown to increase exponentially as stunting prevalence increases (UNICEF, 2009). On the basis of findings from a number of studies on the effect of vitamins supplementation in vitamin deficient households, mortality in pre-school children and pregnant women fell by 27 and 40 % respectively, while Malaria attacks decreased by 30% (Caulifield *et al*., 2004).

Hundreds of millions of children and adults continue to suffer or die as a direct or indirect consequence of preventable food and nutrition related diseases and other factors (Muyonga *et al*., 2008; Academic Research, 2012). The poor are unable to access adequate amounts of nutrient-rich foods to meet dietary requirements and this is the major reason for the high prevalence of malnutrition (Muyonga *et al*., 2008). It is rightly said that the destiny of nations is determined by what they eat. Africa needs new agriculture options. Identification of nutrient-rich crops that can be easily cultivated is needed. Such crops should be fast growing, high yielding and naturally

acclimatized to the drought prone variable weather conditions common in most parts of Sub-Saharan Africa.

Amaranth is one of such crops; it has attracted increasing interest over recent decades because of its nutritional, functional and agricultural characteristics (Queiroz *et al*., 2009).

The role of amaranth as an under exploited plant with promising economic and nutritional value has been recognized by the National Academy of Science (NAS) (National Academy of Science, 1975; 1984). One of the motivating factors for the initiation of the amaranth research evolves from the perceived need to broaden the food base by the utilization of underdeveloped food materials (National Academy of Science, 1975).

Amaranth seed is high in protein (17% of dry weight) and its amino acid composition is close to the optimum amino acid balance required in the human diet (Silva-Sanchez *et al*., 2008). It also has some medicinal qualities (contains lysine, methionine and tryptophan) that help prevent cold sores, reduce disease infection and boost the body's immune system. Both seed and leaves are edible, the leaves are eaten as a vegetable while the seeds are used as cereals or flour to make confectionary (Kauffman and Weber, 1990). In addition, amaranth, quinoa and buckwheat seeds are also naturally gluten-free and thus, they are currently emerging as healthy alternatives to gluten-containing grains in the gluten-free diet (Kupper, 2005).

Amaranth species exhibit tremendous diversity related to their wide adaptability to different eco-geographic situations (Lee *et al*., 2008). The high genetic diversity in the amaranth family offers opportunities for increasing desirable characteristics, such as yield and essential nutrient contents (McKell, 1983).

A prerequisite to crop improvement is the study of its genetic diversity which is based on pedigree, morphological characterization, sexual compatibility, and taxonomic classification (Renganayaki *et al*., 2001).

Morphological observations have been made, but these are inherently weak identifiers as they are influenced by environmental factors (Dey, 1997). The biochemical characterization through the pattern analysis of isozymes and seed protein gel electrophoretic bands has also been done on crops like potato (Douchers and Ladlam, 1991). This is, however, not adequate enough to distinguish closely related genotypes/species due to small number of bands produced by such techniques. The past limitations associated with pedigree data and morphological, physiological

and cytological markers for assessing genetic diversity in cultivated and wild plant species have largely been circumvented by the development of DNA markers. Such markers include restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980); and in recent times, Polymerase Chain Reaction (PCR) based DNA markers such as random amplified polymorphic DNAs (RAPDs); amplified fragment length polymorphisms (AFLPs); and simple sequence repeats (SSRs, microsatellites) (Williams *et al*., 1990; Tragoonrung *et al*., 1992). DNA markers have the advantage of directly detecting sequence variations among cultivars and have proven to be extremely effective tools for distinguishing between closely related genotypes (Ribeiro-Carvalho *et al*., 2004). However, these molecular markers have technical differences in terms of cost, speed, amount of DNA needed, technical labor, degrees of polymorphism, precision of genetic distance estimates and the statistical power of tests.

In general, selection of promising genotype in a breeding programme is based on various criteria, with the most important being crop yield and seed quality (Kozak *et al*., 2008). The future of grain amaranth is dependent on the careful recombinant detection of genotypes from the germplasm collection.

As part of an effort to popularize grain amaranth consumption in Nigeria, improved germplasms were obtained from the North Central Regional Plant Introduction Station (NCRPIS), part of the United States National Plant Germplasm System, USA. Evaluation of this germplasm collection became necessary to assess variability within the *Amaranthus* species for important agronomic and nutritional traits with a view to identifying promising accessions for future breeding studies.

By convention, the ‘quality’ of seed protein refers to how far its amino acid composition matches the balanced amino acid composition recommended for human diet by the World Health Organization (FAO/WHO, 1973).

Both genetic and environmental factors can strongly affect the seed protein composition of amaranth as has been recorded for soybean seeds which contain an average of 36–38% protein and 19% oil, on a dry weight basis (Brumm and Hurburgh, 2002; Zarkadas, *et al*., 1993, 1999; Krishnan, 2000). The composition of soybean storage proteins also varies with maturity, nutrient supply from the soil, fertilizer treatment (Krishnan, *et al*., 2005) and with environmental factors (Fehr, *et al*., 2003). The high protein composition of amaranth compared with those of major cereals would indicate that it deserves a quantitative study of the chemical properties.

Aside from nutritional components, amaranth seeds also contain other substances that play various biological roles in the diet such as protease inhibitors, antimicrobial peptides, amino acids, lectins, saponins, flavonoids, phytate, polyphenols and antioxidant compounds (Berghofer and Schoenlechner, 2002; Taylor and Parker, 2002; Wijngaard and Arendt, 2006; Duarte-Correa *et al*., 1986; Brockaert *et al*., 1992; Valdes-Rodriguez *et al*., 1993). Recent findings have also supported the growing evidence that vegetables, grains, spices and fruits exhibit protective effects against chemicals and toxins (Gbadegesin and Odunola, 2010; Biswas *et al*., 1999; Thompson, 1994; Rastogi *et al*., 2007). This has been hypothesized to be because they contain phytochemicals that combat oxidative stress in the body by helping to maintain a balance between oxidants and antioxidants.

Aqueous extracts of *Amaranthus gangeticus* leaves have been reported to possess anticancer activity on liver, breast and colon cancer cell lines (Sani *et al*., 2004). Vegetable parts of *Amaranthus tricolor* have likewise been found to have antitumor and anti-cell proliferation activities (Jayaprakasm, 2004). Lunasin is a unique 43 amino acid peptide whose cancer preventive properties have been demonstrated in a mammalian cell culture model and in a skin cancer mouse model against chemical carcinogens, oncogenes and inactivators of tumour suppressor proteins (De Lumen, 2005). Its carboxyl-end contains nine Asp (D) residues, an Arg-Gly-Asp (RGD) cell adhesion motif, and a helix with structural homology to chromatin-binding proteins. Lunasin-like peptide, an anticancer bioactive compound has previously been found in soy (Gonzalez de Mejia *et al*., 2004), barley (Jeong *et al*., 2002); wheat (Jeong *et al*., 2007) and more recently in amaranth (Silva-Sanchez *et al*., 2008). Information on the phytochemical constituent and hepatoprotective potential of grain amaranth species are scarce.

This study was therefore designed to characterize accessions of grain amaranth, identify promising accessions with desirable traits for future breeding work, assess the seed protein quality as well as evaluate the hepatoprotective acitivity of the seed extracts *in vivo*.

1.1 AIM OF THIS STUDY:

To characterise grain amaranth accessions using phenotypic and molecular markers and determine the quality of their seed protein and the hepatoprotective activity of the seed ethanol extract.

1.2 OBJECTIVES

The specific objectives of this study are:

(1) Characterisation of 29 accessions of grain amaranth (*Amaranthus* species) using phenotypic (agronomic and nutritional) traits and molecular (RAPD) marker.

(2) Assessment of the seed protein quality of 29 accessions of grain amaranth using storage protein subunit and amino acid composition.

(3) Evaluation of the phytochemical profile (total phenolics, flavonoids, phytate, tannin and antioxidant activities) in the 29 accessions of grain amaranth.

(4) Investigation of the hepatoprotective activity of the ethanol seed extract of the amaranth accessions on sodium arsenite induced toxicity in male rats.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Early history of amaranth

Amaranth is among the oldest of American crops (Sauer, 1950; 1967). Archaeological evidence suggests that grain amaranths have been cultivated in Mexico since as early as 5000 B.C.E. The earliest known record of the pale-seeded, cultivated grain form of *A. cruentus* was discovered in Tehuacan, Puebla, Mexico and is dated at about 4000 B.C.E. However, because *A. cruentus* is not native to this region, it is likely that it was introduced after being cultivated elsewhere earlier (Sauer, 1976).

Pre-Columbian History. Amaranth was cultivated for centuries by the indigenous people who resided in present-day Latin America. Amaranth was sacred to the Aztecs and was also cultivated by the Mayas, Incas and other Pre-Columbian civilizations. In addition to cultivating it for food uses, these people also bred the plant for its rich color, which was used as dyes in religious rites. As a grain crop, the indigenous people prepared and cultivated amaranth in a manner similar to that of maize (*Zea mays* L.). The seeds were ground into flour and used to make tortillas, tamales, etc. (Iturbide and Gispert, 1994). It was also popped, parched or made into gruel (Sauer, 1967).

*Huatli*as as the Aztecs collectively termed amaranth and its relative quinoa (*Chenopodium quinoa* Willd.) was important in Aztec ritual worship as well as in their diets. The Spaniards recorded that Moctezuma II required a tribute of approximately 200,000 bushels per year of *huatli*, an amount nearly equal to the maize tribute (about 280,000 bushels) (Sauer, 1950; 1967; 1993). As part of their daily diets, the Aztecs prepared a drink called *atole* by mixing amaranth flour with water. Dough, known as *tzoalli*, was made from amaranth flour and honey extracted from the maguey (*Agave Americana* L.*)* plant. According to the month of the year, the dough was made into small pyramids or was formed into the shapes of Aztec deities, such as their god of war, Huitzlipochtli. The dough idols were worshipped and then broken into pieces and eaten by the participants. The Spanish Catholics who witnessed these practices considered them a satanic facsimile of the Christian Eucharist (Iturbide and Gispert, 1994). Thus, because of the

deeply rooted use of *huatli* in Aztec religious practices, the Spaniards, who viewed the grain as a symbol of paganism, suppressed its cultivation (Sauer, 1976; 1993).

In addition to religious reasons, the reduction in amaranth cultivation in post- Columbian Mesoamerica and South America is also attributed to its replacement by Old World grains and to a possible dislike for its flavor (Iturbide and Gispert, 1994).

Amaranth outside of the Americans. After the Spanish conquest, amaranth cultivation dwindled in the Americas. However, the Spaniards introduced the crop into Europe where, by the 18th century, all three grains were widely distributed. A dark-seeded form of *A. cruentus* was introduced into Africa during the 1800s. In West Africa, it became an important vegetable crop. It was also found and grown in gardens in various parts of Asia.

Once introduced into the Old World, the common ornamental form of *A. caudatus* became popular and is now cosmopolitan; one popular ornamental form is known by the common name "love-lies-bleeding." Both dark- and pale-seeded *A. hypochondriacus* were introduced into Europe. Unlike its relatives, *A. hypochondriacus* varieties continued to be cultivated as a grain outside of the Americas, in India and a few places in China. The Indians and Chinese adopted the light-seeded types of *A. hypochondriacus* during the 1700s. These have now become widespread throughout India where they are sometimes sewn with *A. caudatus*. In the middle of the 20th century, *A. hypochondriacus* was introduced in Kenya (Sauer, 1993). Although amaranth grain use has spread since the Spanish Conquest, amaranth has remained essentially neglected.

Amaranth in the Modern Era. Lehmann (1996) noted two milestones in amaranth's history in the modern era that revived amaranth from its dormancy: 1) Sauer's (1950) rediscovery of the sacred Aztec crop and 2) the discovery of its advantageous protein content by Dowton (1973). Following these discoveries, the cause of amaranth was taken up by Robert Rodale. Rodale, a well-to-do American publisher and philanthropist, who advocated preventive medicine and organic gardening, was intrigued by amaranth's favorable properties and inadvertently became its "germplasm collector, 'public' breeder and promoter" (Lehmann, 1996). Through his magazine *Prevention and Organic Gardening* and other publications, Rodale disseminated information on amaranth to production farmers as well as backyard gardeners (Lehmann, 1996). This introduction of amaranth as an American crop is singular when contrasted with more

common procedures for the introduction of new crops by landgrant universities or via the plant introduction system (Lehmann, 1996).

Through his Rodale Research Institute, Rodale procured 1,200 accessions for an amaranth germplasm (Kauffman and Reider, 1986) and developed breeding techniques along with advanced breeding lines which he donated to public institutions. The Institute was also instrumental in orchestrating several major research projects on amaranth in various countries, including Mexico, Guatemala, Peru, Thailand and Kenya and in convening national meetings for amaranth farmers, researchers, and advocates (Lehmann, 1996).

Following the impetus Rodale provided, amaranth production began to rise in the US in the late 1970s and increased throughout the 1980s. In the 1990s, however, production rates of amaranth fluctuated (Brenner *et al*. 2000). The 1980s and 1990s have seen much improvement in the understanding of grain amaranths, especially in the areas of its nutritive value and production requirements. In addition, improved cultivars have been released, although much room for improvement remains (Brenner *et al*., 2000; Brenner, 2002).

Recently, the pseudocereal crop (amaranth) has received attention because it contains many of the nutrients essential for the human diet (Bressani *et al*., 1992; Tucker, 1986). It is further praised for its tenacity under harsh growing conditions and its variety of uses (Gupta and Gudu, 1991; Breene, 1991). Thus, some believe the study and improvement of amaranth may be useful as an option for alleviating hunger in developing nations, especially in overpopulated and undernourished areas (Pal and Khoshoo, 1974; Sauer, 1993).

2.2. Basic Botany of the Species

The genus *Amaranthus* (L.) belongs to the family *Amaranthaceae* and comprises of about 60 to 70 species grouped into three subgenera (Kalac and Moudry, 2000; Mosyakin and Robertson 2003). The most economically important is the subgenus *Amaranthus*, which includes the three species domesticated for grain production: *Amaranthus hypochondriacus, Amaranthus cruentus, and Amaranthus caudatus* (Brenner *et al*., 2000). Other species of amaranths have been domesticated as leaf-vegetables, for fodder, as potherbs, or as ornamentals; among these species, *A. tricolor*, from South Asia, is probably the most important (Sauer, 1967; Stallknecht and Schulz-Schaeffer, 1993).

The *Amaranthaceae* family also consists of hardy, weedy, herbaceous, fast-growing, cereal-like plants (Opute, 1979); with a seed yield of up to 3 tons/ hectare when grown in monoculture for 3-4 months, and a vegetable yield of 4.5 tons dry matter/hectare after 4 weeks (Grubben and van Sloten, 1981).

2.2.1 Subgenus *Amaranthus*

The subgenus *Amaranthus* consists of 20 species of annual herbs that are monoecious (Mosyakin and Robertson, 2003), that is, they have separate male and female flowers. The species are native to the Americas, with the exception of only one species of possible European origin (Mosyakin and Robertson, 2003). Monoecious amaranths are primarily self-pollinated, as female and male flowers are arranged in close proximity (Trucco and Tranel, 2011). Stems are usually erect and both axillary and terminal inflorescences are arranged in cylindrical spikes or panicles (Mosyakin and Robertson, 2003). Much of the difficulty in taxonomic discrimination of species within the group can be attributed to attempts at recognizing taxa based on pigmentation or growth forms, which are extremely variable within amaranths (Sauer, 1967). However, examination of floral parts can result in constant characters from which discontinuities can be used to define well-established taxa. In this sense, tepal (petals and sepals are combined in a single floral whorl) number and morphology are commonly used in taxonomic keys.

2.2.1.1 Vegetable species

Amaranth species are cultivated and consumed as a leaf vegetable in many parts of the world. No clear separation between vegetable and grain species exists, because the leaves of young grain varieties may be used as potherbs. There are 4 species of *Amaranthus* documented as cultivated vegetables in eastern Asia: *Amaranthus cruentus*, *Amaranthus blitum*, *Amaranthus dubius*, and *Amaranthus tricolor*. *Amaranthus cruentus* is cultivated as both a vegetable and a grain. Its relative's *A. tricolor*, *A. dubius* and *A. lividus* are grown as vegetables (Stallknecht and Schulz-Schaeffer, 1993); some examples are shown in Figures 2.1 to 2.3. In English, they are known by the common names Chinese spinach, Malabar spinach and tampala respectively (Sauer, 1993). Amaranth greens can be used both in cooking and for salads and has a delicious, slightly sweet flavor (Herbst, 2001). In East Africa, amaranth leaf is known in Swahili as *mchicha*. It is

sometimes recommended by some doctors for people having low red blood cell count. In West Africa, such as in Nigeria, it is a common vegetable, and goes with all Nigerian carbohydrate dishes. It is known in Yoruba as *efo tete* or *arowo jeja* ("we have money left over for fish"). In Congo, it is known as *lenga lenga* or *biteku teku* (Enama, 1994).

2.2.1.2. Grain species

The three amaranths principally grown as grains consist of *A. cruentus*, *A. caudatus*, and *A. hypochondriacus*. *Amaranthus hypochondriacus* and *Amaranthus cruentus* are native to Mexico and Guatemala; *Amaranthus caudatus* is native to Peru and other Andean countries (Figures 2.4 to 2.6). *Amaranthus edulis*, which is grown in the northern Andes of Argentina, is also sometimes cited as a grain species. However, *A. edulis* may be more appropriately considered *A. caudatus* spp. (Sauer, 1976). The wild putative progenitor species of the grains include *A. powellli*, *A. hybridus* and *A. quitensis* (Sauer, 1950; 1967; 1976). *A. hybridus* is also sometimes cultivated as a grain. Some of the wild relatives of the grains are fairly tall with large inflorescences; however the cultivated species are taller and more robust, with enormous inflorescences.

Amaranths are broad-leafed plants and are one of the few nongrasses that produce significant amounts of edible cereal grain (Figures 2.7 to 2.10).

2.2.1.2.1. *Amaranthus caudatus*

This species is a crop in the Andean highlands of Argentina, Peru, and Bolivia. It has pendulous, blazing-red inflorescences and it's commonly sold in Europe and North America as an ornamental under names such as love-lies-bleeding. Other forms of the species give much better grain yields. One good variety that has club-shaped inflorescences is *Amaranthus caudatus* sometimes classified as *Amaranthus edulis.*

The crop contains a great deal of genetic diversity in South America, and although only a small sampling has been introduced to other continents, much genetic diversity has been observed in the germplasm collections from Northern India (NRC, 1984).

2.2.1.2.2. *Amaranthus cruentus*

This Mexican and Guatemalan species is useful both as a grain or a leafy vegetable. The grain types have white seeds; the vegetable types (as well as those used to extract red dye) usually are dark seeded. It is probably the most adaptable of all amaranth species, and its flowers, for example, have a wider range of daylengths than the others.

Amaranthus cruentus is an ancient food and a crop used to extract a red dye for coloring corn-based foods in the Indian pueblos.

2.2.1.2.3. *Amaranthus hypochondriacus*

The most robust, highest yielding of the grain types, *Amaranthus hypochondriacus* was probably domesticated in central Mexico. Some types of *Amaranthus hypochondriacus* are bushy; others are tall and unbranched. *Amaranthus hypochondriacus* is a crop naturally resistant to water deficit and have agronomic features identifying it as an alternative crop where cereals and vegetables cannot be grown (dry soils, high altitudes and high temperatures) (Omami *et al*., 2006). Additionally, the species are widespread ornamentals and they have a potential as forage crops and as a source of red food colorants (Cai *et al*., 1998). The species is particularly useful for tropical areas, high altitudes, and dry conditions. It has excellent seed quality and shows the greatest potential for use as a food ingredient. It pops and mills well and has a pleasing taste and smell.

Figure 2.1 *A. tricolor* "merida"

Figure 2.2 *A. tricolor* "Greenleaf"

Figure 2.3 *A. tricolor* "tigerleaf"

Figure 2.4 *A. hypochondriacus* "burgundy"

Figure 2.5 *A. cruentus* "R104"

Figure 2.6 *A. hypochondriacus* "manna"

Source: ECHO (Educational Concerns for Hunger Organization) 1983. Technical Note.

Figure 2.7 seeds of *A. cruentus*

Figure 2.8 seeds of *A. hypochondriacus*

Figure 2.9 seeds of *A. hypochondriacus*

Figure 2.10 seeds of *A. hybridus*

Photos taken at NIHORT (National Horticultural Research Institute), Ibadan, in October, 2009

2.2.1.3. Weedy species

Not all amaranth plants are cultivated. Most of the species from *Amaranthus* are summer annual weeds and are commonly referred to as pigweeds (Bensch *et al*., 2003). These species have an extended period of germination, rapid growth, and high rates of seed production (Bensch *et al*., 2003) and have been causing problems for farmers since the mid-1990s. This is partially due to the reduction in tillage, reduction in herbicidal use, and the evolution of herbicidal resistance in several species where herbicides have been applied more often (Wetzel *et al*., 1999). The following 9 species of *Amaranthus* are considered invasive and noxious weeds in the United States and Canada: *A. albus, A. blitoides, A. hybridus, A. palmeri, A. powellii, A. retroflexus, A. spinosus, A. tuberculatus,* and *A. viridis* (Wassom and Tranel, 2005). The species *Amaranthus palmeri* (Palmer amaranth) causes the greatest reduction in soybean yields and has the potential to reduce yields by 17 to 68 percent in field experiments (Bensch *et al*., 2003). Palmer amaranth is among the "top five most troublesome weeds" in the southeast and has already evolved resistances to dinitroanilines and acetolactate synthase inhibitors (Culpepper *et al*., 2006). This appearance of the new strain of the Palmer amaranth that is glyphosate-resistant means that it cannot be killed by the widely used Roundup herbicide. Also, this plant can survive in tough conditions. This makes the proper identification of *Amaranthus* species at the seedling stage essential for agriculturalists. Proper herbicide treatment needs to be applied before the species successfully colonizes in the crop field and causes significant yield reductions.

2.3. Morphological Description of Amaranth

While grain amaranth species may be difficult to distinguish from one another on the basis of morphology, the features they share in common separate them from other amaranths. The vegetable amaranths have smooth leaves and exhibit an indeterminate growth habit. The grain amaranths are annuals and have a main stem axis with a large branched inflorescence at the apex (Stallknecht and Schulz-Schaeffer, 1993). The grain species usually range from 0.4 to 3.0 m in height. The grain amaranths are dicotyledonous, and are therefore not true cereals.

Leaves, inflorescences and flowers

Grain amaranth leaves are petiolate and oval to ovulate-oblong and lanceolate in shape with acute apices. The inflorescence is a dichasial cyme with unisexual flowers, which develop in a variety of colors, including red, purple, orange, or gold (Iturbide and Gispert, 1994). The first flower of each of the numerous cymes is staminate followed by an indefinite number of pistillate flowers, frequently over a hundred (Pal and Khoshoo, 1974; Sauer, 1993). Some pistillate flowers on the cyme develop early before the staminate flower has opened, while others become receptive following the abscission of the male flower. However, because cymes at different developmental stages are present on each indeterminate inflorescence branch, self-pollination is more likely than out crossing, although both types of fertilization are possible (Sauer, 1976).

Fruit

Unlike other cereals, grain amaranths have retained the dehiscent fruits of their wild progenitors (Sauer, 1993). The fruits are pyxides, meaning that they house their seeds in circumscissile capsules, which are subtended by colorful bracts and sepals. The top half of the papery utricle surrounding each seed acts as a lid-like section, which pops off at the equator of the utricle to reveal the enclosed seed. Thus, although the majority of seeds remain in the densely packed inflorescences, some seeds are lost during the harvest (Sauer, 1993). However, in recent years non-shattering grain amaranth populations have been developed (Brenner, 2002).

Seeds

The seed heads, some as long as 50 cm, resemble those of sorghum. The seeds of the grain amaranths although barely bigger than a mustard seed, are lens-shaped and approximately 1 to 1.5mm in diameter. The seeds occur in massive numbers sometimes more than 50,000 to a plant and come in a variety of colors, ranging from cream to golden to pink (Iturbide and Gispert, 1994; Sauer, 1993). These colors are governed by simple Mendelian recessive alleles. All three grains produce both dark- and light-colored seed.

Although the dark grains, which are dominant to the light-colored grains, are edible and were eaten by prehistoric hunter-gathers, the lighter grains have been selected for due to their

improved flavor and popping. Furthermore, the pale color also seems to be linked to a loss of dormancy in the seed (Sauer, 1976; 1993).

The seeds exhibit epigeal germination, in which the cotyledons emerge above ground as in common beans (*Phaseolus vulgaris* L.). Seedlings emerge three to four days following sowing and after about two and half months the panicle appears and flowering occurs. The seeds maintain viability for over five years at ambient temperature and <5% humidity (Iturbide and Gispert, 1994).

Yield

As yet, amaranth agronomists have paid little attention to improving seed yield. The plants are already quite productive, and researchers are concentrating on characteristics such as ease of harvest and taste and food-processing qualities that are more fundamental at this early stage. In Pennsylvania, test plots of productive strains of grain amaranths routinely yield 1,800 kg of seed per hectare. In California and elsewhere, small trial plots have yielded up to nearly twice that amount, and at four locations in the Himachal Pradesh and Uttar Pradesh hills of India, lines selected from the local land races have yielded 3,000 kg of grain per hectare. The grain yield of *Amaranthus cruentus* ranged between 400 to 1,500 kg per hectare in Nigeria (Olaniyi, 2007). Therefore, researchers suspect that grain amaranths will eventually match the yields of most other cereals. As with any new crop, there are many production uncertainties. Problems reported at the various research locations include the lygus bug (an insect that sucks nutrients out of the immature seeds), fast-growing weeds that in some environments overwhelm the slow starting amaranth seedling, and strains of amaranth that produce seed heads so heavy that they flop over during a summer thunderstorm. These, however, are not insurmountable difficulties.

2.4. Cultivation Practices of Amaranth species

Traditionally, amaranth is planted either by direct sowing or by sowing in seed beds and transplanting to irrigated land (Iturbide and Gispert, 1994; Tapia, 1997). Amaranth is often sown together with maize or as a border. A mixture of amaranths is usually sown in order to ensure a harvest. Seeds are sown in pre-prepared ground under dry conditions in furrows spaced 80cm apart and fed with a constant stream of water. The plants are harvested before they are fully

matured in order to prevent seed fall. When the lower leaves show signs of yellowing, the plants are cut about 20cm above the soil. The sheaves are usually allowed to dry on the ground above the furrows. To remove the seeds, the sheaves are placed on sheets on top of the ground and beaten with sticks. The chaff is removed from the grain by sifting or winnowing (Tapia, 1997).

2.5. Adaptations

Evidence indicates that amaranths adapt to many environments and tolerate adversity because they use an especially efficient type of photosynthesis to convert the raw materials of soil, sunlight, and water into plant tissues. The grain amaranths exhibit C4 photosynthesis. Thus, they grow rapidly in bright sunlight, high temperatures, and low moisture conditions. Other cultivated crops that exhibit C4 photosynthesis include maize, sorghum (*Sorghum* spp. L.) and sugarcane (*Saccharum officinarum* L.). The C4 pathway is particularly efficient at high temperature, in bright sunlight, and under dry conditions. Plants that use it tend to require less water than the more common C3 carbon-fixation pathway plants. For this reason, grain amaranth may be a promising crop for hot and dry regions. Amaranth is better adapted to semiarid environments than these plants, however, because it can make osmotic adjustments that allow it to tolerate dry conditions without wilting or drying (Tucker, 1986). Amaranths can also tolerate a variety of unfavourable soil conditions such as high salinity, acidity, or alkalinity (Tucker, 1986). Grain amaranths have also been reported to adapt readily to new environments, including some that are inhospitable to traditional grain crops (Gupta and Gudu, 1991).

2.6. Amaranth Development

Despite the growing evidence in amaranth's favour, much research needs to be done before the crop can be commercially produced. Agronomists are starting almost from scratch in adapting it to modern needs. Nevertheless, the researchers are learning the crop's responses to climate, soil conditions, pests, and diseases. Also, they are breeding short-statured plants of uniform height with sturdy, wind-resistant stalks and high-yielding seed heads that hold their seeds until they are harvested. Leading amaranth's development is the Rodale Research Center near Emmaus, Pennsylvania, where more than a thousand different accessions collected from all parts of the world are being bred, grown' and evaluated. Further collaboration has been initiated with

scientists in Africa, Asia, and Latin America; as a result, plant lines have been selected to overcome tendencies toward lodging, seed shattering, indeterminate growth, succulence at harvest time, and day length dependence. This research effort has produced strains with improved baking, milling, popping, and taste qualities, as well as machinery adapted to planting, cultivating, harvesting, and threshing the crop. Lines of uniform color and height that bear their seed heads above the leaves, thus making them suitable for mechanical harvest, are now available. The crop can be said to be on the threshold of limited commercial production in the United States, where twenty or so farmers are growing the crop. Several companies are testing the grain in their products, and an amaranth-based breakfast cereal is available.

2.7. Genetic diversity in Amaranth species

Amaranthus hypochondriacus varies widely, as indigenous cultivars can be found which have red, green or pink ears; the seed may be cream, white, golden or black. The leaf colouring matches that of the ears; however, there are different tones on the stem. Crossings between cultivars of *A. hypochondriacus* are viable, although they are self-fertile: the same is the case with *A. cruentus*. In this species, different ear colours are found: red, green, orange, pink and two-colour (red and green). The colouring of the leaves and petioles matches that of the ear and in some cases the stem has a similar colour; the seed may be white, translucent cream or golden. Interspecific crossings have proved viable, which suggests that coincidence in flowering may produce hybrids.

2.8. Taxonomic challenges.

Because the species within the *Amaranthus* genus are very closely related, literature shows that misclassifications among the grains as well as their weedy and wild relatives occur frequently. Genetic and environmental factors, as well as ambiguous or atypical morphology in some accessions, can lead to classification errors among these closely related species. Several studies have demonstrated the utilitization of molecular markers such as RAPDs and AFLPs for correcting these types of errors in grains and weeds (Transue *et al*., 1994, Chan and Sun, 1997; Wetzel *et al*., 1999; Sun *et al*., 1999; Xu and Sun, 2001; Wassom and Tranel, 2005).

2.9. Molecular markers

With the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc. Ever since their development, they are constantly being modified to enhance their utilization and to bring about automation in the process of genome analysis. The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker-based gene tags, map-based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping, marker-assisted selection of desirable genotypes, etc. thus, giving new dimensions to concerted efforts of breeding and marker-aided selection that can reduce the time span of developing new and better varieties and will make the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively.

Molecular markers are important tools in the investigation and improvement of plants. For example, marker-assisted selection (MAS) has greatly improved the efficiency and effectiveness of breeding programs. While traditional breeding programs base selection on phenotype, MAS is based on genotype. Thus, in MAS, selection is not influenced by the environment. In addition, MAS is much faster because selection can be done as soon as the plant is old enough to provide DNA for examination, therefore significantly reducing the number of generations required to produce new varieties of crops (Yousef and Juvik, 2001). Marker assisted selection is also more cost effective than traditional breeding.

Markers can also be used to assess genetic diversity (Diwan *et al*., 1995; Tanksley and McCouch, 1997). This is important for many reasons. For example, fingerprinting and identifying different species and cultivars can aid in assigning taxonomic designations and can also allow breeders to know where to go for sources of important genes. In addition, assessing genetic diversity aides in the maintenance of genetic diversity and helps prevent genetic erosion (Wilkes, 1989).

Molecular markers include biochemical constituents (e.g. secondary metabolites in plants) and macromolecules, viz. proteins and deoxyribonucleic acids (DNA). Analysis of secondary metabolites is, however, restricted to those plants that produce a suitable range of metabolites which can be easily analyzed and which can distinguish between varieties. These metabolites which are being used as markers should be ideally neutral to environmental effects or management practices. Hence, amongst the molecular markers used, DNA markers are more suitable and ubiquitous to most of the living organisms.

Properties desirable for ideal DNA markers

- Highly polymorphic nature
- Codominant inheritance (determination of homozygous and heterozygous states of diploid organisms)
- Frequent occurrence in genome
- Selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices)
- Easy access (availability)
- Easy and fast assay
- High reproducibility
- Easy exchange of data between laboratories.

It is extremely difficult to find a molecular marker which would meet all the above criteria. Depending on the type of study to be undertaken, a marker system can be identified that would fulfill atleast a few of the above characteristics (Weising *et al*., 1995).

Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and

banding patterns are detected by different methods such as staining and autoradiography. PCR is a versatile technique invented during the mid-1980s (Saiki *et al*., 1985). Ever since thermostable DNA polymerase was introduced in 1988 (Saiki *et al*., 1988), the use of PCR in research and clinical laboratories has increased tremendously. The primer sequences are chosen to allow base-specific binding to the template in reverse orientation. PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology.

Markers commonly used in plant studies include restriction fragment length polymorphisms (RFLPs), random amplified DNA polymorphisms (RAPDs), amplified fragment length polymorphisms (AFLPs), single-nucleotide polymorphisms (SNPs), and simple-sequence repeats (SSRs).

2.9.1. Types and description of DNA markers

2.9.1.1. Restriction fragment length polymorphism (RFLP)

RFLPs are simply inherited naturally occurring Mendelian characters. They have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over (Schlotterer and Tautz, 1992). In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted (Southern, 1975) on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with labelled probe. These probes are mostly species-specific single locus probes of about 0.5–3.0 kb in size, obtained from a cDNA library or a genomic library. The genomic libraries are easy to construct and almost all sequence types are included; however, a large number of interspersed repeats are found in inserts that detect a large number of restriction fragments forming complex patterns. In plants, this problem is overcome to some extent by using methylation-sensitive restriction enzyme *Pst*I. This helps to obtain low copy DNA sequences of small fragment sizes, which are preferred in RFLP analysis (Figdore *et al*., 1988; Liu and Knapp, 1990; Miller and Tanksley, 1990a). On the other hand cDNA libraries are difficult to construct, however, they are more popular as actual genes are analyzed and they contain fewer repeat sequences. The

selection of appropriate source for RFLP probe varies, with the requirement of particular application under consideration. Though genomic library probes may exhibit greater variability than gene probes from cDNA libraries, a few studies reveal the converse (Miller and Tanksley, 1990b; Landry and Michelmore, 1987). This observation may be because cDNA probes not only detect variation in coding regions of the corresponding genes but also regions flanking genes and introns of the gene.

RFLPs were first developed for use in the production of genetic maps in humans (Botstein *et al*., 1980), but were quickly used in plants to assess genetic diversity (Helentjaris *et al*., 1985) and produce genetic maps (Helentjaris *et al*., 1986). RFLPs are codominant markers and can therefore identify heterozygotes. They are very reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual, information highly desirable for recessive traits (Winter and Kahl, 1995). However, their utility has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. The assay is time-consuming and labour-intensive and only one out of several markers may be polymorphic, which is highly inconvenient especially for crosses between closely-related species. Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism.

2.9.1.2. Randomly amplified polymorphic DNA (RAPD)

RAPDs were introduced in 1990 (Williams *et al*., 1990) as a quick way of detecting genetic markers that can easily be mapped. RAPDs are PCR-based and are much quicker, easier, and cheaper than RFLPs. They employ random primers that do not require prior sequence knowledge. This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermocyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide

sequence polymorphism between individuals. However, due to the stoichastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. However, RAPDs are not highly reproducible due to sensitivity to changes in tissue source, DNA extractions protocol, and/or PCR conditions (Staub *et al*., 1996). In addition, RAPDs are generally dominant markers, meaning they do not allow discrimination between heterozygotes and homozygotes. RAPD assay has been used by several groups as efficient tools for identification of markers linked to agronomically important traits, which are introgressed during the development of near isogenic lines. The application of RAPDs and their related modified markers in variability analysis and individual-specific genotyping has largely been carried out. RAPD technology has being well suited to DNA fingerprinting (Dos Santos *et al.,* 1994; Thormann *et al*., 1994) although it does suffer from a certain lack of reproducibility due to mismatch annealing (Neale and Harry, 1994; Demeke *et al.*, 1997; Karp *et al.,* 1997). In RAPD analysis, the intervening region is amplified when the single primer sequence is present in the opposite orientation on opposite genomic DNA strands at distances of up to 5000bp (Williams *et al*., 1990). Due to the technical simplicity and speed of RAPD methodology, it has been successfully used for the generation of genetic similarities and phylogenetic analysis (Gepts, 1993). In this study therefore, RAPD molecular marker was employed in the assessment of genetic diversity.

2.9.1.3. Simple Sequence Repeats

Simple sequence repeats (SSRs) or microsatellites are based on differences in repeat number of simple sequences. These simple sequences generally consist of one to four nucleotide sequences that are repeated in tandem and are surrounded by conserved sequences. These conserved regions are sequenced, and PCR primers are subsequently designed to amplify the repeat region within the flanking conserved sequences. Microsatellite markers are short tandem repeats of nucleotides that are usually one to four base pairs in length, although motifs as long as seven or eight bp in length may be classified as microsatellites. Microsatellites are thus distinguished from minisatellite (10 to 30bp long motifs) and satellite (>30bp long motifs) DNA exist. Unlike their larger counterparts that tend to be found mainly in telomeric regions of chromosomes, microsatellites are relatively evenly dispersed throughout eukaryotic genomes (Ellegren, 2004),

making them useful for obtaining a glimpse of an organism's entire genome when complete sequencing is not an option.

Microsatellites are also known as Short Tandem Repeats (STRs) or Simple Sequence Repeats (SSRs). Microsatellites are thought to be generated by the slippage of one DNA strand during replication of double-stranded DNA or by unequal crossing over during meiosis (Ellegren, 2004).

Polymorphisms in repeat number are caused by slippage during DNA replication and/or by unequal crossing over (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992). These polymorphisms can be detected by running the PCR products on either agarose or polyacrylamide gels and visualizing using eithidium bromide or radioisotopes, respectively. Because SSRs require sequencing, they are relatively expensive; however, operating costs are low after sequencing is accomplished. Primers can easily be developed from published sequences and are therefore easily transferable to other laboratories. In addition, because the sequences surrounding the repeat regions are highly conserved, SSRs are easily transferable to other species with relatively high success. Thus, because they are highly reproducible, polymorphic, codominant markers, SSRs are an excellent choice for use in any plant system. Indeed, plant studies employing SSRs are abundant. SSRs have been used in mapping and genetic diversity studies in numerous minor crops as well as all the major crops including rice (McCouch *et al*., 1997), corn (Taramino and Tingey, 1996), soybeans (Maughan *et al*., 1995), and wheat (Cuadrado and Schwarzacher, 1998).

2.9.1.4. Amplified fragment length polymorphism (AFLP)

A recent approach by Zabeau and Vos, (1993) known as AFLP is a technique based on the detection of genomic restriction fragments by amplification and can be used for DNAs of any origin or complexity. AFLPs were introduced in 1995 as a new fingerprinting technique (Vos *et al*., 1995) and were quickly put to use in mapping and other applications in plants (Haanstra *et al*., 1999). The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction

conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques (Saiki *et al*., 1988; Ehrlich *et al*., 1991) and is extremely useful in detection of polymorphism between closely related genotypes. AFLP procedure mainly involves 3 steps:

(a) Restriction of DNA using a rare cutting and a commonly cutting restriction enzyme simultaneously (such as *Mse*I and *Eco*RI*)* followed by ligation of oligonucleotide adapters, of defined sequences including the respective restriction enzyme sites.

(b) Selective amplifications of sets of restriction fragments, using specifically designed primers. To achieve this, the 5' region of the primer is made such that it would contain both the restriction enzyme sites on either sides of the fragment complementary to the respective adapters, while the 3' ends extend for a few arbitrarily chosen nucleotides into the restriction fragments.

(c) Gel analysis of the amplified fragments.

AFLP analysis depicts unique fingerprints regardless of the origin and complexity of the genome. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping (Vos *et al*., 1995). AFLPs are extremely useful as tools for DNA fingerprinting (Hongtrakul *et al*., 1997) and also for cloning and mapping of variety-specific genomic DNA sequences (Yong *et al*., 1996; Paglia *et al*., 1998). Similar to RAPDs, the bands of interest obtained by AFLP can be converted into SCARs. Thus AFLP provides a newly developed, important tool for a variety of applications. AFLPs require small amounts of high quality DNA. The technique is very demanding and expensive, but results in a large amount of data (generally 100-200 bands per marker). AFLPs are PCR-based and require no sequence knowledge. As with RAPDs, AFLPs are generally dominant markers.

2.9.2. Applications of molecular markers in plant genome analysis and breeding

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions

to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it.

2.9.3 Advantages of molecular markers in Plant Breeding

The use of microsatellites, as well as other molecular markers, in plant breeding has advantages over conventional breeding methods in that it can significantly decrease the time required to breed an improved cultivar (Yousef and Juvik, 2001). Microsatellite markers, for example, can be used at any stage of a plant's development. They do not vary with the environment, and can be used to detect heterozygosity that may not be apparent in the plant's phenotype. They are also useful in finding genes that contribute to polygenic traits (Prasad *et al*., 2003). DNA molecular markers such as random amplified polymorphic DNA (RAPD) (Tao *et al*., 1993; Huang, 2004), simple sequence repeats (SSR) (Ghebru *et al*., 2002; Menz *et al*., 2004) and amplified fragment polymorphism (AFLP) markers (Vos *et al*., 1995) have been successfully used to estimate genetic diversity in crops.

2.9.4. Polymerase chain reaction (PCR) technique

2.9.4.1. Principle and Procedure

Polymerase chain reaction is one of the most important ingenious scientific research tools of the 20th century in molecular biology that allows exponential amplification of short DNA sequences within a longer double stranded DNA molecule. It was invented by Kary Mullis in association with Fred Faloona, Henry A. Erlich, and Randall K. Saiki in the year 1983, while he was working in Emeryville, California for Cetus Corporation. Mullis summarized the procedure: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules within a short period. The reaction is easy to execute, it requires no more than a test tube, a few simple reagents, and a source of heat" (Mullis, 1990). In 1993, Kary Mullis won the chemistry Nobel Prize for developing PCR.

Principle

Polymerase chain reaction is an *in vitro* technique based on the principle of DNA polymerization reaction by which a particular DNA sequence can be amplified and made into multiple copies. It relies on thermal cycling consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs. It thus can amplify a specific sequence of DNA by as many as one billion times. Most PCR methods can amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

Procedure

Polymerase chain reaction is built on 20-40 repeated cycles where the temperature changes in each cycle. The cycling starts with a single temperature step (called hold) at a high temperature (>90 degree Centigrade), and followed by one hold at the end for final product extension or for brief storage (Rychlik *et al*., 1990). The various steps of PCR are shown in Figure 2.11:

1. Initiation step.

It is the first step of the cycle which consists of raising the temperature of the reaction to 94–96 °C or 98 °C if extremely thermostable polymerases are used, which is held for 1–9 minutes. This process activates the DNA polymerase used in the reaction (Sharkey *et al*., 1994).

2. Denaturation step.

It consists of heating the reaction to 94-98 degree centigrade for 20-30 seconds. This helps in breaking of the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

3. Annealing step.

The mixture is now cooled to a temperature of 50–65 degree centigrade for 20-40 seconds which helps in annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence that permits annealing of the primer to the complementary sequences in the DNA. As a rule, these sequences are located at the 3′-end of the two strands of the segment to be amplified. The duration of annealing step is usually 1 min during the first as well as the subsequent cycles of PCR. Since the primer concentration is kept very high relative to that of the template DNA, primer-template hybrid formation is greatly favored over re-annealing of the template strands.

4. Extension/elongation step.

It is a DNA polymerase dependent process. Taq polymerase has its optimum activity temperature at 75-78 degree centigrade. The temperature at this step depends on the DNA polymerase used; the temperature is so adjusted that the DNA polymerase synthesizes the complementary strands by utilizing the 3′-OH of the primer. The primers are extended towards each other so that the DNA segment lying between the two primers is copied; this is ensured by employing primers complementary to the 3′-ends of the segment to be amplified. The duration of primer extension is usually 2 min at 72°C. Taq polymerase usually amplifies DNA fragments of up to 2 Kb; special reaction conditions are necessary for the amplification of longer segments. As a rule of thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute, leading to exponential (geometric) amplification of the specific DNA fragment (Chien *et al*., 1976, Lawyer *et al*., 1993).

5. Final elongation.

This step is performed at a temperature of 70-74 degrees centigrade for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

6. Final hold.

In this step the mixture is allowed to cool to a temperature of 4-15 degree centigrade for short-term storage of the reaction.

PCR Stages

1. Exponential amplification.

As a result of each cycle, the number of copies of the desired segment becomes twice the number present at the end of the previous cycle. The more times the three PCR cycles are repeated the more DNA you can obtain. This is because every cycle of a PCR reaction theoretically doubles the amount of target copies, so we expect a geometric amplification. In other words PCR is an exponential process.

2. Leveling off stage.

The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

2.9.4.2. Uses of PCR:

1. It is appropriate for **forensic testing** procedures because only a very small sample of DNA is required as the starting material which can be obtained from a single strand of hair or a single drop of blood or semen.

2. **Tissue typing** for organ transplantation

3. DNA based phylogeny or functional analysis of genes useful for **research**

4. Detection of **genetic diseases**: Prenatal genetic diagnosis, hereditary diseases

5. Detection of **infectious diseases**: HIV, TB, etc.

6. **Parental testing**, where an individual is matched with their close relatives

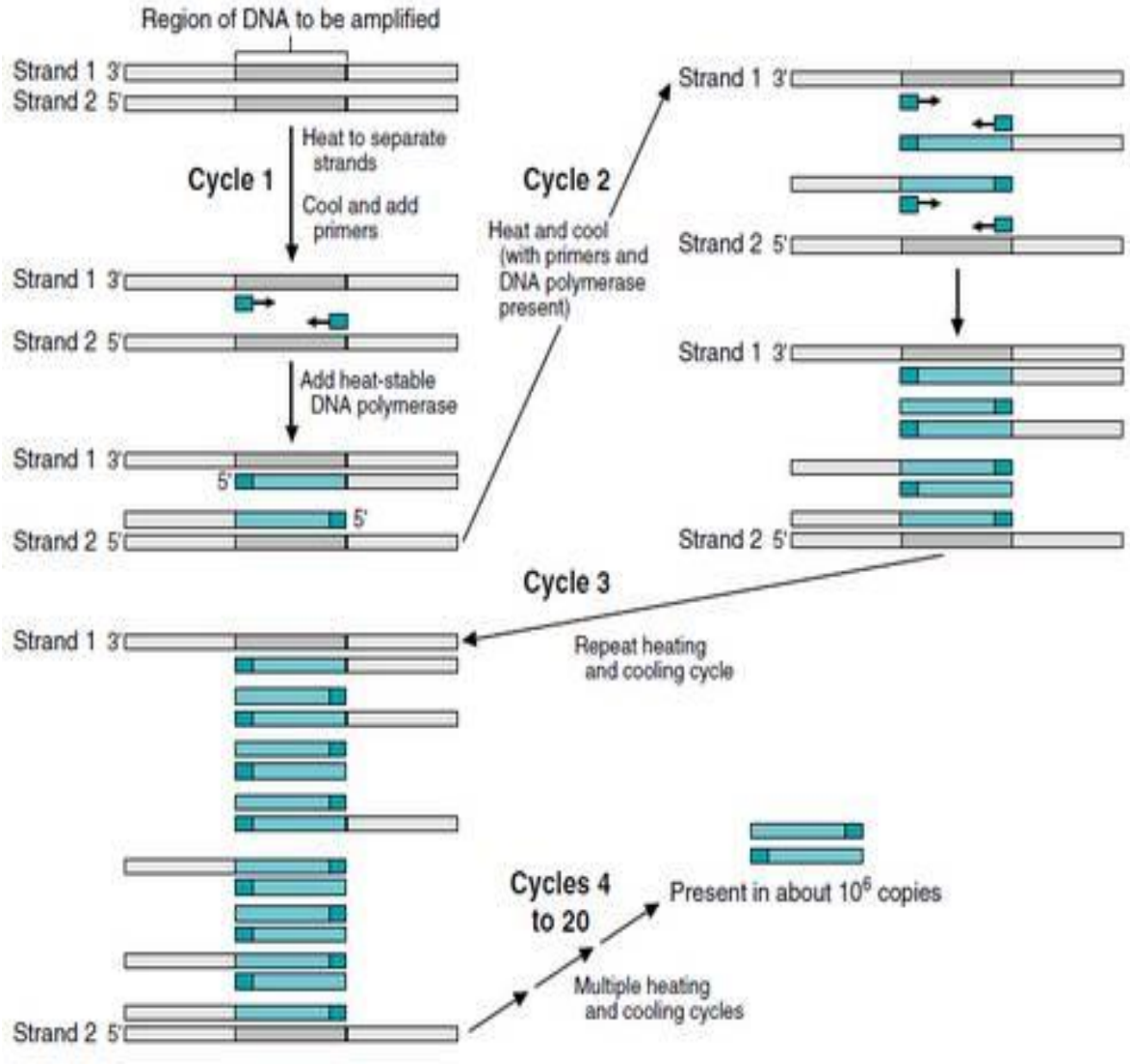


Figure 2.11. Description of the schematic diagram for PCR technique: Strand 1 and Strand 2 are original DNA strands. The short dark blue fragments are the primers. After multiple heating and cooling cycles, the original strands remain, but most of the DNA consists of amplified copies of the segment (shown in lighter blue) synthesized by the heat stable DNA polymerase.

Source:http://medchrome.com/basic-science/biochemistry/polymerase-chain-reaction-principles-and uses-of-pcr/ accessed 4th September, 2011.

2.10. Nutritional value of grain Amaranth

Amaranth is not a true grain or cereal because the plant does not belong to the grass family (Poaceae), but like quinoa, it is known as a "pseudograin" because the flavor, appearance, and cooking of many species exhibit similarities to grains. Amaranth seeds can be used as a cereal or ground into flour and they contain exceptionally complete protein for plant sources (Whole Health, 2005). Amaranths have 50 to 60% starch, with higher protein (15 to 16%) and more fat (7 to 8%) than most cereals (Table 2.0); (Breene, 1991). Like quinoa, amaranth contains the essential amino acid lysine, which is deficient in wheat and barley. Besides protein, amaranth grain provides a good source of dietary fiber about 8% for pale-seeded types, while the black-seeded grain types may have twice that (Pedersen *et al*., 1987). Amaranth seeds and leaves are a very good source of vitamins, including vitamin A, vitamin K, vitamin B6, vitamin C, riboflavin, and folate, and dietary minerals including calcium, iron, magnesium, phosphorus, potassium, zinc, copper, and manganese (Becker *et al*., 1981).

2.10.1. Starch content of grain Amaranth

In amaranth, 78 to 100% of the starch content is found in the branched-chain amylopectin form, while the remaining 0 to 22% of starch content is in the amylose or unbranched form (Tomita *et al*., 1981; Okuno and Sakaguchi, 1982). Overall, amaranth's starch composition shows a low gelatinization temperature and good stability during freezing and thawing (Yanez *et al*., 1986). The granules of starch isolated from the seeds of *A. hypochondriacus* were found to be small (1-3 um in diameter) and angular and polygonal in shape (Lorenz, 1981; Saunders and Becker, 1984; Stone and Lorenz, 1984), while those of *A. cruentus* were reported to be spherical as well as angular and polygonal (McMasters *et al*., 1955; Stone and Lorenz, 1984). Rice (*Oryza sativa* L.*)* for example, has starch granules of about 3 to 8μm, while potato's (*Solanum tuberosum* L.) are 100μm in diameter. It has thus been suggested that the small granule size might make amaranth starch useful as a food thickener, dusting powder in cosmetics, a laundry starch, etc. (Yanez *et al*., 1986).

The existence of both nonglutinous and glutinous starches has been identified in *A. hypochondriacus* (Tomita *et al*., 1981; Okuno and Sakaguchi, 1981), with starch granules

consisting of nearly 100% typical amylopectin. Sugimoto *et al*., (1981) reported that starch granules of two types of *A. hypochondriacus* contained 0 and 14% amylose, while Becker *et al*., (1981) found 7.2% amylose. X-ray diffraction analysis of *A. hypochondriacus* starches showed that they were identical to maize and rice starches, indicating a type of crystalline structure (Sugimoto *et al*., 1981). The *A. caudatus* starch was reported to be completely nonglutinous (Okuno and Sakaguchi, 1981; 1982), while *A. cruentus* starch was reported to be glutinous (McMasters *et al*., 1955). Lorenz (1981) reported that, compared to wheat starch, the starch of *A. hypochondriacus* has a much lower amylose content, a lower swelling power, a higher solubility, a greater water uptake, a lower amylograph viscosity, and a higher gelatinization temperature range. Becker *et al*., (1981) suggested that the very small size of the starch granules and residual amylase activity were presumably responsible for the observed differences in swelling power and solubility. The higher viscosity of wheat starch after cooling to 35°C is due to the higher amylose content's causing the development of aggregated structures with increased viscosity. Compared to corn starch, *A. cruentus* and *A. hypochondriacus* starches had higher swelling power, lower solubility, greater water uptake, lower susceptibility to a-amylase, higher amylograph viscosity, and much lower amylose content (Stone and Lorenz, 1984). High susceptibility of *A. hypochrondriacus* and *A. caudatas* starch granules to amylases was reported by Tomita *et al*., (1981).

Table 2.0. The nutritional composition of grain and uncooked leaves of amaranth (100 g portions).

Component	Vegetable	Grain
Moisture	86.9 g	9.0 g
Protein	**3.5 g**	**15.0 g**
Fat	0.5 g	7.0 g
Total carbohydrates	6.5 g	63.0 g
Fiber	1.3 g	2.9 g
Calories	36	391
Phosphorus	67 mg	477 mg
Iron	3.9 mg	--
Potassium	411 mg	--
Vitamin A (beta carotene)	**6100 IU**	**0**
Riboflavin	0.16 mg	0.32 mg
Niacin	1.4 mg	1.0 mg
Ascorbic acid (C)	**80 mg**	**3.0 mg**
Thiamin (B1)	0.08 mg	0.14 mg
Ash	2.6 g	2.6 g
Calcium	267 mg	490 mg

Source: Cole (1979).

2.10.2. Oil content of grain Amaranth

Several studies have shown that like oats, amaranth seed oil may be of benefit for those with hypertension and cardiovascular disease; regular consumption reduces blood pressure and cholesterol levels, while improving antioxidant status and some immune parameters (Czerwinski *et al*., 2004; Martirosyan *et al*., 2007). The 7-8% oil content found in amaranth seeds may be too low and expensive to compete with other oils commercially available, although it is similar in content to corn and cotton seed oils (Bressani *et al*., 1987). Table 3 shows the ranges of fatty-acids observed for the oil content based on Breene's (1991) summary of various studies (Fernando and Bean, 1984; Saunders and Becker, 1984; Bressani *et al*., 1987). The saturated/ unsaturated fatty acid ratio has been observed to range from 0.29 to 0.43; this ratio is favorable from a nutritional standpoint because unsaturated fatty acids are predominant in amaranth oil (Breene, 1991). High levels of tocopherols (vitamin E) and tocotrienols have been reported in amaranth oil as well (Lehmann *et al*., 1994). Amaranth oil has been noted for its relatively high concentration of squalene (Bressani *et al*., 1987). Squalene is a lucrative ingredient used in cosmetics, skin penetrants, lubricants and is a precursor to cholesterol. The traditional source of squalene for commercial use is liver oil extracted from threatened sea animals such as whales (*Physeter macrocephalus*) and sharks (*Centrophorus squamosus*). Therefore, there is interest in other potential alternative sources. The use of amaranth oil as a squalene source may further its commercialization (Brenner *et al*., 2000).

2.10.3. Protein content of grain Amaranth

The protein content of the grain amaranth has been extensively studied. Crude protein content from pale-seeded grain types has been reported to range from 12.5 to 22.5%, with an average of about 15% (Becker *et al*., 1981, Saunders and Becker, 1984; Teutonico and Knorr, 1985; Bressani *et al*., 1987; Bressani, 1989). The protein content of the seed compares well with the conventional varieties of wheat (12-14 %), rice (7-10%), maize (9-10%) and other widely consumed cereals (NRC, 1984).

Amaranth is one of the few plants whose protein content approaches animal protein quality on the basis of bioavailability and amino acid content (Bressani, 1989). Other examples of plants

with essential amino acid patterns that come close to satisfying the needs of the human diet include soybean, high-quality protein maize and quinoa (Bressani, 1989). Furthermore, amaranth is relatively rich in the essential amino acid lysine, which is usually limiting in other cereal crops. Lysine content ranges from 0.73 to 0.84% of amaranth's total protein content (Bressani *et al*., 1987). Amaranth protein, however, has nearly twice the lysine content of wheat protein, three times that of maize, and in fact as much as is found in milk the standard of nutritional excellence. Seed storage proteins from amaranth have been introduced successfully through transgenics into other crop species. Species such as potato and maize that have been modified to express amaranth seed proteins show improved amino acid composition (Chakraborty *et al*., 2000).

2.10.3.1. Protein digestibility corrected amino acid score (PDCAAS)

For humans, amino acid requirement values have been used to develop reference amino acid patterns for the purpose of evaluating the quality of food proteins or their capacity to efficiently meet both the nitrogen and indispensable amino acid requirements of an individual. Protein quality is a measure of protein bioavailability, and its evaluation is a means of determining the capacity of food proteins and diets to satisfy the metabolic demands of amino acids and nitrogen. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) Joint Expert Consultation's Protein Quality Evaluation Report (FAO/WHO, 1991) recommended the protein digestibility-corrected amino acid score (PDCAAS), which is a simple, scientific and rational procedure for assessing protein quality. The United States Institute of Medicine (IOM) in its 2005 discussion of Daily Reference Intakes (DRIs) reaffirmed the use of PDCAAS for evaluating the relative nutritional quality of different protein sources (Institute of Medicine, 2005). Additionally, the US Food and Drug Administration (USFDA) adopted the use of PDCAAS for the protein quality evaluation of foods in 1993. Table 2.1 shows the PDCAAS of some selected food source.

The PDCAAS methodology takes into consideration three important parameters:

1) The food protein's essential amino acid profile, 2) corrected for its digestibility and 3) its ability to supply the FAO/WHO's amino acid requirements for 2-5 year olds, (FAO/WHO, 1991).

Although there are relatively small differences in amino acid requirements for age-sex groups beyond infancy, the 2-5 year old requirement is used because it is the most demanding pattern of any age group excluding that of infant requirements. For a given protein, the essential amino acid that is present in the relatively lowest quantity when compared to the standard is used for calculation of the final PDCAAS value. The highest possible score using the PDCAAS method is 1.00 (FAO/WHO, 1991). The digestibility of protein is defined as the proportion of ingested protein that is absorbed. True digestibility of protein corrects for endogenous fecal nitrogen (measured as nitrogen loss that occurs on a protein-free diet) and is expressed as a true digestibility percent. The true digestibility of Solae isolated soy proteins is 97% (internal data), and comparable to other high quality proteins such as milk, meat and eggs that range between 94-97% (WHO/FAO/UNU, 2007). Other plant proteins are of lower digestibility. The use of Protein Efficiency Ratio (PER) to assess protein quality, although still used by some, measures the ability of a protein to support growth in young growing rats. It over-estimates the value of some animal proteins and underestimates the value of some vegetable proteins for human growth (FAO/WHO, 1991; Henley and Kuster, 1994). On the other hand, PDCAAS considers the full contribution of vegetable proteins to the diet. Additionally, vegetable proteins such as high quality soy protein have economic and sustainability advantages when compared to animal proteins.

2.10.3.2. Meeting Human Protein Requirements

Soy protein meets the essential amino acid requirements of children and adults (National and International Organizations, FAO/WHO (1991). Institute of Medicine (2005), WHO/FAO/UNU (2007), FAO/WHO/UNU (1985) and individual researchers like Young and Pellett, (1990) have published protein and amino acid requirement recommendations. These recommendations are used to assess whether a protein source is nutritionally complete based on its amino acid composition. The most recent estimations of the indispensable amino acid requirements for various age groups were published by the WHO/FAO/UNU (2007). Values for children and adults were published by the U.S. (Institute of Medicine, 2005) as part of the DRIs for protein and amino acids (Table 2.2). Isolated soy proteins commercially available from Solae meet the published estimated indispensable amino acid requirements for children and adults (Table 2.3).

Table 2.1 PDCAAS of Selected Proteins

Food Source	PDCAAS
Wheat Gluten	0.25
Whole Wheat	0.40
Peanut Meal	0.52
Lentils canned	0.56
Rolled oats	0.63
Kidney beans canned	0.68
Pea flour	0.69
Beef	0.92
Egg white	1.0
Casein	1.0
Isolated soy protein	1.0

Sources: FAO/WHO (1991).

Table 2.2 FAO/WHO/UNU amino acid requirement pattern based on amino acid requirements of preschool-age child[1]

Amino acid	**Requirement**
	mg/g crude protein
Histidine	19
Isoleucine	28
Leucine	66
Lysine	58
Total sulfur amino acids	25
Total aromatic amino acids	63
Threonine	34
Tryptophan	11
Valine	35
Total protein EAA_9	339

Source: [1]FAO/WHO/UNU, (1985)

Table 2.3 True fecal digestibility, amino acid score and PDCAAS for selected proteins[1]

Protein	PER	Digestibility	AAS	PDCAAS
		%		
Egg	3.8	98	121	118
Cow's milk	3.1	95	127	121
Beef	2.9	98	94	92
Soy	2.1	95	96	91
Wheat	1.5	91	47	42

[1]Data from FAO/WHO, (1990).

2.11. Amaranth Uses

2.11.1. Food Uses

Although amaranth is cultivated on a small scale in various parts of the world, including parts of Mexico, Guatemala, Peru, India, and Nepal, and various African nations such as Nigeria, there is potential for further cultivation in the United States and tropical countries and it is often referred to as "the crop of the future" (Marx, 1977). It has been proposed as an inexpensive native crop that could be cultivated by indigenous people in rural areas for several reasons: 1) it is easily harvested, 2) it produces a lot of fruits (and thus seeds), which are used as grain, 3) it is highly tolerant of arid environments, which are typical of most subtropical and some tropical regions, and 4) it contains large amounts of protein and essential amino acids, such as lysine (De Macvean and Poll, 2002).

From an economic stand point, amaranth has the capacity to play four main roles in improving the rural farmer's welfare: First, it may provide impoverished households with an alternative source of income. Secondly, it could improve the nutritional content of food consumed by the household members, especially the vulnerable groups (children, women and immuno-compromised individuals). Thirdly, amaranth could reduce unemployment rates through the cultivation, processing and marketing activities. And fourth, by lowering the relative price of cereals through increased output, amaranth could make livestock products and fruits more affordable (Tagwira and Okumu, 2006).

Due to its weedy life history, amaranth grains grow very rapidly and their large seed heads can weigh up to 1 kilogram containing half a million seeds (Tucker, 1986). *Amaranthus* species are reported to have thirty percent higher protein value than cereals such as rice, wheat flour, oats, and rye (De Macvean and Poll, 2002). A traditional food plant in Africa, amaranth has potential to improve nutrition, boost food security, foster rural development, and support sustainable land care (NRC, 2006).

2.11.2. Traditional Uses. Amaranth seed can be used in breakfast cereals or as an ingredient in confections. In Mexico, the preparation of the sacred Aztec dough, *tzoalli*, by mixing amaranth flour with maguey honey led to the current use of amaranth for preparing *alegria*, a sweet snack.

However, because the use of amaranth flour was discouraged, the modern process has been altered so that *alegria* is currently made with popped amaranth seeds instead of flour (Iturbide and Gispert, 1994). The popped form is also used in cereals. The seed is milled into flour to make a variety of snacks like biscuits, bread, cakes and other baked goods. Amaranth grain, however, contains little functional gluten, so that it must be blended with wheat flour to make yeast-leavened baked goods rise. The leaves are used as a vegetable, particularly in soups. The stems are useful as animal feed (Iturbide and Gispert, 1994).

2.11.3. Commercial Uses. Industrial food uses of amaranth are similar to its traditional uses in Latin America (Breene, 1991). Amaranth seed is packaged and sold as a whole grain or is milled into whole, high-bran and low-bran flour. Amaranth grain can be difficult to mill, however, due to the grain's unusual morphological characteristics and small seed size. Many studies have focused on the beneficial use of amaranth as a replacement for wheat and corn flour. Malted flours are also produced from amaranth seeds that are allowed to germinate to produce "malt," which is then dried and ground into flour. It has been noted that processing amaranth in this manner resulted in an increase of 25 to 30% in true protein content likely due to the decrease in total fat and carbohydrates during the malting process. Another common cereal preparation method applied to amaranth is extrusion. Extrusion involves exposing the food product to intense pressure and heat within an apparatus known as an extruder and cooking it in such a way that the product acquires a particular desired shape or greater uniformity. Amaranth prepared in this manner has been used as an ingredient in beverages, baby formula, atole, croutons, snacks, breakfast cereals, and as a textured vegetable protein (Breene, 1991).

While the active ingredient in oats appears to be water-soluble fiber, amaranth appears to lower cholesterol via its content of plant stanols and squalene. Because of its valuable nutrition, some farmers grow amaranth today and it is seen as a potentially important agricultural crop of the future. However, their moderately high content of oxalic acid inhibits the absorption of calcium and zinc, and also means that they should be avoided or eaten in moderation by people with kidney disorders, gout, or rheumatoid arthritis. Reheating cooked amaranth greens is often discouraged, particularly for consumption by small children, as the nitrates in the leaves can be converted to nitrites, similarly for spinach.

2.11.4. Other uses

Dyes

The flowers of the 'Hopi Red Dye' amaranth were used by the Hopi Amerindians as the source of a deep red dye. There is also a synthetic dye that has been named "amaranth" for its similarity in color to the natural amaranth pigments known as betalains. This synthetic dye is also known as Red No. 2 in North America and E123 in the European Union.

Ornamentals

The genus contains several well-known ornamental plants, such as *A. caudatus* (love-lies-bleeding), a native of India and a vigorous, hardy annual with dark purplish flowers crowded in handsome drooping spikes. Another Indian annual, *A. hypochondriacus* (prince's feather), has deeply-veined lance-shaped leaves, purple on the under face, and deep crimson flowers densely packed on erect spikes.

Medicinal

Amaranth has been used widely by the Chinese for its healing chemicals, used to treat illnesses such as infections, rashes, and migraines.

2.12. Bioactive phytochemicals and functional foods

It is well recognized that foods are the main sources of nutrients used to meet our nutritional needs. However, foods, particularly those of plant origin, contain a wide range of non-nutrient phytochemicals that are elaborated by plants for their own defence and for other biological functions (Narasinga Rao, 2003).

Phytochemicals are plant chemicals or bioactive non-nutrient plant compounds found in fruits, vegetables, grains and other plant foods that have been linked to reducing the risk of major chronic diseases (Temple, 2000). It is estimated that more than 5000 individual phytochemicals have been identified in fruits, vegetables and grain (Liu, 2003); and some classes of

phytochemicals are shown in Figure 2.12. Phytochemicals present in commonly consumed plant foods are normally non-toxic and have the potential for preventing chronic diseases.

For the last two to three decades, however, there has been a surge of interest in plant foods as a source of phytochemicals that may have a useful role in the prevention of chronic diseases such as cancer, diabetes and cardiovascular disease (Greenwald *et al*., 2001). Such an association between consumption of certain foods and low prevalence of non communicable disease was derived initially from epidemiological observations (Liu, 2004; Temple, 2000). Foods that have disease-preventing potential are designated 'functional foods'. Functional foods are foods that provide health benefits beyond basic nutrition (Anon, 1999).

The phytochemicals present in functional foods that are responsible for preventing disease and promoting health have been studied extensively to establish their efficacy and to understand the underlying mechanism of their action. Such studies have included identification and isolation of the chemical components, establishment of their biological potency both by *in vitro* and *in vivo* studies in experimental animals and through epidemiological and clinical-case control studies in man. These studies have been very extensive in the case of plant foods and the phytochemicals present therein in relation to their cancer-preventing potential. Disease-preventing phytochemicals present in plant foods are shown in Table 2.4.

Properties of functional foods are shared by several phytochemicals also present in plant foods. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen containing compounds and organosulfur compounds. It is interesting to note that several nutrients like vitamin E (tocopherols), provitamin A (β-carotene), ascorbic acid and selenium also have disease-preventing and health promoting potentials, just like phytochemicals (Kris-Etherton *et al*., 2002) as shown in Table 2.5.

The potency of a given functional food in preventing a disease is the sum of the bioactivities of all phytochemicals present in that food with a common property. Whether there is synergism between these chemicals, they are additive or only one or two components account for most of the functions of the food are questions that remain unanswered. These phytochemicals are reported to prevent disease mainly through their functions as antioxidants, detoxifiers, neuropharmacological and immunopotentiating agents, and as a source of dietary fibre (non-starch polysaccharide, NSP) (Narasinga Rao, 2003).

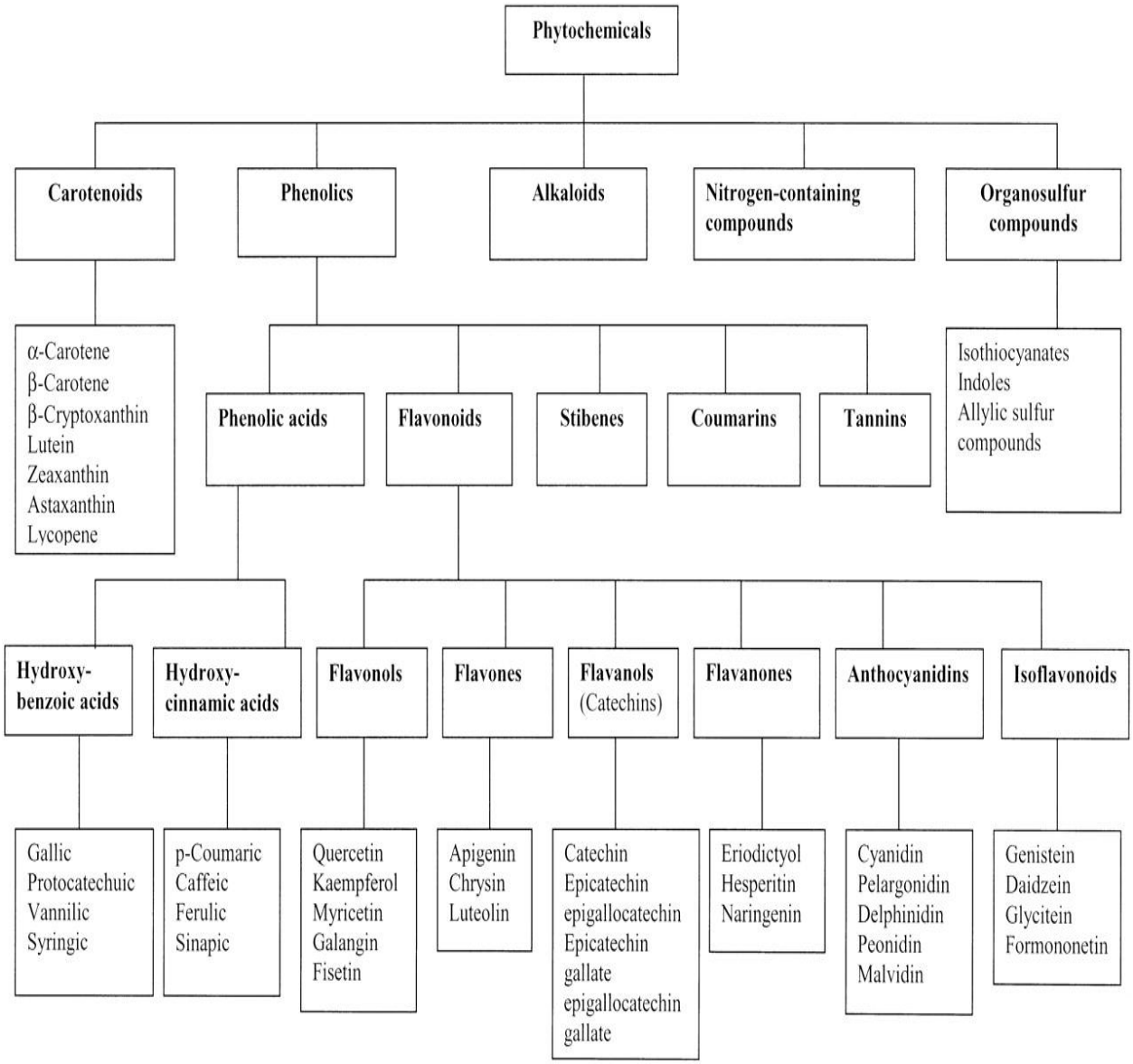


Figure 2.12. Classification of dietary phytochemicals (Liu, 2004)

Table 2.4. Disease-preventing phytochemicals in plant foods

Specific foods/food groups	Main health promoting chemical	Useful in the prevention of
Whole cereal, grains	Dietary fibre, tocopherols†	Cancer, diabetes, cardiovascular disease, hypercholesterolaemia
Vegetables, fat	α-linoleic acid, linoleic acid, tocopherols,†	Cancer, diabetes, cardiovascular disease, sterols hypercholesterolaemia
Palm oil	Tocopherols,† tocotrienols,† carotenoids	Cancer, heart diseases, atherosclerosis, cataract, pulmonary diseases, muscle injury
Yellow or green leafy vegetables, yellow fruits	Carotenes,† ascorbic acid,† dietary fibre, omega-3 fatty acids, pectins	Cancer, heart diseases, atherosclerosis, cataract, pulmonary diseases, muscle injury
Rice bran oil	Sterols, PUFA	Hypercholesterolaemia, diabetes, cardiovascular disease
Linseed oil, fish oil	Omega-3 fatty acid	Hypercholesterolaemia, diabetes, cardiovascular disease
Spices, fenugreek seeds, turmeric	Gums, curcumin, eugenol, capsicin	Cancer, cardiovascular disease, detoxification of drugs and toxins

†Antioxidant. PUFA, polyunsaturated fatty acid. (Narasinga Rao, 2003)

Table 2.5. Bioactive phytochemicals in foods

Classification	Main groups of compounds	Biological function
NSA	Cellulose, hemicellulose, gums, mucilages, pectins, lignins	Water holding capacity, delay in nutrient absorption, binding toxins
Antioxidants	Polyphenolic compounds, flavonoids, carotenoids, tocopherols, ascorbic acid, anthocyanine, phenolic indoles	Oxygen free radical quenching, inhibition of lipid peroxidation
Detoxifying agents	Reductive acids, tocopherols, phenols, indoles, aromatic isothiocyanates, coumarins, flavones, diterpenes, carotenoids, retinoids, cyanates, phytosterols, methyl xanthines, protease inhibitors	Inhibitors of procarcinogen activation, inducers of drug metabolising enzymes, binding of carcinogens, inhibitors of tumourogenesis
Others	Alkaloids, volatile flavour compounds, biogenic amines, terpenoid and other isoprenoid compounds	Neuropharmacological agents, Anti-oxidants, cancer chemoprevention

NSA, non-starch polysaccharides (Narasinga Rao, 2003)

2.12.1. Phenolics

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally are categorized as phenolic acids, flavonoids, coumarins and tannins. Phenolics are the products of secondary metabolites in plants, providing essential functions in the reproduction and the growth of the plants; acting as defense mechanisms against pathogens, parasites and predators as well as contributing to the color of plants (Harborne and Williams, 2000). Polyphenols are found ubiquitously in plants and their regular consumption has been associated with a reduced risk of a number of chronic diseases, including cancer, cardiovascular disease (CVD) and neurodegenerative disorders (David *et al*., 2010).

The increased interest in polyphenols in the past decade has been brought by results from epidemiological studies linking the consumption of diets rich in plant foods with decreased risk of diseases associated with oxidative stress, such as cancer and cardiovascular disease (Scalbert *et al*., 2005).

Several hundred molecules with polyphenol structure (i.e. benzene rings with one or more hydroxyl groups) have been identified in edible plants (Manach *et al*., 2004). Fruit and beverages, such as tea, red wine and coffee are the main sources of polyphenols, however, vegetables, cereals and leguminous plants are also good sources (Manach *et al*., 2004). It is estimated that flavonoids account for approximately two thirds of the phenolics in our diet and the remaining one third are from phenolic acids.

2.12.2. Flavonoids

Flavonoids are a group of phenolic compounds with antioxidant activity that have been identified in fruits, vegetables and other plant foods and that have been linked to reducing the risk of major chronic diseases. They commonly have a generic structure consisting of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring or C rings (Figure 2.13). Differences in the generic structure of the heterocycle C ring classify them as flavonols, flavones, flavanols (catechins), flavonones, anthocyanidins and isoflavonoids (Figure 2.14). Human intake of all flavonoids is estimated at a few hundred milligrams to 650mg/d.

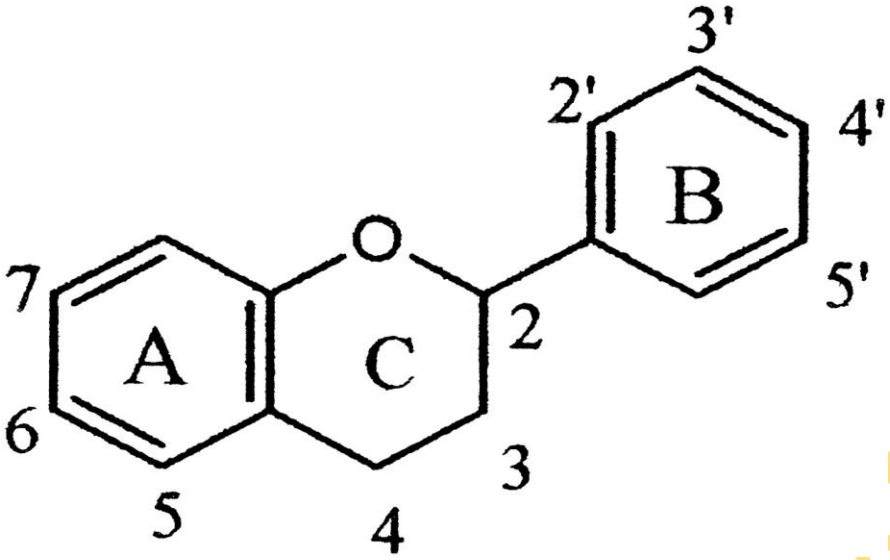


Figure 2.13 The generic structure of Flavonoids. (Liu, 2004)

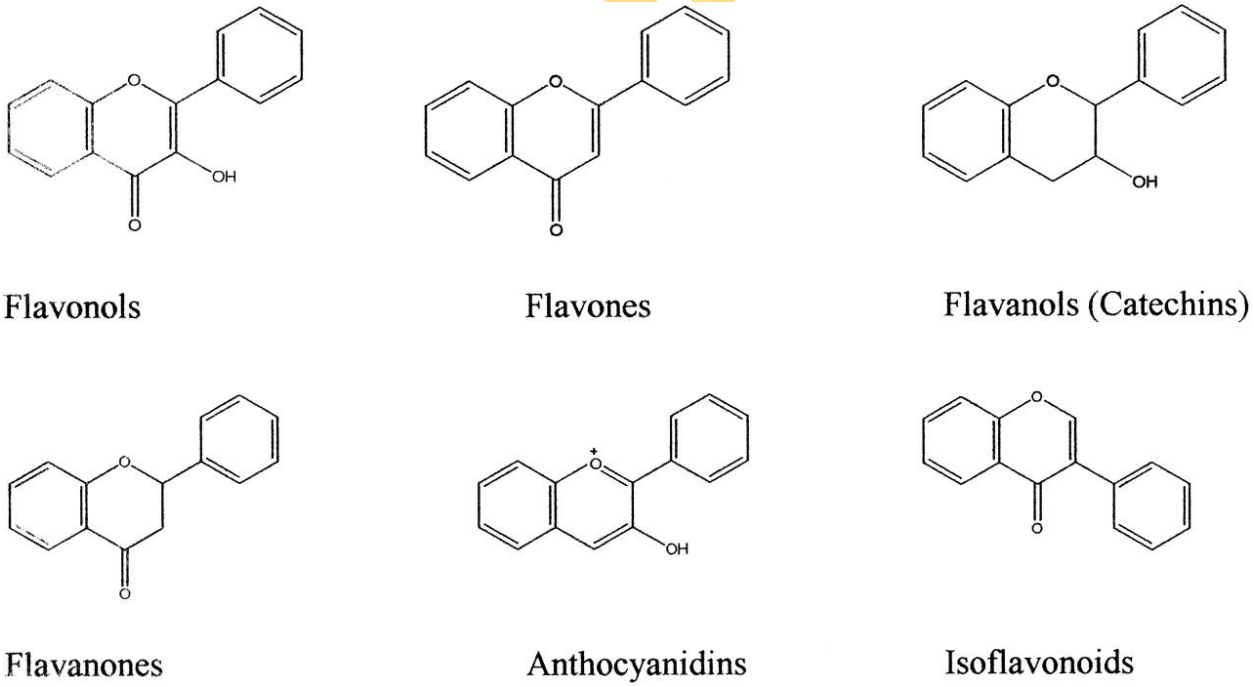


Figure 2.14 Structures of main classes of dietary Flavonoids. (Liu, 2004)

2.12.3. Phytochemical composition in Amaranth.

The pseudocereals amaranth, quinoa and buckwheat have attracted much interest in recent years because of their excellent nutrient profile. They are important energy sources due to their starch content and provide good quality protein, dietary fat, minerals and unsaturated fats (Alvarez-Jubete *et al*., 2010). Moreover, they contain significant amounts of other bioactive components such as phytosterols, squalene and polyphenols (Berghofer and Schoenlecher, 2002; Taylor and Parker, 2002). Unlike its relative quinoa, amaranth does not contain high amounts of bitter saponins that must be washed away before consumption (Tapia, 1997). Low levels of saponin around 0.1% of total seed dry weight that have been observed for *A. cruentus* showed low toxicity in animal tests (Oleszek *et al*., 1999). Furthermore, amaranth grain shows low levels of some other antinutrients. For example, Lorenz and Wright (1984) studied the tannin and phytate content of *A. hypochondriacus, A. cruentus, A. hybridus* and some interspecific crosses and found that tannins were localized in seed coat and were present at 0.04-0.12%, while phytates dispersed throughout the kernel were observed at 0.5-0.6%. However, amaranth seeds and leaves are known to accumulate high levels of trypsin inhibitors as well as α-amylase inhibitors (Sanchez-Hernadez *et al*., 2004).

2.12.4. Phytochemicals: their role in the prevention of cancer

Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These agents may be present in air, food and water or they may be produced by metabolic activity within cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions. Over production of oxidants can cause an imbalance leading to oxidative stress, especially in chronic bacterial, viral and parasitic infections (Liu and Hotchkiss, 1995).

Oxidative stress can cause oxidative damage to large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk for cancer and cardiovascular disease (Ames and Gold, 1991; Ames *et al*., 1993). It is currently believed that reactive oxygen species (ROS) have an important role in the aetiology of several non communicable diseases. Oxidants and free radicals such as singlet molecular oxygen ($-O_2$), superoxide (–O), hydroxyl (OH), peroxide (O-O-H) and lipid peroxides (LOO) are known to cause tissue damage (Packer and Glazer, 1990; Halliwell, 1996).

Such free radicals also include nitrous oxide radicals that are generated in the gastrointestinal tract. Tissue damage caused by free radicals, when it becomes cumulative, is considered to play an important role in the pathogenesis of several degenerative diseases, for example, cancer, cataract, coronary heart disease, dementia, diabetes mellitus, rheumatic arthritis, muscular degeneration, pulmonary dysfunction and radiation sickness. Lipid peroxidation of membrane lipids, circulating lipoprotein lipids (including cholesterol), oxidative damage of cellular proteins and DNA, and lens proteins in the retina are all considered mechanisms by which the oxygen free radicals and peroxides lead to these diseases. These oxidants and free radical species are generated in cells during utilisation of oxygen, which is essential for life's sustenance, and they can also be derived from external sources (Narasinga Rao, 2003).

There are, however, protective mechanisms within the body that protect against dangerous oxygen free radical species generated *in situ*, and those derived from external sources. Superoxide dismutase (SOD) can convert the O_2 radical to hydrogen peroxide, which can be further destroyed by catalase. Glutathione reductase, for which riboflavin is the coenzyme, is also a part of the *in vivo* defence system against oxidation damage in the cells. Selenium too plays a role in this system through glutathione peroxidase. This *in vivo* system of detoxifying oxygen free radicals may not be capable of neutralizing all of the free radicals produced in the body as well as those derived from the environment, and there is therefore a need for an external source of antioxidants to neutralise the free radical load in the body (Narasinga Rao, 2003).

A large number of antioxidants, both nutritive and non-nutritive, occur in foods and need to be consumed to prevent or slow the oxidative stress induced by free radicals. Fruits, vegetables and whole grains contain a wide range of antioxidants such as β-carotene, vitamin C, vitamin E, carotenoids, phenols and flavonoids. Vitamin C and vitamin E prevent formation of nitrosamine, which is carcinogenic. Vitamin E also protects selenium against reduction and protects Polyunsaturated Fatty Acids (PUFA) in the membrane against oxidative damage. Spices are also rich in phenolic compounds that have been shown to act as antioxidants. Turmeric (*Curcuma domestica*), which is widely used in Indian cooking, contains a yellow colouring principle, curcumin, which is a powerful antioxidant and can offer protection against cancer (El-Demerdash *et al*., 2009). The total antioxidant potential of a food or diet can be determined by its capacity to prevent lipid peroxidation in an *in vitro* system. However, the potency of antioxidants

present in foods *in vivo* will depend not only on their levels in the foods but also on their bioavailability, that is, the extent to which the active forms of antioxidants are released from the food and absorbed through the gut. Some flavonoids and phenolic antioxidants are rather poorly absorbed; they often form insoluble complexes with metals such as iron. Their antioxidant potency will also depend on the oxidant level in the food, for example, high PUFA content, which is prone to lipid peroxide formation, can reduce the antioxidant potency of a food. The beneficial influence of antioxidants and antioxidant-rich foods in the prevention of diseases such as cancer has been studied extensively through *in vitro* cell culture, animal and human studies. The association between the intake of dietary antioxidants and protection against non-communicable diseases in humans (cancer, cardiovascular diseases, and cataract) has been largely based on epidemiological studies. However, there have been more systematic human studies involving nutrient antioxidants like vitamin A (β-carotene), vitamin C and selenium in the prevention and treatment of cancer caused by several carcinogenic agents. Based on these studies, the amount of these antioxidants recommended for cancer prevention is 2–20 times the recommended dietary allowance (RDA) for their vitamin function. There have been several recent epidemiological studies that implicate dietary antioxidant phytochemicals like carotenoids (Krinsky, 1993; Packer, 1964), phenolic compounds (Harborne, 1964) and flavonoids (Kuhnau, 1976) as protective agents against cancer and cardiovascular disease. Zeigler *et al.*, (1992) have shown that intake of carotenoids besides β-carotene, like lutein, zeaxanthin and lycopene, through fruits and vegetables resulted in elevated levels of blood carotenoids. This, in turn, was associated with a reduced risk of lung cancer. Hertog *et al.*, (1993) have shown in an epidemiological study in the Netherlands that regular intake of flavonoids (quercetin and kaemferol) by the elderly, through vegetables and fruit (26 mg/day), reduces the risk of death from coronary heart disease.

These flavonoids act by scavenging superoxide anions, singlet oxygens and lipid peroxyradicals, and through sequestering metal ions that promote oxyradical formation. Quercetin, the major flavonoid, inhibits oxidation and cytotoxicity of low-density lipoprotein. Flavonoids also inhibit cyclooxygenase, leading to lower platelet aggregation and reduced thrombotic tendencies.

Polyphenols are widely common secondary metabolites of plants, the content of which varies greatly between different species, and cultivars, and with maturity, season, region and yield.

Polyphenols are classified according to their structure as phenolic acids derivatives, flavonoids, stilbenes or lignans (Harborne, 1988). They are present in many beverages (e.g. red wine and green tea) and foods (e.g. chocolate, grapes, and apples). Several recent studies have reported physiological functionalities of polyphenols (Frankel *et al*., 1993; Hertog *et al*., 1993; Eberhardt *et al*., 2000; Richelle *et al*., 2000). For example, epidemiological studies have indicated that the consumption of red wine might prevent coronary heart disease, because it contains polyphenols that protect against the oxidation of LDL cholesterol. These several findings have led to extensive research on the polyphenol content of human foods and beverages (Shoji *et al*., 2004). The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities (Liu, 2004).

2.13. CANCER

Cancer refers to a group of diseases that are associated with a disturbance in the control of cell growth and metabolism (Hanahan *et al*., 2000, David *et al*., 2010). Indeed, the unbalanced control of cellular proliferation is a primary characteristic of cancer cells and as such, any molecule capable of inhibiting cancer cell proliferation may also be useful as a potential chemo-preventive agent (D'Archivio *et al*., 2008). Cancer remains a leading cause of death in Europe. Each year there are 2.7 million new cases and 1.7 million deaths from the disease (Ferlay *et al*., 2007). In recent years, age-standardised cancer incidence has generally been declining in most European countries, with the notable exception of lung cancer in women, which is continuing to increase and reflects trends in cigarette smoking. It has long been recognised that diet and lifestyle factors can influence the risk of cancer, with associations with some forms of cancer being stronger than with others. It has been estimated that approximately 30% of cancers could be prevented by dietary means in European countries (Key *et al*., 2002). Excess body weight and physical inactivity are estimated to account for approximately 20-30% of the incidence rate of those cancers commonly found in developed countries (Key *et al*., 2004).

Cancer is the common term for all malignant tumors. Malignant cells are characterized by; unrestrained growth, invasion of local tissues and potential metastasis to other tissues of the body (Murray, 1990). The process of Carcinogenesis, which is the transformation of normal cell into initiated cells subsequently leading to cancer cell, can be divided into three stages namely;

(1) Initiation; during which normal cells are initiated after exposure to a carcinogen.
(2) Promotion; a secondary effect following initiation that results in enhancement of cellular growth and division.
(3) Progression; during which already initiated cells continues to develop.
(4) Metastasis; during which malignant cells spread from initial site to invade other sites (Miaskowski and Buchsel, 1999).

Carcinogens are cancer causing substances which damage DNA and induce transmittable mutations and cancer in both humans and animals (Boutrif, 1995). Their effect arises from their interaction with tissues macromolecules such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) thereby altering the replication and repair mechanisms of DNA (Ames, 1979). They can be either genotoxic or non-genotoxic based on their mechanisms of cancer induction.

Genotoxic carcinogens are those for which a primary biological activity of chemical (or a metabolite) leads to alteration in the information encoded in DNA (Butterworth, 1990)

Non-genotoxic carcinogens are those that can cause cancer without causing damage or alteration to the DNA (Butterworth, 1990).

Generally, carcinogens are broadly classified into three major groups: biological carcinogens, physical carcinogens, and chemical carcinogens.

Biological Carcinogens: These are mainly oncogenic viruses. These viruses are of two types: the DNA tumor viruses e.g. hepatitis B virus, papilloma virus and the RNA tumor viruses also called retroviruses e.g. hepatitis C virus (Laterjet, 1960). Some parasites are also implicated as biological carcinogens e.g *Schisotoma haematobium* and *Clonorchis sinensis*.

Physical Carcinogens: These are mainly ionizing radiations such as X-rays or gamma radiations (Heuper, 1961) and atomic particles e.t.c. Physical carcinogens are known to cause cancers in man and animals, such cancers include skin carcinoma, ovarian, pulmonary and leukemia (Darnell *et al.*, 1990).

Chemical Carcinogens: These are classified into:

Direct acting (ultimate carcinogen): these chemical carcinogens are highly reactive compounds which do not require metabolic activation to cause cancer but instead react directly with cells and induce cancer e.g. nitrogen benzyl chloride (Currie and Currie, 1982).

Indirect acting: (pro-carcinogen): these chemical carcinogens require metabolic activation in order to become active e.g. poly aromatic hydrocarbons (PAH).

Co-carcinogens: (promoting agents): These are chemical carcinogens that show little or no intrinsic activity of direct acting carcinogens or pro-carcinogens (Currie and Currie, 1982).

2.13.1. MOLECULAR BASIS OF CARCINOGENESIS

The molecular basis of carcinogenesis is found in the defect or even absence of highly important genes that are involved in regulation of cell growth and apparently those involved in cell death that is growth promoting (proto-oncogenes), growth inhibiting (tumor suppressor genes), anti-oncogenes and genes controlling apoptosis (Bishop, 1995).

- Oncogenes; these are derived from proto-oncogenes. They can either be viral oncogenes (v-onc) or cellular oncogenes (c-onc). Oncogenes encode protein products called oncoproteins which resemble the normal products of the proto-oncogenes, with the exception that oncoproteins are devoid of important regulatory elements and their production in the transformed cells does not depend on growth factors or other external signals (Weinberg, 1989). Oncogenes products include; growth factors (c-sis), growth factor receptors (v-erb B), signal-transducing protein (ras protein) and nuclear regulatory protein (c-myc) (Weinberg, 1989).
- Tumour suppressor gene; these are genes whose products apply ‘brake’ to cell proliferation. Mutation or deletion of these genes is like brake failure, permitting unrestrained cellular growth. Examples of these genes include; retinoblastoma (Rb) gene, p53 gene, adenomatous polyposis coli (APC) gene and necrosis factor-1 (NF-1) gene (Russo and Russo, 1998).

- Genes regulating apoptosis; Genes that prevent or reduce programmed cell death are also important in carcinogenesis. The prototypic gene in this category is bcl-2 and it is found to be over-expressed in certain cancers (Korsmeyer, 1992).

2.13.2. FACTORS INFLUENCING CARCINOGENESIS

Carcinogenesis is a protracted process that depends upon two main factors:

- Internal factors
- External factors.

INTERNAL FACTORS

These include immune function and genetic predisposition (Ames *et al*., 1995). Several immune agents such as macrophages, natural killer cells, helper T lymphocytes, recognize some strange cell surface antigens on malignant cells and therefore destroy them, such that in the immunocompromised people cancer develop more freely (Cooper, 1995). Genetic predisposition is also noteworthy as certain factors like the location of specific proto-oncogenes within the genome is different and may provide an increased susceptibility to mutation or activation (Cooper, 1995). Also, the position of the oncogene may be normal but the genes controlling the oncogenes activity, the suppressor (or regulatory) gene may be abnormal or out of place (Calzone, 1997). BRCA1 and BRCA2 are both ubiquitous cell cycle-regulated proteins that localize to the nucleus in normal cells. Heterozygous carriers of loss-of-function germline mutations in the *BRCA1* or *BRCA2* breast cancer susceptibility genes have a predisposition to breast and ovarian cancer (Vidarsson *et al*., 2002). Persistently increased blood levels of estrogen are associated with an increased risk of breast cancer, as are increased levels of the androgens androstenedione and testosterone (which can be directly converted by aromatase to the estrogens estrone and estradiol, respectively). Increased blood levels of progesterone are associated with a decreased risk of breast cancer in premenopausal women. A number of circumstances which increase exposure to endogenous estrogens including not having children, delaying first childbirth, not breastfeeding, early menarche (the first menstrual period) and late menopause are suspected of increasing lifetime risk for developing breast cancer (Yager and Davidson, 2006).

EXTERNAL FACTORS

External (environmental) factors may be physical (radiation), biological (viral) or chemical (as with clastogens like arsenic). (Chai *et al*., 2003)

2.14. CLASTOGENS AND CLASTOGENESIS

A clastogen is a material that can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. Therefore, Clastogenicity is described as the microscopically visible damages or changes to chromosomes (Testoni *et al*., 1997). This can cause a mutation, and lead to cancer development, as cells that are not killed by the clastogen may become cancerous. Known clastogens include acridine yellow, benzene, ethylene oxide, arsenic, phosphine and mimosine (Testoni *et al*., 1997).

Clastogens generally have low molecular weight (< 10,000 Daltons). Apart from causing chromosomal breakage, they can cause gene mutation, sister chromatid exchanges and other chromosomal aberrations. They were first described in the plasma of irradiated persons, but they are also found in hereditary breakage syndromes and chronic reactions (Testoni *et al*., 1997).

Clastogenesis is the formation of chromosomal aberrations in the cell, DNA strand breakage and gene mutation by the agents called clastogens (Testoni *et al.*, 1997)

Some compounds have been identified to be carcinogenic, mutagenic as well as being clastogenic. For instance, Melq-2-amino-3,4-dimethylimidazo-(4,5-F) quinoline is a potent clastogen and carcinogen having been shown to develop fore-stomach and liver tumor (Ramsey *et al*., 1995). Also 2-acetylaminofluorene, besides acting as a mutagen has the capacity of causing chromosomal damage (Ames *et al*., 1972). During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. This process is disrupted by clastogens, which also cause chromosomal damage. When this occurs, the genetic material is not incorporated into a new nucleus, and then may form its own micronucleus which is clearly visible with a microscope (NTP, 2001). A positive correlation between the degree of clastogenicity and carcinogenicity has been established (Natarajan, 1984). Another potent and common clastogen is arsenic which may be present in water as contaminants

and is an environmental pollutant (Odunola *et al*., 2011; El-Demerdash *et al*., 2009; Chowdhury *et al*., 2001; Chiou *et al*., 1995).

2.15. ARSENIC

Arsenic is a naturally occurring element found in any of four valence states: -3 (arsine), 0 (elementary arsenic), +3 (arsenites), and +5 (arsenates). Elementary arsenic is a grey metallic-looking crystalline powder; arsine is a colorless gas; arsenites and arsenates are white crystalline powders. The term "arsenic" is used when the valence state is not specified and generally refers to arsenite and/or arsenate. The alkali salts are highly soluble in water but the calcium and lead salts are not. The pentavalent form, As^{5+} (arsenate or organic form) is less toxic than trivalent form, As^{3+} (arsenite or inorganic form) based on lower solubility. Both forms are found in arsenic-contaminated water, and they are inter-convertible once absorbed. Arsenic belongs to the group V elements of the periodic table and has atomic number of 33 and mass number of 76 (Chemistry world, 2008; Hughes, 2002).

In chemistry arsenite is a chemical compound containing an arsenic oxoanion where arsenic has oxidation state +3. Examples of arsenites include sodium arsenite which contains a polymeric linear anion, $[AsO_2^-]_n$, silver arsenite, Ag_3AsO_3, which contains the trigonal, AsO_3^{3-} anion, sometimes called ortho-arsenite (Greenwood *et al*., 1997). In fields that commonly deal with ground water chemistry, arsenite are commonly referred to as As_2O_3.

Compounds in this class are acutely toxic, carcinogenic, teratogenic, and mutagenic. They are readily absorbed by various body tissues through the skin, respiratory and intestinal tracts, and transplacentally. They may cause severe irritation of tissues (skin, eyes, mucous membranes, and lungs). All arsenic compounds are moderately toxic (arsine is highly toxic), mutagenic in some but not all test systems, and teratogenic (ECRP, 1988).

Major uses of arsenic in various forms are as pesticides (insecticides, herbicides, and sheep and cattle dips) and in drugs. Sodium arsenite is used in the water gas shift reaction to remove carbon dioxide.

2.15.1. FORMS AND OCCURRENCE

Arsenic compounds are ubiquitous in nature and most metallic ores contain small quantities. Mispickel or arsenical pyrites ($FeS_2.FeAs_2$), the sulphides realgar (As_2S_2) and Orpiment (As_2S_3) form the natural sources of arsenicals (Clarke and Clarke, 1975). Arsenic trioxide is normally produced in the roasting of metallic ores. This may be carried as a dust along with the smoke, and hence serve as a contaminant of soil, herbage and waste.

ARSENIC TRIOXIDE (As_2O_3)

This is most common in general use. It occurs as an amorphous or crystalline, tasteless and odorless, white powder. It is sparingly soluble, volatile on heating, forming distinct glistering octahedral crystals on sublimation. It resembles flour and French chalk, which has led to several accidents. Copper arsenite, which was initially used as a cheap pigment for coloring wall-papers and artificial flowers, was stopped due to deaths that resulted from its use. However, it is being used as an insecticide antihelminthic and slug bait (Clarke and Clarke, 1975).

SODIUM ARSENITE ($NaAsO_2$)

Sodium arsenite (Sodium meta arsenite) is a trivalent inorganic compound belonging to general class of compound called arsenicals. It is toxic and the most effective agent of the arsenicals (Hodgson *et al*., 1988). Sodium arsenite is produced from arsenious acid (As_2O_3) by reacting with alkali such as arsenite and water (Lenihan and Fletcher, 1977).

$$As_2O_3 + H_2O \longrightarrow 2HAsO_2$$

$$As_2O_3 + 2NaOH \longrightarrow 2NaAsO_2 + H_2O$$

Sodium arsenite is structurally represented as;

$$O = As - O^- \quad Na^+$$

2.15.2 SOURCES OF ARSENIC COMPOUNDS/EXPOSURE AND USES

Arsenic being a potent environmental toxic agent, leads to development of various hazardous effects on human health. All human populations are exposed to arsenic and its compounds through occupational or environmental processes (Ramanathan *et al*., 2003). More than 80% of arsenic compounds are used to manufacture products like glass and semiconductors, dyestuffs and as an additive to metal alloys. Also, it is used in agricultural applications such as insecticides, herbicides, fungicides, algaecides, sheep dips and wood preservatives (Tchounwou *et al*., 2002). Low level arsenic exposure continues to take place in the general population (as do some cases of high dose poisoning) through the commercial use of inorganic arsenic compounds in common products, such as wood preservatives, ant-killers, herbicide and fungicides. As well as through the consumption of foods and smoking of tobacco treated with arsenic-containing pesticides and through burning of fossil fuels in which arsenic is a contaminant (Jain and Ali, 2000).

Human exposure to arsenic is associated with cancer, organ injury and immunotoxicity (Patrick, 2003). There is an evidence suggesting that arsenic toxicity involves oxidative damage (Izquierdo-Vega *et al*., 2006), mainly by the interaction of arsenic with protein thiols that are central components of redox-sensitive proteins in redox signaling and control pathways (Hansen *et al*., 2006). Several studies have demonstrated that liver is the primary arsenic metabolizing organ (Hughes *et al*., 2003). Metabolic conversion of arsenic into methylated products is a multistep process that yields mono–di and trimethylated arsenic forms that have different toxic potential than that of the parent compound arsenic. Many biological processes have been identified as involved in arsenic-induced toxicity and carcinogenicity. These include induction of micronuclei, alterations in gene expression, induction of oxidative stress, alteration in enzyme activities, change in carbohydrate metabolism, inhibition of DNA repair, perturbation of DNA methylation, alteration of signal transduction pathways, altered cell cycle control, aberrant differentiation, and altered apoptosis (Kitchin, 2001; Manna *et al*., 2007; Bagnyukova *et al*., 2007). Previous studies in our laboratory has also shown that arsenite induced toxicity in rats has been reduced by administration of extracts of garlic and green tea known to exhibit hepatoprotective and clastogenic activity (Odunola *et al*., 2008, Gbadegesin *et al*., 2009).

Unbound inorganic arsenic generates reactive oxygen species (ROS) during redox cycling and metabolic activation processes (Bashir *et al*., 2006). Various studies reported that arsenic could participate in the cellular oxidation–reduction reactions resulting with the formation of excess ROS such as superoxide anion (O_2) and hydroxyl radical (OH.) via a chain reaction (Liu *et al*., 2001; Garcia-Shavez *et al*., 2006) causing oxidative stress. Toxic effects of arsenic are mediated primarily by triggering the production of reactive oxygen species (ROS), inhibiting the activity of enzymes like superoxide dismutase and catalase, leading to alterations in cells' intrinsic antioxidant defenses; and resulting in oxidative stress or disturbed antioxidant/pro-oxidant ratio (Liu *et al*., 2001). Endogenous antioxidants, including vitamins, trace minerals, antioxidant enzymes, tripeptides and reductants, may quench reactive oxygen species (ROS) or suppress lipid peroxidation.

2.16. BIOLOGICAL EFFECTS

2.16.1. ARSENIC ABSORPTION AND FATE

The rate of absorption of inorganic arsenicals from digestive tract depends upon their solubility. Sodium arsenite is readily soluble, rapidly absorbed and highly toxic (Calesnick *et al.*, 1966). The soluble arsenic compounds get easily absorbed by contact with intact skin and absorption from a fresh wound is very rapid.

Accumulation of arsenic is seen in the liver with slow release and distribution to other tissues. Continued administration can cause its disappearance from soft tissues and its long term storage in bones, skin and keratinized tissues (for example, hair and hoof) (Grollman and Slaughter, 1947). Arsenic deposited in hair is irremovable and moves slowly along the hair as it grows. Arsenic is excreted in the urine, faeces, sweat and milk. The rate of excretion depends on the compound and it is inversely related to the toxicity (Sabeh *et al*., 1993).

Pentavalent arsenic is well absorbed through the gut, but the trivalent form is more lipids soluble. Toxicity results from the arsenite form (As^{3+}), especially by dermal absorption. Arsenic compounds are well absorbed peritoneally within 24 hours. Sodium arsenite is readily soluble, rapidly absorbed and highly toxic (Sabeh *et al*., 1993).

Arsenites and arsenates are absorbed by ingestion and peritoneal injection; sodium arsenite, arsenic trioxide, and arsine are absorbed by inhalation. Sodium arsenite and arsenate are absorbed through the placenta.

2.16.2 DISTRIBUTION

Sodium arsenite is distributed to other tissues from the liver where it accumulates and excess can be stored in bone, skin, and keratinized tissues such as hair and hooves. Arsenic initially localizes in the blood bound to globulin. Redistribution occurs within 24 hours to the liver, lungs, intestinal wall, and spleen, where arsenic binds to sulfhydryl groups of tissue proteins, only small amounts of arsenic penetrates the blood-brain barrier (Winsk and Carter, 1995). There is also a significant accumulation of arsenate in the skeleton, presumably by exchange with phosphate (Lindgren *et al*., 1982). Application of pentavalent arsenic to skin results first in an accumulation of arsenic in the skin, followed by distribution to other organs, followed by urinary excretion. Significant deposition in hair and nails has been demonstrated in man and animals.

2.16.3 BIOTRANSFORMATION

Arsenic is biotransformed *in vivo* by methylation to monoethyl and dimethyl arsenic. Dimethylarsenic is the principal transformation product and it appears to be the terminal metabolite which is rapidly formed. Dimethylarsenic promotes lung and skin tumor by way of the metabolic production of free radicals such as dimethyl arsenic peroxyl radical [$(CH_3)_2AsO$]. Dimethylarsenic may play an important role in arsenic carcinogenesis through induction of oxidative damage, particularly of base oxidation especially in the target organs of arsenic carcinogenesis; skin, lung, liver, and urinary bladder (Jayanthika *et al*., 2001). Sodium arsenite produces alteration in the RNA and DNA synthesis in cells, this inhibition is said to be dose-dependent (Goering *et al*; 1999).

The possible mechanism of this cytotoxic effect of arsenite might be due to its reactivity with intracellular Sulfhydryl groups.

The biotransformation of arsenic is based on several generally accepted steps. For methylation to occur arsenate (As^{5+}) species must be reduced to arsenite (As^{3+}) in a process that occurs through reactions

involving glutathione (GSH). The arsenite is then methylated to monomethylarsonic acid (MMA). The MMA is then methylated to dimethylarsonic acid (DMA) (Goering *et al*., 1999).

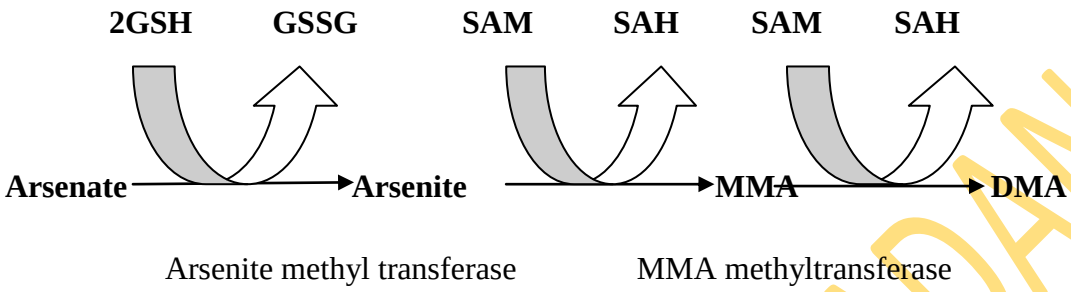


Figure 2.15: The biotransformation pathway for arsenic (Goering *et al*., 1999)

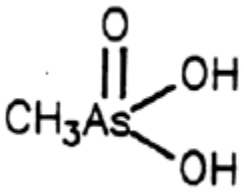
Oxidative addition of methyl groups to arsenic occurs by methyltransferase enzymes, with S-adenosyl-methionine (SAM) as the methyl-donating cofactor (Aposhian *et al*., 1997).

In addition to the pentavalent metabolites, the trivalent metabolites, monomethylarsonic acid [MMA (111)] and dimethylarsonic acid [DMA (111)], has been identified as intermediary metabolites in the methylation of arsenic compounds and have been detected in cultured human cells treated with inorganic arsenic (Thomas *et al*., 2001). Mandal *et al.*, (2001) reported the presence of MMA (III) and DMA (III) in the urine of people chronically exposed to inorganic arsenic via drinking water in West Bengal, India.

2.16.4. EXCRETION

The pathways of arsenic metabolism vary with the type of arsenic compound administered, route of administration, and animal species. Some aspects have been reviewed (Peoples, 1983). Urinary and fecal excretion products are inorganic arsenic and the result of successive methylation to Monomethylarsonic Acid (MAA) and Dimethylarsonic Acid (DMA) in most species including man (Marafante *et al*., 1985).

Monomethylarsonic Acid (MMA)



Dimethylarsonic Acid (Cacodylic acid, DMA)

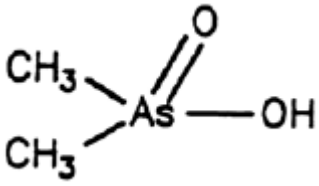


Figure 2.16: Structure of arsenic metabolism products.

Source: http://pubs.rsc.org/en/content/articlelanding/2008/ja/b713145c. Accessed 23rd Feb, 2011.

2.17. TOXIC EFFECTS

The toxicology of arsenic is a complex phenomenon as arsenic is considered to be an essential element also. Two types of toxicity are known from long time, acute and chronic. The acute arsenic poisoning requiring prompt medical attention usually occurs through ingestion of contaminated food or drink. The acute toxicity of arsenics is related to its chemical form and oxidation state. A basic tenet is that the acute toxicity of trivalent arsenic is greater than pentavalent arsenic (Hughes, 2002). In general, arsenites are much more toxic than arsenates; the oral LD_{50} of arsenates in rats and mice is about 100 mg/kg and that of arsenites about 10 mg/kg; the acute oral LD_{50} of arsenic trioxide is 15 mg/kg in rats and 39 mg/kg in mice. Although rare, acute poisoning is reported in children (Gullen *et al.*, 1995). Acute and chronic effects of arsenic intoxication in man have been summarized (IARC, 1980). The major early manifestation due to acute arsenic poisoning include a burning sensation of mouth and throat (Done and Peart, 1971; Armstrong *et al*., 1984); metallic, garlicky odor of breath and faeces (Chatterjee *et al*., 1995; Kiping, 1977); difficulty in swallowing (Fairchild *et al*., 1977); vomiting (Mueller and Benowitz, 1989); diarrhea and cyanosis (Jain and Ali, 2000). Chronic effects include hyperpigmentation

and keratosis (characteristics of prolonged treatment with Fowler's solution) (Yeh *et al*., 1968; Pinto and Mcgill, 1953; Cebrian *et al*., 1983), vascular effects ("blackfoot disease") (Pershagen 1983; Tseng, 1977), cirrhosis of the liver (and effects on the hematopoietic system (Lerman *et al*., 1980). The chief toxic effect of inhaled arsenic is due to its binding to hemoglobin, resulting in extensive hemolysis and hematuria followed by jaundice; the usual cause of death is renal failure (Gullen *et al.,* 1995).

2.17.1. BASIS OF ARSENITE-INDUCED TOXICITY

Arsenite interacts with thiol-containing amino acids, peptides and proteins (Winsk and Carter, 1995). Arsenite exerts its cellular toxicity by binding to sulflhydryl groups which results in enzyme inhibition. During arsenic metabolism, oxygen radical may be produced, possibly leading to damage of DNA, proteins, lipids and other molecules. There is a positive correlation between lipid peroxidation and arsenic tissue concentrations in the livers, kidneys and heart of arsenite treated rats (Rasmos *et al.*, 1995). Arsenite induces the body's antioxidant activities in human fibroblasts (Lee *et al*., 1995). It induces heme oxygenase, leading to the heme degradation, iron release and decrease in the cytochrome p450 biotransformation enzymes important in both endogenous and xenobiotic metabolism (Albores *et al*., 1989). Because of arsenite affinity for protein sulfhydryls, many side effects can occur from enzyme inhibition. Chronic arsenite toxicity results in mitochondrial changes that block lipoic acid-dependent dehydrogenase, which in turn inhibits glycolysis and results in demand for glucose and subsequently hypoglycemia (Cobo *et al*., 1995). Arsenicals also inhibit pyruvate dehydrogenase in gluconeogenesis (Szinicz *et al*., 1988). Carbohydrate depletion caused by gluconeogenesis depletion may therefore aggravate arsenic toxicity.

Increasing evidence indicates that arsenic acts on signaling pathways that regulate cell proliferation rather than causing direct DNA damage because arsenic exhibits its mutagenic activities only at concentrations high enough to also produce cell damage (Jacobson-Kram and Montalbano, 1985). Arsenic has been shown to modulate the mitogen-activated protein AP-1 (Cavigelli *et al*., 1996). AP-1 mediates many biological effects of tumor promoters and is an important regulator of cell growth. The ability of arsenic to interact with protein thiol groups on key regulatory proteins and subsequently alters their activities is likely to contribute to this

effects (Cavigelli *et al*., 1996). Accordingly, it has been demonstrated that arsenic can induce a moderate increase in keratinocyte cell proliferation, as evidenced by increases in thymidine incorporation (Germolec *et al*., 1998), cell cycling (Klimecki *et al*., 1997), labeling of the proliferating cell marker Ki-67 (Klimecki *et al*., 1997), ornithine decarboxylase activity (Brown and Kitchin, 1996) and expression of oncogenes and growth factors such as c-*fos*, c-*jun*, c-*myc*, and transforming growth factor (Germolec *et al*., 1998).

2.18. CARCINOGENIC EFFECTS

The International Agency for Research on Cancer (IARC) classify arsenic as a carcinogen for which there is sufficient evidence from epidemiological studies to support a causal association between exposure and skin cancer. Chronic arsenic exposure has also been associated with a greatly elevated risk of skin cancer and possibly of cancer of the lung, liver, angiosarcoma, bladder, kidney and colon cancers (ECRP, 1988). They also detected elevation of serum globulins and development of oesophaged varices at follow up studies. There is also some evidence suggesting changes in choleostatic function of the liver as shown by conjugated hyperbilirubinemia and elevated alkaline phosphatase activity, which directly relates to the concentration of total arsenic in urine (Ellenhorn, 1997).

Pulmonary findings of chronic arsenic toxicity include both obstructive and restrictive patterns of pulmonary function tests (Ellenhorn, 1997). Arsenicals have been shown to cause enzymatic inhibition of the tricarboxylic acid (TCA) cycle (Webb, 1966). Pyruvate dehydrogenase system has been shown to be especially sensitive to trivalent arsenicals because of their apparent interaction with disulphydryl lipoic acid moiety of this system as shown below:

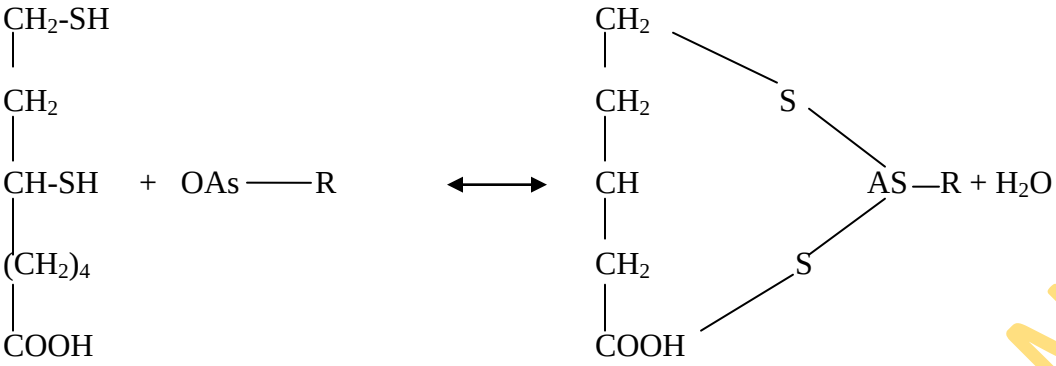


Figure 2.17: Enzymatic inhibition reaction of Sodium Arsenite (Webb, 1966).

Sodium arsenite was found to inhibit methyl thymidine uptake in human cells invitro, consistent suppression of DNA synthesis. Chromosomal aberrations were observed in human leucocyte exposed to sodium arsenite. Arsenic has also been suggested to substitute for phosphorus in DNA, causing a weak bond in DNA chain (Petres *et al*., 1977). The toxicity of trivalent arsenic to animals and human beings has also been thought to be caused by its binding to thiol ions, thus inhibiting enzymatic reactions. As late as 1980 it was believed that arsenic compounds were not carcinogenic in experimental animals, and this conclusion was drawn from a summary of largely negative results (IARC, 1980). Since that time evidence has appeared which indicates carcinogenicity in rats (Ivankovic *et al*., 1979). The evidence for carcinogenicity of arsenic compounds in man is more positive, and this has been reviewed (Landrigan, 1981; IARC, 1980). A correlation was established between the appearance of skin cancer and arsenic concentration in the well water in certain regions of Taiwan (Tseng, 1977). Skin cancers were also noted repeatedly in patients after prolonged treatment with Fowler's solution (potassium arsenite) and in vineyard workers employing arsenical pesticides. Lung cancers have been noted in men involved in the production of arsenicals (Mabuchi *et al*., 1980). Other studies (involving workers in copper smelters and mines) are not as clear-out since exposure to other materials occurred concomitantly.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1 Experiment 1: Evaluation of genetic variation of important agronomic and nutritional traits of twenty nine accessions of grain amaranth.

3.1.1. Plant Materials

Twenty nine accessions of *Amaranthus* belonging to five species *A. caudatus, A. cruentus, A. hybrid, A. hypochondriacus* and *A. hybridus* were used in this study and their descriptions are given in (Table 3.1). Twenty seven of the accessions were obtained from the United States Department of Agriculture – Agricultural Research Station (USDA-ARS), North Central Regional Plant Introduction Station (NCRPIS) in Ames, part of the United States National Plant Germplasm System, USA; and two were obtained from the National Horticultural Research Institute (NIHORT) germplasm, Ibadan.

3.1.2. Expermental Design

Seeds were first raised in trays in the nursery for 21 days before transplanting to the experimental field of National Horticultural Research Institute (NIHORT), Ibadan; in May 2008 and June 2009. The experimental design was a randomized complete block with three replications (Figure 3.1). The plot size of each accession for each replication was 2 x 2 m. The plot to plot distance was 1 m and the plant to plant distance was 50 cm. The total rainfall, temperature and relative humidity for May, 2008 were 234.4mm, 27.9% and 89%, respectively. The total rainfall, temperature and relative humidity for June, 2009 were 183.1mm, 27% and 88%, respectively. Prior to sowing on the beds, the sand was turned to loosen up particles two days before tansplanting. Thinning was done to maintain plant density within rows and normal cultural practices were followed during the experiment as suggested by Shukla *et al*., (2006) and Grubben and Van Sloten, (1981).

Table 3.1 Species and accessions of 29 grain amaranth used in this study and their passport data.

Species	Accession Number[a]	Accession code	Origin	Plant name	Leaf colour	Stem colour	Inflorescence colour	Seed colour
Amaranthus caudatus	A1	P1 490458	Bolivia	LSK 38	Normal green	Pink base to mid stem and green mid stem to tip	Green	Cream+black
	A2	PI 511679	Argentina	RRC 551	Normal green	Green	Golden yellow	Cream
	A3	PI 553073	United States, New Jersey	LOVE-LIES-BLEEDING	Normal green	Pink	Pink	Pink
	A4	P1 642741	Bolivia	Oscar Blanco	Normal green	Pink base to mid stem and green mid stem to tip	Green	Cream
Amaranthus cruentus	A5	PI 477913	Mexico	RRC 1011	Normal green	Green	Green	Cream
	A6	PI 511719	Guatemala	Niqua, alegria, chang	Normal green	Green	Green, Pink, Green with Pink	Cream
	A7	PI 515959	United states, Montana	Montana-3	Normal green	Green	Green	Cream
	A8	PI 538319	United states, Pennsylvania	K266	Normal green	Green	Green	Cream
	A9	PI 590992	China	TIBET	Normal green, with pink veins and margin	Wine, green	wine, green, speckled pink	Cream
	A10	P1 604666	United states, Pennsylvania	RRC 1027	Normal green	Green, Orange	Green, Orange	Cream
	A11	PI 641047	Nigeria, Oyo	CEN/IB/97/AMA008	Normal green with pink veins and margin	Pink	Pink	Cream
	A12	PI 641045	Nigeria, Oyo	CEN/IB/97/AMA005	Normal green with pink veins and margin	Pink	Pink	Cream

[a]Accession Number used for figures in this study.

Species and accessions of 29 grain amaranth used in this study and their passport data (contd.)

Amaranthus hybrid	A13	PI 538325	United states, Pennylsyvania	K593	Normal green with pink vein and margin	Pink	Green, wine	Cream
	A14	PI 538326	United States, Pennysylvania	D70-1	Normal green with pink vein and margin	Pink	Green, wine	Cream
	A15	PI 538327	United States, Pennysylvania	DI36-1	Normal green	Green	Green	Cream
	A16	Ames 1974	Nigeria	RRC 18C	Pink, Green, wine	Green, Pink	Normal green, with pink veins and margin	Black + Cream
	A17	Ames 2256	Nigeria	SP 12C	Pink, green, wine	Green, Pink	Normal green, with pink veins and margin	Black + Cream
	A18	Ames 5644	Nigeria	RRC 1044	Normal green with pink veins and margin	Pink	Wine	Cream
	A19	Ames 5647	Nigeria	RRC 1047	Pink, wine	Green, Pink	Normal green, with pink veins and margin	Black + Cream
Amaranthus hypochondriacus	A20	PI 337611	Uganda	P 373	Normal green	Green	Green	Cream
	A21	PI 511731	Mexico	RRC 646	Normal green with pink veins and margin	Pink	Green	Cream
S	A22	PI 558499	United States, Nebraska	PLAINSMAN	Normal green with pink veins and margin	Pink	Wine	Cream
Nz	A23	PI 590991	China, Shanxi	ZHEN PING	Normal green	Pink	Green	Gold

[a]Accession Number used for figures in this study.

Species and accessions of 29 grain amaranth used in this study and their passport data (contd.)

	A24	PI 615696	India, Himachal Pradesh	Amapurna	Normal green	Green	Green	Cream
	A25	P1 619250	United States, Pennsylvania	K 116	Normal green with pink veins and margin	Pink	Green, wine	Gold
	A26	PI 633596	Nepal	Jumla	Normal green with pink veins and margin	Pink	Pink	Cream
	A27	Ames 1972	Nigeria	RRC 18A	Normal green	Green	Green	Cream
Amaranthus hybridus	A28	NH 84/444-4	Nigeria	NH Purple	Normal green	Purple	Green	Cream
	A29	NAC 3	Nigeria	NH Green	Normal green	Green	Green	Cream

[a]Accession Number used for figures in this study.

Figure 3.1 Experimental field of National Horticultural Research Institute (NIHORT) where the 29 accessions of grain amaranth were planted (June, 2009)

3.2. Methodology

3.2.1. Morphological/Phenotypic characterization

Plants were grown to maturity and characterized based on taxonomic keys using amaranth descriptors as suggested by the International Board for plant Genetic Resources (Grubben and Van Sloten, 1981). A total of 27 characters were collected in this experiment. They include plant height, leaf length, leaf width, number of branches per plant, number of leaves per plant, stem diameter, number of days to 50% flowering, 100-seed weight, and yield per plant. Data from the field was taken at two stages of growth, the first was at the onset of flowering when 50% of the plants had produced infloresence and the second was at plant maturity just before harvesting. Collection of data was based on 10 randomly selected plants per accessions per plot.

Leaf area (LA) was determined using the formula of Pearcy *et al*., (1989).

LA = 0.5(L x W)

L = leaf length; W = leaf width

Other phenotypic parameters that were determined by chemical analyses included moisture content, protein, crude fibre, ash, starch, sugar, crude fat and some minerals (iron, zinc, aluminium, calcium, potassium, phosphorus, magnesium, magnesium, copper and selenium).

3.2.2. Data analysis

Descriptive statistics such as means, standard deviation and coefficient of variation were done on data collected in 2009 for the various quantitative characters using the generalized linear model of SAS (Statistical Analysis System) (2003). The degree of association among the different quantitative characters was done by Pearson's correlation. An estimate of analysis of genetic diversity was carried out using the non-hierarchical k-means technique of cluster analysis to generate a dendogram.

Principal component analysis was computed using PROC PRINCOMP (SAS, 2003) on correlation matrices.

3.3. Chemical analysis

3.3.1. Sample material and preparation

Seeds of the twenty nine grain amaranth accessions harvested at maturity as described in experiment I, were milled into flour to obtain a homogenous particle size material and made to pass through a 40-mesh screen. The milled samples were kept at 4 ^{o}C prior to analysis.

3.3.1.1. Determination of moisture content

Principle: Representative portion of weighed samples were dried at 103±2 ^{o}C for 16 hours in a draft air oven according to the method of AOAC, (1990). The loss in weight was reported as moisture content of that sample.

Procedure

Approximately 2-5 g of flour was weighed into a pre-weighed clean, dry aluminium can. They were dried in an oven maintained at 105 ^{o}C until constant weight was obtained. The dried samples and can were transferred to a desiccator and allowed to cool before weighing. Moisture content was calculated as loss in weight.

Calculation: % Moisture content = $\frac{M_1 - M_2}{M_1 - M_0} \times 100$

Where M_0 = weight of can in g

M_1 = weight of can and sample before drying

M_2 = weight of can and sample after drying

% Dry matter content = 100 - % moisture content

3.3.1.2. Determination of ash content

The determination of inorganic substances as residue after ignition of samples at a specific temperature is the basis of ashing (AOAC, 1990).

Procedure

Two grams of milled flour was weighed into a clean pre-weighed porcelain crucible and pre-ashed by charring on a hot plate inside a fume hood until all the dark fumes were eliminated. The sample was then transferred into a pre-heated muffle furnace at 600 ^{o}C and ashed for 6 hours. The ash content was calculated as follows:

$$\% \text{ Ash} = \frac{\text{Weight of crucible + ash – Weight of empty crucible}}{\text{Weight of sample}} \times 100$$

3.3.1.3. Determination of free sugars and starch content

Principle: For the quantitative determination of free sugars and starch (Dubois *et al*., 1956 and McCready, 1970), 95% ethanol was used first to extract sugars from the starch. The residue was then hydrolyzed with perchloric acid to its monosaccharides. The sugars were quantified colorimetrically using phenol and sulphuric acid. Sugars give an orange color when treated with phenol and sulphuric acid. Sugars extracted with the solvent were directly analyzed to determine the free or soluble sugar content of the sample, while sugars obtained from the hydrolysis of the residue was converted to starch by multiplying by 0.9. The addition of sugar and starch gives the total carbohydrate content.

Procedure

Preparation of standard curve

To a 100 ml volumetric flask containing some distilled water was added 0.01 g of D-glucose and the volume made up to mark. From the glucose stock solution (100 µg/ml); 0, 0.1, 0.2, 0.3 and 0.4 ml were pipetted into test tubes, and made to 1 ml with distilled water. This corresponds to 0, 10, 20, 30 and 40 µg glucose per ml. To this was added 0.5 ml of phenol followed by the addition of 2.5 ml concentrated H_2SO_4. The mixture was vortexed, allowed to cool and read at 490 nm. A glucose standard curve of absorbance against concentration was plotted (Figure 3.2).

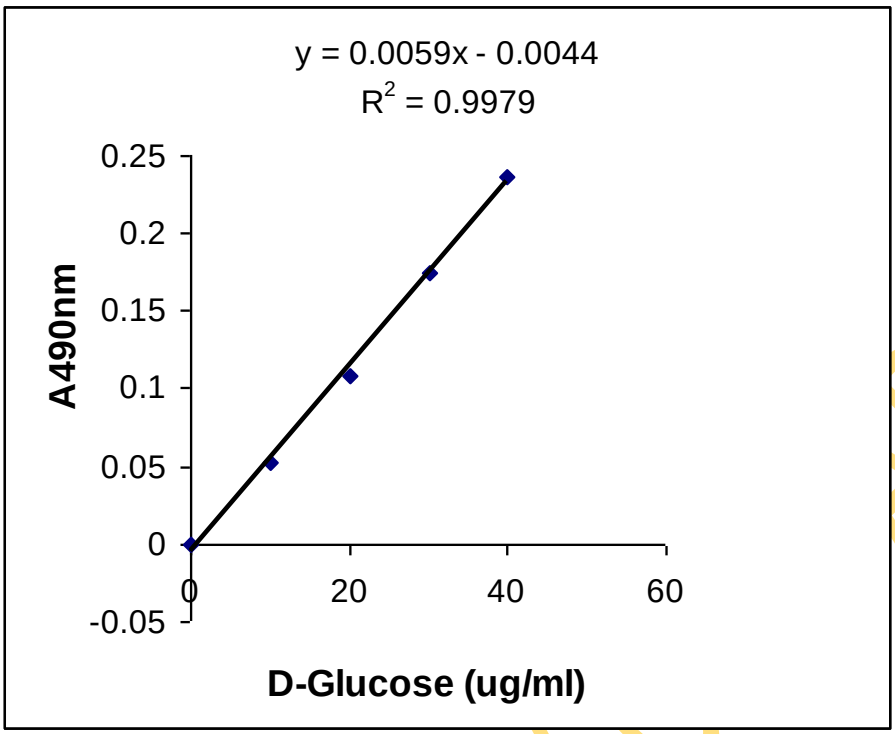


Figure 3.2 D-Glucose standard curve

Sample analysis

Finely ground sample approximately 0.020-0.025 g was weighed into centrifuge tubes and wetted with 1 ml of ethanol. Two millilitres of distilled water was added, followed by 10 ml hot ethanol. The mixture was vortexed and centrifuged at 2000 rpm for ten minutes. The supernatant was collected and used for free sugar analysis, while the residue was used for starch analysis. To the residue was added 7.5 ml of perchloric acid and allowed to hydrolyse for 1 hour. It was diluted to 25 ml with distilled water and filtered through Whatman No. 2 filter paper. From the filtrate, 0.05 ml was taken, made up to 1 ml with distilled water and vortexed.

The supernatant was made up to 20 ml with distilled water and an aliquot of 0.2 ml was taken. To this was added 0.5 ml of 5% phenol and 2.5 ml concentrated sulphuric acid. The mixture was vortexed, left to cool and the absorbance was read at 490 nm.

Calculation

$$\% \text{ Sugar} = \frac{(A-I) \times D.F \times V}{B \times W \times 10^6} \times 100$$

$$\% \text{ Starch} = \frac{(A-I) \times D.F \times V \times 0.9}{B \times W \times 10^6} \times 100$$

Where A is absorbance of sample, I is intercept of the standard curve, D.F is dilution factor based on aliquot of sample extract taken for assay, V is total extract volume, B is slope of the standard curve and W is sample weight.

3.3.1.4. Determination of protein content

Principle: The method of Hach, (1990) was used. The procedure involves digesting the material with conc. H_2SO_4 and H_2O_2 a process that converts the nitrogen to ammonium salts. On treatment with a dispersing agent (polyvinyl alcohol), the ammonium salt decomposes to liberate

ammonia, which in the presence of Nessler's reagent gives an orange colour which is read at 460 nm.

Procedure

About 0.25 g sample was weighed into a Hach digestion flask and 4 ml of conc. H_2SO_4 was added. The sample was transferred to the fume hood and heated for 5 minutes at 440 ^oC. To the charred sample was added 16 ml of H_2O_2 to clear off the brown fumes and make the digest colourless. The H_2O_2 was boiled off by heating for one more minute. The flask was taken off the heater, allowed to cool and the content made up to the 100 ml mark with deionized water and mixed. To 1 ml of the digest, 3 drops of mineral stabilizer was added and 3 drops of polyvinyl alcohol dispersing agent. It was mixed, made up to 25 ml and 1 ml of Nessler's reagent was added. The colour was read within 5 minutes at 460 nm on the Hach spectrophotometer against deionized water blank. The absorbance gives mg/l apparent Nitrogen. The true Kjeldahl nitrogen is calculated as follows:

Calculation

$$\% N = \frac{0.0075}{B \times C} \times A$$

Where A = mg/l (reading displayed); B = weight (mg) of sample digested and C = volume (in mls) of digest analyzed.

% Protein = % N x 6.25

3.3.1.5. Determination of crude fibre content

Reagents

- TCA digestion reagent - 500ml glacial acetic acid is mixed with 450ml water and 50ml concentrated nitric acid. 20g trichloroacetic acid is then dissolved in this mixture
- Industrial spirit

Procedure

The method of Entwistle and Hunter, (1949) was used. To 1g of defatted sample in a digestion beaker was added 100ml of TCA digestion reagent. This was placed on the heating unit of digester and water supply was opened to reflux condenser.The mixture was brought to boiling and refluxed for exactly 40 minutes counting from the time boiling commenced. The beaker was removed from the heater, cooled a little bit and filtered through Whatman No.4 (15.0cm diameter) filter paper. The residue was washed six times with hot water, once with industrial spirit and transfered to a previously ignited and pre-weighed dish. The residue was dried overnight in an oven at 105 ^{o}C, transfered to a dessicator and weighed after cooling. It was ashed in a muffle furnace at 600 ^{o}C for 6 hours, allowed to cool and re-weighed.

Calculation: % Crude fibre = Loss in weight on ashing x 100

3.3.1.6. Determination of crude fat content

Procedure (Automated method-Soxtec System HT2)

The methods of AOAC, 1970 and Josyln, 1970 were used. Dried and ground sample weighing about 2-3g was loaded into each thimble and plugged with cotton wool. To the dried and weighed extraction cups containing boiling chips, 25-50ml of the solvent was added into each cup and inserted into the Soxtec HT and extracted for 15 mins in boiling position and for 30-45 mins in "Rinsing "position. The solvent was evaporated; the cups were released and dried at 100 ^{o}C for 30 mins. Cups were allowed to cool in a dessicator and then weighed.

Calculation

Weight of the cup with the extracted oil = W_3.

Weight of the emty cup = W_2

Weight of sample = W_1

$$\% \text{ fat/oil} = \frac{(W_3 - W_2)}{W_1} \times 100$$

3.3.1.7. Analyses of minerals

Dried and milled grain amaranth samples were analyzed for mineral elements according to the methods of Dahlquist and Knoll, (1978); Havlin and Soltanpour, (1980); Zarcinas *et al*., (1987), using an Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES).

Procedure

To 0.6 g milled powder weighed into 50 ml digestion tubes, 11 mls of $HNO_3/HClO_4$ (10:1) mixture was added. The samples were first cold digested overnight in the tube before heating. The temperature was ramped up over time (4-5 hrs) and through various stages to prevent bumping and to achieve the best recovering of elements. Content of digest in tube was made up to a final volume of 25 ml and mineral elements analyzed.

3.3.2. Data analysis

In this study, all chemical analyses were done in duplicate on the three replications of each accession. Analyses were carried out in the Crop Utilisation Laboratory of the International Institute of tropical Agriculture (IITA), Ibadan. Data were analysed using Analysis of Variance (ANOVA) of the Generalized Linear Model (GLM) procedure of SAS (Statistical Analysis System) (2003).

3.4. Molecular characterisation

3.4.1. Plant material

Samples of young fresh leaves from the 29 accessions of amaranth were collected in ice three weeks after planting in the nursery.

3.4.1.1. DNA extraction materials and reagents

Materials: Microcentrifuge tubes, plastic pestles, eppendorf table top centrifuge, water bath, ice maker machine, micro shaker, spectrophotometer, gel electrophoresis box, gel doc system.

Reagents: Liquid nitrogen, extraction buffer (100Mm Tris-HCl; 500mM EDTA-ethylenediaminetetraacetate, sodium dodecyl sulphate, polyvinyl pyrrolidene, beta-mercaptoethanol), isopropanol, RNAase, potassium acetate and agarose gel.

3.4.1.2. Extraction of genomic DNA.

Genomic DNA was extracted from the leaves of young seedlings by the method of Doyle and Doyle (1990). Approximately, 0.15g – 0.2g leaves were collected from each accession and placed in labeled 1.5ml eppendorf tubes, frozen in liquid nitrogen, and ground with a micropestle. The ground powder was suspended in 800 µl of 2 x cetyltrimethylammonium bromide (CTAB) buffer containing 100 µl of 20% sodium dodecyl sulphate. The mixed sample was incubated at 65 ^{o}C for 15 mins, allowed to cool to room temperature (approximately 2mins) and 300µl of ice cold 5M Potassium acetate was added and gently mixed by inverting 5-6 times. The mixture was incubated on ice for 30 min and centrifuged at 12,000rpm for 10 min. The supernatant was carefully transferred to new eppendorf tubes in duplicate; approximately 700µl of ice-cold iso-propanol was added and mixed by inverting 8-10 mins. The mixture was incubated at -80^{0}C for 1h and centrifuged at 12,000rpm for 10min. The supernatant was discarded and last drops of the iso-propanol were removed by placing face down on paper towels for another 1hr and 100-200µl of 1x TE was added. The tube was stored at 4^{0}C overnight to dissolve pellet. DNA was stored at -20^{0}C for longtime storage.

DNA quality check: The quality of the DNA samples was checked by running 2µl of the DNA on 1% agarose gel in 0.5M TBE buffer at 100 volts for 1 - 2 hours. The DNA bands were viewed

on the UV light after staining in 0.5µg/ml ethidium bromide and destaining in distilled water. This was viewed with UV transiluminator and photographed with Polaroid film, smearing or discrete bands were checked for.

DNA quantification: Genomic DNA was quantified using the UV spectrophotometer. A blank containing only 1x TE was prepared together with three standards (500ng/µl, 250ng/µl and 100ng/µl) of DNA solution from the calf thymus DNA stock. The spectrophotometer was calibrated using the blank, and the three standard DNA solutions were read at 260 nm and 280 nm. Samples of the extracted genomic DNA were read at same wavelength and recorded. Final concentrations of DNA samples were diluted to 25ng/µl and stored at -20^0C.

3.4.1.3. Polymerase chain reaction (PCR)

Components of PCR

The basic components and reagents required to set up a PCR reaction are:

1. Microfuge tube.

These are small cylindrical plastic conical containers with conical bottoms with a snap cap. They are made up of polypropylene, thus can withstand a wide range of temperature.

2. Thermal cycler.

It is an apparatus used to amplify segments of DNA. It has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler works on the principle of Peltier effect, which raises and lowers the temperature of the block in a pre-programmed manner by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.

3. DNA template.

4. Primer.

These are oligonucleotides that define the sequence to be amplified. Two primer that are complementary to the 3′ (three prime) ends of each of the sense and anti-sense strand of the DNA target (Tm 52-58 degree centigrade preferred). Primers with melting temperatures above 65 degree centigrade have a tendency for secondary annealing. The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.

5. Tris-HCl.

The recommended buffer solution is 10 to 50 mM Tris-HCl (pH 8.3-8.8) at 20 degree centigrade.

6. $MgCl_2$.

It is the cofactor of the enzyme. It is beneficial to optimize the magnesium ion concentration. The magnesium ion affects the primer annealing, strand dissociation temperatures of template and PCR product, product specificity, formation of primer-dimer artifacts and enzymatic activity and fidelity. Taq DNA polymerase requires free magnesium that binds to template DNA, primers, and dNTPs.

7. KCl is to be used for the reaction to facilitate primer annealing.

8. Gelatin or bovine serum.

Aautoclaved gelatin or nuclease-free bovine serum albumins are included to help stabilize the enzyme.

9. Autoclaved distilled water is used. The volume depends on the reaction.

10. Deoxyneuclotide triphosphates (DNTP).

These are the DNA building blocks. DTTP (TTP-thymidine triphosphate), dCTP (deoxycytidine triphosphate), dATP (deoxyadenosine triphosphate) and dGTP (deoxyguanosine triphosphate) solutions neutralized to pH 7.0. Primary stock solution are diluted to 10 mM, aliquoted, and

stored at -20 ^{0}C. A working stock containing 1 mM each of dNTPs is recommended. The stability of dNTPs during repeated cycles of PCR is such that approximately 50% remains as dNTP after 50 cycles. DNTP concentration between 20 and 200 uM is best for the reaction. The 4 dNTPs should be at equivalent concentrations to minimize mis-incorporation error.

11. DNA polymerase.

It is an enzyme that catalyzes the reaction. Taq DNA polymerase isolated from *Thermus aquaticus* growing in hot springs acts best at 72 degree centigrade and the denaturation temperature of 90 degree centigrade does not destroy its enzymatic activity. Other thermostable enzyme like Pfu DNA polymerase isolated from *Pyrococcus furiosus* and Vent polymerase isolated from *Thermococcus litoralis*, were discovered and were found to be more efficient. A recommended concentration of Taq polymerase (Perkin-Elmer Cetus) is between 1 and 2.5 units (SA=20 units/pmol) per 100 uL reaction. However enzymatic activity will vary with respect to individual target templates or primers.

Materials and reagents

RAPD oligonucleotide primers (from Operon Technologies Inc, Alameda, CA, USA) were used. Total of 40 RAPD 10-mer primers were screened (B, N and U kits) and 16 primers that generated polymorphic profiles were selected for use in the PCR reaction. Their names and sequences are given in Table 3.2. Other materials such as deoxynucleoside triphosphates (dNTPs), PCR buffer, $MgCl_2$, Taq polymerase were from Promega* technology (IDT), USA.

3.4.1.4. The PCR-RAPD reaction

The following method was optimized for the amplification reaction (Williams *et al.*, 1990). All amplification reactions were performed in a 25 µL volume overlaid with 50 µL of light mineral oil (BDH) and using an MJR thermocycler. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH=9 at 25°C), 200 µM of each of the dNTPs, 0.25 µM primer, 25 ng template DNA, 2.0 U *Taq* polymerase and 2.5 mM $MgCl_2$. The amplification program consisted of denaturation at 94°C for 2 min, followed by 35 cycles comprising a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72°C for 2 min. The cycling program

was terminated by a final extension step at 72°C for 9 min. The amplification products were separated by loading 12.5 µL of each reaction onto a 1% agarose gel containing ethidium bromide (0.17 ng/mL) in TBE buffer (0.089M Tris-borate, 0.002M EDTA, pH 7.8) and running at 5V/cm for 4 h. The sizes of the fragments obtained were estimated by running alongside a 1 kb ladder marker (Gibco-BRL). Bands were viewed using an Alpha imager gel doc system with UV transilluminator (Maniatis *et al*., 1982).

Separation of amplified DNA*:* PCR products were separated on 1.5% agarose (BioRad) gels in TAE buffer (Sambrook *et al*., 1989) at 80 V constant for 3 hours, and were stained with ethidium bromide and photographed over a transilluminator.

3.4.2. Data analysis on RAPD

The bands of equal molecular weight and mobility generated by the same primer were considered to be of identical locus. Gels were scored as 1 for presence and 0 for absence of amplification fragments or bands from each of the 16 RAPD markers. Each polymorphic fragment was treated as a unit character and compared between each pair of accessions. At the intraspecific level, RAPD polymorphism was measured as the proportion of polymorphic loci to the total number of loci scored in all populations of the same species.

The band matrices were analyzed using Numerical Taxonomy System of Statistics (NTSYS) version 2.01, Roulph (2000). The similarity data matrix was used for cluster analysis based on Unweighted Pair Group using Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). This was used to generate a dendrogram. Similarity indices between pair of grain amaranth accessions were calculated for the combination of data from 16 RAPD primer pairs by selecting similarity for qualitative analysis (SIMQUAL) using Jaccard's (1908) similarity coefficient.

Polymorphic Information Content (PIC) or expected heterozygosity scores for each RAPD marker were calculated based on the formula:

$Hn = 1 - pi^2$, where *pi* is the allele frequency for the i^{th} allele (Nei, 1973).

Table 3.2: Oligonucleotide Adapters and Primer Combinations used for RAPD Analysis

No	Name	Sequence
1	OPB-17	AGGGAACGAG
2	OPH-12	ACGCGCATGT
3	OPH-17	CACTCTCCTC
4	OPI- 09	TGGAGAGCAG
5	OPI- 06	AAGGCGGCAG
6	OPQ- 07	CCCCGATGGT
7	OPR- 02	CACAGCTGCC
8	OPT- 08	AACGGCGACA
9	OPU-13	GGCTGGTTCC
10	OPU-14	TGGGTCCCTC
11	OPV-19	GGGTGTGCAG
12	OPG-12	CAGCTCACGA
13	OPI-10	ACAACGCGAG
14	OPV- 9	TGTACCCGTC
15	OPV- 04	CCCCTCACGA
16	OPV-10	GGACCTGCTG

3.5. Experiment 2: Evaluation of the seed protein quality of twenty nine grain amaranth accessions through the determination of storage proteins by SDS-PAGE and amino acid profile.

3.5.1. Plant material

Twenty nine accessions of grain amaranth harvested at maturity as described in experiment 1 were used. The seeds were separated from the heads upon rubbing between the hands and milled into flour to obtain a homogenous particle size. The milled samples were kept at 4 ^{o}C prior to analysis.

3.5.1.1. Sample preparation for protein fractionation

Milled flour was defatted with n-hexane at a flour/hexane ratio of 1:10 (w/v) i.e. 1 g in 10 ml of solvent prior to protein extraction and fractionation. The resulting suspension was stirred for 4 h at 4 ^{o}C and slurries were centrifuged at 9,000 x g for 20 min. The flour was air-dried at room temperature and stored at 4 ^{o}C (Barba de la Rosa *et al*., 1992) in plastic bags until analysis.

3.5.1.2. Extraction of total grain amaranth proteins

Grain amaranth seed meals (100 mg) were suspended in 2 ml of prechilled Tris buffer (50mM Tris-HCl, ph 7.6; 1mM DDT, 150 mM NaCl and 1mM EDTA), for 2h (20 ^{o}C) in an orbital shaker and centrifuged for 10 min at 15,000 x g at 4 ^{o}C. The supernatant was diluted with an SDS sample buffer at a final concentration of 60mM Tris-HCl, pH 6.8, 2% w/v SDS, 3.33% v/v β-mercaptoethanol, 10% glycerol and a trace of bromophenol blue, heated at 98 ^{o}C for 5 min prior to application (Zarkadas *et al*., 2007a).

3.5.1.3. Procedure for protein fractionation.

The protein fractions were extracted according to solubility in different solvents. Proteins were extracted stepwise following the method of Landry and Moureaux (1980), as outlined in Table 3.3.

1g of each sample was mixed with 10 ml of extractant under magnetic stirring. Fractionation sequences were performed first in distilled water, followed by sodium chloride. The time (min) and number of extractions were as follows:

Fraction I (distilled water) 60min, 30min is albumin.
Fraction II (0.5 M NaCl) 60min, 30min is globulin.
Fraction III (55% 2-propanol) 60min, 30min, 15min is prolamin-like.
Fraction IV (55% 2-propanol with 0.6% 2-mercaptoethanol (w/v) 30min, 30min is prolamin.
Fraction V (sodium borate buffer (pH 10) with 0.6% 2-mercaptoethanol and 0.5 M NaCl) 60min, 30min, 15min is glutelin-like.
Fraction VI (borate buffer (pH 10) with 0.6% 2-mercaptoethanol and 0.5% sodium dodecyl sulfate (w/v) is glutelin.
The residue represents the insoluble protein. Fractions I and II were extracted at 4 ^{o}C, while the rest were extracted at room temperature. After extraction, the mixtures were centrifuged for 15 min at 10000 x g and the supernatants were combined for each solvent.
The supernatant was diluted with an SDS-sample buffer at a final concentration of 60 mM Tris-HCl, pH 6.8, 2% w/v SDS, 3.33% v/v β-mercaptoethanol, 10% glycerol and 0.05% of bromophenol blue, heated at 98 ^{o}C for 5 min before being loaded on the gel.

Table 3.3 Procedure for protein fractionation (Landry and Moureaux, 1980)

Fraction	Solvent	agitation (minute)	Time	Temperature (ºC)	Protein fraction
I	Distilled water	60		4	Albumin
		30		4	
II	0.5M NaCl	60		4	Globulin
		30		4	
III	Isopropanol 55% (v/v)	60		20	Prolamins
		30		20	
IV	Isopropanol 55% v/v + 2-ME 0.6% (v/v)	60		20	Prolamin-like
		30		20	
V	Borate buffer (pH10) + 2-ME 0.6 % (v/v) + 0.5M NaCl	60		20	Glutelin-like
		30		20	
VI	Borate buffer (pH10) + 2-ME 0.6% + SDS 0.5%	60		20	Glutelin
		30		20	

3.5.1.4. Reagents and Gel Preparation for SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) on Vertical Slab

Stock Solutions

Acrylamide/bis (30% T, 2.67% C)

29.2g/100 ml

0.8g/100ml N'N-bis-methylene-acrylamide

Make up to 100ml with distilled water. Filter and store at 4 ^{o}C in the dark (30 days maximum).

1.5 M Tris-HCl pH 8.8

18.15 g/100 ml

Adjust to pH 8.8 with 1N HCl. Make to 100 ml with distilled water.

0.5 M Tris-HCl, pH 6.8

6g Tris base

Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water.

10% SDS

Dissolve 10g SDS in water with gentle stirring and bring to 100 ml with dH_2O

Sample buffer (SDS reducing buffer)

Distilled water

60 mM Tris-HCL pH 6.8

10% Glycerol

2% (w/v) SDS

3.33% (v/v) 2-β-mercaptoethanol

0.05% (w/v) bromophenol blue

Make up to 100 ml with dH_2O

5X electrode running buffer pH 8.3

Tris base 9g

Glycine 43.2g

SDS 3g

Make up to 600 ml with dH_2O

Dilute 60 ml 5X stock with 240 ml dH_2O for one electrophoresis run.

12% separating gel preparation

Distilled water 3.5 ml

1.5M Tris-HCl pH 8.8 2.5 ml

10% SDS stock 100 ul

Acrylamide/Bis (30% stock) 4.0 ml

10% ammonium persulfate 100ul (prepare fresh daily)

TEMED 5ul

4% Stacking gel preparation

Distilled water 6.1 ml

0.5 M Tris-HCl pH 6.8 2.5 ml

10% (w/v) SDS 100 µl

Acrylamide/Bis (30% stock) 1.3 ml

10% ammonium persulfate 50 ul

TEMED 10ul

3.5.1.5. One-dimensional gel electrophoresis (SDS-PAGE)

One dimensional electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli (1970), on a vertical slab gel (Mini-PROTEAN II Electrophoresis Cell) using a 12% gradient separating (acrylamide/bis acrylamide) gel and a 4% (w/v) stacking gel containing 0.1% SDS. Aliquots (10-15 µl) of each sample were applied onto each of the 10 gel wells. Low range marker (7.5 to 66 kDa) was loaded onto the first or last well and was used as the standard. 1-DE SDS-PAGE was performed at constant voltage of 125 V for about 2 h until the tracking

bromophenol blue dye migrated to the bottom of the gel. At the end of the run, the gels were stained with Coomasie Brilliant Blue R250 in methanol/water/acetic acid (4:5:1 v/v/v) and destained with methanol/water/acetic acid (4:5:1 v/v/v) over night. Low range molecular weight marker obtained from Sigma Chemical Co. was used for the molecular weight estimation of the protein subunits.

3.5.2. Amino acid analysis.

3.5.2.1. Plant material

Of the 29 grain amaranth accessions used for total protein determination in experiment 1, two accessions each from the five amaranth species used in this study were selected representing accessions with the highest and lowest protein content within each species. A soybean cultivar (TGX 1448-2E) obtained from International Institute of Tropical Agriculture (IITA) germplasm was also included as a reference (high protein source).

Procedure

The methods of Spackman *et al*., (1958) and Barrett, (1985) were adopted. Samples were hydrolyzed in 6M HCl in tubes sealed under vacuum for 24 h at 110 ^{o}C; one or two phenol crystals were added as an oxygen scavenger. Subsequently, samples were derivatized with ninhydrin reagent. The ninhydrin reaction takes place in a heated coil at 125 °C for 5mins situated in a modified column heater and the derivatised amino acids were detected in a Waters 2487 variable wavelength UV/VIS detector. The amino acids were separated by ion exchange chromatography on a strong cation exchange resin by means of citrate buffer gradients of increasing pH. Acidic amino acids such as aspartic acid elute first, followed by the neutrals, then the basic amino acids such as arginine. The 18 protein amino acids obtained after acid hydrolysis were separated using a series of sodium citrate buffers. After separation, the amino acids were reacted post column with a stream of ninhydrin reagent for 5 minutes at 125°C, and then the absorbance of the coloured complex was detected at both 660nm and 460nm.

Cysteine is usually converted to the acid stable cysteic acid before standard acid hydrolysis.

Tryptophan is stable in alkali and so was hydrolysed in barium hydroxide at 110 ^{o}C for 24h under vacuum.

After detection, the data was transferred to an 'Atlas' integration and data handling system. The peaks were integrated and the areas compared against those from a separation of a standard calibration mixture.

3.5.3. Amino acid data analysis

Data was analysed by analysis of variance of the generalized linear model procedure using SAS, (2003) and represents the mean values from 3 sub-samples per accession.

Chemical score of amino acids was calculated using the FAO/WHO, (1973) reference pattern. Essential Amino acid (EAA) was calculated according to Oser, (1959) using the amino acid composition of the whole egg protein published by Hidve' gi and Be' ke's (1984). The proposed method by Young and Steinke (1992) for calculating the protein digestibility-corrected amino acid score (PDCAAS) of foods was used to calculate the PDCAAS. This was expressed as the proportion or percentage of the concentration of the limiting amino acid in a standard or reference amino acid pattern as follows:

$$\text{PDCAAS} = \frac{\text{AA content (mg/g of protein) of food protein x digestibility}}{\text{AA content of FAO/WHO/UNU (1985) pattern for 2-5-year-old child}}$$

Amino acid scores have been defined as the concentration of the limiting amino acid in the food protein, which is expressed as a proportion or percentage of the concentration of the same amino acid in a standard or reference pattern, such as for the diet of the 2- to 5-year-old child (Zarkadas *et al*., 2007a). Digestibility was included in this amino acid scoring procedure to allow for differences in the digestibility between plant and animal sources (Zarkadas *et al*., 1993, 1999, 2007a). The true protein digestibility value for grain amaranth has not been established and so for the purpose of this study, true protein digestibility values established for cereal crops and their derivatives taken from the US Federal Register's Appendix B, pp. 2193-2195 (USFDA, 1993) were used to determine the PDCAAS for grain amaranth since it is a pseudocereal.

3.6. Experiment 3a. Determination of the phytochemical composition of 29 grain amaranth accessions

3.6.1. *In vitro* evaluation of phytochemicals and antioxidant activity

3.6.1.1. Plant material and sample preparation

Seeds obtained from the twenty nine accessions of grain amaranth harvested at maturity were used. The seeds were milled into flour to obtain a homogenous particle size.

3.6.1.2. Determination of Phytic acid content

Phytic acid was determined by a combination of two methods. The extraction and precipitation of phytic acid were performed according to the method of Wheeler and Ferrel (1971) and Fe in the precipitate was measured using the method of Makower (1970). A 4:6 Fe/P atomic ratio was used to calculate the phytic acid content.

Procedure:

Milled grain sample weighing 5 g was transferred into a 125 mls Erlenmeyer flask containing 50 mls of 3% TCA. Extraction was done by mechanical shaking of the mixture for 30 mins and then centrifugation at 7,500 rpm for 15 mins. A 10 ml aliquot of the supernatant was transferred into a 40 ml conical centrifuge tube and 4 mls $FeCl_3$ solution (containing 2 mg ferric iron per ml 3% TCA) was added by blowing rapidly from the pipette. The tube and contents were heated in a boiling water bath for 45 mins. The suspension was centrifuged for 15 mins and the supernatant was carefully decanted. The precipitate was washed twice by dispersing well in 25 mls 3% TCA, heating in a boiling water bath for 5 mins and centrifuging. The wash was repeated once with water and the precipitate was dispersed in 3 mls of 1.5 N NaOH with mixing. The volume was made up to 30 mls with distilled water and the mixture was heated in boiling water bath for 30 mins. The suspension was filtered hot, and precipitate washed with 60 mls hot water. The filtrate was discarded. The precipitate from the paper was dissolved with 40 mls of hot 3.2 N HNO_3 into 100 ml volumetric flask and the paper was washed with several portions of water, collecting the washings in the same flask and diluting to volume. A 5 ml aliquot was transferred to another 100 ml volumetric flask and diluted to 70 mls. To this was added 20 mls of 1.5M KSCN and volume

was made to 100 mls. Absorbance was read (within 1 min) at 480 nm. A reagent blank was run with each set of samples. Iron nitrate standard was prepared and absorbance read at 480 nm. This was used to prepare a standard curve, from which the iron concentration was calculated and expressed as a percentage. The phosphorus content was calculated using a constant Fe:P atomic ratio of 4:6. Phytic acid was calculated by multiplying the inorganic phosphorus by a conversion factor of 3.55.

3.6.1.3. Determination of Tannin content.

Procedure

Tannin content was determined spectrophotometrically by the acidified vanillin method of Burns (1971), as modified by Chang *et al*., (1994) using tannic acid as the tannin standard.

Approximately 2 g sample was extracted in 10 ml 0.1% acidified (HCl) methanol in a capped tube at 24 ^{0}C with 20 min continuous shaking using a wrist-action shaker (model 75, Burrell Corp. at position 6). The mixture was centrifuged at 17,000 x g for 10 min.

To 1 ml of extract supernatant, 5 ml 2% vanillin-HCl reagent (2 g vanillin was dissolved in 100 ml 4% acidified methanol) was added and the mixture was vortexed for 30 min. After standing for 20 min, absorbance was read at 500 nm. A standard curve was also prepared using tannic acid.

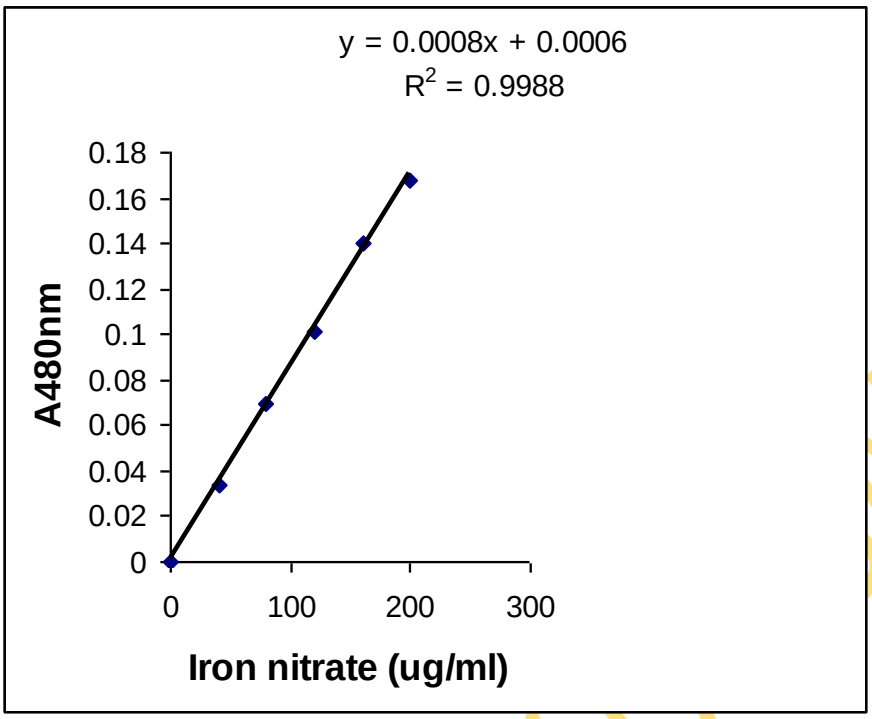


Figure 3.3 Phytate standard curve **(**Using iron nitrate as standard)

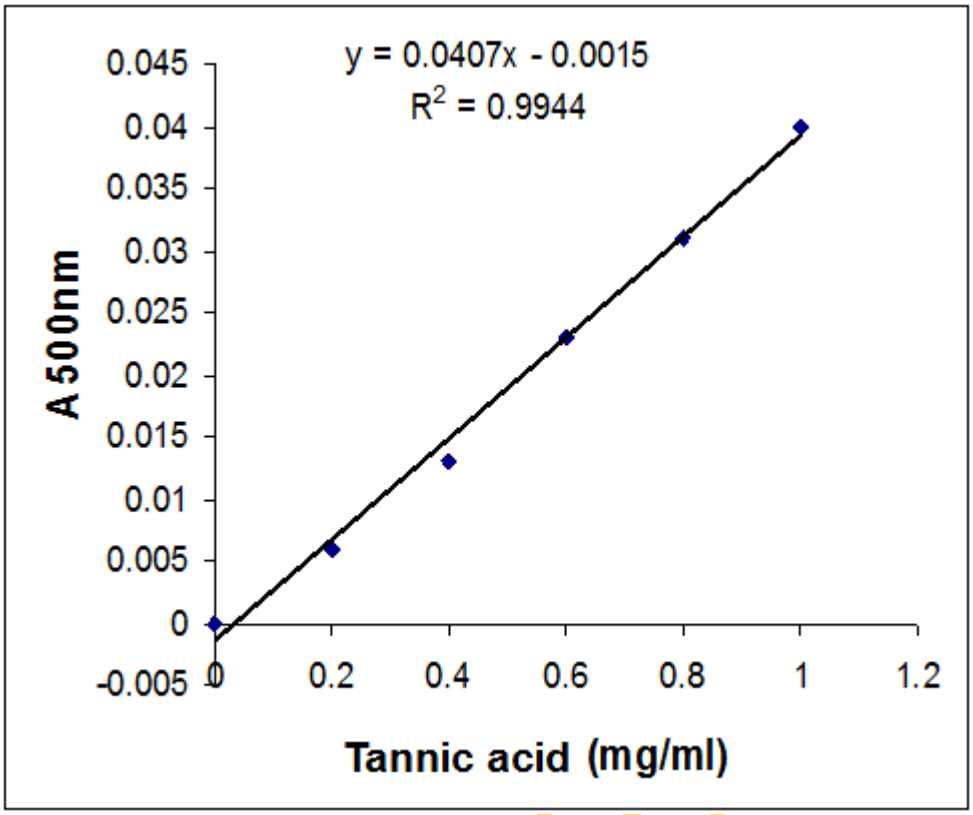


Figure 3.4 Tannin standard curve (using tannic acid equivalent as standard)

Extraction of plant materials

Amaranth flour weighing 1g of each accession was extracted with 25 ml of ethanol containing 1% HCl for 24h at 24 ^{o}C according to the method of Shen *et al*., (2009). The procedure was repeated twice. The ethanolic extracts were centrifuged at approximately 4000 x g for 15 min; the supernatants were pooled together and stored at 4 ^{o}C prior to use.

3.6.1.4. Determination of Total Phenolic content

Total phenolic content was assayed by the Folin-Ciocalteu colorimetric method with slight modification (Bao *et al*., 2005; Cai *et al*., 2004). Briefly, aliquots (1.0 ml) of diluted extracts or standard solutions were mixed with 2.5 ml 10% Folin-Ciocalteu reagent, and the reaction was neutralized by adding 2.0 ml of saturated sodium carbonate (75 g/l). The samples were vortexed for 20 sec. The absorbance of the resulting blue color was recorded using a UV-Vis spectrophotometer at 760 nm after incubation for 2 hr at 23 ^{o}C. A calibration curve was prepared using gallic acid solution. Total phenolic contents were expressed as milligrams of gallic acid equivalent (mg GAE) per 100g of dry weight.

3.6.1.5. Determination of Total Flavonoid content

This was determined by a colorimetric method of Bao *et al*., (2005), with minor modification.

Reagents

1. **1M NaOH**
   4g NaOH (M.wt 40 g/mol) was dissolved in distilled water and the mixture was made up to 100 ml
2. **10% $AlCl_3.6H_2O$**
   10g of $AlCl_3$ was dissolved in distilled water and the solution made up to 100 ml.
2. **5% $NaNO_2$**
   5g of **$NaNO_2$** was dissolved in distilled water and the solution made up to 100 ml.

Procedure

Aliquots (0.5 ml) of diluted extracts or standard solutions were pipetted into 15-ml polypropylene conical tubes containing 4.5 ml of double distilled water and mixed with 0.3 ml 5% $NaNO_2$. After 5min, 0.6 ml 10% $AlCl_3.6H_2O$ solution was added, the mixture was allowed to stand for another 5min, and then 2 ml 1 M NaOH was added, followed by 2.1 ml of distilled water. The reaction solution was well mixed, kept for 15 min, and the absorbance was determined at 510 nm. Total flavonoid content was calculated using the standard catechin curve, and expressed as mg catechin equivalent (mg CE) per 100g of dry weight or micromoles of catechin equivalent per 100g of grain.

3.6.1.6. Determination of Reducing Power

This was estimated using the method described by Oyaizu (1986) and Duh and Yen (1997) with some modifications (Hsu *et al*., 2003).

Principle

This assay is based on the fact that radical chain reactions could be terminated when substances exhibiting reducing power donate electrons which can react with free radicals converting them to more stable products in the process.

Reagents

1. **0.1% Iron III Chloride ($FeCl_3$)**

   0.1g of $FeCl_3$ was dissolved in distilled water and the solution, made up to 100ml.

2. **1% Potassium ferricyanide (K_3FeCN)**

   1g of K_3FeCN was dissolved in distilled water and the solution made up to 100ml

3. **10% Trichloroacetic acid (TCA)**

   10g of TCA was dissolved in distilled water and the solution was made up to 100ml

4. **0.2M phosphate buffer (pH 6.6)**

2.4g of NaH_2PO_4 (M.wt. 120 g/mol) was weighed and made up to 100 ml with distilled water. The pH was adjusted to 6.6 using HCl or NaOH as required.

Procedure

To 1ml of the grain extract in a test tube, 0.5ml of 0.2 M phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide (K_3FeCN) were added. The mixture was left to incubate for 20 minutes at 50 ^oC. The tube was immediately cooled over crushed ice and then an aliquot of 0.5ml 10% trichloroacetic acid was added. After centrifugation at 3000 x g for 10 minutes, 1 ml of the supernatant was mixed with 1ml of distilled water and 0.Iml of 0.1% $FeCl_3$. The mixture was left to stand for 10 minute and absorbance was measured at a wavelength of 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

3.6.1.7. Determination of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity (RSA)

This was estimated according to the method described by Hsu *et al*. (2003).

Principle

Freshly prepared $DPPH^{\bullet}$ solution exhibits a deep purple colour with an absorption maximum at 517mm. This purple colour generally disappears when an antioxidant molecule can quench $DPPH^{\bullet}$ and convert them to a bleached product i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine, resulting in a decrease in absorbance. The disappearance of the radical can be measured spectrophotometrically at 517 nm and expressed as radical scavenging ability.

Reagents

1. Methanolic $DPPH^{\bullet}$ Solution

0.1mM DPPH (0.0394g of DPPH was weighed and dissolved in methanol and then made up to 1000ml with the same solvent).

Procedure

To 1ml of the extract, 5ml of freshly prepared methanolic solution of $DPPH^{\bullet}$ radical was added. The mixture was shaken vigorously and left to stand for 50 minutes in the dark until stable absorption values could be obtained. The reduction of the $DPPH^{\bullet}$ radical was determined by measuring the absorption at 517nm. Methanol (1ml), replacing the extract, was used as the blank. DPPH solution only served as control. The experiment was performed in duplicates. The DPPH radical scavenging activity was calculated according to the following equation:

Calculation

$$\% \text{ RSA} = [1 - (A_{sample}/A_{blank})] \times 100.$$

A_{sample} = absorbance of the solution when the sample was added

A_{blank} = absorbance of the blank containing methanol only

1 is the absorbance of the control containing DPPH only

Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity.

3.6.1.8. Determination of Total Antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo V complex at acid pH (Prieto *et al*., 1999).

Reagents

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

Procedure

To 0.2ml of extract was added 3.8ml reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate), the mixture were incubated at 95 ^{o}C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank containing dH_2O and the reagent solution. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

3.6.1.9. Determination of the antioxidant scavenging activity using 2, 2–Azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt), ABTS

This assay was carried out according to the method described by Re *et al*., (1999) and modified by Huang *et al*., (2005).

Principle

This method is based on the capacity of a sample to inhibit the ABTS (2, 2-azinobis (3-ethylbenz-thiazoline-6-sulfonic acid, diammonioum salt) radical ($ABTS^{+}$) with a reference antioxidant standard (Trolox).

Reagents

1 **95% Ethanol**

This was prepared by measuring 95ml of Ethanol into a 100 ml standard flask and making up to volume with distilled water.

2 **7 mM 2, 2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt) (ABTS).** This was prepared by dissolving 30 mg equivalent to 3 tablets of ABTS, M.wt. 546.68 (Sigma-Aldrich) in 7.81 ml of distilled water).

3 **2.45 mmol/L Potassium persulfate**

This was prepared by dissolving 6.6 mg of Potassium persulfate in 95% ethanol (Sigma, USA) and solution made up to 10 ml with same.

Procedure

$ABTS^{+}$ radical cation was generated by the interaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate ($K_2S_2O_8$) (Re *et al*., 1999). The solution was held at room temperature in the dark for 12-16h before use. Once the dark green solution was formed it was diluted with 95% ethanol until the absorbance read 0.7 at 734 nm (Huang *et al.*, 2005). For measurements, 2.5 ml of the resulting solution was mixed with 500µl of the extract. The absorbance was read 6 min after mixing at 734 nm.

The percentage decrease of the absorbance at 734 nm was calculated by the formula:

I= [(AB – AA)/AB] x 100

Where I = ABTS Inhibition %

AB = Absorbance of blank sample (t=0)

AA = Absorbance of tested extract solution at the end of the reaction

A standard curve was obtained by using Trolox solutions (0–250 mmol/mL) in ethanol. The absorbance of the reaction samples was compared to that of the trolox standard and results were expressed in terms of micromoles Trolox Equivalents Scavenging Capacity (TEAC) per FW.

3.6.2. Ferrous ion chelating capacity

The method described by Hsu *et al*., (2003) was used to determine the ferrous ion chelating activities of grain flour.

Ethanol grain flour extract (1 ml), 2 mM $FeCl_2.4H_2O$ (0.1 ml), 0.2 ml of 5 mM ferrozine (3-(2-pyridyl)-5,6-bis- (4-phenylsulfonic acid)-1,2,4-triazine, and 3.7 ml methanol were mixed in a test tube, and were reacted for 10 min. The absorbance at 562 nm was measured; a lower absorbance indicated a higher ferrous ion chelating capacity, which was calculated as follows:

Ferrous ion chelating capacity (%) = [1- (A_{562} nm, sample/A_{562} nm, control)] x 100

Where A_{562} nm sample is the absorbance of sample at 562nm

A_{562} nm control is the absorbance of control at 562nm

3.7. Experment 3b. Hepatoprotective effect of ethanolic seed extract of accessions A23 (*A. hypochondriacus*) and A28 (*A. hybridus*) on sodium-arsenite induced toxicity in male albino rats.

3.7.1. Plant material

Of the 29 grain amaranth accessions that were evaluated for nutritional contents, phytochemicals and antioxidant activities, A23 and A28 having highest levels of protein and phytochemicals were selected for the *in vivo* experiment.

3.7.1.1. Preparation of grain amaranth extracts

The milled grain amaranth flour was extracted in 80% ethanol using a soxhlet apparatus. The extract was concentrated at 40 ^{o}C on a rotary evaporator (Buchi, USA) and the resulting molten extract was further lyophilized (Freezone 4.5, Labconco, USA) at high vacuum (133 x 10^{-3} mBar) and at temperature -40 ^{o}C. The dried extract was stored in air tight amber bottle and kept at -20 ^{o}C. The dried extract was weighed and percent yield was calculated using the expression:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract x100\%}}{\text{Weight of sample used}}$$

The dried extract was re-suspended in distilled water and used as the test sample.

3.7.2. Experimental animals

Eighty Male albino rats weighing between 125-195 g were purchased from the Department of Veterinary Medicine, University of Ibadan. They were divided into two main groups (representing the two accessions selected for the hepatoprotective evaluation), with each group consisting of eight treatment groups of five rats each.

3.7.2.1. Treatment of animals

The animals were kept in the departmental animal house in well ventilated room and allowed to acclimatize for two weeks prior to administration of the test substances. Each sub group was kept in a metal cage with wood shaven bedding under standard environmental conditions of 50 ± 10%

relative humidity, 29 ± 2 ^{0}C temperatures and 12 h dark/light periodicity. They were fed with standard commercial pellets purchased from Vita Feeds, Mokola, Ibadan, Nigeria and water ad-libitum.

3.7.3. Procedure

Each of the 2 main groups comprised of eight treatment groups, having five rats each as summarized in Tables 3.4.

Group 1 received daily only distilled water orally, Group II received 2.5 mg/kg body weight of sodium arsenite only on the 7th and 14th day, this was administered by oral intubation, Groups 3 to 5 were orally administered graded doses (100, 200 and 300 mg/kg body weight) of ethanol extract of grain amaranth daily for two weeks, Groups 6 to 8 were administered graded doses of 100, 200, 300 mg/kg ethanol extract of grain amaranth daily and 2.5 mg/kg sodium arsenite on the 7th and 14th day. Treatment was stopped on the 14th day and animals were fasted overnight.

Table 3.4: Test samples administered: *A. hypochondriacus* (A23) and *A. hybridus* (A28))

Group	A23	A28
	Treatments	
I	DH_2O only	DH_2O only
II	NaAS only (2.5 mg/kg body weight)	NaAS only (2.5 mg/kg body weight)
III	Ethanolic extract of AHP (100 mg/kg body weight)	Ethanolic extract of AHB (100 mg/kg body weight)
IV	Ethanolic extract of AHP (200 mg/kg body weight)	Ethanolic extract of AHB (200 mg/kg body weight)
V	Ethanolic extract of AHP (300 mg/kg body weight)	Ethanolic extract of AHB (300 mg/kg body weight)
VI	Ethanolic extract of AHP (100 mg/kg body weight) + NaAS	Ethanolic extract of AHB (100 mg/kg body weight) + NaAS
VII	Ethanolic extract of AHP (200 mg/kg body weight) + NaAS	Ethanolic extract of AHB (200 mg/kg body weight) + NaAS
VIII	Ethanolic extract of AHP (300 mg/kg body weight) + NaAS	Ethanolic extract of AHB (300 mg/kg body weight) + NaAS

DH_2O – Distilled water, NaAS – Sodium arsenite, AHP – *Amaranthus hypochondriacus,* AHB – *A. hybridus*

3.7.4. Apparatus and Instrumentation

3.7.4.1. Materials for *In vivo* study

Materials for Preparation of test substances and administration

I ml syringe and needle, 10 ml collection bottles, hand gloves, Oral cannolae

Materials for sacrificing animals, collection of blood serum and organs

Dissecting board, surgical sets, disposable syringes and needles, non-heparinised bottles

Materials for enzyme assays

Test tubes and test tube racks, automatic pipette and automatic pipette tips, cuvettes, incubator, pH meter, spectrophotometer, centrifuge.

Materials for staining.

Cover glasses, rectangular tray, staining jar, precleaned slides and petri dishes

Apparatus for scoring of slides.

Compound microscope and Tally counter.

Apparatus for Liver Function Test.

Micropipette, timer, test-tubes, test-tube rack, incubator, spectrophotometer

Chemicals/reagents for analysis

Sodium arsenite, Ethanol, Formalin, Phosphate buffer, Rinsing buffer, NaOH, HCl, KCl, Giemsa's stain, xylene e.t.c. are all of analytical grade. Liver function tests kits was purchased from Randox Chemicals, UK,

Preparation of reagents

Sodium arsenite ($NaAsO_2$) (BDH chemicals Ltd, Poole, England) was dissolved in distilled water.

1.15% KCl

1.15 g of potassium chloride (KCl) was dissolved in distilled water and the volume made up to 100 mL. This was used as a rinsing buffer during the harvesting of liver samples.

10% Formal saline

8.5g of sodium chloride was dissolved in 900ml distilled water and made up to 1000ml with formalin.

0.4M NaOH

16g of NaOH was dissolved in distilled water and the volume made up to 1000mL. It was used for ALT and AST assays.

0.01M PHOSPHATE BUFFER (PH 6.8)

0.71 g of disodium hydrogen phosphate (Na_2HPO_4) and 0.68 g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in water and made up 1000 ml, and then the pH was adjusted to 6.8. This was used to prepare 5% Giemsa and to rinse the slides while washing.

5% GIEMSA STAIN

5g of Giemsa was dissolved in phosphate buffer (pH 6.8) and the volume made up to 100 ml. It was used for micronucleus assay.

0.04% COLCHICINE

0.04g Colchicine was dissolved in distilled water and the volume made up to 100 mL. It was used to inject the rats (1ml/100g body weight) two hours prior to the sacrifice to arrest the metaphase.

0A.4% MAY-GRUNWALD STAIN 1

0.4ml of May-Grunwald stain was dissolved and made up to 100ml with absolute methanol.

0.4% MAY-GRUNWALD STAIN 2

1:1 dilution was made using stain 1 and distilled water. They were used in staining of slides.

3.7.4.2. Administration of test substances

Sodium arsenite was administered by means of oral intubation, at a dose of 2.5mg/kg body weight corresponding to $1/10^{th}$ of the oral LD_{50} of the salt (Preston *et al.*, 1987), once a week for two weeks.

The test extract (ethanolic extract of grain *amaranth*) was given at 3 graded doses of 100, 200 and 300 mg/kg body wt according to Zeashan *et al.*, (2008) and was administered by oral intubation, every day for two weeks. All animals were fasted for 24 h after withdrawal of treatment and Colchicine (1ml/100g body weight) was administered 2 hours prior to sacrifice. The rats were sacrificed by cervical dislocation.

3.7.4.3. Collection and preparation of blood samples

The blood sample was collected through occular puncture using capillary tubes. Each blood sample was collected into a new clean and labeled bottle. All blood samples collected were allowed to clot for two hours after which they were centrifuged at 3,000 x g for 30 minutes to separate cells from serum. The supernatant (serum) was aspirated with the aid of micropipettes into a clean labeled serum bottle and immediately stored in ice for the liver enzyme assays.

3.7.5. Enzyme assays

3.7.5.1. Aspartate amino transferase (AST) assay

Aspartate Aminotransferase (AST) was assayed for according to the method of Reitman and Frankel, (1957). AST like ALT is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from aspartate to α-Ketoglutarate to form oxaloacetate and glutamate.

The diagnostic implications of this enzyme in the serum include liver cirrhosis, myocardial infarction (becoming evident 4-8 hours after the onset of pain and peaking after 24-36 hours), muscular dystrophy and paroxysmal myoglobinuria in which the level is often higher than that of ALT. As in ALT, elevated level is also found in metastatic or primary liver neoplasm.

Principle:

α-oxoglutarate + L-aspartate = L-glutamate + Oxaloacetate

Reagents for AST:

(1.)-Phosphate buffer, -L-aspartate, -α-oxoglutarate.-2,4-dinitrophenylhydrazine

The AST measurement was done against reagent blank, with 1cm light path cuvette, a wavelength of Hg 546nm and 37^{o}C incubation

To 0.5ml of R1 was added 0.1ml of serum sample. The mixture was incubated for 30mins at 37 ^{o}C, 0.5ml of R2 was added and incubated for 20mins at 20-25^{o}C. 5ml of NaOH was added to the mixture and the absorbance of the sample was read against sample blank after 5mins.

3.7.5.2. Alanine aminotransferase (ALT) assay

Alanine Aminotransferase (ALT) was assayed for according to the method of Reitman and Frankel, (1957). ALT is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from alanine to α-Ketoglutarate to form pyruvate and glutamate. The diagnostic implications of this enzyme in the serum include hepatitis and other liver diseases in which the level is often higher than that of AST. Elevated level is also found in metastatic or primary liver neoplasm**.**

Principle:

α-oxoglutarate + L-alanine = L-glutamate+Pyruvate

Reagents for ALT:

(1.)-Phosphate buffer, -L-alanine,-α-oxoglutarate -2,4-dinitrophenylhydrazine

The ALT measurement was done against reagent blank, with 1cm light path cuvette. Wavelength was 546 nm (530-550) and incubation temperature was 37 ^{o}C.

0.5ml of R1 was added to 0.1ml of serum sample, the mixture was incubated for 30mins at 37 ^{o}C. 0.5ml of R2 was added and incubated for 20mins at 20-25 ^{o}C.5ml of NaOH was added to the mixture and the absorbance of the sample was read against sample blank after 5mins.

3.7.5.3. Alkaline phosphatase assay (ALP)

Alkaline phosphatase (ALP) was assayed according to the method of Williamson, (1972).

Principle:

P-nitrophenylphosphate + H_2O = Phosphate + P-nitrophenol

Reagents for ALP:

(1.)-Diethanolamine buffer, $MgCl_2$, P-nitrophenylphosphate

The ALP measurement was done against air, with 1cm light path curvette, wavelength of Hg 405nm and 25, 30 and 37 ^{o}C temperature. The working reagent was prepared by reconstituting R2 with R1. To 3ml of the working reagent was added 0.05ml of serum sample, the initial absorbance was read. The timer was set and read again after 1, 2 and 3 min.

Calculation

The ALP activity (U/L) = 2760 x ΔA 405 nm/min

Normal value in serum is 60-170 U/L

3.7.5.4. Gamma glutamyl transferase (GGT)

γ-GT activity was determined following the principle described by Szasz, (1974).

Principle

Gamma glutamyl transferase (γ-GT) catalyses the transfer of the glutamyl group from glutamyl peptide to an amino acid of another peptide, glylglycine to yield a cleavage product, p-nitro-

anilide, which absorbs UV light at 405nm, thus making a direct kinetic determination possible. γ-Glutamyl transferase (E.C. 2.3.2.2.) has been known to be more sensitive and hence more reliable than the serum amino transferases.

L-γ-glutamyl-p-nitoranilide + glycylglycine $\xrightarrow{\gamma\text{-GT}}$ L-γ glutamylglycylglycine + p-nitroaniline

Procedure

Wavelength: Hg 405nm

Cuvette: 1cm light path

Temperature: 25^0C, 30^0C, 37^0C

Measurement: against air

The 0.1ml of sample was added into cuvette followed by the 1.0ml of reagent, the reaction was mixed and initial absorbance was read. A timer was put on simultaneously and absorbsance read again after 1, 2 and 3 minutes.

Calculation

The GGT activity (U/L) = 1158 x ΔA 405 nm/min

Normal value in serum

8-38 U/L

3.8. Histopathological examination

The rats were sacrificed and their liver tissues were quickly excised and blotted dry. They were then rinsed in potassium chloride solution (1.15% w/v) in order to remove all traces of haemoglobin which could contaminate the liver tissues. The tissues were then preserved in 10% formalin for histological analysis.

Tissues of about 5 mm thickness obtained from the liver were fixed in 10% neutral buffered formalin. These tissues were processed for histopathological examination using a routine

paraffin-wax embedding method. Sections of 5µm thickness were stained with haematoxylin and eosin. Photomicrographs of the haematoxylin-eosin stained tissue sections prepared on slides were taken with a camera attached to the compound light microscope in the Department of Anatomy, University of Ibadan.

3.9. The micronuclei assay

Micronucleus refers to a chromosomal fragment which is lagging in anaphase for various reasons. It is therefore not incorporated into the daughter nuclei at the time of cellular division. In telophase stage of cell division, this material becomes inserted into one of the daughter cells, and either fuses with the main nucleus or forms one or several secondary nuclei (if any). These are significantly smaller than the main nucleus and hence are called micronuclei. They can be observed in any dividing cell population, which has lost some chromosomal fragments (Heddle and Salamone, 1981).

3.9.1. Principle of micronuclei

The principle of the micronucleus assay is based on the fact that polychromatic erythrocyte (PCE) cells have a staining property that is different from the normal nature erythrocyte (normocyte). The polychromatic staining property results from the presence of ribosomal RNA 24 hours prior to the formation of the cell. As PCEs develop into mature erythrocytes, they lose the ribosomal RNA and the staining property. In mammals, mature erythrocytes expel their nuclei 8-12 hours after the last mitosis preceding the formation of an erythrocyte. The micronuclei for some reasons are not expelled completely. Micronuclei are not normally found in the circulating erythrocytes in blood because they are filtered out by the spleen (Schalm, 1965).

3.9.2. Detection of micronucleated PCEs

The micronuclei assay is developed for detection of *in vivo* chromosomal breakage more conveniently than the traditional cytogenetic methods (Heddle, 1973). It has been used to detect *in vivo* genetic activity in bone marrow cells (Sai *et al*., 1992). The micronucleus test has also been employed to detect *in vitro* chromosomal aberration (Sasaki *et al*., 1980). The majority of the micronuclei are found in the polychromatic erythrocyte (PCE) cells and this offers an

advantage for the use of the micronucleus assay for screening mutagens (Von Le deBur and Schmidt, 1973).

Positive result with the micronucleus test is just an indication of chromosomal damage; it does not conclude the tested agent as a mutagen or carcinogen. But most studies have proved the agent being tested as mutagenic or carcinogenic agents (Salamone *et al*., 1980). Also negative results may suggest that there is no *in vivo* chromosomal damage.

3.9.3. Reagents for micronuclei assay

- May-Grunwald stain
- 0.01M phosphate buffer (pH 6.8)
- Fetal calf serum
- Bovine serum
- Absolute methanol
- Xylene
- Depex (DPX) mountant.
- 5% Giemsa Stain.

3.9.4. Preparation of bone marrow smears

Procedure:

The method of Schmidt (1975) was adopted in the preparation of the bone marrow smears. After the rats were sacrificed, the femur of each rat was removed and cleaned of muscle. A pair of scissors was used to make an opening in the iliac region of the femur, A small pin was then introduced into the marrow canal at the epiphyseal end, as the pin was pushed inside the canal, the marrow exude through the hole at the iliac end. The marrow was placed into a slide and a drop of fetal calf serum was added to the smear using a Pasteur pipette. The whole content was mixed to become homogenous by using a clean edge of another slide. This homogenous mixture was then spread on the slide as a smear and allowed to dry.

3.9.5. Fixing and staining of the slides

The stepwise procedure includes;

- Fixing the slides in methanol for 5 minutes.
- Air drying to remove the methanol.
- Staining 0.4% May Grunwald stain 1
- Then in stain 2 May Grunwald
- Air drying of the slides
- Staining in 5% Giemsa for at least 30 minutes.
- Rinsing in phosphate buffer for about 30 seconds.
- Rinsing in distilled water.
- Air drying of the slides.
- Fixing the slides in xylene for 20 minutes
- Air drying of the slides.
- Mounting in DPX (a natural mountant) with cover slips.

3.9.6. Scoring of the slides

The fixed cells on the slides were viewed under light microscope to detect the presence of polychromatic erythrocytes (PCE), which contain micronuclei. Tally counter was used to make scoring easier. The slides were first screened at medium magnification to get suitable regions for scoring. These are often located close to the end of the smear.

PCEs and micronuclei stain blue while normal mature erythrocytes stain red, with this one can distinguish between micronuclei and normocytes.

3.9.6.1. Frequency of micronuclei in polychromatic erythrocytes.

Studies on clastogenic agents have shown that variation occurs in the frequency of micronuclei that are formed during cell division (Salmone *et al*., 1980), sampling after 8-12 hours of treatment do not produce effective micronuclei. Sampling after 24 hours of injection is known to produce micronuclei. Multiple injections of clastogens are also known to produce high frequency of micronuclei when samples are taken after 48, 72 or 96 hours of first injection.

3.10. Assessment of the Biomarkers of oxidative stress

3.10.1. Lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Varshney and Kale, (1990).

Principle

Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of membrane fatty acid and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA), to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm. The pink chromophore is readily extractable into organic solvents such as butanol.

Reagents

1. Trichloroacetic acid (TCA, 30%)

TCA (9 g) was dissolved in distilled water and made up to 30 ml with same.

2. Thiobarbituric acid (0.75%)

This was prepared by dissolving 0.225 g of thiobarbituric acid (TBA) in 0.1 M HCl and made up to 30 ml with same.

3. Tris-KCl buffer (0.15 M, pH 7.4)

KCl (1.12 g) and 2.36 g of Tris base were dissolved separately in distilled water and made up to 100 ml with same. The pH was then adjusted to 7.4.

Procedure

An aliquot (0.4 ml) of the post mitochondria fraction (PMF) was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000 *g*. The clear supernatant was collected and absorbance measured against a reference blank

of distilled water at 532 nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi, (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532}\text{nm x volume of sample x mg protein}}$$

3.10.2. Determination of catalase activity

Catalase activity was determined according to the method of Sinha, (1972).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570 - 610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture

Reagents

1. Dichromate Solution (5%)

   $K_2Cr_2O_7$ (5 g) was dissolved in 80 ml of distilled water and made up to 100 ml with same.

2. Hydrogen peroxide (0.2M)

   H_2O_2 (0.67 g) was mixed with distilled water in a 100 ml volumetric flask and the solution made up to the mark with same.

3. Dichromate/acetic acid

This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and it is stable for about one month.

4. Phosphate buffer (0.01M, pH 7.0)

$Na_2HPO_4.12H_2O$ (3.5814 g) and 1.19 g $NaH_2PO_4.2H_2O$ dissolved in 900 ml of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

3.10.2.1. Colorimetric determination of H_2O_2

Procedure

Different volumes of H_2O_2, ranging from 10 to 100 μmoles was taken in small test tubes and 2 ml of dichromate/acetic acid was added to each (Table 3.5). Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3 ml and the optical density measured with a spectrophotometer at 570 nm. The concentrations of the standard were plotted against absorbance.

3.10.2.2. Procedure for catalase activity

Sample (1 ml) was mixed with 49 ml distilled H_2O to give 1 in 50 dilution of the sample. The assay mixture contained 4 ml of H_2O_2 solution (800 μmoles) and 5 ml of phosphate buffer in a 10 ml flat bottom flask. 1ml of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

Table 3.5 Composition of hydrogen peroxide standard

Test tube	1	2	3	4	5	6	7
H_2O_2 (ml)	0.00	0.10	0.20	0.30	0.40	0.50	0.60
Dichromate/acetic acid (ml) `	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	1.00	0.90	0.80	0.70	0.60	0.50	0.40

3.10.3. Determination of superoxide dismutase activity.

The level of superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of adrenaline (epinephrine) at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O^{-}_{2}) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O^{-}_{2} introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide (O^{-}_{2}) radical and hence inhibitable by SOD.

Reagents

1. Carbonate buffer (0.05 M, pH 10.2)

   $Na_2CO_3.10H_2O$ (14.3 g) and 4.2 g of $NaHCO_3$ were dissolved in 900 ml of distilled water. The pH was adjusted to 10.2 and then made up to 1 litre.

2. Adrenaline (0.3 mM)

   Adrenaline (0.0137 g) was dissolved in 200 ml distilled water and then made up to 250 ml. This solution was prepared just after the experiment.

Procedure

Sample (1 ml) was diluted in 9 ml of distilled water to make a 1 in 10 dilution. An aliquot (0.2 ml) of the diluted sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette

contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

3.10.4. Protein determination

The protein concentrations of the various samples were determined by means of the Biuret method as described by Gornall *et al.*, (1949) with some modifications. Potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions as cuprous oxide.

Principle

Proteins form a colored complex with cupric ions in an alkaline solution as exemplified by the Biuret reagent containing CuSO4, KI and sodium potassium tartarate. The protein and Biuret reagent form complex with maximum absorbance at 540nm. The procedure is usually calibrated with a standard BSA curve (Table 3.6).

Reagents

1. 0.2M sodium hydroxide

8g of NaOH (BDH, England) was dissolved in distilled water and made up to 1litre.

2. Stock Bovine Serum Albumin (standard)

20mg of BSA (Sigma Chemical Co., USA) was dissolved in 2ml distilled water to give a stock solution of 10mg protein/ml.

3. Biuret Reagent

3g of $CuSO_4.5H_2O$ (BDH Chemicals, England) were dissolved in 500ml of 0.2M NaOH, 9g of Sodium tartarate and 5g of potassium iodide, KI (BDH Chemicals, England) was added and the solution made up to a litre with 0.2M NaOH.

3.10.4.1 Standard BSA curve by the Biuret method

Several dilutions of the stock solution containing 2-10mg protein/ml were made. Into 1ml of each protein standard solution in a test tube was added 4ml of biuret reagent. The mixture was allowed to stand at room temperature for 30 minutes and the optical densities of the resulting

violet solutions were read in a spectrophotometer at 540nm. A curve of the optical densities against protein concentration was plotted.

3.10.4.2 Estimation of protein in the samples

0.1ml of the liver supernatants was added to 0.9ml of distilled water to make a 1 in ten dilution in order to reduce the sensisitivity of the biuret reagent. 1ml of the diluted sample was taken and added to 3ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples were usually extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

Table 3.6: Protocol for Protein estimation

Test tube no.	1	2	3	4	5
Stock BSA (ml)	0.1	0.2	0.3	0.4	0.5
Distilled water (ml)	0.9	0.8	0.7	0.6	0.5
Biuret reagent (ml)	4	4	4	4	4
BSA Concentration (mg/ml)	1	2	3	4	5
Absorbance (540 nm)	0.160	0.310	0.462	0.651	0.790

3.10.5. Assay for glutathione peroxidise (GP_X) activity

GPx activity was determined according to the method of Rotruck *et al.*, 1973.

Reagents

1. **Sodium azide (NaN_3 10mM)**

   0.0325g of sodium azide was dissolved in 50ml of distilled water.

2. **Reduced glutathione (GSH 4mM)**

   0.0123g of reduced GSH was dissolved in 10ml of phosphate buffer

3. **Hydrogen peroxide (H_2O_2 2.5mM)**

   28µL of hydrogen peroxide was dissolved in 100ml of distilled water.

4. **Trichloroacetic acid (10%)**

   2g of TCA was dissolved in 20ml of distilled water

5. **Dipotassium hydrogen orthophosphate, K_2HPO_4, (0.3M)**

   5.23g of dipotassium hydrogen orthophosphate was dissolved in 100ml of distilled water

6. **5'-5'-dithiobis-(2-dinitrobenzoic acid) [DTNB]**

   0.04g of DTNB was dissolved in 100ml of phosphate buffer

7. **Phosphate buffer**

   0.992g of K_2HPO_4 and 1.946g of KH_2PO_4 were dissolved with 200ml of distilled water and adjusted to pH of 7.4

Table 3.7: Assay protocol for glutathione peroxidase

Phosphate buffer	500µL
NaN_3	100µL
GSH	200µL
H_2O_2	100µL
Sample	500µL
Distilled Water	600µL

The whole reaction mixsture was incubated at 37°C for 3 minutes after which 0.5ml of TCA was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 1ml of each of the supernatants, 2ml of K_2HPO_4 and 1ml of DNTB was added and the absorbance was read at 412nm against a blank.

Calculation

Glutathione peroxidise activity was observed by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve.

$$\text{GSH consumed} = 245.84 - \text{GSH remaining}$$

$$\text{Glutathione peroxidase activity} = \frac{\text{GSH consumed}}{\text{mg protein}}$$

3.11. Data analysis

The data was analyzed by Statistical Analysis Software (SAS, 2003) using the one-way Analysis of Variance (ANOVA) of the Generalized Linear Model procedure. Duncan multiple range test was used to compare differences between means and were considered to be statistically significant at $p < 0.05$.

CHAPTER FOUR

4.0. RESULTS

4.1. Genetic variation of important agronomic traits among 29 grain amaranth accessions

The analysis of variance for two separate vegetative stages (at 50% flowering and at harvest) revealed significant differences among the 29 grain amaranth accessions for all the 9 agronomic characters evaluated indicating the presence of a high degree of variation among the accessions.

The mean performance of the 29 accessions for the quantitative traits taken at 50% flowering is presented in Table 4.1. The mean value of a number of accessions for each of the trait was higher than their corresponding arithmetic means. Significant variation was observed for all characters ($p < 0.01$) at 50% flowering.

Plant height and leaf length ranged from 54.10 to 155.00 cm and 11.00 to 22.10 cm, respectively, with accession A11 having the highest value for plant height; leaf length and second highest in leaf breadth and leaf area.

Leaf width and leaf area ranged from 4.80 to 13.00 cm and 26.40 to 128.90 cm, respectively. Stem diameter ranged from 10.80 to 21.90 mm. Accession A10 had the highest value for leaf width, leaf area and was second highest in stem diameter. Accession A9 had the highest value for stem diameter.

Number of leaves ranged from 25.50 to 167.60 and accession A13 had the highest value.

Days to 50% flowering ranged from 102.67 to 125.33 days among the 29 accessions of grain amaranth and had an interval of 22.67 days from the earliest to the latest maturing accessions. Significant variation was observed among all the accessions ($p < 0.01$). Accessions A24, A25 and A16 were observed to be early maturing as days to 50% flowering were lowest in these accessions.

Table 4.1: Mean values of different morphological characters for 29 grain amaranth accessions at flowering

Accession	PHt	NOL	SD	LL	LW	LA	DTF
1	82.46±12.21	69.22±15.76	19.61±1.23	17.74±1.67	8.57±1.47	78.35±21.16	123.00±2.00
2	123.79±8.54	125.36±37.41	18.32±0.87	17.87±2.24	8.15±0.98	74.86±17.44	116.67±2.33
3	64.47± 8.83	106.70±22.18	15.10±2.03	18.13±3.87	7.04±0.71	66.50±21.20	114.67±3.71
4	123.79±8.54	125.36±37.41	18.32±0.87	17.87±2.24	8.15±0.98	74.86±17.44	116.67±2.33
5	54.12±4.47	48.54±5.16	14.55±1.73	15.35±1.75	8.72±0.60	67.91±12.64	119.00±4.04
6	90.78±16.96	127.22±30.81	17.49±1.67	15.70±0.56	7.37±0.74	58.27±7.76	121.33±0.88
7	126.10±17.79	67.32±9.59	19.01±0.99	20.19±1.55	9.94±0.81	101.61±16.33	120.67±0.67
8	93.80±6.60	57.05±5.51	19.63±0.99	15.55±0.32	8.88±0.38	68.90±1.45	116.33±1.45
9	124.99±20.16	116.31±28.42	21.92±1.15	17.93±1.30	8.24±1.02	75.10±14.60	121.67±0.88
10	93.77±23.88	46.78±6.80	20.86±0.66	20.48±1.90	13.00±2.34	128.94±10.82	117.67±1.20
11	155.02±3.28	57.35±8.70	19.35±2.74	22.13±1.16	11.48±1.60	128.88±24.96	116.67±2.60
12	112.39±22.60	69.35±36.46	16.91±3.54	19.08±1.31	9.78±1.93	95.74±24.98	125.33±2.96
13	81.70±16.88	167.59±61.77	13.38±2.07	11.82±0.74	5.40±0.15	32.02±2.80	110.67±4.84
14	99.93±16.68	151.14±24.19	15.02±1.48	13.52±0.11	6.02±0.40	40.67±2.67	105.67±1.67
15	65.58±3.88	89.49±10.49	11.88±0.68	20.88±3.18	9.86±2.00	109.08±39.35	108.00±2.08
16	92.95±6.89	166.74±31.11	15.65±1.94	15.46±2.24	6.44±0.69	51.28±13.17	103.67±0.88
17	101.36±22.40	87.03±14.37	17.53±1.32	18.35v1.63	8.51±0.63	79.04±12.41	122.67±0.88
18	93.25±1.85	100.24±7.73	14.39±0.96	13.54±2.77	6.28±1.22	45.88±15.29	111.67±2.91
19	84.63±14.37	120.21±24.15	14.99±1.64	17.06±1.31	7.44±0.48	63.80±7.77	111.67±0.67
20	70.64±8.83	101.50±19.18	15.46±0.71	11.84±1.05	6.70±0.58	39.13±1.73	118.33±1.20
21	90.09±21.90	60.21±4.37	19.54±1.66	16.31±3.06	8.61±1.50	74.75±27.56	120.00±3.79
22	77.14±4.37	75.36±14.91	11.59±0.95	11.08±0.52	5.73±0.45	31.90±3.86	108.00±2.65
23	68.55±12.89	81.48±27.31	12.23±2.17	10.99±0.62	4.78±0.28	26.42±2.94	112.00±2.00

PHt = Plant height (cm), NOL = Number of leaves, SD = Stem diameter (mm), LL = Leaf length (cm), LW = Leaf width (cm), LA = Leaf area (cm^2), DTF = Days to 50% flowering. *P<0.05%, **P<0.01%, ***P<0.001%.

Table 4.1: Mean values of different morphological characters for 29 grain amaranth accessions at flowering (contd.)

Accession	PHt	NOL	SD	LL	LW	LA	DTF
24	74.18±11.72	103.85±15.40	11.20±1.01	16.43±2.96	7.45±1.35	65.19±23.24	102.67±1.20
25	88.92±11.37	82.00±9.54	10.82±0.66	16.26±1.98	8.36±0.43	67.39±6.34	102.67±0.33
26	69.90±10.75	97.12±11.70	10.97±1.41	13.30±1.95	6.22±1.39	44.07±16.60	106.33±2.85
27	87.99±10.82	106.96±25.61	14.21±1.48	13.07±0.38	6.46±0.53	42.34±4.44	113.00±1.15
28	119.40±8.64	48.13±9.33	18.24±1.60	19.27±0.31	8.64±0.62	83.28±6.50	122.00±1.15
29	69.10±3.71	25.47±0.17	11.29±0.81	16.26±0.50	8.20±0.17	66.67±2.94	122.33±0.67
Mean	92.44±3.21	92.45±5.22	15.84±0.42	16.33±0.42	7.94±0.25	68.37±3.73	114.86±0.79
LSD	35.43	65.16	4.31	5.28	3.08	46.60	6.35
Min	54.10	25.50	10.80	11.00	4.80	26.40	102.67
Max	155.00	167.60	21.90	22.10	13.00	128.90	125.33
CV (%)	23.40	43.10	16.60	19.80	23.70	41.30	3.38
Pr>F	***	**	***	**	***	***	***

PHt = Plant height (cm), NOL = Number of leaves, SD = Stem diameter (mm), LL = Leaf length (cm), LW = Leaf width (cm), LA = Leaf area (cm^2), DTF = Days to 50% flowering. *P<0.05%, **P<0.01%, ***P<0.001%.

At harvest, all of the characters measured exhibited broad variability and were highly significant ($p<0.001$), though number of leaves showed significant variation at $p<0.01$ (Table 4.2).

Accessions A7, A8, A9, A10, A11, A13, A22 and A23 exhibited high mean performance for most of the morphological characters.

Accession A11 had the highest value for plant height and leaf area. Accession A7 had the highest value for leaf length and leaf width and was second highest for leaf area, 100-seed weight and seed yield.

Accessions A2, A4, A7, A8, A9, A10, A11, A13, A14, A16, A17, A19, A23 and A28 had high mean values than arithmetic mean for all the morphological traits studied.

Number of branches was recorded at time of harvest and it ranged from 0 to 13.1 with significant variation observed at $p < 0.001$. Accessions A1, A8, A10 and A12 had no branches. Highest number of branches was observed for accession A22, followed by accessions A2 and A4.

Seed yield at harvest ranged from 15.20 to 209.94g and was significant at $p < 0.001$. Accession A10 had the highest value, followed by accession A7.

100-seed weight ranged from 0.0036 to 0.0091g, with highest value recorded for accession A23, followed by accession A7.

Table 4.2: Mean values of different morphological characters for 29 grain amaranth accessions at maturity

Accession	PHt	NOB	NOL	SD	LL	LW	LA	Yld	100-SW
1	147.65±18.82	0.00±0	101.28±24.79	22.75±1.77	16.93±1.36	6.64±0.58	56.76±8.78	15.20±6.91	0.006±0.00
2	143.19±17.78	12.23±0.96	116.08±21.78	19.44±0.49	14.68±3.78	5.74±1.32	47.09±19.28	54.30±2.72	0.006±0.01
3	125.63±20.31	7.85±0.23	169.33±35.70	18.3±1.45	14.98±4.07	6.16±1.91	53.84±30.04	61.59±2.75	0.0055±0.01
4	143.19±17.78	12.23±0.96	116.08±21.78	19.44±0.49	14.68±3.78	5.74±1.32	47.09±19.28	17.12±8.10	0.0058±0.01
5	87.25±10.31	3.03±0.19	63.51±13.31	16.54±2.13	15.83±0.15	8.31±1.03	65.81±8.58	157.96±8.50	0.0081±0.01
6	112.58±18.81	2.41±0.18	134.10±31.64	20.29±2.41	17.56±1.47	7.41±0.8	66.22±12.53	183.10±46.97	0.0081±0.00
7	136.39±12.21	5.35±0.74	104.82±21.72	20.79±1.26	21.69±0.47	10.42±0.84	113.35±11.42	199.57±37.08	0.0083±0.00
8	134.70±10.06	0.00±0.00	63.87±11.40	24.98±0.57	19.41±1.64	6.94±2.71	71.75±34.18	75.27±10.09	0.007±0.00
9	155.90±15.11	4.54±0.30	163.39±43.43	25.36±1.22	19.23±2.78	9.31±2.03	95.15±31.48	109.40±15.25	0.0065±0.00
10	148.86±19.00	0.00±0.00	77.10±16.3	24.76±1.71	20.15±2.35	7.56±1.48	79.59±23.53	209.94±17.14	0.0073±0.00
11	162.76±11.14	3.53±0.04	72.95±20.15	23.86±4.23	21.58±2.62	9.94±2.42	113.48±38.52	158.87±30.82	0.0077±0.00
12	142.58±15.55	0.00±0.00	95.97±40.12	18.41±3,56	19.21±3.58	9.87±2.27	102.76±39.43	141.65±18.83	0.0075±0.00
13	93.55±24.70	6.48±0.25	185.47±53.97	15.05±3.41	8.74±2.26	3.88±1.10	19.33±8.42	56.57±7.87	0.0071±0.00
14	122.08±17.80	8.86±0.21	109.25±19.75	15.61±0.92	11.21±1.51	4.51±0.56	26.12±6.10	101.52±7.06	0.0078±0.00
15	75.93±6.99	9.45±0.66	70.99±14.39	12.61±0.22	15.24±1.02	6.09±0.53	46.94±6.95	102.86±8.23	0.0072±0.07
16	116.40±13.91	3.14±0.08	166.47±26.77	16.15±1.64	11.68±1.18	4.92±0.32	29.10±4.81	52.92±0.43	0.0036±0.00
17	154.94±9.87	5.13±0.08	95.2±16.45	20.04±1.89	18.99±1.18	8.76±0.66	83.90±11.46	68.16±0.51	0.005±0.00
18	107.85±11.57	5.17±0.36	85.41±12.24	15.65±1.06	11.48±1.50	5.30±0.67	31.34±8.04	90.32±3.89	0.0047±0.00
19	121.24±5.03	5.15±0.21	113.66±32.28	16.33±1.39	14.15±1.06	5.80±0.85	41.85±9.11	48.94±0.24	0.0048±0.00
20	60.00±4.35	10.50±0.00	118.42±4.46	19.6±1.27	10.88±1.24	4.49±0.61	25.15±5.87	69.79±11.99	0.0059±0.00
21	108.04±16.38	6.61±0.13	92.86±25.56	23.07±3.72	17.66±3.09	8.33±1.83	78.99±29.97	60.77±14.81	0.006±0.00
22	74.57±0.50	13.19±0.19	55.42±14.84	13.45±0.86	9.06±1.34	4.76±0.75	22.48±6.13	90.00±7.72	0.007±0.00
23	71.65±6.02	7.75±0.32	171.95±27.91	13.65±2.45	7.46±0.48	2.74±0.34	10.37±1.98	98.07±12.42	0.0091±0.00
24	85.95±17.38	8.95±0.58	65.57±12.32	14.06±2.22	9.91±1.16	4.89±0.61	24.93±5.49	89.60±6.34	0.0073±0.00

PHt = Plant height (cm), NOB= Number of branches, NOL = Number of leaves, SD = Stem diameter (mm), LL = Leaf length (cm), LW = Leaf width (cm), LA = Leaf area (cm^2), YLD = Yield (g), 100-SW = 100 Seed weight (g). *P<0.05%, **P<0.01%, ***P<0.001%.

Table 4.2: Mean values of different morphological characters for 29 grain amaranth accessions at maturity (contd.)

Accession	PHt	NOB	NOL	SD	LL	LW	LA	Yld	100-SW
25	115.17±14.07	7.51±0.28	99.92±8.63	13.57±0.67	12.43±0.00	5.61±0.00	34.86±0.00	99.18±16.01	0.0078±0.00
26	90.06±11.92	7.16±0.49	104.76±15.13	13.27±2.76	10.62±2.57	4.54±1.27	27.31±13.75	95.88±2.87	0.0072±0.00
27	97.79±6.37	5.62±0.20	100.05±16.72	16.20±2.28	12.75±0.34	6.19±0.45	39.59±3.71	63.85±11.94	0.0063±0.00
28	133±4.79	3.46±0.40	62.86±17.97	21.09±1.52	15.65±0.75	8.51±2.87	67.93±24.86	114.48±0.11	0.0079±0.00
29	110.87±6.08	3.46±0.39	31.98±1.46	13.97±0.97	13.52±1.58	6.31±1.21	44.6±14.02	82.66±0.52	0.0051±0.00
Mean	116.54±3.68	5.82±0.40	103.75±5.53	18.22±0.52	14.74±0.53	6.54±0.30	54.06±4.20	95.50	0.0067
LSD	39.24	0.95	67.44	5.38	5.39	3.29	47.30	41.42	0.0082
Min	60.00	0.00	32.00	12.60	7.50	2.70	10.40	15.20	0.0036
Max	162.80	13.19	185.50	25.40	21.70	10.40	113.50	209.94	0.0091
CV (%)	20.60	9.98	39.70	18.10	22.40	30.80	53.50	26.40	8.53
Pr.>F	***	***	**	***	***	***	***	***	***

PHt = Plant height (cm), NOB= Number of branches, NOL = Number of leaves, SD = Stem diameter (mm), LL = Leaf length (cm), LW = Leaf width (cm), LA = Leaf area (cm^2), YLD = Yield (g), 100-SW = 100 Seed weight (g). *P<0.05%, **P<0.01%, ***P<0.00

4.1.1. Correlation among Characters

The Pearson-correlation coefficient was used to indicate the linear relationship among the characters. They are considered significant when probability level is less than 0.05 (95% confidence level). Of all the correlation coefficients, 15 were positive and highly significant at $p < 0.01$ (Table 4.3). Plant height highly correlated with stem diameter, Leaf Length, Leaf width, Leaf area and days to 50% flowering. Stem diameter was highly correlated with Leaf Length, Leaf width, Leaf area and days to 50% flowering. Leaf length was highly correlated with leaf width, leaf area and days to 50% flowering. Leaf breadth was highly correlated with Leaf area and Days to 50% flowering. Leaf area was highly correlated with days to 50% flowering. Seed yield exhibited significant and positive association with 100-seed weight ($p < 0.01$).

The highest correlation was between leaf breadth and leaf area ($R = 0.97$). Leaf length was also highly correlated with leaf area ($R = 0.96$).

Plant height was negatively correlated with number of branches ($p<0.05$). Generally, number of branches was negatively correlated with most characters like stem diameter, leaf length, leaf breadth, leaf area, days to 50% flowering and seed yield ($p < 0.05$).

4.1.2. Descriptive analysis

The summary of descriptive statistics for all the 27 quantitative traits (morphological and nutritional) is given in Table 4.4. A particularly high coefficient of variation was observed for leaf area (53.5%), number of leaves (39.7 %), leaf width (30.8%), seed yield (26.40%), leaf length (22.4%) and plant height (20.6%) indicating high variability for these characters.

Table 4.3 Pearson-correlation matrix of selected agronomic characters in 29 accessions of grain amaranth.

Character	PHt	NOB	NOL	SD	LL	LW	LA	DTF	YLD
NOB	-0.451*								
NOL	-0.007	0.128							
SD	0.703***	-0.476*	0.002						
LL	0.753***	-0.566*	-0.234	0.793***					
LW	0.651***	-0.524*	-0.296	0.661***	0.918***				
LA	0.712***	-0.530*	-0.207	0.740***	0.960***	0.973***			
DTF	0.486***	-0.467*	-0.125	0.668***	0.678***	0.693***	0.694***		
YLD	0.113*	-0.369*	-0.230	0.203	0.452*	0.512*	0.519*	0.208	
100-SW	-0.146	-0.034	-0.100	-0.007	0.077	0.140	0.165	0.010	0.627**

PHt = Plant height, NOB= Number of branches, NOL = Number of leaves, SD = Stem diameter, LL = Leaf length, LW = Leaf width, LA = Leaf area, DTF = Days to 50% flowering, YLD = Yield, 100-SW = 100 Seed weight
*P<0.05%, **P<0.01%, ***P<0.001%.

Table 4.4 Descriptive statistics of 27 quantitative traits among 29 accessions of grain amaranth

No.	Characters	Mean	LSD	CV	Min	Max	Pr.>F
1	PHt cm	116.54	39.24	20.60	60.00	162.80	***
2	NOB	5.82	0.95	9.98	0.00	13.19	***
3	NOL	103.75	67.44	39.70	32.00	185.50	**
4	SD mm	18.22	5.38	18.10	12.60	25.40	***
5	LL cm	14.74	5.39	22.40	7.50	21.70	***
6	LW cm	6.54	3.29	30.80	2.70	10.40	***
7	LA cm^2	54.06	47.30	53.50	10.40	113.50	***
8	DTF	114.86	6.35	3.38	102.67	125.33	***
9	Yld (g)	95.50	41.42	26.40	15.20	209.94	***
10	100-sd wt (g)	0.0067	0.0082	8.53	0.0036	0.0091	***
11	MC %	10.28	0.41	2.31	7.43	15.46	***
12	Sugar %	1.62	0.16	6.22	0.37	3.10	***
13	Starch %	32.67	0.52	0.98	24.63	48.89	***
14	Fat %	6.80	0.32	2.90	1.43	10.39	***
15	Ash %	4.03	0.31	4.73	2.35	6.98	***
16	Protein %	15.18	0.65	2.62	11.77	19.01	***
17	Crude fibre %	3.03	0.23	4.58	1.04	6.27	***
18	Al mg/kg	86.29	8.75	6.21	17.24	250.73	***
19	Fe mg/kg	128.41	14.79	7.05	61.66	189.04	***
20	Zn mg/kg	49.37	4.63	5.74	34.73	100.07	***
21	Cu mg/kg	4.75	0.57	7.36	2.29	7.65	***
22	Mn mg/kg	101.74	3.70	2.23	45.74	213.73	***
23	Mg mg/kg	2125.13	75.15	2.16	1582.11	2624.40	***
24	Ca mg/kg	1482.86	87.25	3.60	888.24	1980.32	***
25	K mg/kg	4870.41	342.83	4.31	3157.00	10346.60	***
26	P mg/kg	5162.41	218.92	2.59	3531.00	7462.40	***
27	Se mg/kg	0.54	0.07	7.90	0	1.73	***

4.1.3. Variation in Proximate composition

The mean chemical composition of all the 29 accessions differed significantly ($p < 0.001$) for all the characters evaluated and is presented in Table 4.5.

Moisture content ranged from 7.43 to 15.46% with variety A21 having the highest value followed by variety A27.

The sugar and starch contents ranged from 0.37 to 3.1% and 24.63 to 48.89% respectively, with variety A25 having the highest value followed by variety A24 for both characters.

Fat content ranged from 1.43 to 10.39% and highest value was observed for variety A11 followed by variety A6.

Ash content ranged from 2.35 to 6.98%, with variety A15 having the highest value followed by variety A23.

Protein content ranged from 11.77 to 19.01%, highest value was observed for variety A28 followed by variety A9.

Crude fibre ranged from 1.04 to 6.27%, highest value was observed for variety A2 followed by variety A9.

4.1.4. Variation in Mineral Composition

The mineral composition varied significantly ($p < 0.001$) amongst all the 29 accessions evaluated with variety A23 having the highest values for potsassium, phosphorus and second to the highest in calcium (Table 4.6).

Potassium was the most abundant mineral element present, followed by phosphorus. Selenium was the least mineral element present followed by copper.

Aluminiun ranged from 17.24 to 250.73%, with variety A14 having the highest value followed by variety A15.

Iron content ranged from 61.66 to 189.04 mg/kg with variety A21 having the highest value followed by variety A15.

Zinc content ranged from 34.73 to 100.07 mg/kg, with variety A11 having the highest followed by variety A7. Copper ranged from 2.29 to 7.65 mg/kg with variety A12 having the highest followed by variety A8.

Manganese ranged from 45.74 to 213.73 mg/kg with variety A27 having the highest value followed by variety A6.

Magnesium ranged from 1582.11 to 2624.4 mg/kg with variety A3 having the highest value followed by variety A13.

Calcium ranged from 888.24 to 1980.32 mg/kg, variety A19 had the highest value followed by variety A23.

Potassium ranged from 3157 to 10346.6 mg/kg, variety A23 had the highest followed by variety A1.

Phosphorus ranged from 3531 to 7462.4 mg/kg, variety A23 had the highest value followed by variety A3.

Selenium was the least abundant mineral element ranging from 0 to 1.73 mg/kg. Variety A17 had the highest value followed by variety A18.

Table 4.5 Proximate composition of 29 accessions of grain amaranth

Varieties	% MC	% Sugar	% Starch	% Fat	% Ash	% Protein	% Crude Fibre
A1	11.04 ± 0.25	1.66 ± 0.07	29.59 ± 0.736	8.51 ± 0.10	3.49 ± 0.121	14.12± 0.256	3.56± 0.266
A2	12.27 ± 0.05	1.91 ± 0.03	29.25 ± 0.201	8.59 ± 0.21	3.44 ± 0.141	17.30± 1.12	6.27± 0.241
A3	10.61 ± 0.05	2.23 ± 0.10	29.67 ± 0.284	7.19 ± 0.17	4.17 ± 0.085	11.77±0.26	3.25± 0.181
A4	9.48 ± 0.05	0.71 ± 0.13	28.91 ± 0.142	8.44 ± 0.08	3.56 ± 0.466	13.83± 0.15	3.07± 0.246
A5	9.62 ± 0.22	2.55 ± 0.087	31.90 ± 0.281	8.70 ± 0.198	3.47 ± 0.78	16.1± 0.20	1.91± 0.081
A6	11.73 ± 0.29	1.75 ± 0.065	34.55 ± 0.274	9.49 ± 0.161	3.45 ± 0.086	15.04± 0.59	2.74± 0.105
A7	9.64 ± 0.22	2.28 ± 0.123	32.49 ± 0.204	7.43 ± 0.33	3.28 ± 0.14	15.39± 0.114	2.91± 0.070
A8	9.52 ± 0.09	3.10 ± 0.153	35.35 ± 0.320	8.63 ± 0.092	2.48 ± 0.081	16.03± 0.087	3.67± 0.080
A9	13.01 ± 0.13	0.99 ± 0.095	33.67 ± 0.317	7.63 ± 0.136	4.36 ± 0.23	18.42± 0.167	5.37± 0.060
A10	13.66 ± 0.09	0.37 ± 0.057	30.40 ± 0.125	8.28 ± 0.178	2.81 ± 0.06	14.45± 0.130	3.57± 0.162
A11	9.24 ± 0.086	0.73 ± 0.351	24.98 ± 0.486	10.39 ± 0.147	2.35 ± 0.32	15.71± 0.062	4.41± 0.076
A12	12.98 ± 0 193	1.50 ± 0.061	26.3 ± 0.208	8.86 ± 0.091	2.95 ± 0.12	15.36± 0.083	3.25± 0.111
A13	10.54 ± 0.191	1.74 ± 0.091	36.04 ± 0.373	5.60 ± 0.140	3.43 ± 0.14	16.57±0.081	2.36±0.086
A14	9.75 ± 0.191	1.06 ± 0.140	25.84 ± 0.191	5.62 ± 0.516	6.26 ± 0.17	13.63± 0.092	1.63± 0.129
A15	10.44 ± 0.125	0.59 ± 0.091	24.63 ± 0.38	5.68 ± 0.123	6.98 ± 0.08	17.75 ± 0.09	3.36± 0.101
A16	9.88 ± 0.176	1.28 ± 0.149	27.81 ± 0.387	5.41 ± 0.723	4.42 ± 0.25	16.46± 0.075	2.54± 0.114

MC = Moisture content, LSD = Least significant difference, CV= Coefficient of variation, Min = minimum, Max = maximum

Table 4.5 Proximate composition of 29 accessions of grain amaranth (contd.)

Varieties	% MC	% Sugar	% Starch	% Fat	% Ash	% Protein	% Crude Fibre
A17	10.47 ± 0.234	1.17 ± 0.075	29.38 ± 0.155	5.94 ± 0.208	4.78 ± 0.10	17.96± 0.618	1.04± 0.147
A18	9.45 ± 0.107	1.01 ± 0.059	32.37 ± 0.530	7.59 ± 0.398	4.53 ± 0.30	12.81± 0.297	1.13± 0.113
A19	9.53 ± 0.160	1.42 ± 0.029	27.24 ± 0.110	5.61 ± 0.182	4.18 ± 0.10	15.27± 0.815	1.13± 0.201
A20	8.67 ± 0.267	1.21 ± 0.066	35.88 ± 0.04	4.45 ± 0.065	5.93 ± 0.32	13.04± 0.203	5.13± 0.126
A21	15.46 ± 0.10	1.66 ± 0.091	33.23 ± 0.22	1.43 ± 0.095	3.38 ± 0.30	12.76± 0.277	2.50± 0.068
A22	8.58 ± 0.131	1.56 ± 0.205	34.17± 0.627	3.77 ± 0.095	3.65 ± 0.14	12.82± 0.042	3.66± 0.142
A23	7.43 ± 0.095	2.07 ± 0.137	29.36 ± 0.115	4.46 ± 0.255	6.47 ± 0.16	14.01± 0.346	2.38± 0.073
A24	13.19 ± 0.257	2.83 ± 0.135	43.46 ± 0.216	5.40 ± 0.085	3.46 ± 0.09	15.16 ± 0.118	2.16± 0.161
A25	13.26 ± 0.162	3.04 ± 0.050	48.89 ± 0.426	6.41 ± 0.07	3.82 ± 0.18	14.51± 0.512	1.70± 0.062
A26	11.06 ± 0.322	2.51 ± 0.139	35.65 ± 0.071	6.32 ± 0.131	3.53 ± 0.19	12.72± 0.559	4.14± 0.177
A27	14.70 ± 0.679	0.69 ± 0.057	40.53 ± 0.135	6.59 ± 0.154	3.37 ± 0.07	15.53± 0.43	4.33± 0.087
A28	9.33 ± 0.45	1.77 ± 0.032	28.84 ± 0.271	7.39 ± 0.240	5.26 ± 0.19	19.01± 0.213	3.09± 0.114
A29	9.27 ± 0.707	1.46 ± 0.075	47.17 ± 0.131	7.53 ± 0.215	3.54 ± 0.13	16.77± 0.729	1.57± 0.093
Mean	10.28	1.62	32.67	6.8	4.03	15.18	3.03
Min	7.43	0.37	24.63	1.43	2.35	11.77	1.04
Max	15.46	3.1	48.89	10.39	6.98	19.01	6.27
LSD	0.41	0.16	0.52	0.32	0.31	0.65	0.23
CV	2.31	6.22	0.98	2.9	4.73	2.62	4.58

MC = Moisture content, LSD = Least significant difference, CV= Coefficient of variation, Min = minimum, Max = maximum

Table 4.6 Mineral contents of 29 grain amaranth accessions (mg/kg)

Variety	Al	Fe	Zn	Cu	Mn	Mg	Ca	K	P	Se
A1	72.88±1.65	136.30±1.52	35.62±1.06	4.29±0.21	47.57 ± 1.05	2264.6 ± 42.04	935.28 ± 20.99	7910.88 ± 42.54	6133.90 ± 105.02	0.37 ± 0.03
A2	168.41±0.52	121.67±4.38	50.16±1.13	2.37±0.16	63.74 ± 2.70	2043.5 ± 48.04	1285.46 ± 46.79	3775.66 ± 151.87	5232.90 ± 173.36	0 ± 0
A3	129.48±3.18	169.49±2.86	37.72±0.69	4.53±0.10	45.74 ± 0.65	2624.4 ± 9.04	1063.91 ± 4.99	4448.33 ± 51.75	6363.01 ± 32.14	0 ± 0
A4	73.57±2.27	149.10±1.36	49.24±0.41	5.37±0.27	97.16 ± 1.03	1944.2± 73.0	1866.44 ± 138.65	4415.42 ± 678.66	5333.19 ± 206.76	0.60 ± 0.09
A5	63.34±1.36	107.49±1.49	51.45±12.32	7.04±0.51	81.90 ± 0.40	2411.4 ± 129.0	1326.16 ± 55.75	7218.19 ± 360.35	5752.76 ± 280.42	1.45 ± 0.05
A6	24.43±0.73	83.28±1.94	50.06±1.46	4.98±0.65	203.38 ± 5.63	2169.3 ± 54.9	1803.56 ± 7.82	3877.84 ± 15.45	4355.02 ± 114.64	0.87 ± 0.03
A7	26.48±0.70	89.34±4.45	63.55±2.94	6.86±0.44	187.89 ± 7.00	2095.5 ± 103.8	1803.45 ± 49.05	4112.48 ± 97.83	4503.07 ±235.50	1.32 ± 0.02
A8	18.28±0.39	62.22±1.28	54.71±1.59	7.29±0.25	153.64 ± 1.39	2185.3 ± 29.9	1613.37 ± 27.27	3641.42 ± 18.69	4534.74 ± 64.74	0.53± 0.03
A9	50.88±1.23	128.77±3.50	51.19±1.80	6.65±0.18	93.80 ± 3.29	2278.7 ± 74.1	1795.58 ± 58.29	4520.66 ± 30.99	4922.92 ± 216.77	0.65 ± 0.03
A10	74.63±3.08	162.96±5.81	48.35±1.42	5.30±0.20	97.54 ± 3.65	1940.2 ± 69.3	1866.44 ± 138.65	4415.42 ± 678.66	5333.19 ± 206.76	0.53 ± 0.03
A11	68.95±0.68	152.93±3.76	100.07±5.58	7.17±0.05	130.1 ± 2.22	1619.5 ± 16.6	1263.10 ± 23.02	3157.00± 41.33	3530.98 ± 239.77	0 ± 0
A12	53.45±1.91	156.50±1.95	56.56±1.94	7.65±0.49	143.3 ± 1.20	1582.1 ± 20.8	1667.96 ± 7.66	3305.48 ± 39.90	4017.44 ± 33.99	0.89 ± 0.04
A13	20.30±1.49	81.40±0.93	50.59±1.39	6.36±0.75	69.64 ± 0.68	2605.1 ± 13.0	1682.51 ± 12.12	4423.44 ± 22.11	5574.11 ± 5.21	1.48 ± 0.07
A14	250.73±16.27	130.25±21.94	47.31±0.61	5.15±0.41	109.71 ± 0.89	2042.6 ± 11.2	1081.68 ± 15.63	5313.65 ± 30.44	5189.05 ± 131.83	0 ± 0
A15	223.92±14.85	182.57±15.30	36.67±1.71	2.29±0.17	103.58 ± 2.83	1871.2 ± 51.3	1841.29 ± 54.09	4650.46 ± 121.49	4964.54 ± 140.39	0.04 ± 0
A16	20.98±1.29	68.03±4.32	48.53±0.91	4.62±0.04	128.21 ± 0.20	1930.5 ± 10.6	1653.60 ± 14.18	5023.72 ± 11.03	4460.29 ± 32.37	0.45 ± 0.03

LSD = Least significant difference, CV= Coefficient of variation, Min = minimum, Max = maximum

Table 4.6 Mineral contents of 29 grain amaranth accessions (mg/kg) (contd.)

Variety	Al	Fe	Zn	Cu	Mn	Mg	Ca	K	P	Se
A17	20.37±1.02	76.94±1.82	61.07±1.18	6.09±0.60	62.01 ± 0.54	2377.5 ± 32.8	1562.18 ± 23.66	5081.10 ± 115.60	5948.78 ± 149.21	1.73 ± 0.04
A18	52.62±1.19	113.41±1.90	51.47±1.59	3.53±0.44	89.04 ± 1.10	2142.2 ± 19.0	1542.06 ± 8.47	4008.26 ± 27.88	5586.42 ± 78.39	1.57 ± 0.07
A19	17.23±0.94	61.66±0.85	34.73±0.57	4.42±0.28	47.75 ± 0.48	2382.9 ± 26.6	1980.32 ± 155.4	4698.62 ± 202.90	5958.82 ± 223.81	0.45 ± 0.01
A20	56.05±2.51	122.90±5.27	42.29±0.37	3.33±0.41	117.44 ± 0.58	2245.6 ± 17.5	1541.77 ± 2.73	4556.29 ± 87.94	5343.84 ± 37.90	0.32 ± 0.06
A21	83.61±2.22	189.04±4.51	41.93±0.85	4.46±0.24	121.15 ± 0.83	2380.5 ± 20.9	1275.98 ± 11.41	4716.66 ± 44.58	5836.18 ± 57.58	0.35 ± 0.07
A22	20.55±0.56	139.86±1.63	55.19±2.74	3.38±0.40	83.03 ± 1.96	1856.0 ± 14.5	970.30 ± 13.33	4698.62 ± 202.90	5958.82 ± 223.81	0 ± 0
A23	173.67±2.60	125.43±1.11	42.11±0.99	2.60±0.14	87.33 ± 1.03	2604.2 ± 18.4	1920.22 ± 6.01	10346.58 ± 181.08	7462.41 ± 48.04	0± 0
A24	132.93±13.04	156.05±36.49	41.91±0.66	2.82±0.10	83.08 ± 1.92	2051.2 ± 35.6	1352.25 ± 37.50	5453.22 ± 21.06	4823.05 ± 124.86	0.35 ± 0.04
A25	129.09±4.22	162.09±9.05	41.15±0.62	2.60±0.14	47.70 ± 0.41	2142.9± 19.0	1560.18 ± 24.51	3603.58 ± 56.77	4858.83 ± 16.94	0.30 ± 0.06
A26	156.53±1.33	162.22±2.48	41.75±1.46	2.69±0.15	53.27 ± 0.91	2381.9 ± 28.3	1544.06 ± 9.26	4112.81 ± 97.57	3530.98 ± 33.45	0.36 ± 0.07
A27	129.10±7.05	140.07±5.76	61.86±1.01	5.04±0.42	213.73 ± 1.21	1935.2 ± 18.9	971.97 ± 15.82	5411.00 ± 98.13	5237.59 ± 33.45	0.49 ± 0.07
A28	35.31±2.02	140.88±1.00	43.76±0.67	5.27±0.14	52.98 ± 0.48	1853.7 ± 10.7	1343.56 ± 12.11	3599.91 ± 53.09	4858.83 ± 16.94	0.45 ± 0.05
A29	154.64±6.32	151.00±1.60	40.64±0.65	3.73±0.32	135.12 ± 1.42	1666.8 ± 10.2	888.24 ± 9.10	6745.30 ± 45.08	4104.76 ± 15.75	0 ± 0
Mean	86.29	128.41	49.37	4.75	101.74	2125.13	1482.86	4870.41	5162.41	0.54
LSD	8.75	14.79	4.63	0.57	3.7	75.15	87.25	342.83	218.92	0.07
CV	6.21	7.05	5.74	7.36	2.23	2.16	3.6	4.31	2.59	7.9
Max	250.73	189.04	100.07	7.65	213.73	2624.4	1980.32	10346.6	7462.4	1.73
Min	17.24	61.66	34.73	2.29	45.74	1582.11	888.24	3157	3531	0

LSD = Least significant difference, CV= Coefficient of variation, Min = minimum, Max = maximum

4.1.5. Principal Component Analysis (PCA)

In order to assess the patterns of variation, PCA was done by simultaneously considering all 27 characters. The percentage of total variance that each variable represents and the coefficients used in the weighted sum (loadings or eigenvectors) are presented in Table 4.7.

The first four principal components (PCs) contributed 57.5% of the variability present among the 29 accessions for the 27 traits studied. PC_1 accounting for 30.23 % of the variation had leaf lenght, leaf area, leaf width, copper, plant height and stem diameter as the variables with the largest positive coefficient.

PC_2 accounting for an additional 11.87% of the total variation depicted primarily the patterns of variation in magnesium, selenium, iron, aluminium, number of leaves and phosphorus. All variables except iron and aluminium contributed positively towards PC_2.

PC_3 contributing 8.2% of the variability among the 29 accessions for the 27 traits had high positive coefficient of variates for crude fibre, selenium, while starch and sugar had high negative coefficients.

PC_4 having eigen value of 1.931 and contributing to 7.2% of the variability had high coefficient of variates observed for ash, seed yield, 100-seed weight, moisture, starch and calcium.

Table 4.7 Eigen values, proportion of variability and agronomic traits contributing to first four PCs of *Amaranthus* species

	PC1	PC2	PC3	PC4
Eigen value	8.162	3.207	2.213	1.931
% variance	0.3023	0.119	0.082	0.072
Cumulative variance	0.3023	0.421	0.503	0.575
	Coefficient of variates			
Plant Height cm	0.262	0.036	0.225	-0.155
Number of Branches	-0.224	-0.117	0.145	0.071
Number of leaves	-0.076	0.298	0.166	0.004
Stem diameter mm	0.266	0.075	0.232	-0.172
Leaf length cm	0.321	-0.009	0.113	-0.041
Leaf breadth cm	0.318	-0.032	0.028	-0.020
Leaf area cm^2	0.326	0.018	0.074	-0.001
Days to 50% flowering	0.237	0.098	0.163	-0.087
Seed yield (g)	0.181	-0.103	-0.245	0.356
100- seed weight (g)	0.040	-0.125	-0.229	0.346
Moisture %	0.073	-0.186	-0.010	-0.374
Sugar %	-0.062	-0.043	-0.414	-0.212
Starch %	-0.084	-0.154	-0.427	-0.314
Fat %	0.231	-0.049	-0.031	0.064
Ash %	-0.197	0.087	0.236	0.381
Protein %	0.139	0.053	0.017	0.163
Crude fibre %	0.073	-0.208	0.276	-0.044
Aluminium mg/kg	-0.175	-0.306	0.137	0.148
Iron mg/kg	0.055	-0.410	0.181	-0.084
Zinc mg/kg	0.219	-0.083	-0.044	0.184
Copper mg/kg	0.280	0.170	-0.064	0.042
Manganese mg/kg	0.145	-0.117	-0.173	0.178
Magnesium mg/kg	-0.133	0.372	-0.020	-0.179
Calcium mg/kg	0.046	0.258	-0.035	0.290
Potassium mg/kg	-0.156	0.157	0.031	0.075
Phosphorus mg/kg	-0.168	0.287	0.214	-0.069
Selenium mg/kg	0.128	0.345	0.252	-0.007

4.1.6. Cluster analysis

Using the k-means non-hierarchical clustering, the amaranth accessions could be broadly divided into three groups at 85% similarity coefficient which can be further subdivided into 5 clusters at 87.5% similarity coefficient as shown in the dendogram (Fig 4.1). The mean values of accessions falling in each cluster are presented in Table 4.8. The five clusters showing the number of accessions and their accession number as they fall into each group are presented in Table 4.9.

Cluster 1 included 3 accessions (A1, A3 and A21) having the highest mean values for moisture (12.37%) and iron (164.94 mg/kg). In this group, mean values for seed weight, seed yield, protein, zinc, magnesium and calcium were lowest. Also value for days to 50% flowering was highest, indicating late maturity.

Cluster II comprised of ten accessions (A2, A20, A22, A24, A25, A26, A14, A15, A27 and A29) having high mean values for number of branches per plant, starch content and lowest mean value for days to 50% flowering indicating that they are early maturing. There was no lowest mean value observed among accessions in this group.

Cluster III comprising of 14 accessions (A4, A16, A19, A18, A13, A5, A17, A6, A7, A8, A9, A10, A12 and A28) had the highest mean values for protein (15.91%) and selenium (0.76 mg/kg) and lowest mean value for aluminium and iron.

Cluster IV having only one accession (A11) in its group had the highest mean value for plant height, stem diameter, leaf length, leaf width, leaf area, seed yield, fat, crude fibre, zinc, copper and manganese content. Accession A11 had the lowest mean value for number of branches, number of leaves, sugar and starch, ash, magnesium, potassium and phosphorus.

Cluster V comprising only one accession (A23), was separated from other clusters owing to its high mean values for number of leaves per plant, 100-seed weight, sugar, ash, aluminium, magnesium, calcium, potassium and phosphorus. It had lowest mean values for plant height, stem diameter, leaf length, leaf breadth, leaf area, moisture, fat, fibre and copper.

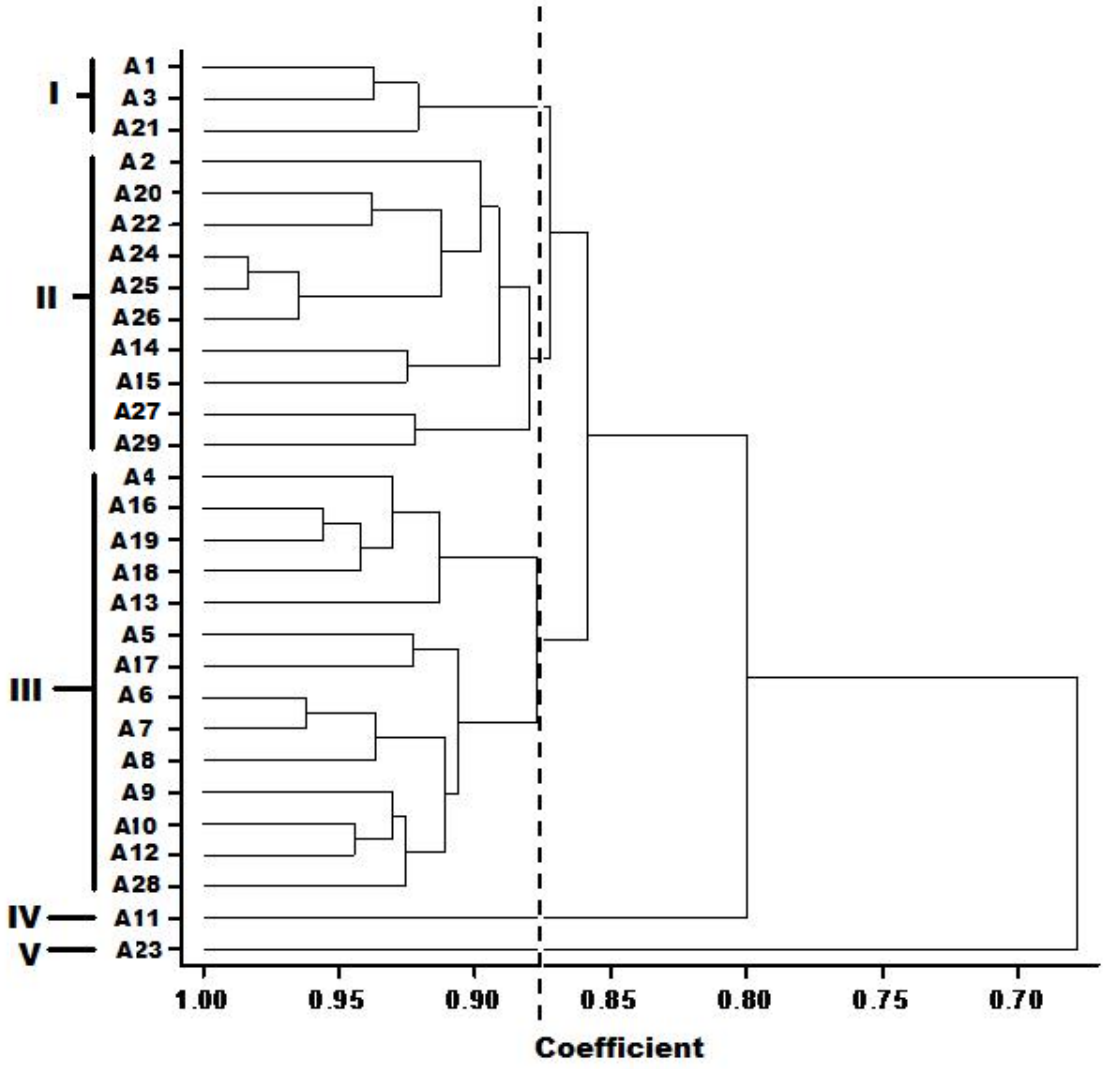


Figure 4.1 Dendogram of 29 accessions of grain amaranth species from morphological data

Table 4.8 Cluster analysis for 27 traits in 29 accessions of grain amaranth

	Cluster				
	I	II	III	IV	V
No of accessions in each cluster	3	10	14	1	1
Character					
Plant height (cm)	126.44	97.56	127.74	162.76	71.65
Branches per plant	4.82	8.69	4.01	3.53	7.75
Leaves per plant	121.16	87.24	109.13	72.95	171.95
Stem diameter (cm)	21.38	15.18	19.64	23.86	13.66
Leaf length (cm)	16.53	12.03	16.31	21.58	7.46
Leaf width (cm)	7.04	5.31	7.34	9.94	2.74
Leaf area (cm2)	63.20	33.91	65.37	113.48	10.37
Days to 50% flowering	119.22	110.36	117.21	116.67	112
Seed yield (g per plant)	45.85	84.96	108.96	158.87	98.07
100- seed weight (g)	0.0058	0.0068	0.0066	0.0077	0.0091
Moisture (% w/w)	12.37	11.12	10.63	9.24	7.43
Sugar (g/100g)	1.85	1.69	1.55	0.73	2.07
Starch (g/100g)	30.83	36.55	31.09	24.98	29.36
Fat (g/100g)	5.71	6.04	7.50	10.39	4.46
Ash (g/100g)	3.68	4.40	3.78	2.35	6.47
Protein (g/100g)	12.88	14.92	15.91	15.71	14.01
Crude fibre (g/100g)	3.10	3.40	2.70	4.41	2.38
Aluminium(mg/kg)	95.32	142.19	68.28	68.95	173.67
Iron (mg/kg)	164.94	146.87	120.01	152.93	125.43
Zinc (mg/kg)	38.42	45.89	55.30	100.07	42.11
Copper (mg/kg)	4.43	3.34	5.65	7.17	2.60
Manganese (mg/kg)	71.49	101.04	123.82	130.1	87.33
Magnesium (mg/kg)	2423.17	2023.69	2083.36	1619.5	2604.2
Calcium (mg/kg)	1091.72	1303.72	1555.33	1263.10	1920.22
Potassium (mg/kg)	5691.95	4832.06	4490.81	3157.0	10346.58
Phosphorus (mg/kg)	6111.03	4924.44	4965.05	3530.98	7462.41
Selenium (mg/kg)	0.24	0.19	0.76	0	0

Table 4.9 Cluster composition of 29 *Amaranthus* accessions

Cluster	No of accessions	Accession number	Accession code
1	3	A1	PI490458
		A3	P1553073
		A21	PI511731
2	10	A2	PI511679
		A20	PI337611
		A22	PI558499
		A24	PI615696
		A25	PI619250
		A26	PI633596
		A14	PI538326
		A15	PI538327
		A27	Ames 1972
		A29	NAC3
3	14	A4	PI642741
		A16	Ames 1974
		A19	Ames 5647
		A18	Ames 5644
		A13	PI538325
		A5	PI477913
		A17	Ames 2256
		A6	PI511719
		A7	PI515959
		A8	PI538319
		A9	PI590992
		A10	PI604666
		A12	PI641045
		A28	NH84/444-4
4	1	A11	PI641047
5	1	A23	PI590991

EXPERIMENT 1b

4.2. Molecular characterization

4.2.1. Genetic variation among 29 Amaranth accessions as revealed by RAPD Analysis

In this study, 40 primers were tested by initial screening on one plant from each population resulting in the selection of 16 decamer primers that produced clear polymorphic RAPD profiles. An analysis of individual plants of 29 populations was conducted with the selected 16 primers to study the phylogeny among the amaranth accessions. The extent of RAPD polymorphism can be observed among the amaranth populations using primer OPT-08 (Figure 4.2) and OPV-10 (Fig 4.3).

The dendrogram constructed (Figure 4.4) reflects that in spite of the apparent phenotypic similarity, RAPD markers were able to detect sufficient polymorphisms to distinguish the same plant species collected from different phytogeographic regions.

Most of the accessions of a species were observed to cluster together, with the exception of accessions A2 and A21 which did not cluster together with other members of their species. The RAPD-based UPGMA analysis clearly grouped the grain amaranth into 2 major groups at 71.9% coefficient of similarity. One of the major groups consisted mostly of *A. caudatus, A. hybrid* and *A. hypochondriacus* while the other group consisted of *A. cruentus, A. hybridus* and *A. hybrid*.

At 77.4% coefficient of similarity the dendogram can be observed to have 9 groups or clusters. Accessions A28 and A29 are genetically identical at 89% similarity coefficient and were the two accessions obtained from the NIHORT germplasm. The most distant are accessions A1 and A17 which are found to be at the extreme loci on the dendrogram. It is also evident from the RAPD-based clustering that *A. caudatus* and *A. hybrid* are closely related to *A. hypochondriacus* as they are found on neighbouring branches.

This study showed some geographic cohesiveness, as represented by accessions A13, A14, A15; and A16, A17, A18, A19; which are all *Amaranthus hybrid* collected from

close phytogeographic regions and found to be on neighboring branches in the dendrogram.

Amplicons were scored for an estimation of genetic relationships among *Amaranthus* accessions. A total of 193 loci were generated (Table 4.10), of which 36 were monomorphic (18.65%) resulting in 81.35% polymorphism at the interspecific level. An average of 12.06 loci per primer was produced, ranging from a minimum of 7 loci using primer OPG-12 to a maximum of 16 loci using primer OPV-04. The average polymorphic information content (PIC) was 0.872, ranging from 0.739 to 0.923. The lowest and the highest PIC values were recorded for primer OPG-12 and OPH-17, respectively.

At the intraspecific level, the percentages of RAPD polymorphism were found to be 41.97%, 48.19%, 61.14%, 66.32% and 10.88% in *A. caudatus*, *A. cruentus*, *A. hybrid*, *A. hypochondriacus* and *A. hybridus*, respectively (Table 4.11).

4.2.2. Similarity matrix

A similarity matrix shows the genetic distance among all the 29 accessions of grain amaranths in this study (Table 4.12). High correlation was observed for all accessions of grain amaranth studied, with *A. caudatus* exhibiting highest correlation with *A.cruentus* than with others ($P<0.01$). The genetic distance observed was between 0.61 and 0.88%.

OPT 08

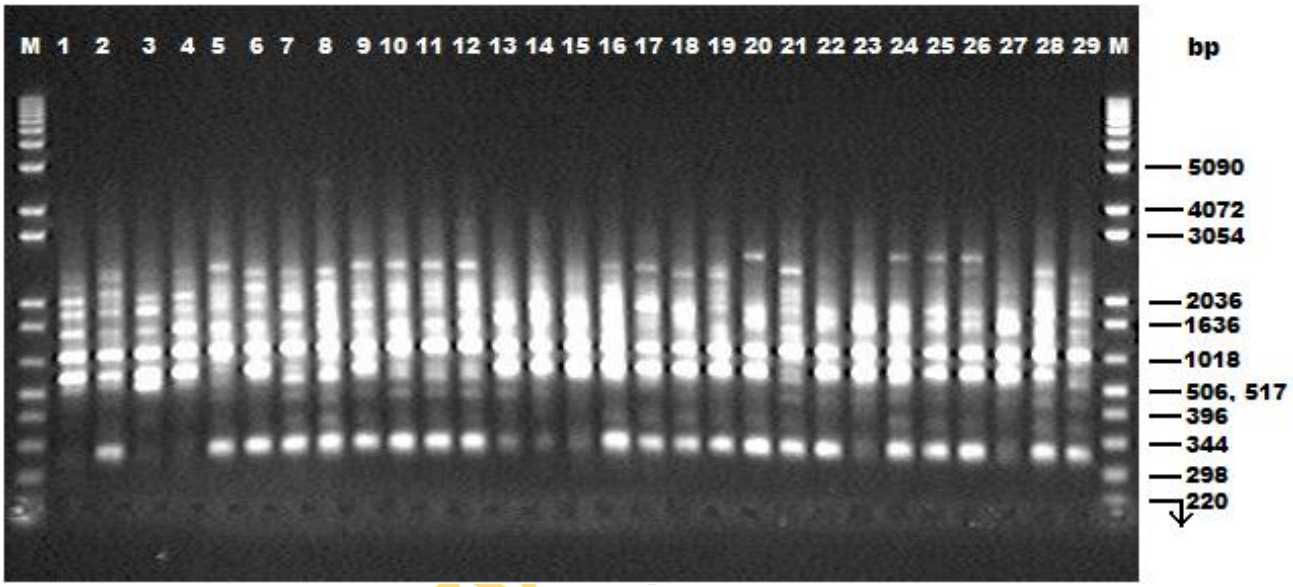


Figure 4.2 RAPD profiles of grain amaranth accessions using primer OPT 08. Lane M is 1kb ladder marker DNA. Lanes 2-30 (varieties 1-29 of grain amaranth accessions)

OPV10

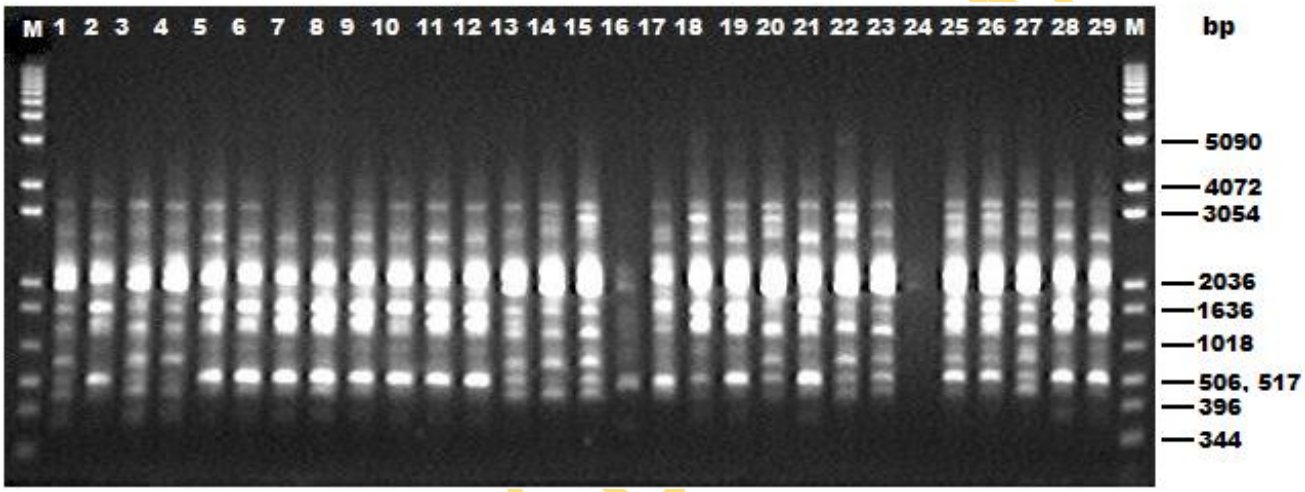


Figure 4.3 RAPD profiles of grain amaranth accessions using primer OPV 10. Lane M is 1kb ladder marker DNA. Lanes 2-30 (varieties 1-29 of grain amaranth accessions)

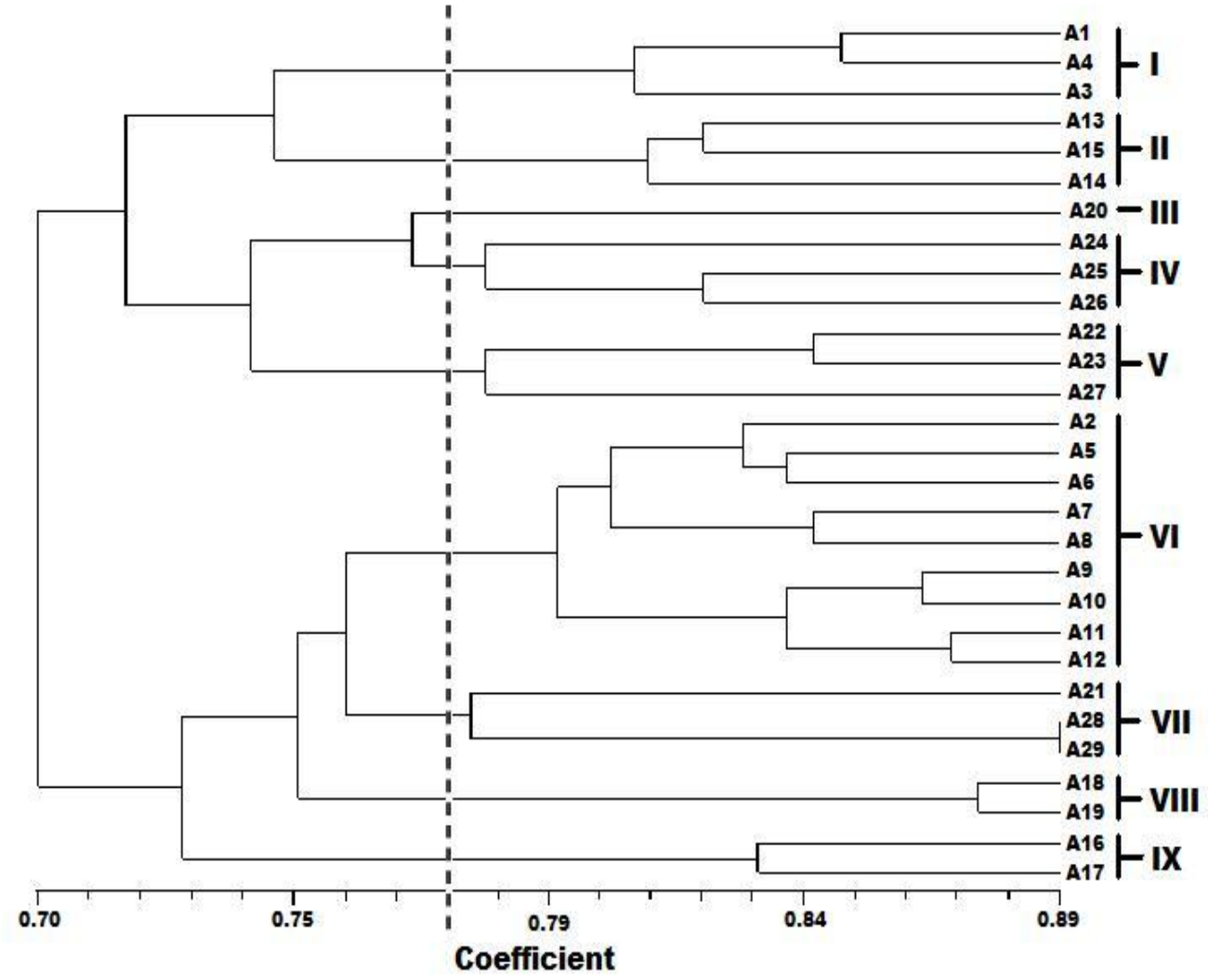


Figure 4.4 UPGMA-based dendogram derived from RAPD analyses of 29 accessions of grain Amaranth.

Table 4.10 RAPD Polymorphism among 29 accessions of *Amaranthus*

Primer	Total No. of fragments	Polymorphic fragments	Polymorphism (%)	Polymorphic Information Content (PIC)
OPT08	12	10	83.33	0.904
OPV10	13	8	61.54	0.909
OPB17	10	10	100.00	0.893
OPQ07	15	11	73.33	0.901
OPV9	12	11	91.67	0.831
OPU13	12	8	66.67	0.893
OPU14	14	12	85.71	0.897
OPH12	10	6	60.00	0.857
OPH17	14	11	78.57	0.923
OPR02	8	4	50.00	0.797
OPI09	12	9	75.00	0.883
OPV19	11	9	81.82	0.896
OPG12	7	5	71.43	0.739
OPV04	16	16	100.00	0.899
OPI10	12	12	100.00	0.812
OPI06	15	15	100.00	0.913
All Primers Total	193	157	81.35	13.947
Mean	12.06	9.81	5.08	0.872

Table 4.11. RAPD Polymorphism at the Intraspecific Level Detected with 16 Primers

Species	No. of accessions	No. of polymorphic fragments	Polymorphism (%)
A. Caudatus	4	81	41.97
A. Cruentus	8	93	48.19
A. Hybrid	7	118	61.14
A. hypochondriacus	8	128	66.32
A. hybridus	2	21	10.88
Mean	5.8	88.2	45.70

Table 4.12 Similarity matrix of *Amaranthus* populations based on Jaccard's similarity coefficient

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25	A26	A27	A28	A29
A1	1.00																												
A2	0.75	1.00																											
A3	0.79	0.72	1.00																										
A4	0.85	0.70	0.83	1.00																									
A5	0.66	0.83	0.70	0.70	1.00																								
A6	0.71	0.83	0.73	0.68	0.84	1.00																							
A7	0.72	0.83	0.76	0.72	0.80	0.82	1.00																						
A8	0.69	0.81	0.73	0.72	0.79	0.79	0.84	1.00																					
A9	0.71	0.80	0.73	0.73	0.77	0.77	0.82	0.80	1.00																				
A10	0.74	0.84	0.75	0.70	0.79	0.78	0.83	0.81	0.87	1.00																			
A11	0.73	0.82	0.67	0.69	0.79	0.81	0.80	0.76	0.84	0.84	1.00																		
A12	0.73	0.80	0.70	0.67	0.78	0.78	0.79	0.78	0.82	0.85	0.87	1.00																	
A13	0.74	0.73	0.76	0.74	0.72	0.74	0.72	0.71	0.75	0.74	0.72	0.78	1.00																
A14	0.69	0.68	0.77	0.70	0.67	0.69	0.69	0.65	0.72	0.69	0.66	0.70	0.81	1.00															
A15	0.76	0.73	0.75	0.78	0.68	0.69	0.70	0.68	0.76	0.72	0.71	0.68	0.82	0.82	1.00														
A16	0.72	0.71	0.73	0.75	0.70	0.72	0.72	0.73	0.75	0.74	0.73	0.72	0.68	0.67	0.68	1.00													
A17	0.68	0.75	0.66	0.67	0.69	0.75	0.74	0.70	0.75	0.73	0.74	0.69	0.67	0.65	0.65	0.83	1.00												
A18	0.71	0.75	0.70	0.68	0.74	0.75	0.74	0.74	0.74	0.71	0.72	0.68	0.68	0.69	0.68	0.68	0.72	1.00											
A19	0.76	0.78	0.75	0.76	0.77	0.79	0.78	0.76	0.78	0.77	0.77	0.75	0.75	0.73	0.74	0.76	0.76	0.88	1.00										
A20	0.70	0.70	0.74	0.71	0.67	0.68	0.73	0.69	0.73	0.74	0.70	0.70	0.74	0.68	0.74	0.64	0.66	0.74	0.78	1.00									
A21	0.70	0.77	0.67	0.68	0.82	0.76	0.77	0.74	0.76	0.74	0.79	0.76	0.72	0.68	0.66	0.70	0.73	0.72	0.77	0.63	1.00								
A22	0.74	0.67	0.70	0.73	0.68	0.69	0.68	0.69	0.73	0.74	0.75	0.78	0.78	0.69	0.75	0.67	0.65	0.67	0.73	0.78	0.65	1.00							
A23	0.72	0.63	0.73	0.75	0.65	0.68	0.66	0.66	0.72	0.67	0.66	0.73	0.76	0.70	0.74	0.62	0.61	0.68	0.72	0.73	0.64	0.84	1.00						
A24	0.72	0.65	0.68	0.72	0.68	0.67	0.68	0.67	0.70	0.70	0.67	0.66	0.70	0.69	0.71	0.69	0.68	0.70	0.76	0.76	0.64	0.77	0.72	1.00					
A25	0.68	0.62	0.70	0.66	0.65	0.69	0.68	0.65	0.66	0.67	0.66	0.66	0.73	0.63	0.68	0.68	0.69	0.65	0.70	0.77	0.65	0.78	0.69	0.77	1.00				
A26	0.74	0.71	0.70	0.74	0.74	0.74	0.69	0.70	0.75	0.70	0.75	0.73	0.73	0.67	0.70	0.74	0.75	0.72	0.77	0.78	0.68	0.82	0.72	0.80	0.82	1.00			
A27	0.72	0.69	0.71	0.69	0.69	0.70	0.67	0.62	0.70	0.70	0.69	0.69	0.77	0.71	0.72	0.63	0.64	0.68	0.71	0.75	0.65	0.78	0.79	0.68	0.69	0.73	1.00		
A28	0.73	0.77	0.72	0.74	0.73	0.74	0.77	0.73	0.81	0.76	0.76	0.76	0.75	0.70	0.72	0.73	0.74	0.72	0.75	0.70	0.78	0.69	0.69	0.72	0.67	0.75	0.72	1.00	
A29	0.73	0.75	0.69	0.73	0.73	0.68	0.74	0.74	0.78	0.75	0.75	0.77	0.73	0.69	0.70	0.70	0.73	0.70	0.76	0.69	0.78	0.69	0.68	0.70	0.65	0.76	0.72	0.89	1.00

Names are abbreviated according to population code of Table 1.

Experiment 2a

4.3. Assessment of the protein quality of 10 selected grain amaranth accessions by amino acid analysis

4.3.1 Amino acid composition.

Knowledge of the amino acid composition of protein enables accurate assessment of the quality of protein. Results of the amino acid analyses carried out on 10 selected grain amaranth accessions (A2, A3, A9, A10, A17, A18, A21, A23, A27 and A28) representing the five species of 29 accessions under study are presented in Table 4.13. A soybean accession (TGX 1448-2E) was included as a high protein source material for comparison.

The data are expressed as grams of amino acids per hundred gram of protein and are compared with reference amino acid patterns for humans recommended by FAO/WHO (1991) and USFDA (1993). The total protein content among these accessions differs significantly ($p < 0.05$) (Table 4.13). Accession A28 which had the highest protein content (19.01 mg/100g) was significantly higher than any of the other 9 accessions. Although many of the amino acid values appeared to be very similar, accession to accession variations in amino acid content from the mean were significant ($p<0.01$) (Table 4.13). Results show that all grain amaranth cultivars investigated were high in glutamic acid, aspartic acid, arginine, glycine, and lysine. Mean glutamic acid values ranged from 14.10 to 23.70 g/100 g protein and accounted for 17.49% of the total protein. These values are lower than 29.10 g amino acid/100 g total protein obtained for the soybean cultivar used as reference in this study. This was followed by aspartic acid which ranged from 8.6 to 11.50 g/100 g protein, values were lower than 20.57 g/100 g protein obtained for the soybean cultivar used as reference. Arginine ranged from 6.70 g/100 g to 11.80 g/100g protein, this was also lower than value (24.30 amino acid g/100 g protein) obtained for the soybean used as reference. Lysine an essential amino acid usually absent or present in low amounts in most cereals was found to be present in relatively high amounts in all the cultivars of grain amaranth investigated. It ranged from 5.65 to 7.89 g/100 g protein. Accessions A27 and A23 had the highest values for lysine (7.89 and 7.88 g/100 g protein, respectively). Accession A27 had highest values for most of the essential amino acids (Threonine, valine, Isoleucine, Leucine,

Phenylalanine, Histidine and Lysine). Among the amino acid residues present in lowest amounts are tryptophan, (0.72 to 0.91g/100 g protein); methionine (1.77 to 2.74 g/100 g protein) and cysteine (0.98 to 2.00 g/100 g protein). The levels of tryptophan in all varieties of grain amaranth studied are similar.

4.3.1.1. Evaluation of protein quality using amino acid score and the PDCAAS

The essential amino acid profiles and protein ratings of the 10 selected grain amaranth accessions investigated are compared with those of the FAO/WHO, (1991) reference pattern of hen's whole egg and for the diet of a 2- to 5-year-old child, and the results are summarized in Table 4.14.

The calculated amino acid scores for the 10 grain amaranth accessions varied slightly in their EAA_9 contents (Table 4.14). These grain amaranth proteins contain all of the EAA_9 (nine essential amino acids). The essential amino acid (EAA_9) pattern ranged from 31.22 to 44.88% and was higher than the 33.9% reference protein pattern value given by FAO/WHO, (1991) for the diet of a 2- to 5-year-old child and was also comparable to 51.2% given for hen's whole egg. The soybean EAA_9 (60.87%) obtained in this study was also higher than the reference protein value (33.9%) and values (31.22 to 44.88%) obtained for all accessions of grain amaranth.

From the calculated amino acid scores (Table 4.15), tryptophan and leucine were the limiting essential amino acid for all grain amaranth accessions evaluated, while for soybean it was found to be methionine and tryptophan.

Table 4.13: Comparison of the amino acid (AA) composition and protein content (g/100 g of total protein; mean ± SD) of ten accessions of Grain Amaranth with a soybean accession (TGX 1448-2E)

AA	A2	A 3	A9	A10	A17	A18	A21	A23	A27	A28	Soybean	Mean AA	LSD	CV
Aspartic acid	11.20 ± 0.20	8.60 ± 0.60	10.60 ± 0.52	10.80 ± 0.60	10.80 ± 0.36	9.47 ± 0.10	8.87 ± 0.10	11.40 ± 0.20	11.50 ± 0.40	11.00 ± 0.50	20.57 ± 0.45	13.22	0.69	3.09
Threonine	4.82 ± 0.02	3.50 ± 0.44	4.53 ± 0.03	4.58 ± 0.03	4.50 ± 0.30	4.22 ± 0.04	3.86 ± 0.06	4.78 ± 0.03	5.11 ± 0.03	4.79 ± 0.03	6.70 ± 0.30	5.28	0.31	3.52
Serine	7.37 ± 0.02	5.79 ± 0.02	7.11 ± 0.02	7.39 ± 0.10	7.22 ± 0.20	6.20 ± 0.20	5.70 ± 0.60	7.61 ± 0.01	8.14 ± 0.04	7.48 ± 0.06	8.40 ± 0.26	7.89	0.37	2.77
Glutamic acid	22.90 ± 0.40	14.10 ± 0.17	19.90 ± 0.90	22.40 ± 0.20	21.70 ± 0.30	18.40 ± 0.40	16.00 ± 0.57	23.20 ± 0.10	23.70 ± 0.20	21.50 ± 0.50	29.10 ± 0.26	23.82	0.72	1.78
Proline	4.95 ± 0.03	3.42 ± 0.02	4.64 ± 0.04	5.02 ± 0.03	5.05 ± 0.02	4.14 ± 0.03	3.39 ± 0.02	5.22 ± 0.11	5.23 ± 0.10	4.87 ± 0.07	7.60 ± 0.20	5.56	0.14	1.46
Glycine	8.82 ± 0.02	6.96 ± 0.17	8.03 ± 0.03	8.81 ± 0.01	8.67 ± 0.07	7.44 ± 0.04	6.97 ± 0.02	9.11 ± 0.10	9.04 ± 0.03	8.24 ± 0.02	6.10 ± 0	8.58	0.11	0.79
Alanine	4.68 ± 0.08	3.30 ± 0.30	4.30 ± 0.20	4.34 ± 0.01	4.37 ± 0.04	4.06 ± 0.03	3.59 ± 0.20	4.71 ± 0.01	4.70 ± 0.03	4.54 ± 0.04	6.45 ± 0.30	5.04	0.27	3.11
Cystine	2.00 ± 0.20	1.13 ± 0.03	1.57 ± 0.02	1.88 ± 0.08	1.57 ± 0.03	1.32 ± 0.02	0.98 ± 0	1.54 ± 0.04	1.55 ± 0.03	1.47 ± 0.03	1.50 ± 0.07	1.64	0.12	4.41
Valine	5.15 ± 0.04	3.64 ± 0.03	4.69 ± 0.06	4.86 ± 0.05	4.98 ± 0.03	4.61 ± 0.01	4.19 ± 0.01	5.35 ± 0.05	5.38 ± 0.05	5.16 ± 0.02	7.30 ± 0.60	5.69	0.31	3.24
Methionine	2.74 ± 0.04	1.80 ± 0.20	2.35 ± 0.04	2.44 ± 0.04	2.32 ± 0.02	2.49 ± 0.02	1.77 ± 0.10	2.56 ± 0.10	2.45 ± 0.04	2.45 ± 0.02	2.15 ± 0.20	2.52	0.17	3.93
Isoleucine	4.75 ± 0.05	3.31 ± 0.02	4.37 ± 0.02	4.48 ± 0.02	4.65 ± 0.04	4.19 ± 0.02	3.94 ± 0.02	4.89 ± 0.02	4.98 ± 0.03	4.75 ± 0.10	7.25 ± 0.50	5.35	0.26	2.92
Leucine	7.53 ± 0.03	5.33 ± 0.03	6.94 ± 0.04	7.10 ± 0	7.22 ± 0.20	6.55 ± 0.15	6.00 ± 0.87	7.65 ± 0.02	7.74 ± 0.03	7.38 ± 0.03	12.25 ± 0.40	8.54	0.5	3.49
Tyrosine	3.12 ± 0.10	2.12 ± 0	2.82 ± 0.02	3.26 ± 0.05	2.88 ± 0.02	2.44 ± 0.03	2.27 ± 0.02	2.92 ± 0.02	3.06 ± 0.06	3.05 ± 0.05	4.95 ± 0.40	3.44	0.22	3.73
Phenylalanine	5.71 ± 0.02	4.04 ± 0.04	5.01 ± 0.01	5.39 ± 0.05	5.31 ± 0	4.77 ± 0.10	4.43 ± 0.02	5.66 ± 0.06	5.74 ± 0.04	5.44 ± 0.04	8.20 ± 0.20	6.17	0.13	1.21
Histidine	4.49 ± 0.10	3.07 ± 0.02	4.22 ± 0.10	4.48 ± 0.03	4.45 ± 0.03	3.53 ± 0.03	3.36 ± 0.06	4.53 ± 0.03	4.70 ± 0.20	4.30 ± 0.30	5.29 ± 0.11	4.7	0.21	2.65
Lysine	7.77 ± 0.02	5.65 ± 0.08	7.01 ± 0.01	7.42 ± 0.03	7.30 ± 0.30	6.84 ± 0.03	6.06 ± 0.06	7.88 ± 0.08	7.89 ± 0.07	7.55 ± 0.05	10.45 ± 0.70	8.39	0.4	2.8
Arginine	11.5 ± 0.50	6.70 ± 0.50	9.94 ± 0.03	11.30 ± 0	11.20 ± 0.40	9.13 ± 0.02	8.09 ± 0.05	11.40 ± 0.20	11.80 ± 0.70	11.40 ± 0.05	12.15 ± 0.20	11.52	0.57	2.91
Tryptophan	0.91 ± 0.02	0.88 ± 0.04	0.84 ± 0.04	0.91 ± 0.07	0.79 ± 0.04	0.72 ± 0.08	0.78 ± 0.08	0.91 ± 0.03	0.89 ± 0.04	0.81 ± 0.08	1.28 ± 0.26	0.88	0.16	1.09
Total protein g/100g dry matter	17.30	11.77	18.42	14.45	17.96	12.81	12.76	14.01	15.53	19.01	32.63			

Table 4.14 Comparison of the essential amino acid (EAA) scores of ten grain amaranth accessions and a soybean accession with Hen's Whole Egg, and the FAO/WHO EAA Requirements of a 2-5-year-old child

EAA	EAA[a] requirements for a preschool child (2-5-year-old)	A2	A3	A9	A10	A17	A18	A21	A23	A27	A28	Soybean	Egg[a]
	mg of amino acid/g of total protein												
Histidine	19	44.9	30.7	42.2	44.8	44.5	35.3	33.6	45.3	47	43	52.9	22
Isoleucine	28	47.5	33.1	43.7	44.8	46.5	41.9	39.4	48.9	49.8	47.5	72.5	54
Leucine	66	75.3	53.3	69.4	71	72.2	65.5	60	76.5	77.4	73.8	122.5	86
Lysine	58	77.7	56.5	70.1	74.2	73	68.4	60.6	78.8	78.9	75.5	104.5	70
Methionine + cystine	25	47.4	29.3	39.2	43.2	38.9	38.1	18.68	41.0	40.0	39.2	36.5	57
Phenylalanine + tyrosine	63	88.3	61.6	78.3	86.5	81.9	72.1	67.0	85.8	88.0	84.9	131.5	93
Threonine	34	48.2	35	45.3	45.8	45	42.2	38.6	47.8	51.1	47.9	67	47
Tryptophan	11	9.1	8.83	8.44	9.07	7.92	7.2	7.76	9.08	8.93	8.07	12.76	17
Valine	35	51.5	36.4	46.9	48.6	49.8	46.1	41.9	53.5	53.8	51.6	73	66
mg/g Total protein EAA9	339	438.7	312.23	399.64	416.6	415.22	379.2	343.86	442.08	448.83	426.27	608.66	512
% Total protein EAA9	33.9	43.87	31.22	39.96	41.66	41.52	37.92	34.39	44.21	44.88	42.63	60.87	51.2
Chemical score[b]		73	73	73	73	73	73	73	73	73	73	91[c]	97[C]

[a] Data from FAO/WHO (1991)

[b] Based on FAO/WHO (1973) scoring pattern

[c] Protein digestibility corrected amino acid score Taken from Zarkadas *et al*., (1999)

Table 4.15 Amino acid scores of ten accessions of grain amaranth, one soybean accession (TGX 1448-2E) and their limiting amino acid

EAA	EAA Requirement	A2	A3	A9	A10	A17	A18	A21	A23	A27	A28	Soybean
Histidine	19	2.36	1.61	2.22	2.36	2.34	1.86	1.77	2.38	2.47	2.26	2.78
Isoleucine	28	1.70	1.18	1.56	1.60	1.66	1.50	1.41	1.75	1.79	1.70	2.59
Leucine	66	1.14	0.81	1.05	1.08	1.09	0.99	0.91	1.16	1.17	1.12	1.85
Lysine	58	1.34	0.97	1.21	1.28	1.25	1.18	1.04	1.36	1.36	1.30	1.80
Methionine + cysteine	25	1.89	1.17	1.57	1.73	1.56	1.52	0.75	1.64	1.60	1.57	1.46
Phenylalanine + tyrosine	63	1.40	0.98	1.24	1.37	1.30	1.14	1.06	1.36	1.40	1.35	2.09
Threonine	34	1.42	1.03	1.33	1.35	1.32	1.24	1.13	1.41	1.5	1.41	1.97
Tryptophan	11	0.83	0.80	0.77	0.82	0.72	0.65	0.71	0.83	0.81	0.73	1.16
Valine	35	1.47	1.04	1.34	1.39	1.42	1.32	1.20	1.53	1.54	1.47	2.08
Limiting amino acid		Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Leu, Tryp	Met, Tryp

Experiment 2b

4.3.2. Evaluation of the seed storage proteins in 29 accessions of grain amaranth

Electrophoretic patterns of the different protein fractions in amaranth seeds were obtained under denaturing conditions, after extraction of the protein by step wise solubility using different solvents. Proteins that were extracted in each step as described by Landry and Moureaux (1980) and Bressani and Garcia-Vela (1990); were as follows: Fraction I (albumin), fraction II (globulins), fraction III (prolamins), fraction IV (prolamin-like), fraction V (glutelin-like); fraction VI (true glutelin).

Typical electrophoretic patterns of the total proteins obtained from the 29 grain amaranth accessions on 12% SDS-polyacrylamide gradient gel and 4% stacking gel are shown in Fig 4.5. Total proteins under denaturing conditions were present in the range of 14 to 50 kDa and very few protein bands were present in the range of molecular weights below 14 kDa. The patterns among cultivars are similar with good resolution over the entire gel. All the 29 grain amaranth accessions evaluated shared common bands with the soybean cultivar, with distinct similarities observed at the 20, 24, 34, 36 and 50 kDa regions indicating a close identity between amaranth total protein pattern and soybean protein (Figure 4.5). The protein pattern of low molecular weight polypeptides (less than 36 kDa) was almost the same for all examined amaranth accessions. Varieties A16 to A19 belonging to *Amaranthus hybrid* and A20 to A21 (*Amaranthus hypochondriacus*) were observed to have a common protein band slightly below the 34 kDa region. Variety A17 (*Amaranthus hybrid*) which had a high total protein content of about 17.66% was observed to have a unique band just below the 66 kDa molecular weight marker which represents the bovine serum albumin. This band was not found in any of the other amaranth accessions but was present in the soybean cultivar used as reference. The soybean protein pattern has a very characteristic group of polypeptides above the 66 kDa revealed as three thick bands which are absent in all amaranth accessions evaluated.

The electrophoretic pattern of albumin fractions from twenty nine accessions of grain amaranth is shown in Fig 4.6. All subunits migrated between 14 and 66 kDa and all accessions had characteristic bands at 20, 24 and 36 KDa. Some minor fractions were observed in the region

between 45 and 66 kDa. Varieties A3, A4 (*A. caudatus*) and A5, A6 and A7 (*A. cruentus*) differed from others mainly in the region of 24 kDa.

The globulin fractions were in the range of 14 to 36 kDa bands with some minor bands observed above the 45 kDa (Figure 4.7). Electrophoretic pattern of globulin showed characteristic main bands at 24, 34, 36 and just above the 45 kDa region. All the accessions showed similar bands along the 20, 24, 34, 36 and 50 kDa regions and these bands were similar to the soybean cultivar used as reference. Bands were observed in the soybean cultivar just above the 66 kDa region which was distinct and not found in any of the amaranth accessions evaluated.

Prolamins were not detectable in this study when the procedures of Landry and Moureaux (1980) for the extraction of cereal prolamins (alcohol soluble proteins) were adopted as it is observed in Figure 4.8.

The amaranth glutelin-like pattern is similar in all accessions studied and also shows very close similarity with the soybean reference material. The glutelin-like fraction has three main polypeptides groups, one at 3 to 14 kDa, the second at 24 to 34 kDa and the third around 65 kDa region (Figure 4.9). Varieties A5, A7, A14, A15, A18, A19, A20 and A27 have two unique bands at the 66 kDa region.

Electrophoretic separation showed that glutelins were made up of three main sets of polypeptides (Figure 4.10) and the pattern had high similarity with the soybean cultivar. Two of the three polypeptides groups were between the 3 to 14 kDa; 24 kDa region while the third group was around the 66 kDa molecular weight marker. As can be seen from the electrophoretic patterns, all accessions shared common bands at the 14, 24 and 66 kDa regions similar to the soybean.

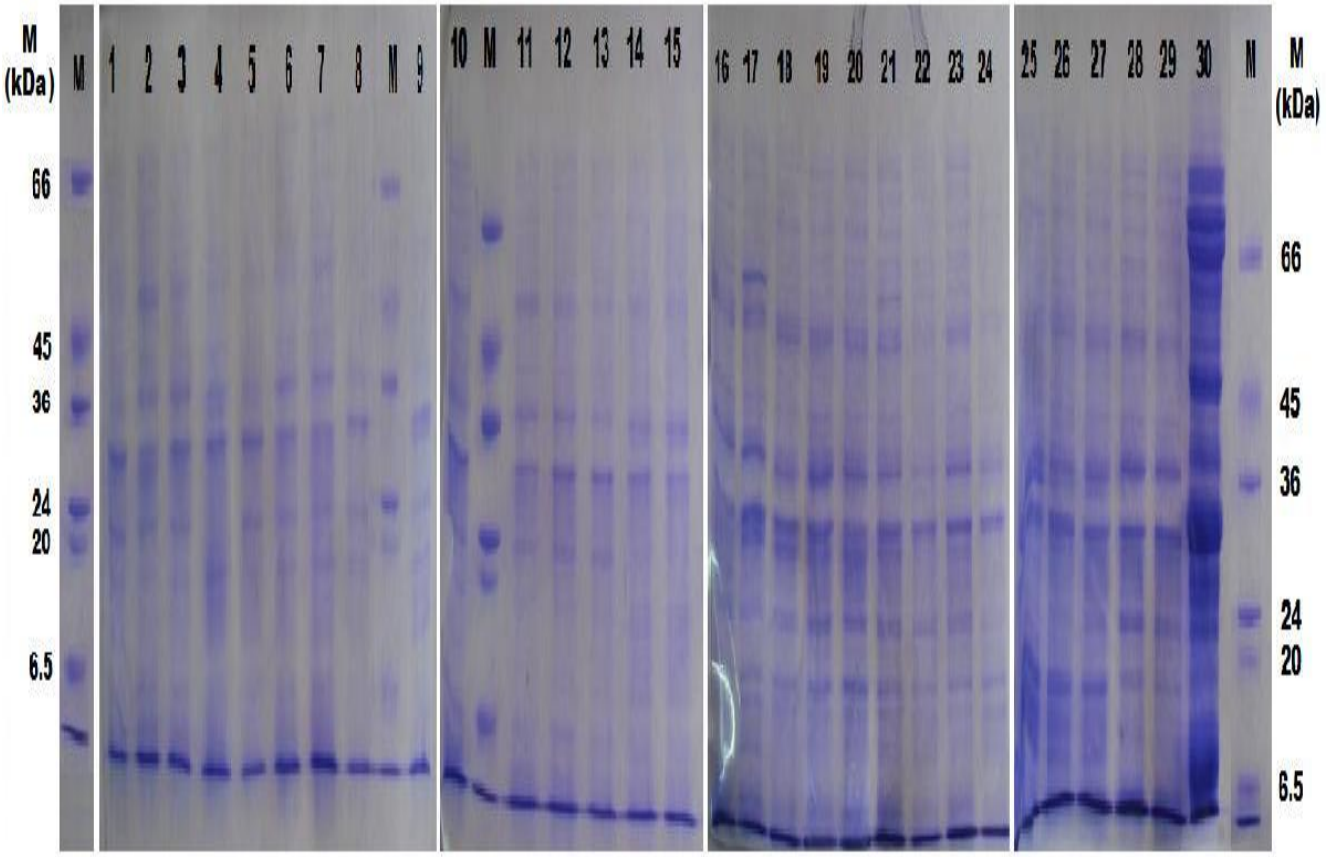


Figure 4.5 SDS-PAGE of total proteins from 29 accessions of grain amaranth A1-A4 *A. caudatus,* A5-A12 *A. cruentus*, A13-A19 *A. hybrid,* A20-A27 *A. hypochondriacus*, A28-A29, *A. hybridus*, A30 Soybean cultivar.

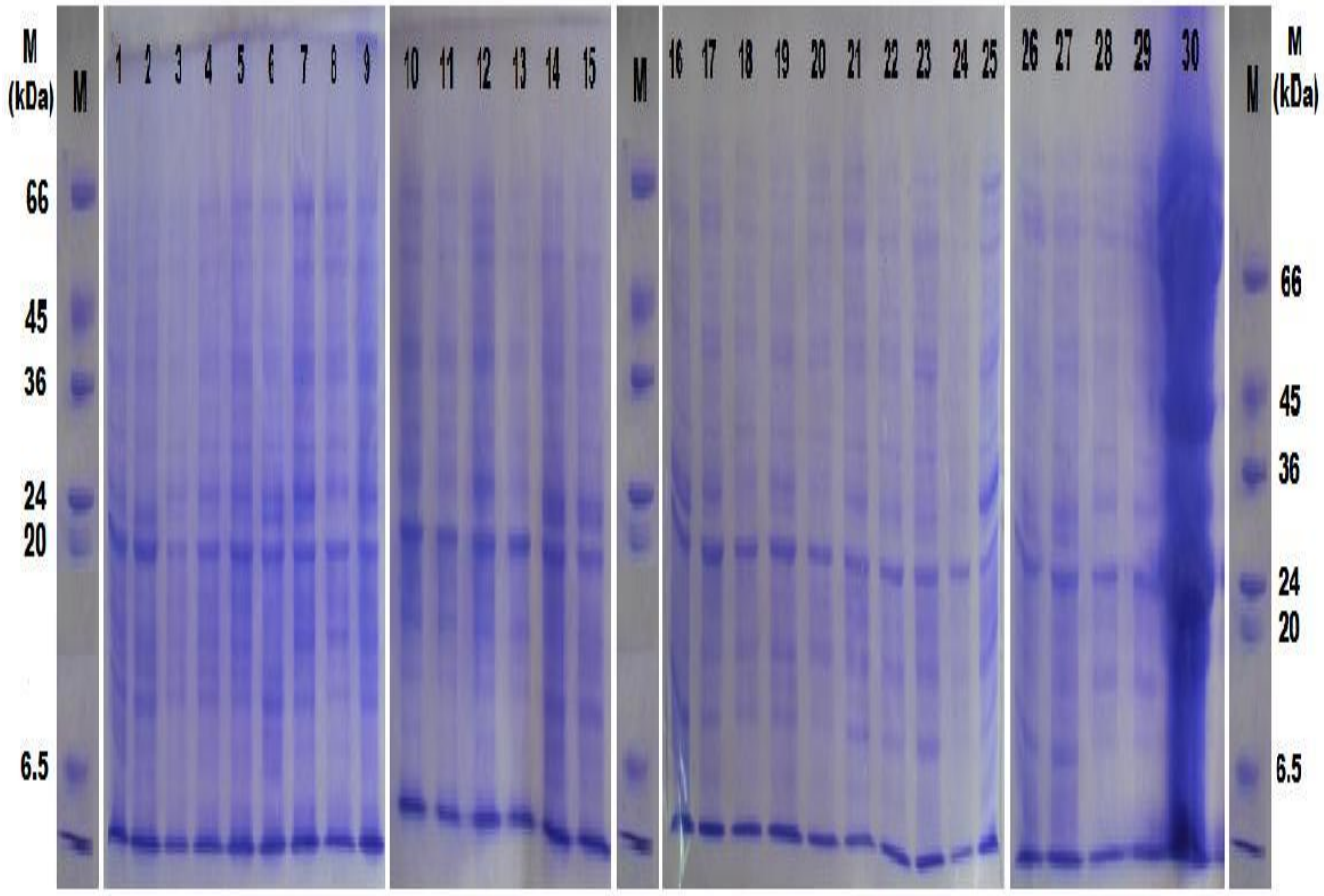


Figure 4.6 SDS-PAGE of albumin from 29 accessions of *Amaranth* seeds. A1-A4 *A. caudatus,* A5-A9, A10-A12 *A. cruentus*, M-marker; A13-A15, A16-A19 *A. hybrid*, A20-A27 *A hypochondriacus*, A28-A29, *A. hybridus*, A30 Soybean.

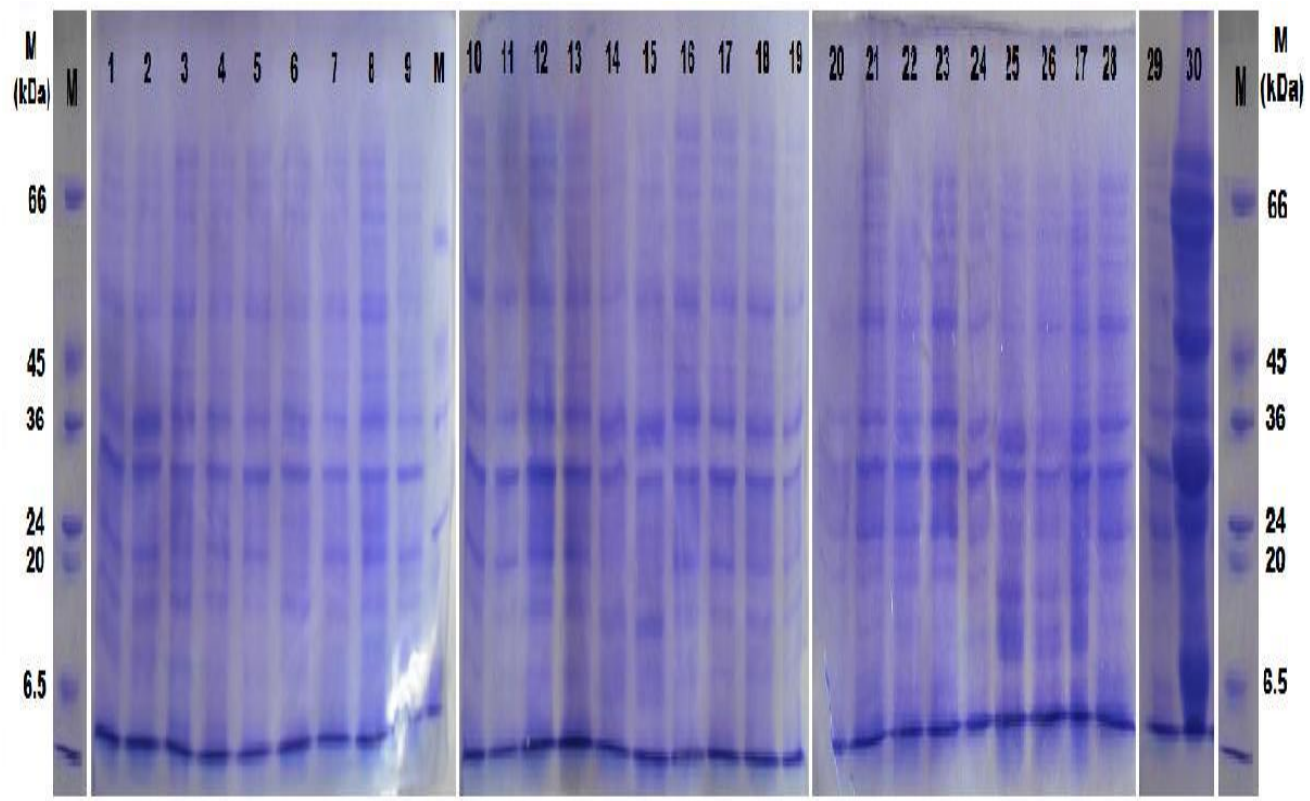


Figure 4.7 SDS-PAGE of Globulin from different species of Amaranth seeds. A1-A4 *A. caudatus,* A5-A9, A10-A12 *A. cruentus*, A13-A19 *A. hybrid*, A20-A27 *A. hypochondriacus*, A28 and A29, *A. hybridus*, A30 soybean.

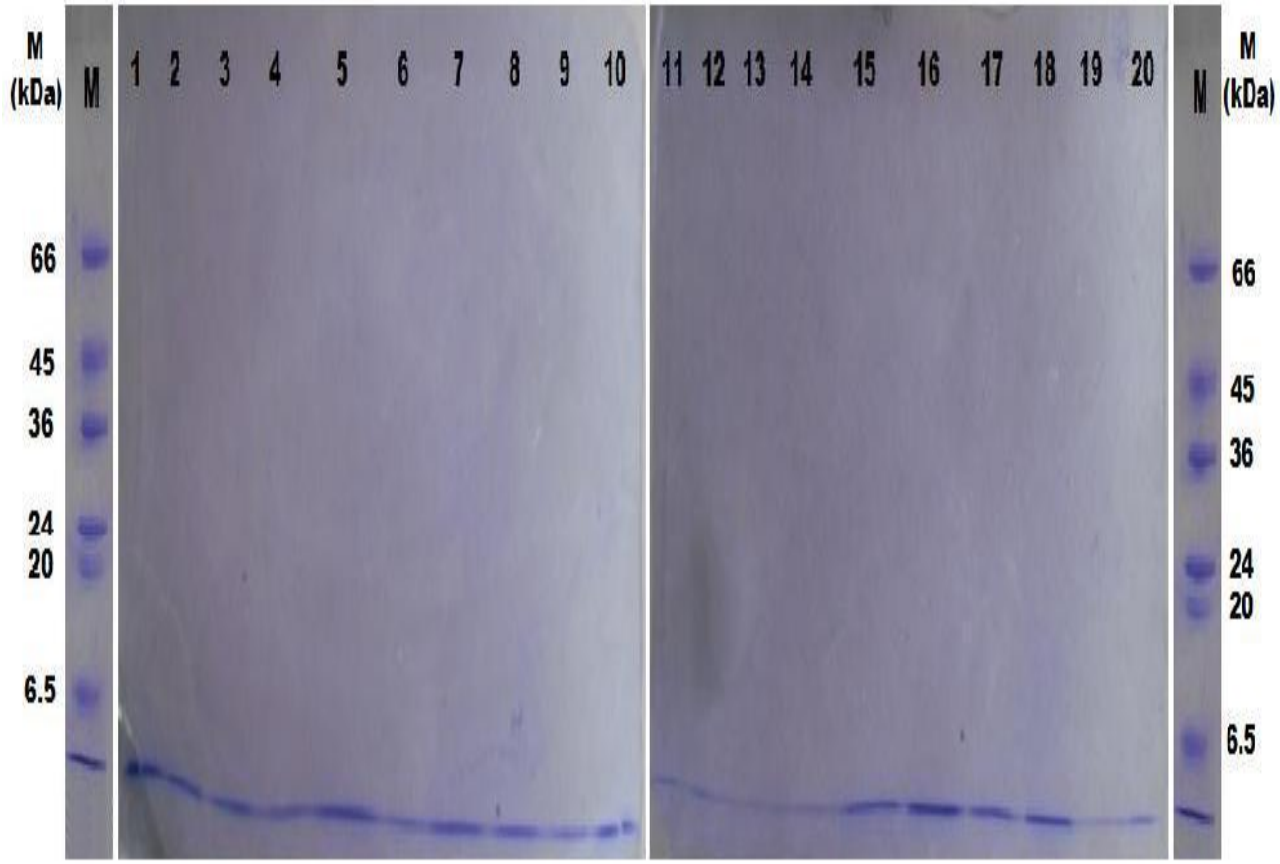


Figure 4.8 SDS-PAGE of Prolamin from different species of Amaranth seeds. Lanes A1-A4 *A. caudatus;* A5-A12 *A. cruentus*; A13-A19 *A. hybrid*.

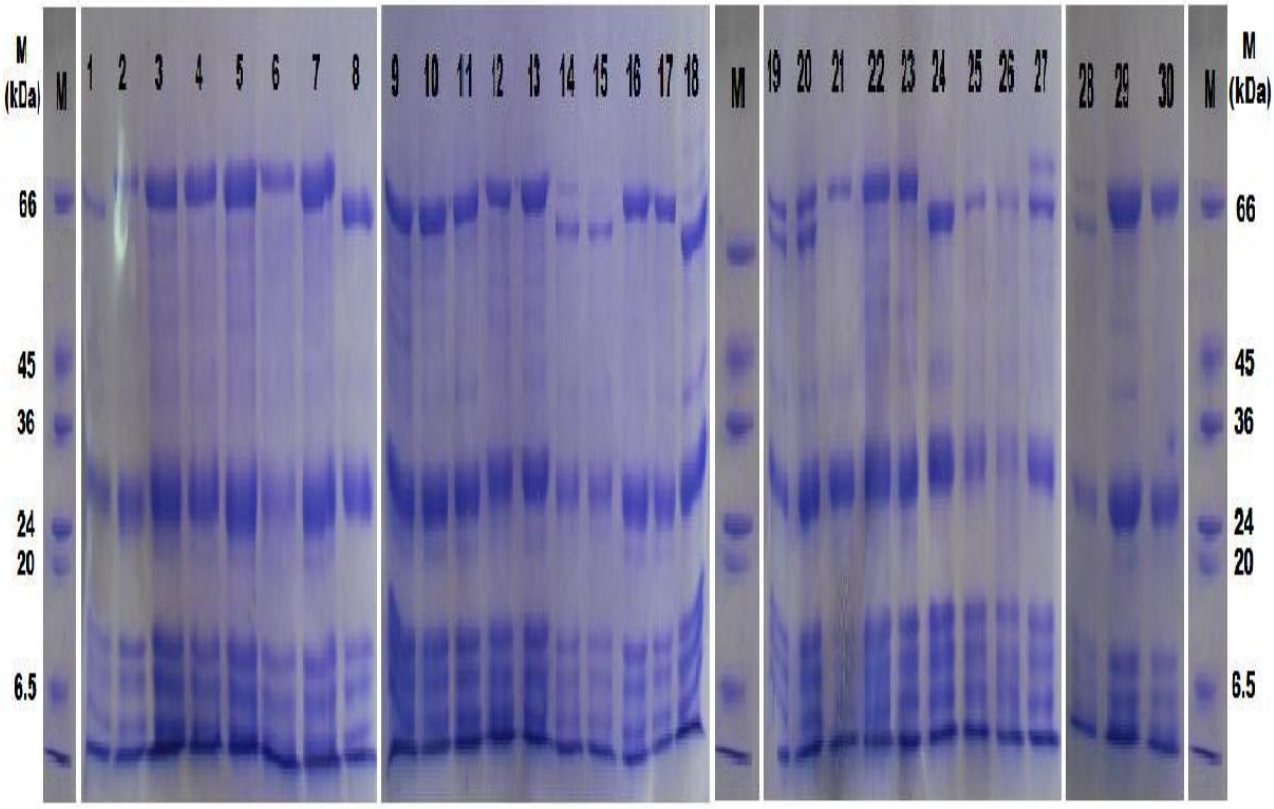


Figure 4.9 SDS-PAGE of Glutelin-like from different species of Amaranth seeds. A1-A4 *A. caudatus;* A5-A8, A9-A12 *A. cruentus*; A13-19A *A. hybrid*; A20-A27 *A. hypochondriacus*; A28-A29, *A. hybridus*; A30 Soybean.

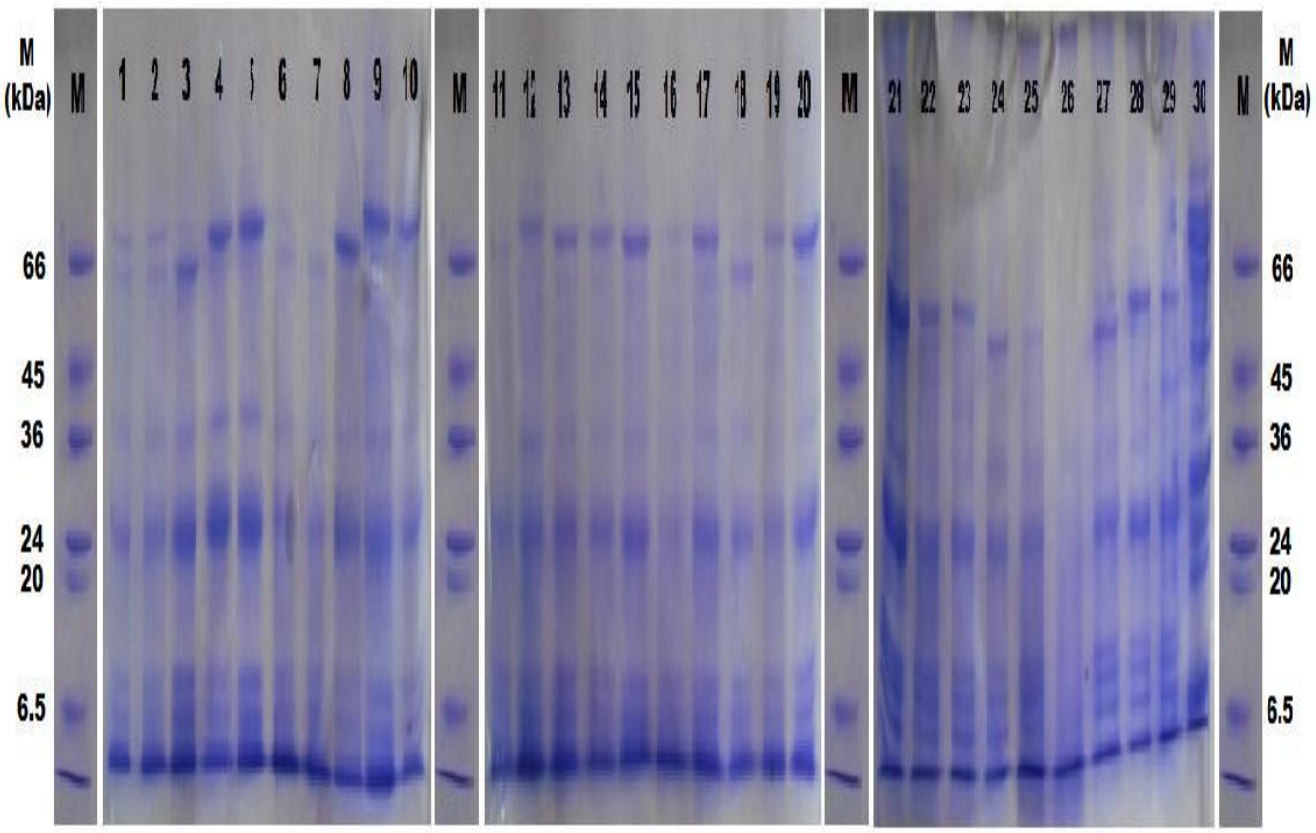


Figure 4.10 SDS-PAGE of Glutelin from different species of Amaranth seeds. A1-A4 *A, caudatus,* A5-A10, A11-A12 *A. cruentus*, A13-A19 *A. hybrid*, A20-A27 *A hypochondriacus*, marker (M), A28-A29, *A. hybridus*, A30 Soybean.

4.4. Experiment 3a. Phytochemical composition of 29 grain amaranth accessions

4.4.1. Phytochemical composition

The results of analysis of variation of the phytochemical composition of 29 grain amaranth accessions are presented in Table 4.16. Significant variation ($p<0.001$) was observed in all the antinutritional factors. Variation in total flavonoid amongst all the accessions was significant at $p<0.05$. Large variation was observed in the reducing power, total antioxidant activity and ABTS as was evident in their high values of coefficient of variation.

The tannin content ranged from 0.08% to 0.16%. Highest value was obtained for accession A5 followed by accession A7.

Phytate content ranged from 0.79% to 1.87%. Highest value was observed for accession A25 followed by accession A15.

Total flavonoid ranged from 7.77 to 10.97 mg/100g CE, with highest value observed for accession A23 followed by accession A6.

Total polyphenol ranged from 23.90 to 35.39 mg/100g GAE. Highest value was recorded for accession A21, followed by accession A27.

DPPH ranged from 82.83 to 95.40%, highest value was recorded for accession A28 followed by accessions A29 and A18.

Reducing power ranged from 0.01 to 0.21A^{o}, highest value was recorded for accession A24 followed by accession A28.

Total antioxidant activity ranged from 111.33 to 271.60 mg/100g AAE, highest value was recorded for accession A1, followed by accession A23.

Fe chelating ranged from 31.51 to 86.66%, highest value was recorded for accession A3 followed by accession A5

ABTS ranged from 157.57 to 208.80 mM TE, highest value was observed for accession A23 followed by accession A27.

4.4.1.1. Correlation analysis of the phytochemical composition

In this study, significant correlation was observed only between DPPH and reducing power (R^2 = 0.64), (Table 4.17). Weak correlation was observed between DPPH and ABTS (R^2 = 0.42); and between total flavonoid and ABTS (R^2 = 0.44). Phytate exhibited weak and negative correlation with iron chelating activity (R^2 = -0.43).

Table 4.16 Phytochemical compositions of 29 grain amaranth accessions

Variety	Tannin %	Phytate %	TF mg/100g CE	TP mg/100g GAE	DPPH %	Reducing Power A^{o}	TA mg/100g AAE	Fe chelating %	ABTS (mM TE)
A1	0.12 ± 0.01	1.29 ± 0.05	9.2573 ± 1.23.57	29.74 ± 5.3381	90.33 ± 2.05	0.12 ± 0.00	271.6 ± 23.06	31.51 ± 4.79	176.76 ± 4.87
A2	0.12 ± 0.00	0.79 ± 0.13	9.0947 ± 0.8415	29.6067 ± 1.4138	89.97 ± 1.88	0.13 ± 0.01	142.37 ± 40.80	73.48 ± 0.22	176.99 ± 20.93
A3	0.12 ± 0.01	1.16 ± 0.01	7.7973 ± 1.1850	26.8433 ± 1.5635	89.37±1.29	0.16 ± 0.03	126.87 ± 20.91	86.66± 3.02	157.57 ± 1.45
A4	0.12 ± 0.01	1.38 ± 0.04	9.7040 ± 1.4896	23.8967± 3.5293	88.47±0.90	0.14 ± 0.01	191.47 ± 22.77	75.22 ± 4.19	167.04 ± 9.30
A5	0.16 ± 0.00	1.27 ± 0.03	10.3173 ± 0.7537	26.9767 ± 2.3245	90.87 ± 1.45	0.11 ± 0.02	139.33 ± 26.43	81.43 ± 0.85	182.26 ± 16.19
A6	0.11 ± 0.01	1.41 ± 0.07	10.744 ± 1.3440	31.3667± 6.7456	91.53 ± 1.70	0.12 ± 0.00	137.27 ± 21.71	54.80 ± 5.16	183.03± 7.12
A7	0.15 ± 0.00	1.22 ± 0.02	9.6173 ± 2.7645	30.42 ± 1.8102	92.2 ± 1.05	0.169 ± 0.02	119.83 ± 9.09	72.96 ± 0.95	167.68 ± 16.19
A8	0.12 ± 0.00	1.16 ± 0.02	9.7973 ± 0.5897	29.9167 ± 1.1451	89.73 ± 1.23	0.15 ± 0.02	238.73 ± 25.21	67.09 ± 2.13	171.90± 19.75
A9	0.10 ± 0.00	1.49 ± 0.04	9.2373 ± 0.8533	30.336 ± 2.7786	92.47 ± 1.58	0.146 ± 0.00	127.5 ± 24.94	70.45 ± 1.06	185.07 ± 11.31
A10	0.11 ± 0.00	1.37 ± 0.06	10.464 ± 1.6506	31.2967 ± 1.0053	91.33 ± 2.45	0.023 ± 0.18	132.23 ± 9.30	59.86 ± 0.22	182.13 ± 15.41
A11	0.11 ± 0.00	1.42 ± 0.03	10.2707 ± 0.7414	32.923 ± 1.1404	82.83 ± 9.13	0.01± 0.16	140.67 ± 35.11	52.37 ± 0.84	179.70 ± 16.28
A12	0.10 ± 0.01	1.28 ± 0.02	9.0307 ± 2.8217	30.6233 ± 1.8405	90.27 ± 0.85	0.01 ± 0.22	127.07 ± 12.46	53.70 ± 0.46	182.64 ± 14.54
A13	0.11 ± 0.00	1.47 ± 0.09	8.2507 ± 1.0304	28.5367 ± 2.0845	88.67 ± 2.59	0.02± 0.16	158.77± 42.58	68.81 ± 0.14	183.54 ± 20.27
A14	0.08 ± 0.01	1.10 ± 0.11	7.9507 ± 0.9636	27.34 ± 2.2001	92.7 ± 2.2	0.16 ± 0.02	132.4± 24.14	71.88 ± 0.55	191.80 ± 2.77
A15	0.11 ± 0.00	1.79 ± 0.02	8.2373 ± 0.3233	31.9933 ± 1.2810	91.87 ± 1.47	0.17 ± 0.00	127.67 ± 14.32	53.31 ± 0.74	176.38 ± 15.73
A16	0.09 ± 0.01	1.42 ± 0.03	8.1473 ± 0.3647	30.476 ± 0.8160	90.63 ± 3.02	0.15 ± 0.02	125.73 ± 11.14	56.54 ± 0.94	178.55 ± 19.68

TP – Total polyphenol, TF – Total flavonoid, CE – Catechin Equivalent, GAE – Gallic Acid Equivalent, AAE – Ascorbic Acid Equivalent

Table 4.16 Phytochemical compositions of 29 grain amaranth accessions (contd.)

Variety	Tannin %	Phytate %	TF mg/100g CE	TP mg/100g GAE	DPPH %	Reducing Power A^{o}	TA mg/100g AAE	Fe chelating %	ABTS (mM TE)
A17	0.13 ± 0.00	1.54 ± 0.04	8.8907 ± 0.7484	30.61 ± 0.3951	93.57 ± 1.00	0.15 ± 0.06	126.17 ± 7.60	54.72 ± 1.17	192.51 ± 1.77
A18	0.13 ± 0.01	1.47 ± 0.07	7.7707 ± 0.2730	31.3097 ± 0.0355	94.2 ± 1.18	0.16 ± 0.04	122.83 ± 6.78	58.67 ± 0.43	182.95 ± 9.61
A19	0.10 ± 0.00	1.57 ± 0.02	8.9173 ± 0.7643	31.1087 ± 3.1798	93.83 ± 1.29	0.151 ± 0.02	119.97 ± 26.92	61.89 ± 0.43	185.10 ± 4.04
A20	0.11 ± 0.00	1.57 ± 0.01	8.0527 ± 0.3939	29.706 ± 4.5453	91.43 ± 1.10	0.14 ± 0.02	118.67 ± 18.75	53.92 ± 0.66	170.60 ± 18.80
A21	0.12 ± 0.01	1.48 ± 0.07	8.956 ± 1.0684	35.3867 ± 2.6699	93.0 ± 3.3	0.17 ± 0.03	121.13 ± 12.93	64.04 ± 0.58	168.44 ± 11.38
A22	0.08 ± 0.00	1.20 ± 0.03	9.0827 ± 1.7621	31.106 ± 1.2151	91.7 ± 4.18	0.19 ± 0.04	135.23 ± 11.18	43.67 ± 3.59	174.15 ± 4.12
A23	0.08 ± 0.01	1.61 ± 0.13	10.9727 ± 0.9463	30.982 ± 1.6939	94.03 ± 0.55	0.186 ± 0.01	240.83 ± 1.55	50.05 ± 1.31	208.80 ± 8.49
A24	0.11 ± 0.00	1.63 ± 0.20	8.444 ± 1.0346	27.6977 ± 0.9574	92 ± 3.21	0.21 ± 0.01	134.8± 15.76	33.31 ± 5.43	168.06 ± 0.80
A25	0.13 ± 0.00	1.87 ± 0.09	8.802 ± 0.5213	28.4573 ± 0.7227	90.5 ± 0.7	0.146 ± 0.04	135.95 ± 4.09	52.59 ± 0.78	177.14 ± 13.47
A26	0.10 ± 0.00	1.55 ± 0.02	8.6113 ± 0.8287	29.506 ± 4.3665	93.07 ± 3.42	0.18 ± 0.02	135.5 ± 20.23	51.23 ± 0.55	159.49 ± 2.55
A27	0.12 ± 0.00	1.35 ± 0.04	10.336 ± 1.7195	34.8847 ± 1.3929	93.9 ± 2.95	0.145 ± 0.03	111.33 ± 11.94	48.84 ± 2.65	207.46 ± 1.17
A28	0.09 ± 0.00	1.58 ± 0.05	10.3353 ± 0.7876	33.8163 ± 0.2654	95.4 ± 3.11	0.197 ± 0.06	209.5 ± 61.63	65.79 ± 1.17	200.54 ± 12.81
A29	0.10 ± 0.00	1.67 ± 0.10	9.038 ± 0.4928	27.849 ± 0.8285	94.2 ± 3.67	0.17 ± 0.04	207.17 ± 17.08	51.38± 0.99	195.94± 8.05
Mean	0.11	1.417	9.1768	30.1625	91.52	0.16	150.3	59.32	180.49
LSD	0.0085	0.1119	1.9851	4.2598	4.52	0.044	38.68	3.63	20.39
CV	4.66	4.83	0.1323	0864	3.02	16.83	15.75	3.75	6.91

TP – Total polyphenol, TF – Total flavonoid, CE – Catechin Equivalent, GAE – Gallic Acid Equivalent, AAE – Ascorbic Acid Equivalent

Table 4.17: Pearson's correlation matrix of phytochemicals

	Tannin	Phytate	Total flav	Total polyphen	DPPH	Reducing power	Total antioxid	Fe chelating
Phytate	-0.1158							
Total flav	0.0695	-0.0672						
Total polyphen	-0.1499	0.1402	0.2904					
DPPH	-0.1769	0.2827	-0.0399	0.2527				
Reducing power	-0.1236	0.1921	-0.1918	-0.0259	0.6376			
Total antioxid	-0.1628	-0.0043	0.3644	-0.1675	-0.0177	0.0966		
Fe chelating	0.3098	-0.4250	-0.0324	-0.3005	-0.1275	-0.0917	-0.1928	
ABTS	-0.3013	0.1766	0.4351	0.3528	0.4223	-0.0355	0.2225	-0.1606

4.4.1.2. Principal Component analysis of the phytochemical composition

Principal component analysis was done on nine variables (Table 4.18). The Results indicated that the first four principal components could explain 72.7% of total variance among the 29 grain amaranth accessions evaluated. The four principal components and their corresponding eigenvalues and variances are listed in Table 4.18.

The first principal component (PC_1) was the most important, explaining 27% of the total variance. The DPPH and ABTS exhibiting high positive values and Fe chelating exhibiting high negative value were the major components contributing to this variance. The second principal component (PC_2) accounted for an additional 19% of the total variance and was mainly attributed to high positive values by total flavonoid and ABTS and high negative value by reducing power. The third principal component (PC_3) accounted for 14% of the total variance; and the variation was mainly contributed to high positive values by total antioxidant activity and reducing power and high negative values by total polyphenol. The fourth principal component (PC_4) explained 13% of the total variance with an eigenvalue of 1.176 and mainly represents contribution from high positive values exhibited by Fe chelating capacity, DPPH and Tannin.

The first three PCs were plotted to observe realationships between the clusters (Figure 4.11). PCA plot grouped the 29 grain amaranth accessions into 5 clusters. Cluster I comprised of accessions A23, A8, A1 and A4. Cluster II comprised of A29, A28 and A14. Cluster III comprised of A5, A2, A6, A13, A27, A12 and A10. Cluster IV comprised of A7, A9, A3, A22, A17, A25, A19, A16, A20, A18, A15, A21, A26 and A24. Cluster V comprised only of accession A11 and showed clear separation on the plot. Accessions A28, A29 and A14 had high positive coefficients for all three components and occupied the extreme right corner of the upper half of the plot. Accessions A23, A8, A1 and A4 had high positive coefficients of PC_1 PC2 and PC_3 and thus occupied the upper right corner of the plot. Accession A11, having the lowest coefficient of PC_1 and PC_2 and a negative coefficient of PC_3, occupied the bottom left corner of the plot.

Table 4.18: Eigen values, proportion of variability and phytochemical contents contributing to first four PCs of 29 accessions of grain amaranth

	PC_1	PC_2	PC_3	PC_4
Eigen value	2.433	1.711	1.224	1.176
% Variance explained	0.2704	0.1901	0.136	0.1307
Cumulative	0.2704	0.4604	0.5964	0.7272
	Coefficient of Variates			
Tannin	-0.33	0.02	-0.07	0.34
Phytate	0.34	-0.22	-0.20	-0.31
Total Flavonoid	0.18	0.60	0.12	0.23
Total Polyphenol	0.34	0.19	-0.54	0.22
DPPH	0.44	-0.33	0.11	0.43
Reducing power	0.27	-0.50	0.37	0.22
Total antioxidant	0.17	0.30	0.68	-0.27
Fe-Chelating	-0.38	0.00	0.18	0.57
ABTS	0.44	0.32	0.02	0.25

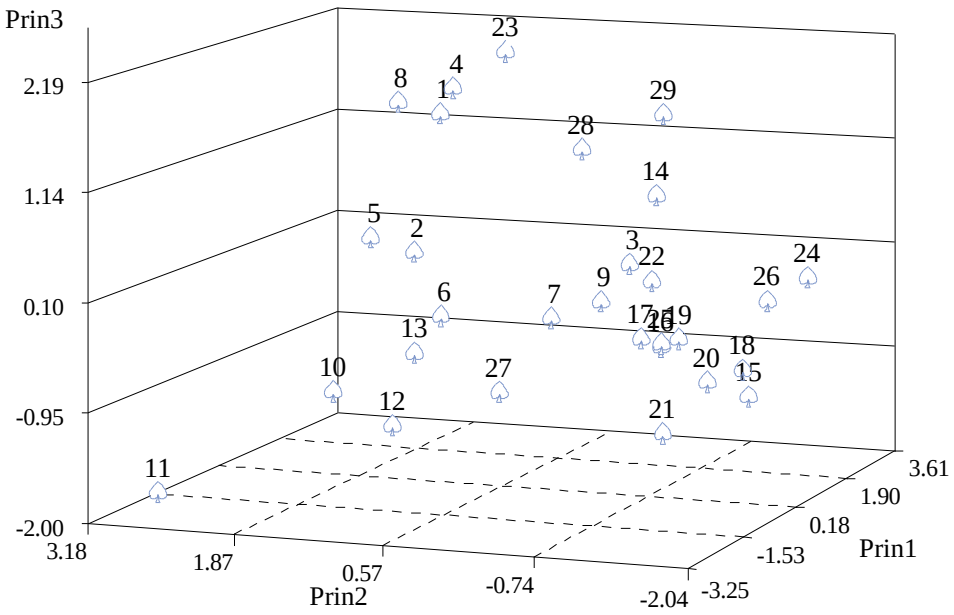


Figure 4.11 Plot of first, second and third component scores for 29 accessions of grain amaranth.

4.4.2. Hepatoprotective effect of ethanol seed extract of Accessions A23 (*A. hypochondriacus*) and A28 (*A. hybridus*) on sodium arsenite-induced toxicity in male rats

4.4.2.1. Effects of treatment on % body weight and relative weight of Liver

4.4.2.1.1 Experimental groups administered *Amaranthus hypochondriacus* (A23) extract

Results indicated a significant decrease ($p<0.05$) in percentage body weight as observed in rats administered sodium arsenite (group II) when compared with control (group I) and all other groups (Table 4.19). Treatment with 100, 200 and 300 mg/kg ethanol extract of *A. hypochondriacus* (groups III, IV and V, respectively) did not cause any significant change in percentage body weight when compared with rats administered distilled water alone (control group I). Similar observation was made in groups VI, VII, and VIII (rats treated with 100, 200 and 300 mg/kg ethanol extract of *A. hypochondriacus* with sodium arsenite) when compared with control (group I).

4.4.2.1.2 Experimental groups administered *A. hybridus* (A28) extract

A significant difference ($p<0.05$) in % body weight was observed in rats treated with sodium arsenite (group II) when compared with group I (rats administered distilled water only) and group VII (rats treated with 200 mg/kg ethanol extract of *A. hybridus* and sodium arsenite), (Table 4.20). Also, no significant change in % body weight was observed in rats administered 100, 200 and 300 mg/kg *A. hybridus* (groups III, IV and V) when compared with group II rats (administered sodium arsenite only). Similarly, % body weight change in rats administered 100 and 300 mg/kg ethanol extracts of *A. hybridus* and sodium arsenite (groups VI and VIII) were not significantly different from group I (rats administered water only).

Table 4.19 Change in body weight of rats administered *A. hypochondriacus* (Accession A23) extract

Group	Treatment	Final body wt (g)	Initial body wt (g)	Difference in body wt (g)	% weight change
I	Distilled water alone	170.00 ± 37.64	125.00 ± 10.21	45	36[a]
II	NaAS alone	179.17 ± 10.21	150.00 ± 0.00	29.17	20[b]
III	100 mg/kg *A. hypochondriacus*	190.00 ± 13.69	150.00 ± 0.00	40	26.67[a]
IV	200 mg/kg *A. hypochondriacus*	195.00 ± 11.18	150.00 ± 0.00	45	30[a]
V	300 mg/kg *A. hypochondriacus*	190.00 ± 12.50	150.00 ± 0.00	40.00	26.67[a]
VI	100 mg/kg *A. hypochondriacus* + NaAS	195.00 ± 11.18	150.00 ± 0.00	45	30[a]
VII	200 mg/kg *A. hypochondriacus* + NaAS	195.00 ± 11.18	150.00 ± 0.00	45	30[a]
VIII	300 mg/kg *A. hypochondriacus* + NaAS	192.50 ± 28.50	150.00 ± 0.00	42.5	28.33[a]

NaAS – sodium arsenite; values are expressed as means ± standard deviation; n = 5 for each treatment; mean values within a column having same letter are not significantly different ($p<0.05$)

Table 4.20 Change in body weight of rats administered *A. hybridus* (Accession A28) extract

Group	Treatment	Final body wt (g)	Initial body wt (g)	Difference in body wt (g)	% weight change
I	Distilled water alone	170.00 ± 37.64	125.00 ± 10.21	45	36[a]
II	NaAS alone	179.17 ± 10.21	150.00 ± 0.00	29.17	20[b]
III	100 mg/kg *A. hybridus*	212.50 ± 13.60	175.00 ± 0.00	37.5	20[b]
IV	200 mg/kg *A. hybridus*	205.00 ± 11.18	175.00 ± 0.00	30	16.67[b]
V	300 mg/kg *A.hybridus*	215.00 ± 22.36	175.00 ± 0.00	40	22.86[b]
VI	100 mg/kg *A. hybridus* + NaAS	212.50 ± 22.81	175.00 ± 0.00	37.5	21.43[b]
VII	200 mg/kg *A. hybridus* + NaAS	195.83 ± 10.21	175.00 ± 0.00	20.83	11.91[c]
VIII	300 mg/kg *A. hybridus* + NaAS	225.00 ± 17.68	195.00 ± 11.18	30	15.71[b]

NaAS – sodium arsenite; values are expressed as means ± standard deviation; n = 5 for each treatment; mean values within a column having same letter are not significantly different ($p<0.05$)

4.4.2.1.3 Effect of ethanol extract of *A.hypochondriacus* (A23) on relative liver weight

Relative liver weight was observed to be higher in rats administered sodium arsenite (group II) when compared with rats administered with water only (group I) (Tables 4.21). Treatment with the three doses of ethanol extract of *A. hypochondriacus* alone (groups III, IV and V) did not cause any significant change in the liver weight when compared with control (group 1). Treatment with the different doses of *A. hypochondriacus* extracts with sodium arsenite (groups VI, VII and VIII) did not cause any significant change in the relative weight of the liver when compared with the control (group 1). Administration of *A. hypochondriacus* extract with sodium arsenite (groups VI, VII and VIII) ameliorated the toxic effect induced by arsenite as the relative liver weight of the rats was observed to be close to the relative liver weight of control (group 1).

4.4.2.1.4 Effect of ethanol extract of *A. hybridus* (A28) extract on relative liver weight

Results indicated that the relative liver weight of rats administered sodium arsenite (group II) was significantly ($p<0.05$) higher than in control group (rats administered water only) (Table 4.22). Treatment with the three doses of *A. hybridus* extracts alone (groups III, IV and V) as well as treatment with the three doses of *A. hybridus* with sodium arsenite (groups VI, VII and VIII) did not cause any significant change in the relative liver weight when compared with control group (group I). A significant change in the relative liver weight was observed in rats administered the three doses of *A. hybridus* extracts with sodium arsenite when compared with rats treated with arsenite alone (group II).

Table 4.21 Relative liver weight of rats administered *Amaranthus hypochondriacus* (Accession A23) extract

Group	Treatment	Mean Liver weight (g)	Relative Liver weight (%)
I	Distilled water alone	6.61 ± 1.45	3.68^{b} ± 0.84
II	NaAS alone	10.51 ± 2.21	6.42^{a} ± 2.06
III	100 mg/kg *A. hypochondriacus*	7.46 ± 0.42	3.93^{b} ± 0.29
IV	200 mg/kg *A. hypochondriacus*	6.99 ± 2.52	3.65^{b} ± 1.60
V	300 mg/kg *A. hypochondriacus*	8.51 ± 3.27	4.57^{b} ± 2.03
VI	100 mg/kg *A. hypochondriacus* + NaAS	7.22 ± 2.28	3.69^{b} ± 1.09
VII	200 mg/kg *A. hypochondriacus* + NaAS	7.16 ± 0.83	3.58^{b} ± 0.41
VIII	300 mg/kg *A. hypochondriacus* + NaAS	6.46 ± 1.03	3.32^{b} ± 0.92

NaAS – sodium arsenite; values are expressed as means ± standard deviation; n = 5 for each treatment; mean values within a column having same letter are not significantly different ($p<0.05$)

Table 4.22 Relative liver weight of rats administered *Amaranthus hybridus* (Accession A28) extract

Group	Treatment	Mean Liver weight (g)	Relative Liver weight (%)
I	Distilled water alone	6.61 ± 1.45	3.68^{b} ± 0.84
II	NaAS alone	10.51 ± 2.21	6.42^{a} ± 2.06
III	100 mg/kg *A. hybridus*	6.57 ± 1.44	3.13^{b} ± 0.65
IV	200 mg/kg *A. hybridus*	6.46 ± 0.59	3.16^{b} ± 0.24
V	300 mg/kg *A. hybridus*	6.45 ± 1.38	2.93^{b} ± 0.33
VI	100 mg/kg *A. hypbridus* + NaAS	5.97 ± 1.07	2.78^{b} ± 0.44
VII	200 mg/kg *A. hybridus* + NaAS	7.13 ± 2.58	3.71^{b} ± 1.63
VIII	300 mg/kg *A. hybridus* + NaAS	6.81 ± 0.54	3.03^{b} ± 0.25

NaAS – Sodium arsenite; Values are expressed as means ± standard deviation, n = 5 for each treatment. Mean values within a column having same letter are not significantly different ($p<0.05$)

4.4.2.2. Effects of ethanol extracts of *A. hypochondriacus* (A23) and *A. hybridus* (A28) on serum Liver marker enzymes intoxicated with sodium arsenite.

The effect of three different doses of ethanol extracts of *A. hypochondriacus* and *A. hybridus* seeds were studied on serum Liver marker enzymes in sodium arsenite intoxicated animals. Administration of sodium arsenite caused significant changes in serum enzyme (ALP, ALT, AST and GGT) activities when compared with the control group in both experiments (Table 4.23 and 4.24).

Accession A23 (*Amaranthus hypochondriacus* ethanol extract)

No significant difference was observed in the level of ALP activity in all the groups (Table 4.23), though treatment with sodium arsenite (group II) caused an increase in the activity of ALP when compared with control (group I). The elevated level of ALP activity caused by sodium arsenite (group II) was observed to be reduced in rats administered 100, 200, and 300 mg/kg *A. hypochondriacus* and sodium arsenite (groups VI, VII and VIII; respectively) close to control (group I) in a dose dependent manner.

The level of ALT activity was significantly ($p<0.05$) increased in sodium arsenite treated group (group II) when compared with control (group I). Treatment with the three doses of *A. hypochondriacus* extracts alone (groups III, IV and V) was significantly ($p<0.05$) different in the level of ALT activity when compared with treatment with arsenite (group II), but were similar to control (group I). Treatment with 300 mg/kg *A. hypochondriacus* and sodium arsenite (group VIII) reduced significantly ($p<0.05$) the NaAS induced elevated level of ALT close to control (group I).

Sodium arsenite treated rats (group II) showed elevated level of AST activity and this was significantly ($p<0.05$) different from control (group I). The level of AST activity in rats treated with the three doses of *A. hypochondriacus* extract alone (Groups III, IV and V) was similar to the control (group I). Treatment with the three doses of *A. hypochondriacus* extracts and NaAS (Groups VI, VII and VIII) caused a significant reduction ($p<0.05$) in the elevated level of AST activity induced by sodium arsenite.

No significant difference in the activity of GGT was observed in all groups, except in group V (rats administered 300 mg/kg of grain amaranth alone). Treatment with sodium arsenite (group II) caused an increase in the level of GGT activity when compared with control. Elevated level of GGT induced by sodium arsenite was reduced in groups treated with the three doses of *A. hypochondriacus* extract plus sodium arsenite (groups VI, VII and VIII) in a dose dependent manner.

Accession A28 (*Amaranthus hybridus* ethanol extract)

No significant difference in the level of ALP activity was observed in all the groups (Table 4.24). An increase in the level of ALP activity was observed in rats treated with sodium arsenite (group II). Groups treated with the three doses of *A. hybridus* and sodium arsenite (groups VI, VII and VIII) caused a significant reduction in the elevated level of ALP activity induced by sodium arsenite.

Treatment with sodium arsenite (group II) significantly ($p<0.05$) increased the level of ALT activity when compared with control (group I) (Table 4.24). The elevated level of ALT activity induced by sodium arsenite was significantly ($p<0.05$) reduced by treatment with 300 mg/kg *A. hybridus* and sodium arsenite (group VIII).

The level of AST activity increased significantly ($p<0.05$) in the sodium arsenite treated group (group II) when compared with control (group I). The elevated ALT activity induced by sodium arsenite was significantly ($p<0.05$) reduced by treatment with 300 mg/kg *A. hybridus* extract and sodium arsenite (group VIII) close to control (group I).

There was no significant difference in the GGT activity in all the groups, however an increase in the GGT level by the sodium arsenite treated group (group II) was reduced in groups treated with the three doses of *A. hybridus* and sodium arsenite (groups VI, VII and VIII) close to the control (group I) in a dose dependent manner.

Table 4.23 Effect of ethanol extract of *A. hypochondriacus* (Accession A23) on serum Liver marker enzymes (ALT, ALP, AST and GGT)

Treatment	**Distilled water alone**	**NaAS alone**	**100mg *A. hypo***	**200mg *A. hypo***	**300mg *A. hypo***	**100mg *A. hypo* + NaAS**	**200mg *A. hypo* + NaAS**	**300mg *A.hypo* + NaAS**
GROUP	**GRP I**	**GRP II**	**GRPIII**	**GRPIV**	**GROUP V**	**GROUP VI**	**GROUP VII**	**GROUP VIII**
Parameter								
ALP(U/L)	$66.93^{a} \pm 10.42$	$71.76^{a} \pm 19.52$	$62.10^{a} \pm 5.28$	$50.37^{a} \pm 15.51$	$43.47^{a} \pm 16.77$	$69.69^{a} \pm 4.14$	$53.82^{a} \pm 17.24$	$49.68^{a} \pm 9.82$
ALT (U/L)	$9.39^{d} \pm 1.32$	$16.74^{a} \pm 1.04$	$10.35^{cd} \pm 0.94$	$10.58^{cd} \pm 2.26$	$8.76^{d} \pm 2.21$	$14.12^{b} \pm 0.80$	$12.62^{bc} \pm 0.33$	$9.18^{d} \pm 0.23$
AST (U/L)	$38.17^{b} \pm 3.61$	$54.61^{a} \pm 11.94$	$38.96^{b} \pm 3.60$	$33.13^{bc} \pm 1.45$	$32.44^{bc} \pm 1.23$	$26.90^{c} \pm 2.89$	$24.30^{c} \pm 2.18$	$23.30^{c} \pm 2.35$
GGT (U/L)	$1.74^{ab} \pm 0.67$	$3.47^{a} \pm 0.95$	$2.32^{ab} \pm 1.64$	$1.74^{ab} \pm 0.67$	$1.16^{b} \pm 0.00$	$3.18^{ab} \pm 0.58$	$2.61^{ab} \pm 1.11$	$1.74^{ab} \pm 0.67$

NaAS – sodium arsenite, *A. hypo* (*A. hypochondiacus*)

Values are expressed as means ± SD; n = 5 for each treatment group

Mean values within a row not sharing same letter are significantly different $p<0.05$

Table 4.24 Effect of ethanol extract of *A. hybridus* (Accession A28) on serum Liver marker enzymes (ALT, ALP, AST and GGT)

Treatment	Distilled water alone	NaAS alone	100mg *A. hyb*	200mg *A. hyb*	300mg *A. hyb*	100mg *A. hyb* +NaAS	200mg *A. hyb* +NaAS	300mg *A.hyb* +NaAS
Group	**GRP 1**	**GRPII**	**GRPIII**	**GRPIV**	**GRPV**	**GRPVI**	**GRP VII**	**GRPVIII**
Parameters								
ALP (U/L)	$66.93^{a} \pm 10.42$	$71.76^{a} \pm 19.52$	$62.10^{a} \pm 6.95$	$62.79^{a} \pm 9.12$	$55.21^{a} \pm 2.70$	$53.82^{a} \pm 5.28$	$48.30^{a} \pm 5.75$	$46.92^{a} \pm 13.14$
ALT (U/L)	$9.39^{d} \pm 1.32$	$16.74^{a} \pm 1.04$	$9.87^{ab} \pm 1.70$	$9.46^{ab} \pm 1.64$	$8.72^{b} \pm 1.54$	$12.52^{a} \pm 1.37$	$11.77^{ab} \pm 0.76$	$8.57^{b} \pm 2.72$
AST (U/L)	$38.17^{b} \pm 3.61$	$54.61^{a} \pm 11.94$	$28.85^{c} \pm 5.66$	$26.59^{c} \pm 8.01$	$25.42^{c} \pm 2.93$	$41.50^{a} \pm 2.26$	$34.43^{ab} \pm 2.81$	$34.13^{b} \pm 2.88$
GGT (U/L)	$1.74^{ab} \pm 0.67$	$3.47^{a} \pm 0.95$	$2.03^{a} \pm 1.11$	$1.93^{a} \pm 0.55$	$1.45^{a} \pm 0.58$	$2.90^{a} \pm 0.66$	$2.32^{a} \pm 0.94$	$1.82^{a} \pm 0.59$

NaAS – sodium arsenite; *A. hyb* (*A. hybridus*), values are expressed as means ± SD; n = 5 for each treatment group

Mean values within a row not sharing same letter are significantly different $p<0.05$

4.4.2.3 Activities of antioxidant enzymes

Ttreatment with sodium arsenite caused a significant decrease ($p<0.05$) in the activities of SOD, CAT and GPx in the liver tissue when compared with control for both *A. hypochondriacus* and *A. hybridus* extracts (Tables 4.25 and 4.26).

Accession A23 (*A. hypochondriacus*)

Treatment with sodium arsenite (group II) caused a significant ($p<0.05$) decrease in the SOD activity when compared with control (group I) (Table 4.25). All other groups were significantly ($p<0.05$) different from the control (group I) in the SOD activity. Treatment with the three doses of *A. hypochondriacus* and sodium arsenite (groups VI, VII and VIII) were able to increase the SOD activity caused by sodium arsenite induced toxicity.

The catalase (CAT) activity was significantly reduced in sodium arsenite treated rats (group II) when compared with control (group I) (Table 4.25). There was no significant difference in the CAT activity between sodium arsenite treated rats (group II) and rats treated with 100 mg/kg *A. hypochondriacus* extract alone (group III). Treatment with 300 mg/kg *A. hypochondriacus* extract and sodium arsenite (group VIII) caused an increase in the reduced CAT activity induced by arsenite, close to the control.

The GPX activity in control (group I) was significantly ($p<0.05$) higher than in groups II, III, IV, V and VI. Treatment with sodium arsenite (group II) caused a significant ($p<0.05$) decrease in the GPx activity when compared with control (group I). The GPx reduced activity caused by sodium arsenite was ameliorated by the increased activity as observed in rats administered 300 mg/kg *A. hypochondriacus* extract and sodium arsenite (group VIII) close to the control (group 1).

There was a significant ($P<0.05$) increase in the activity of MDA and hydrogen peroxide in sodium arsenite treated rats (group II) when compared with all the other groups (Table 4.25). Treatment with 100, 200 and 300 mg/kg *A. hypochondriacus* extract and sodium arsenite

significantly prevented this heave in levels and were able to give some protection as levels obtained were comparable to control (group I).

There was no significant difference between the protein content in group I and group II, however group I was significantly different from groups III, IV, V, VI, VII and VIII.

Accession A28 (*A. hybridus*)

Treatment with sodium arsenite (group II) significantly ($p<0.05$) decreased the SOD activity when compared with control (group I) (Table 4.26). Group II differed significantly ($p<0.05$) from groups III and VIII in the SOD activity. Treatment with 300 mg/kg *A. hypochondriacus* extract and sodium arsenite (group VIII) was able to increase the SOD activity reduced by sodium arsenite induced toxicity close to control.

Treatment with sodium arsenite (group II) caused a significant ($p<0.05$) decrease in CAT activity when compared with control (group I). The CAT activity in control group was significantly ($p<0.05$) higher than in groups III, IV, VI, VII and VIII. No significant difference in CAT activity was observed between group I and group V. The reduced activity of CAT caused by sodium arsenite induced toxicity was increased slightly by treatment with the three doses of *A. hybridus* extract and arsenite (groups VI, VII and VIII).

The GPx activity in group I was significantly ($p<0.05$) higher than groups III, IV, VI, VII and VIII (Table 4.26). Treatment with sodium arsenite (group II) significantly ($p<0.05$) reduced the GPx activity when compared with control (group I). The GPx activity in sodium arsenite treated rats were significantly ($P<0.05$) different from groups IV, V and VI. The slight increase in the activity of GPx by groups VI, VII and VIII indicates the potential to ameliorate the effect caused by arsenite induced toxicity.

A significant ($p<0.05$) increase in MDA and hydrogen peroxide acitivity was observed in sodium arsenite treated group when compared with group I and all other groups (Table 4.26). Control group was similar to group VIII in the Hydrogen peroxide activity, indicating the potential of this treatment to reduce the elevated level caused by arsenite.

Table 4.25 Activities of SOD, CAT, GPx, MDA, H_2O_2 and Protein in liver of rats treated with *A. hypochondriacus* and sodium arsenite

Treatment	**Distilled water alone**	**NaAS alone**	**100mg/kg *A.hypo***	**200 mg/kg *A.hypo***	**300mg/kg *A.hypo***	**100mg/kg *A.hypo*+NaAS**	**200mg/kg *A.hypo*+NaAS**	**300mg/kg *A.hypo*+NaAS**
Group	GRPI	GRPII	GRPIII	GRPIV	GRPV	GRPVI	GRPVII	GRPVIII
Parameters								
SOD	$4.22^{a} \pm 0.40$	$2.82^{dc} \pm 0.33$	$3.32^{bc} \pm 0.47$	$3.52^{b} \pm 0.35$	$3.55^{b} \pm 0.26$	$2.39^{d} \pm 0.11$	$3.12^{bc} \pm 0.21$	$3.31^{bc} \pm 0.47$
CAT	$123.83^{a} \pm 20.50$	$71.38^{e} \pm 8.33$	$71.95^{de} \pm 4.81$	$86.05^{cd} \pm 6.99$	$110.05^{ab} \pm 5.34$	$80.99^{e} \pm 11.69$	$99.20^{bc} \pm 1.08$	$117.63^{a} \pm 7.55$
GPX	$539.75^{a} \pm 55.70$	$398.45^{cd} \pm 31.25$	$361.10^{d} \pm 44.15$	$447.05^{bc} \pm 56.90$	$482.29^{ab} \pm 55.61$	$451.89^{bc} \pm 15.23$	$473.08^{b} \pm 24.02$	$483.80^{ab} \pm 24.99$
MDA	$4.71^{c} \pm 1.48$	$11.15^{a} \pm 0.85$	$4.52^{c} \pm 0.55$	$4.38^{c} \pm 0.62$	$4.15^{c} \pm 0.75$	$6.54^{b} \pm 0.46$	$5.86^{b} \pm 0.53$	$4.40^{c} \pm 0.40$
H_2O_2	$29.87^{c} \pm 6.70$	$41.42^{a} \pm 5.89$	$21.85^{d} \pm 2.57$	$27.33^{c} \pm 2.36$	$29.39^{c} \pm 4.80$	$36.38^{b} \pm 1.33$	$34.19^{b} \pm 2.04$	$31.66^{c} \pm 1.37$
Protein	$0.66^{a} \pm 0.06$	$0.60^{ab} \pm 0.05$	$0.50^{cd} \pm 0.02$	$0.53^{bc} \pm 0.04$	$0.54^{bc} \pm 0.05$	$0.44^{e} \pm 0.05$	$0.49^{cde} \pm 0.02$	$0.43^{e} \pm 0.04$

Values are expressed as means ± SD; n = 5 for each treatment group; mean values within a row not sharing same letter are significantly different $p<0.05$

SOD activity. Unit/mg protein; CAT activity; µmol H_2O_2 consumed/mm/mg protein; GPx µg/mol/mg protein;

MDA unit/g tissue x $10^{-6;}$ H_2O_2 µmol/mg protein/ Protein mg/ml; NaAS – Sodium arsenite

Table 4.26 Activities of SOD, CAT, GPx, MDA, H_2O_2 and Protein in liver of rats treated with *A. hybridus* and sodium arsenite

Treatment	Distilled water alone	NaAS alone	100mg/kg *A.hyb*	200 mg/kg *A.hyb*	300mg/kg *A.hyb*	100mg/kg *A.hyb*+NaAS	200mg/kg *A.hyb*+NaAS	300mg/kg *A.hyb*+NaAS
Group	**GRPI**	**GRPII**	**GRPIII**	**GRPIV**	**GRPV**	**GRPVI**	**GRPVII**	**GRPVIII**
Parameters								
SOD	$4.22^{a} \pm 0.40$	$2.82^{bc} \pm 0.33$	$3.03^{a} \pm 0.66$	$3.63^{ab} \pm 1.13$	$3.85^{ab} \pm 0.56$	$3.13^{ab} \pm 0.09$	$3.57^{ab} \pm 0.14$	$4.10^{a} \pm 0.70$
CAT	$123.83^{a} \pm 20.50$	$71.38^{d} \pm 8.33$	$94.33^{b} \pm 5.67$	$100.27^{b} \pm 1.96$	$109.64^{a} \pm 5.28$	$73.66^{c} \pm 10.24$	$79.85^{c} \pm 4.47$	$94.03^{b} \pm 6.78$
GPX	$539.75^{a} \pm 55.70$	$398.44^{cd} \pm 31.25$	$498.40^{bc} \pm 29.27$	$514.28^{b} \pm 36.87$	$575.91^{a} \pm 25.32$	$410.20^{e} \pm 23.34$	$434.04^{de} \pm 28.38$	$460.53^{cd} \pm 15.29$
MDA	$4.71^{c} \pm 1.48$	$11.15^{a} \pm 0.85$	$5.41^{bc} \pm 0.42$	$4.65^{cd} \pm 0.26$	$4.26^{d} \pm 0.56$	$8.36^{a} \pm 0.29$	$6.09^{b} \pm 0.65$	$5.34^{bc} \pm 0.55$
H_2O_2	$29.87^{c} \pm 6.70$	$41.42^{a} \pm 5.89$	$32.99^{b} \pm 2.79$	$29.09^{c} \pm 6.84$	$27.26^{c} \pm 4.72$	$34.48^{b} \pm 2.75$	$34.29^{b} \pm 1.02$	$32.83^{b} \pm 1.84$
Protein	$0.66^{a} \pm 0.06$	$0.60^{ab} \pm 0.05$	$0.50^{ab} \pm 0.04$	$0.51^{ab} \pm 0.04$	$0.54^{a} \pm 0.03$	$0.46^{ab} \pm 0.03$	$0.45^{ab} \pm 0.07$	$0.40^{b} \pm 0.12$

Values are expressed as means ± SD; n = 5 for each treatment group; mean values within a row not sharing same letter are significantly different $p<0.05$

SOD activity. Unit/mg protein; CAT activity; µmol H_2O_2 consumed/mm/mg protein; GPx µg/mol/mg protein;

MDA unit/g tissue x $10^{-6;}$ H_2O_2 µmol/mg protein/ Protein mg/ml; NaAS – Sodium arsenite

4.5. Micronuleated Polychromatic erythrocytes PCEs

The frequency of micronucleated polychromatic erythrocytes (mPCEs) induced in the rat bone marrow cells following the oral administration of ethanol extract of *A. hypochondriacus* and *A. hybridus* are shown in Tables 4.27 and 4.28. The data were pooled from five animals in each treatment group. The aberrations induced are directly related to the dose. In all cases, however, the frequency of micronucleated erythrocytes is higher in rats administered sodium arsenite alone (group II) when compared with control (group 1).

Administration of sodium arsenite significantly ($p<0.05$) induced mPCEs formation in the rat bone marrow cells as observed in the increased frequency of mPCEs (group II) when compared with control (group I) (Table 4.27). There was no significant difference in the number of micronucleated cells observed in groups III, IV and V rats when compared with the control group I. The increased frequency of mPCEs were significantly reduced ($p<0.05$) in rats administered 100, 200 and 300mg/kg of *A. hypochondriacus* extracts with sodium arsenite (groups VI, VII and VIII) when compared with group II, close to control (group I). Also, groups IV and V (rats administered 100 and 200 mg/kg *A. hypochondracus* exracts alone) were observed to have frequency of mPCEs close to the control.

Table 4.28 shows the frequency of polychromatic erythrocytes in the rat bone marrow cells following the oral administration of the *A. hybridus* extract alone or in combination with sodium arsenite. The micronucleated PCEs count are also directly related to the dose. The mPCE count in rats administered sodium arsenite alone (group II) was significantly ($P<0.05$) higher than all other treatment groups. The frequency of mPCEs observed in rats administered 200 and 300 mg/kg *A. hybridus* extract alone (groups IV and V) were not significantly different when compared with control (group 1). Treatment with 100, 200 and 300 mg/kg *A. hybridus* extract and sodium arsenite (groups VI, VII and VIII) significantly decreased ($p<0.05$) the induction of mPCEs by sodium arsenite close to the control (group I) in a dose dependent manner.

Table 4.27 Micronucleated polychromatic erythrocytes (mPCEs) count of rats administered ethanol extract of *A. hypochondriacus*

Treatment	Group	Number of mPCE/1000PCEs
Distilled water alone	I	$4.43^{d} \pm 0.32$
NaAS alone	II	$10.24^{a} \pm 0.43$
100mg/kg *A. hypo* alone	III	$5.74^{c} \pm 0.70$
200mg/kg *A. hypo* alone	IV	$4.93^{cd} \pm 0.36$
300mg/kg *A. hypo* alone	V	$4.22^{d} \pm 0.44$
100mg/kg *A. hypo*+NaAS	VI	$6.84^{b} \pm 0.39$
200mg/kg *A. hypo*+NaAS	VII	$5.53^{c} \pm 0.45$
300mg/kg *A. hypo*+NaAS	VIII	$4.96^{cd} \pm 0.20$

Values are means ± standard deviation, values with same letters on the same column are not significantly different at $p<0.05$
A. hypo (*A. hypochondriacus*); NaAS (sodium arsenite)

Table 4.28 Micronucleated polychromatic erythrocytes (mPCEs) count of rats administered ethanol extract of *A. hybridus*

Treatment	Group	Number of mPCE/1000PCEs
Distilled water alone	I	$5.56^{e} \pm 0.24$
NaAS alone	II	$11.31^{a} \pm 0.53$
100mg/kg *A. hyb* alone	III	$6.71^{c} \pm 0.38$
200mg/kg *A. hyb* alone	IV	$5.22^{e} \pm 0.18$
300mg/kg *A. hyb* alone	V	$5.09^{e} \pm 0.28$
100mg/kg *A. hyb* + NaAS	VI	$8.64^{b} \pm 0.44$
200mg/kg *A. hyb* + NaAS	VII	$7.13^{c} \pm 0.32$
300mg/kg *A. hyb* + NaAS	VIII	$6.11^{d} \pm 0.28$

Values are means ± standard deviation, values with same letters on the same column are not significantly different at $p<0.05$
A. hyb (*A. hybridus*), NaAS (sodium arsenite)

4.6. HISTOPATHOLOGICAL EVALUATION

Histopathology of liver tissues of experimental rats administered *A. hypochondriacus* ethanol extracts

The results of the histopathology analysis carried out on the liver samples of the experimental animals treated with ethanol extract of *A. hypochondriacus* showed that the control (group 1) had no visible lesions (Figure 4.12). The liver section of rats in the control showed central vein surrounded by hepatic cord of cells (normal liver architecture).

Treatment with sodium arsenite alone (group II), showed diffused hydropic degeneration of hepatocytes, portal congestion, and periportal cellular infiltration by mononuclear cells (Figure 4.13). The liver showed patches of liver cell necrosis with inflammatory collections around central vein.

The liver sections of rats treated with the three doses of *A. hypochondriacus* extract alone, showed no visible lesions (Figures 4.14 to 4.16). The liver sections showed central vein surrounded by hepatic cord of cells as was observed in control group.

Animals treated with the three doses of *A. hypochondriacus* extract and sodium arsenite (groups VI, VII and VIII) showed mild vacuolar degeneration of hepatocytes (Figures 4.17 to 4.19). Liver in these groups showed minimal inflammatory conditions around the central vein. Treatment with 300 mg/kg *A. hypochondriacus* extract and sodium arsenite (group VIII) showed near normal liver architecture having minimal inflammatory conditions thus demonstrating higher hepatoprotective activity than groups VI and VII (Figure 4.19).

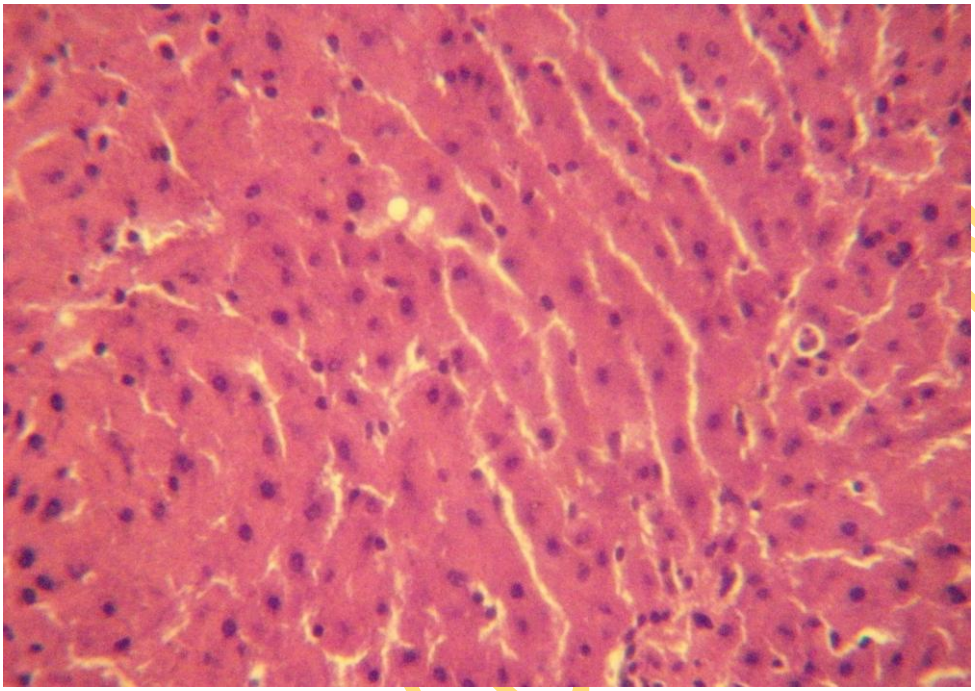


Figure 4.12 Liver section of rat administered distilled water only showing normal architecture (X 400)

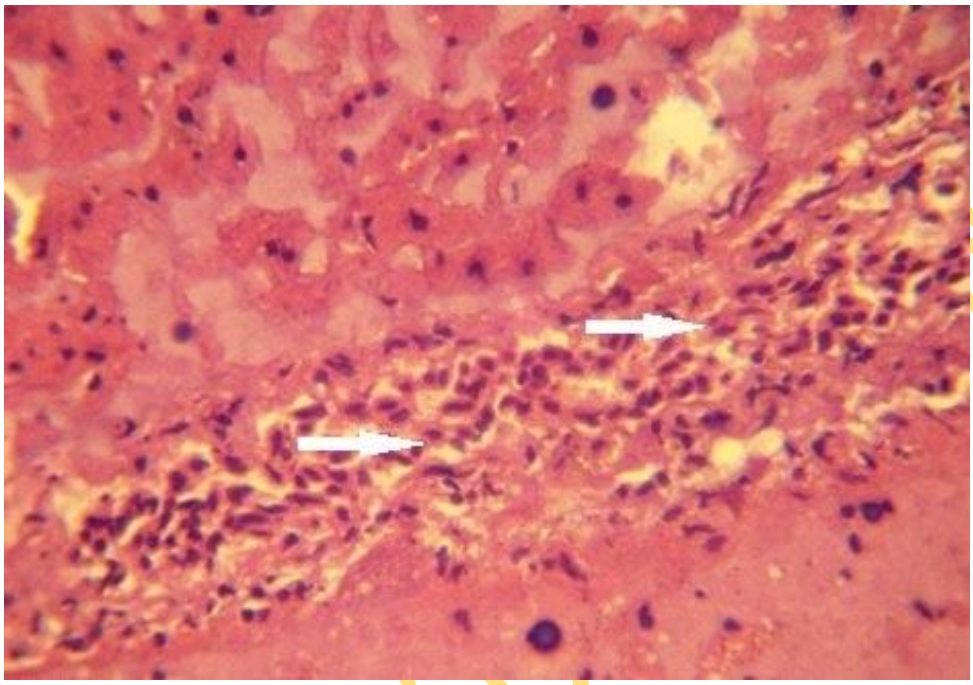


Figure 4.13 Liver section of rat administered sodium arsenite alone showing severe portal congestion, periportal cellular infiltration by mononuclear cells (arrows) X 400

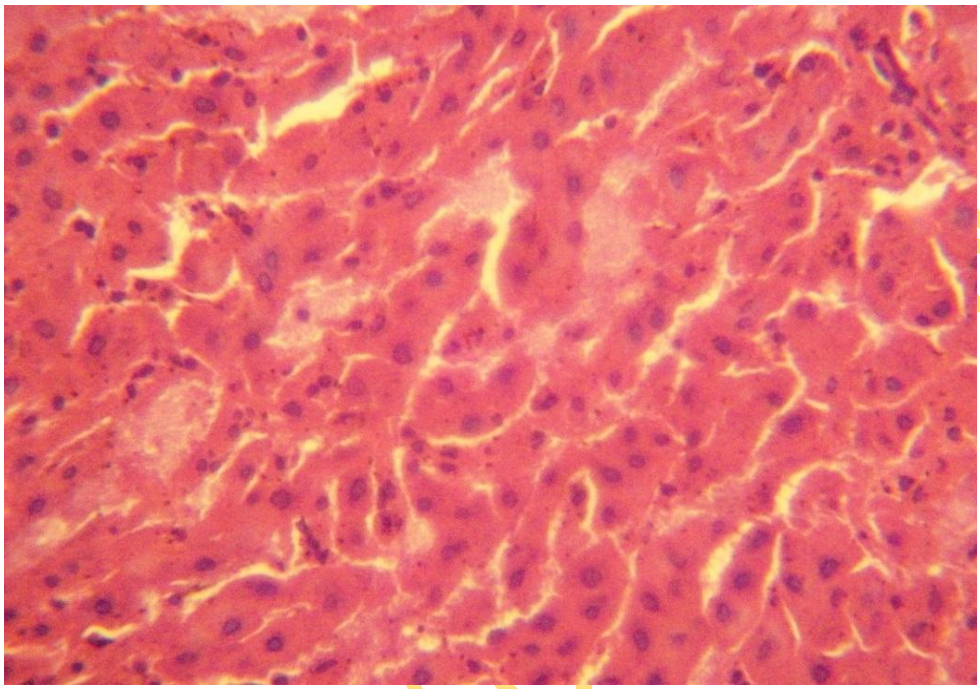


Figure 4.14 Liver section of rat administered 100 mg/kg b.wt of *A. hypochondriacus* extract alone showing no visible lesions (X 400)

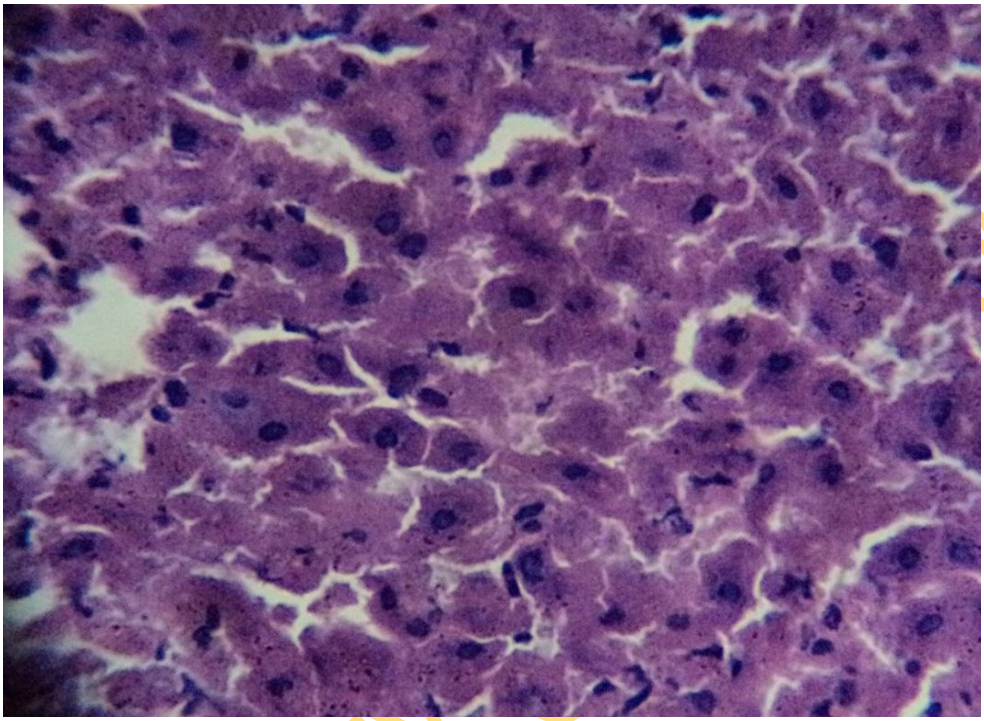


Figure 4.15 Liver section of rat administered 200 mg/kg *A. hypochondriacus* extract showing no visible lesions, cytoplasm and nucleus are intact and well differentiated (X 400)

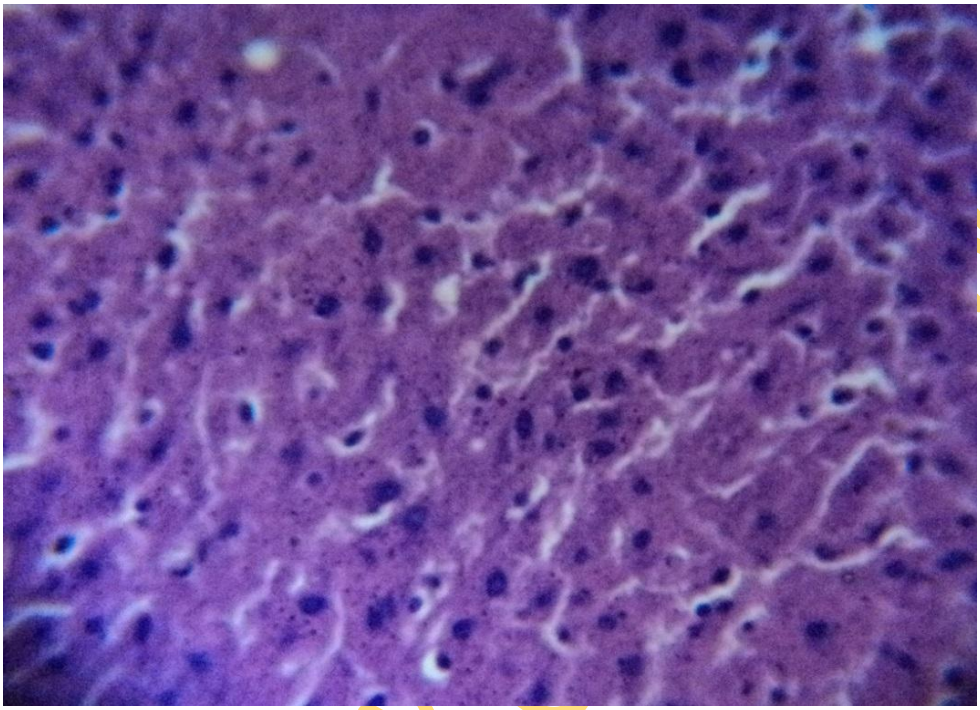


Figure 4.16 Liver section of rat administered 300 mg/kg *A. hypochondriacus* showing no visible lesions, cytoplasm and nucleus are intact and well differentiated (X 400)

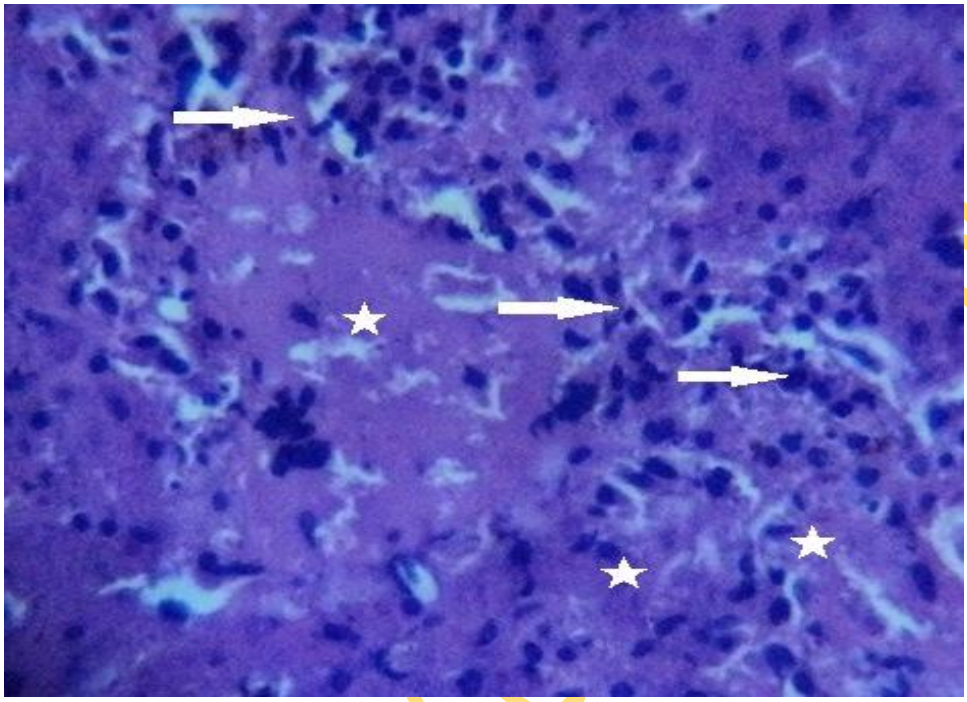


Figure 4.17 Liver section of rat administered 100 mg/kg *A. hypochondriacus* and 2.5 mg/kg b. wt sodium arsenite. Inflammation changes were observed (arrows), with mild hepatic degeneration (stars). X 400

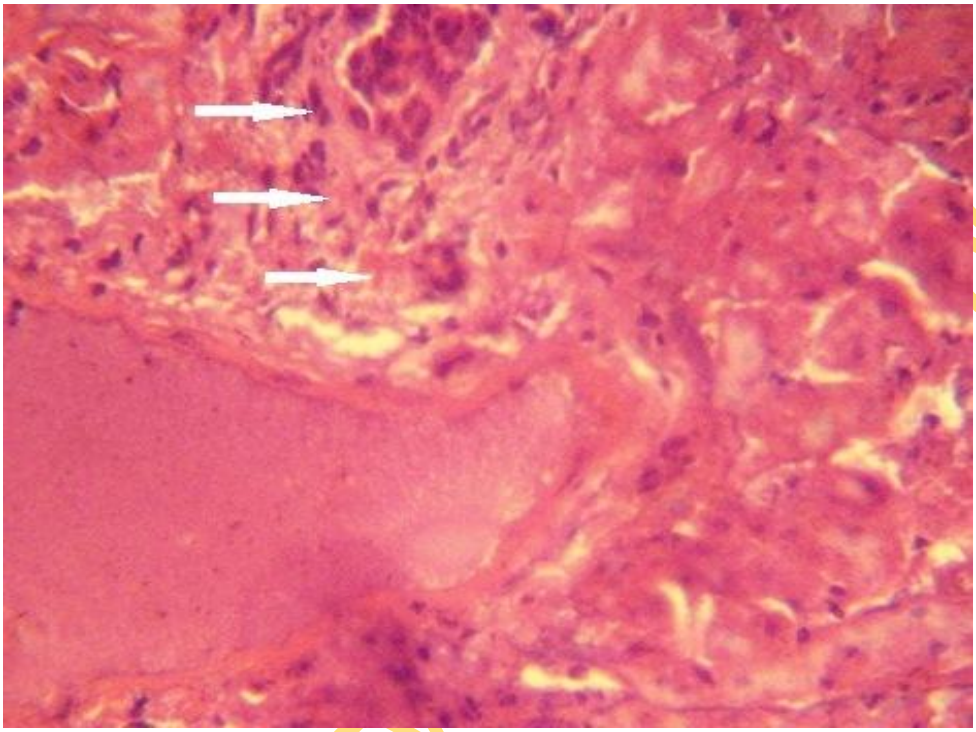


Figure 4.18 Liver section of rat administered 200mg/kg *A. hypochondriacus* and 2.5 mg/kg b. wt sodium arsenite. Cellular infiltration around the portal area is mild (arrow) X 400

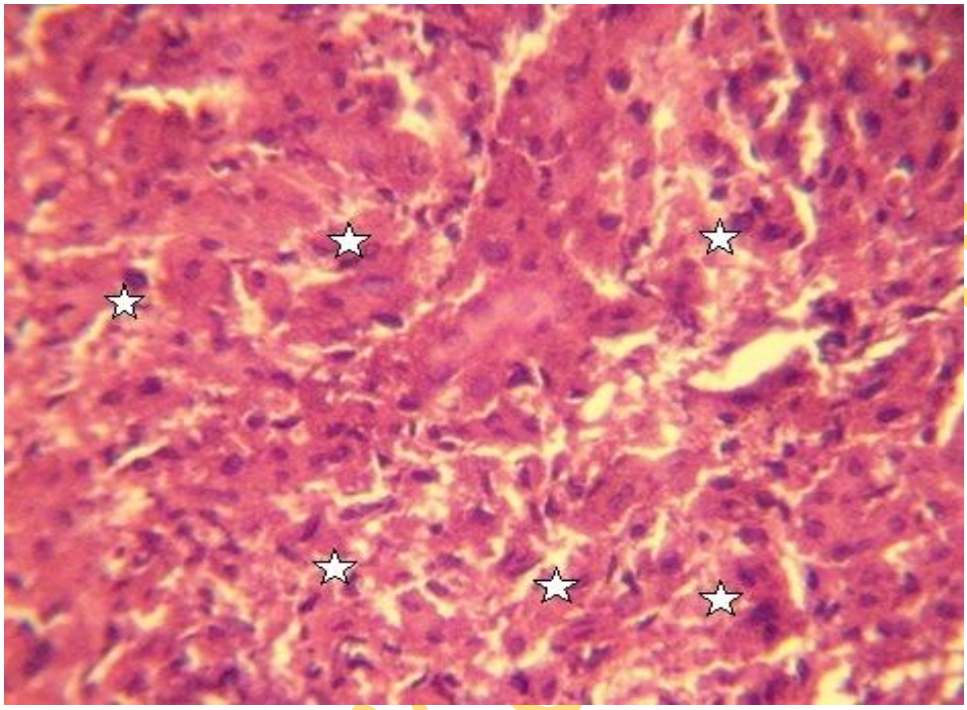


Figure 4.19 Liver section of rat administered 300mg/kg *A. hypochondriacus* and 2.5 mg/kg b. wt sodium arsenite. Showing very mild hepatic degeneration (stars). X 400

Histopathology of liver tissues of experimental rats administered *A. hybridus* ethanol extracts

Similar trend in the results of histopathology analysis was observed for liver samples of the experimental animals treated with *A. hybrdus* ethanol extracts.

The control which is treatment with distilled water alone (group 1) had no visible lesions (Figure 4.20). For rats treated with sodium arsenite alone (group II), the result showed diffused hydropic degeneration of hepatocytes, portal congestion, and periportal cellular infiltration by mononuclear cells (Figure 4.21). Also, liver cell necrosis was observed.

The liver sections of rats treated with the three doses of *A. hybridus* extract alone (groups III, IVand V), showed no visible lesions (Figures 4.22 to 4.24). The liver sections showed central vein surrounded by hepatic cord of cells as is observed in normal liver architecture and is similar to the liver morphology observed in control (group I).

Treatment with the three doses of *A. hybridus* extract and sodium arsenite (groups VI, VII and VIII) showed vacuolar degeneration of hepatocytes (Figures 4.25 to 4.27). Liver in these groups showed minimal inflammatory conditions around the central vein. Rats administered 300 mg/kg *A. hybridus* extract and sodium arsenite (group VIII) showed minimal inflammatory conditions, with near normal liver architecture close to control, thus exhibiting potential of possessing a higher hepatoprotective activity than groups VI and VII (Figure 4.27).

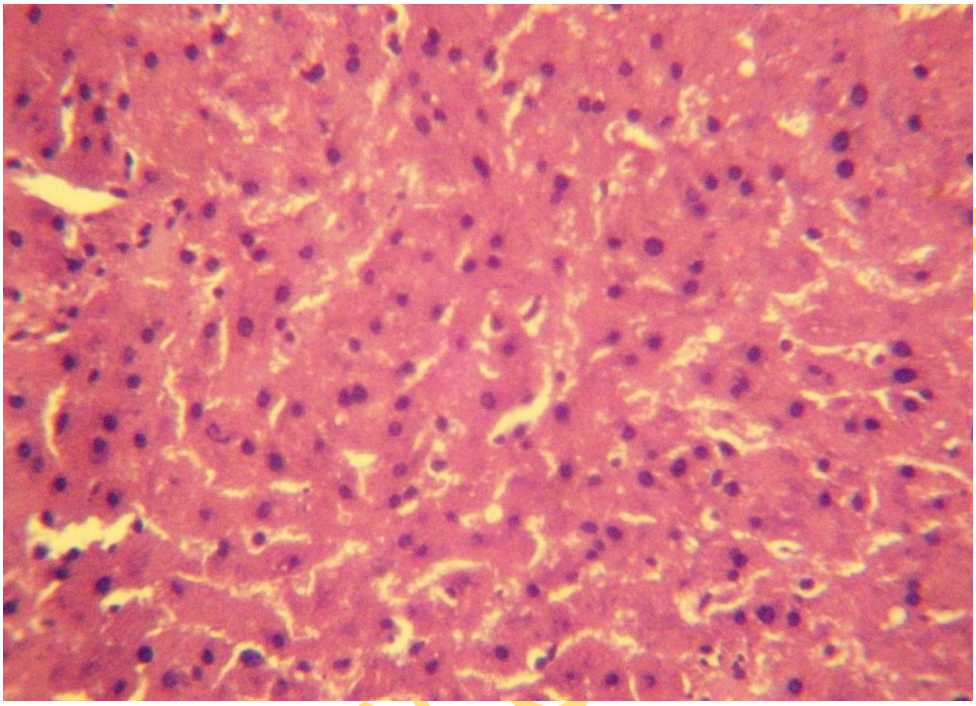


Figure 4.20 Liver section of rat administered distilled water only showing normal architecture (X 400)

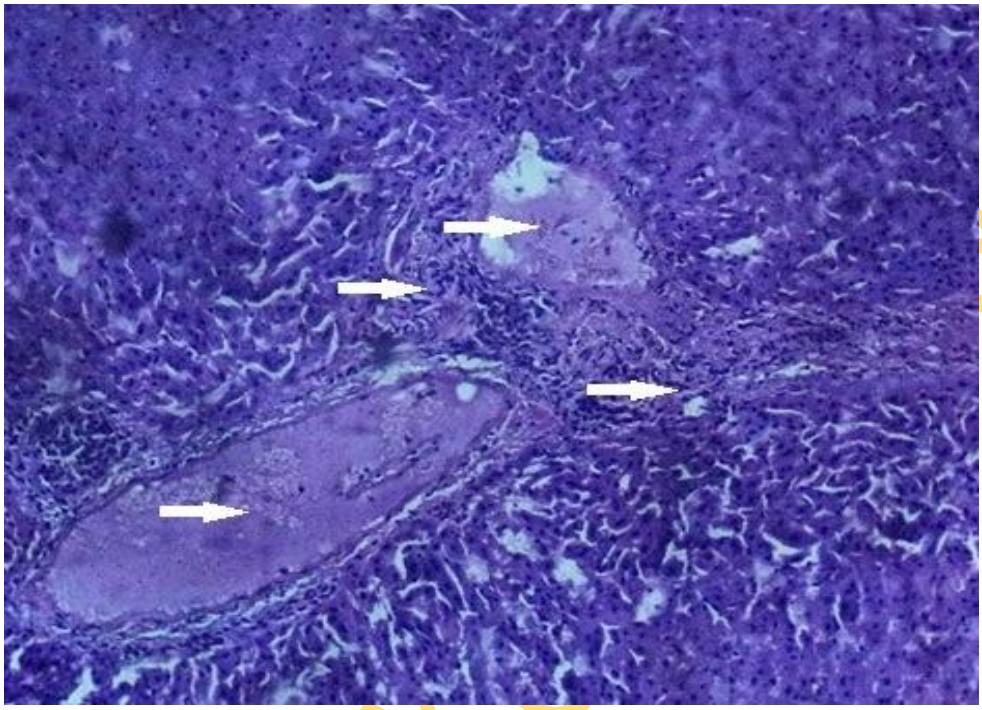


Figure 4.21 Liver section of rat administered sodium arsenite alone showing moderate portal congestion, severe cellular infiltration and periportal hepatic necrosis (arrows) X 400

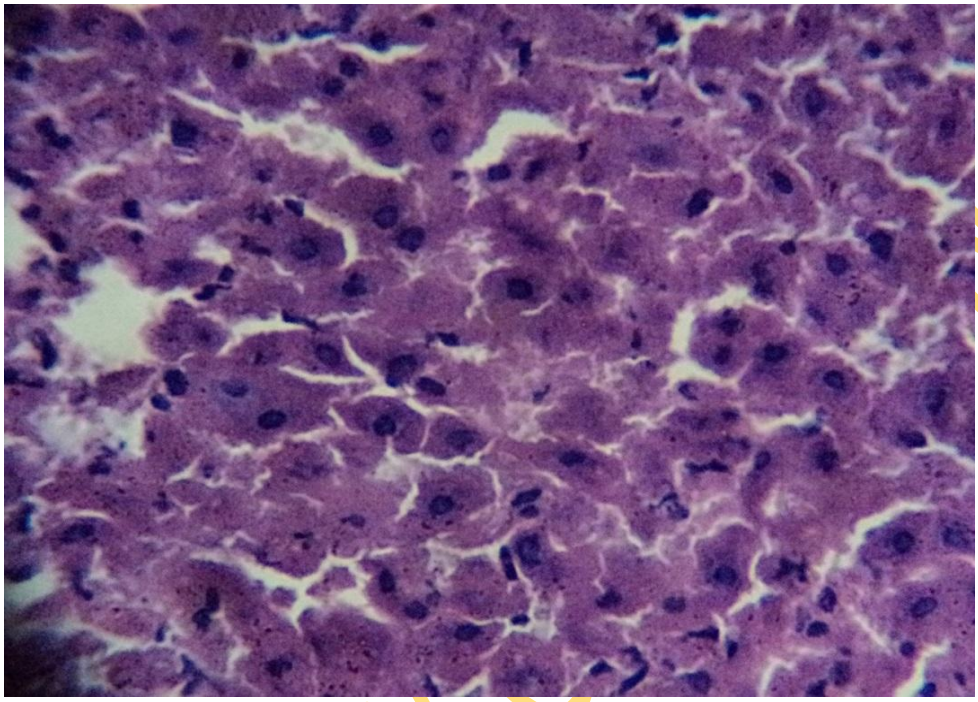


Figure 4.22 Liver section of rat administered 100 mg/kg *A. hybridus* extract only showing no visible lesions (X 400)

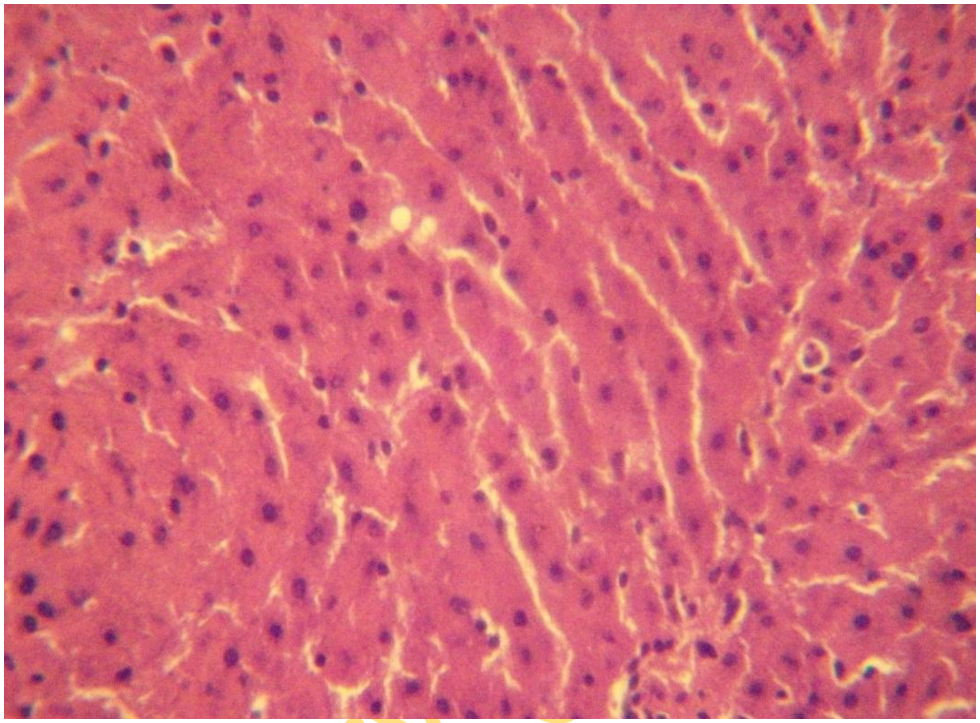


Figure 4.23 Liver section of rat administered *A. hybridus* extract only (200 mg/kg b.wt) showing no visible lesions (X 400)

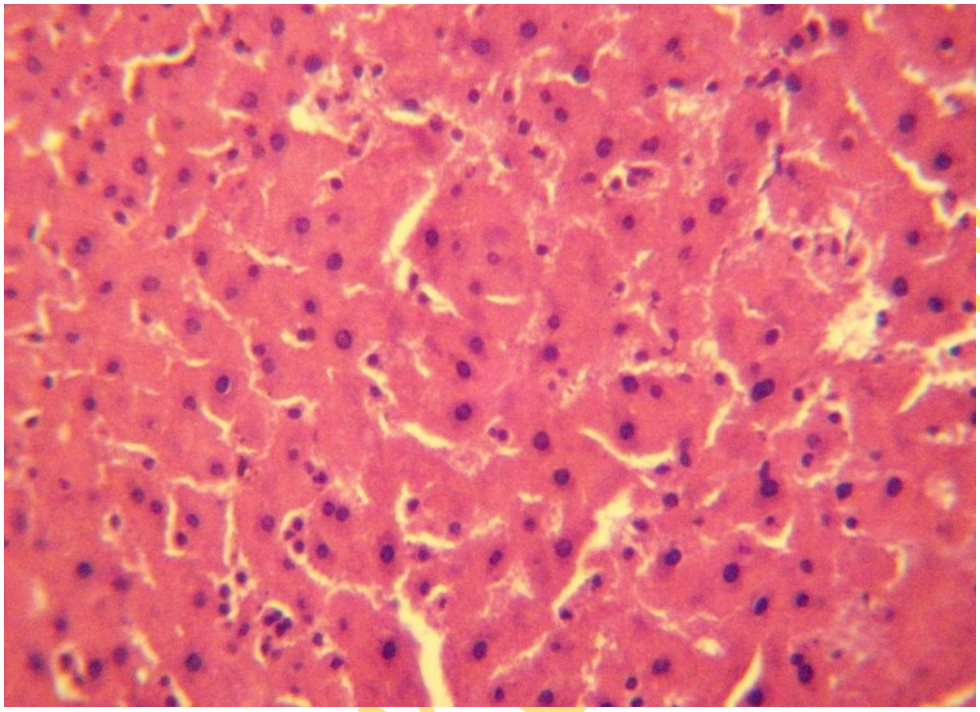


Figure 4.24 Liver section of rat administered *A. hybridus* extract only (300 mg/kg b.wt) showing no visible lesions (X 400)

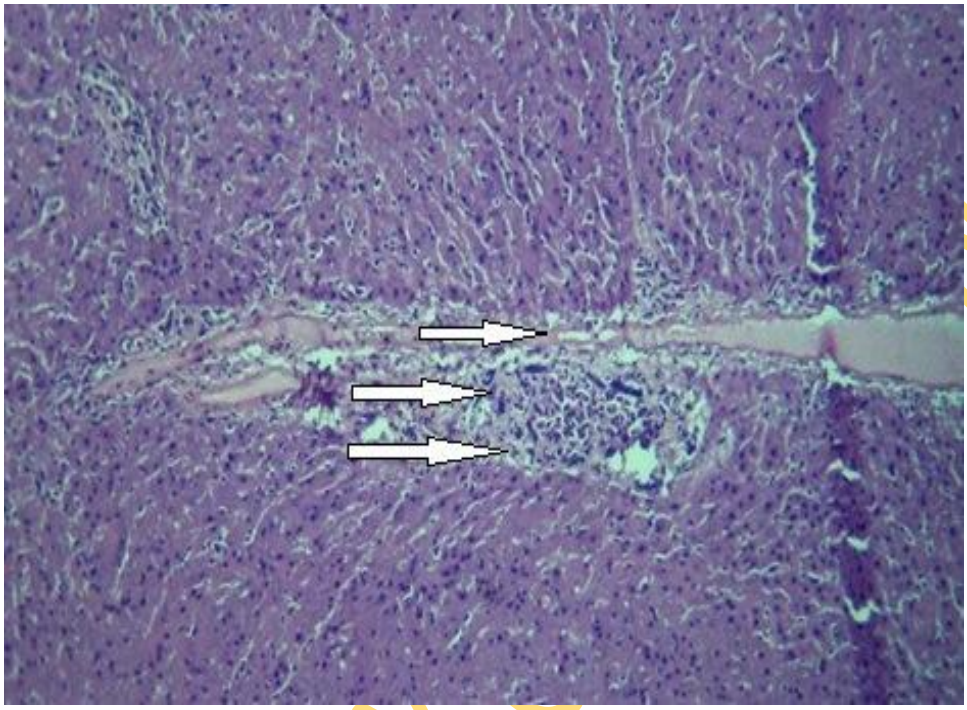


Figure 4.25 Liver section of rat administered *A. hybridus* extract (100 mg/kg b.wt) and 2.5 mg/kg b.wt of sodium arsenite showing cellular infiltration by mononuclear cells (arrows). X 400

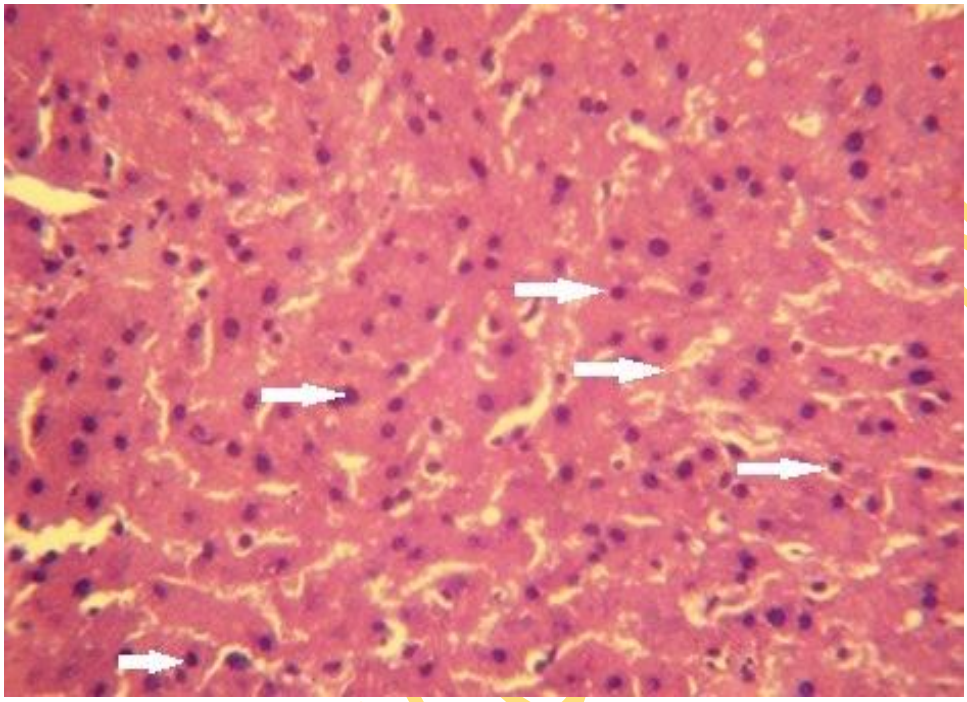


Figure 4.26 Liver section of rat administered *A. hybridus* extract (200 mg/kg b.wt) and 2.5 mg/kg b.wt sodium arsenite showing cellular infiltration by mononuclear cells (arrows). X 400

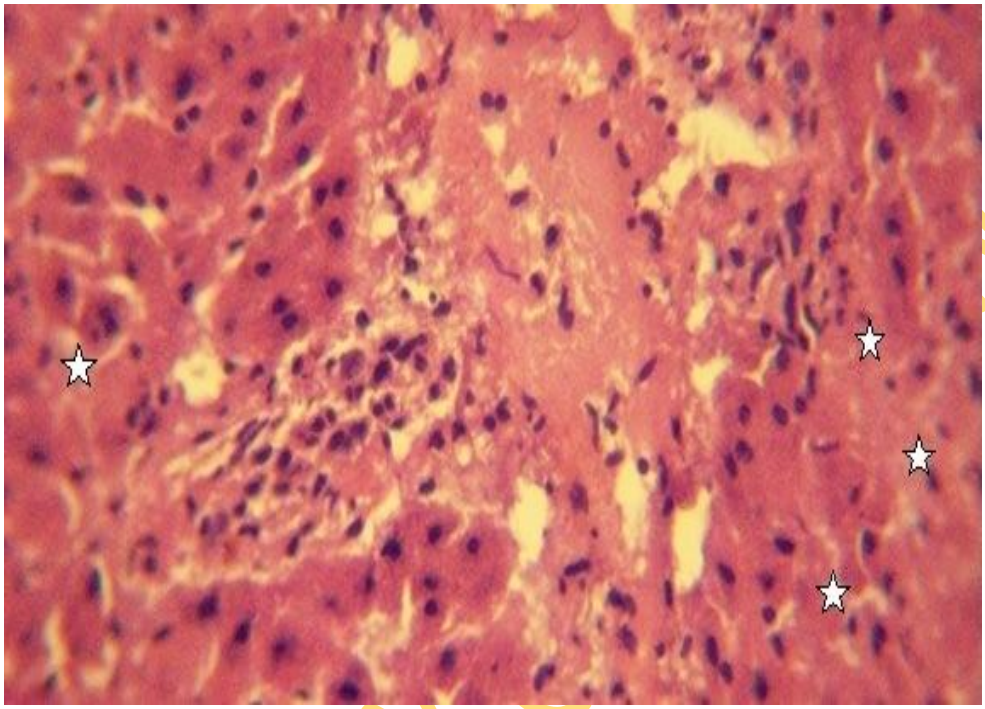


Figure 4.27 Liver section of rat administered *A. hybridus* extract (300 mg/kg b.wt) and 2.5 mg/kg b.wt sodium arsenite showing very mild hepatic degeneration (stars) X 400

CHAPTER FIVE

5.0. DISCUSSION

5.1. Variation of important agronomic and nutritional traits in grain amaranth

Grain amaranth has been cultivated for centuries because of its grain which is rich in protein and mineral components. It is an underutilized crop due to its small seed size and low seed yield per plant as a result of which cultivation is limited.

In view of the enormous nutritional benefits of the crop, there is a definite need for its genetic improvement to develop high yielding varieties with a high content of quality traits. In the process of genetic improvement of any crop, genetic diversity among germplasm plays a major role, since it opens the way to determine the most divergent parents based on the contribution of different qualitative and quantitative traits, for further utilization in any hybridization programme.

Genetic variability in the base population plays a very important role in any crop-breeding programme. The extent of diversity present in the germplasm determines the limit of selection for improvement. The characters of economic importance are generally quantitative in nature and exhibit considerable degree of interaction with the environment. Thus, it becomes imperative to compute variability present in the breeding material and its partitioning into genotypic, phenotypic and environmental ones (Shukla *et al*., 2006; 2010). Previous studies on vegetable amaranth showed the presence of wide range of diversity in both agronomic and qualitative traits especially leaf and stem colour (Wu *et al*., 2000; Xiao *et al*., 2000). Xiao *et al*., (2000) further opined that stem and leaf colour were useful indices in the classification of vegetable amaranths.

In this study, characters such as plant height, stem diameter, number of leaves per plant, leaf length, leaf width, leaf area and seed yield had high values of coefficient of variation (Table 4.4) indicating scope for improvement in these traits through selection to enhance the potentiality of seed yield and plant vigour. Wu *et al*., (2000) in a study of 229 genotypes from 20 *Amaranthus* species observed wide variability which was useful in cultivar improvement for agronomic traits such as plant height, seed yield, stem and leaf colour among genotypes.

Results from traits such as days to 50% flowering, moisture content, starch, fat, protein, magnesium, manganese, and phosphorus had low values of coefficient of variation, which implies that chances of getting substantial gains under selection are likely to be less for these traits.

The traits investigated in this study are important, as they have direct and indirect effects on yield and nutritional components. The PCA showed that the first four PCs accounted for about 57.5% of the total variation encountered among the accessions taking into account all the 27 traits simultaneously (Table 4.7). PC_1 distinguished those accessions that gave high leaf length, leaf breadth, leaf area, copper content and plant height. PC_2 distinguished accessions having high values of iron, aluminium, magnesium, selenium, number of leaves, phosphorus and calcium. Although calcium has some strength in PC_2, it appears more strongly in PC_4. This indicates that the calcium content is not strongly correlated with the rest of the traits and hence it should be possible to select accessions with more calcium without adversely affecting other economically important traits. PC_3 distinguished accessions having high values of sugar, starch and crude fibre content, while PC_4 distinguished ash, seed yield, 100-seed weight, moisture and calcium content.

The germplasm were grouped into five clusters (Figure 4.1), each carrying amaranth accessions sharing common properties and being similar to one another. With few exceptions, the collections from same regions were clustered together. Accessions A11 and A23 were tall, thick stemmed, had high leaf length, leaf breadth, leaf area, number of leaves per plant, high seed yield, 100-seed weight, sugar, fat, ash, crude fibre and mineral contents and were grouped as clusters IV and V respectively. Such strong relationship of diversity and geographical origin has been reported previously in crops such as oats (Rezai and Frey, 1990), maize (Alika *et al*., 1993), mustard (Alemayehu and Becker, 2002), bambara groundnut (Ntundu *et al*., 2006) and vegetable amaranth (Shukla *et al*., 2010). The outcome of this analysis was consistent with the results obtained through PCA, wherby the major differences between the clusters were attributed to the same traits that contributed most to PC_1, PC_2, PC_3 and PC_4.

Accessions showing high mean performance for specific traits can be used as donor parent for improving those traits in component breeding (Shukla *et al*., 2006). Cluster IV with only one member (Accession A11), can be considered as a source of genes for seed yield as well as most of the morphological traits (Table 4.8). Similarly, accession A23 in cluster V can be considered as donor parents for 100-seed weight and most of the nutritional traits (mineral components), while members of

cluster III can be used for introgression of protein and selenium (Table 4.8). Findings from this study are in concordance with earlier reports that both PCA and cluster analysis can disclose complex relationships between taxa in a more understandable way and with equal effectiveness (Rezai and Frey, 1990; Abede and Bjornstad, 1996).

This study also revealed that accession A28 (*A. hybridus*) has the potential of imparting high protein, selenium and high seed yield to grain types through cross breeding. Ugborogho and Oyelana, (1993) observed in their investigations that many accessions of *A. hybridus* exist and they tend to vary in plant height, leaf colour, stem colour and seed inflorescence colour and grain yield. *Amaranthus hybridus* has shown promise as leafy vegetable crop and also as a breeding line for grain amaranth. This variation has led to the opinion that Amaranths were a taxonomically difficult group because of the ease of intra- and inter specific hybridization which resulted in close morphological resemblance and floral complexes.

5.1.1. Evaluation of the nutritional composition of grain amaranth

Proximate (moisture, ash, protein, fat, crude fiber, sugar, starch) and mineral compositions (Aluminium, iron, zinc, copper, phosphorus, calcium, potassium, selenium, magnesium, and manganese) indicates that all the 29 accessions evaluated differed significantly ($p < 0.001$) in these characters.

The moisture content of all the 29 accessions evaluated ranged from 7.43 to 15.46% (Table 4.5) and were higher than values obtained for *A. cruentus* (6.71 to 6.23%), but similar to 11.40 to 12.07% obtained for *A. caudatus* (Repo-Carrasco-Valencia *et al*., 2010) and 13.00% obtained for wheat (Becker *et al*., 1981).

The fat content obtained in this study ranged from 1.43 to 10.39% (Table 4.5). The fat content of most accessions were comparable with 7.71 to 8.13% reported for *A. cruentus, A. hypocondriacus* and *A. edulis* and higher than 2.5% reported for wheat by Becker *et al*., (1981). Repo-Carrasco-Valencia *et al*., (2010) recently reported that the fat content of *A. caudatus* ranged from 6.31 to 7.56%. Pedersen *et al*., (1987) also reported that the fat content of *A. caudatus* was 10% and this was higher than 7.19 to 8.59% obtained for *A. caudatus* in this study.

Ash content ranged from 2.35 to 6.98% with accession A15 having the highest value followed by accession A23. Ash content obtained for *Amaranthus caudatus* and *A. cruentus* in this study ranged from 2.35 to 4.17% and are comparable with 2.5% reported for *A. caudatus* by Pederson *et al*., (1987) and higher than 2.0% reported for wheat by Becker *et al*., (1981). The ash content of some varieties of

A. caudatus has been reported to vary from 2.16 to 2.77% (Repo-Carrasco-Valencia *et al*., 2010) and is lower than values obtained in this study.

The protein contents (11.77 to 19.01%) are in accordance with reports by Bressani and Garcia-Vela, (1990); Segura-Nieto *et al*., (1994); Czerwinski *et al*., (2004) and Gorinstein *et al*., (1998, 1999). Highest value (19.01%) was observed for *Amaranthus hybridus* (variety A28), followed by *Amaranthus cruentus* (variety A9) having 18.42%, *A. hybrid* (variety A17) having 17.96%; *A. caudatus* (variety A2) having (17.3%) and *A. hypochondriacus* (variety A27) having 15.53%. Our results are similar to earlier reports which stated that *A. cruentus* had crude protein content (13.2-18.2%); *A. caudatus* (17.6-18.4%) and *A. hypochondriacus* (17.9%). Results from this study showed that when compared with wheat (13.5-14.5%), maize (10.6-13.8%), barley (10-14.9%), and oats (12.4-12.9%), amaranth had the highest protein content. The nutritional value of pseudocereals is mainly connected to their proteins. Proteins are an important group of biomacromolecules that are involved in physiological functions (Wright, 1987). Natural vegetable proteins are useful materials owing to their safeness, high biocompatibility, nutritional value and low cost.

Crude fiber in the 29 *amaranthus* accessions evaluated in this study ranged from 1.04 to 6.27% and were similar to 3.44-5.34% obtained for *A. cruentus, A. hypochondriacus*; but much higher than 2.6% obtained for wheat, as reported by Becker *et al*., (1981). Results of this study are similar to values (2.68 to 6.73%) obtained in *A. caudatus* (Repo-Carrasco-Valencia *et al*., 2010). Czerwinski *et al*., (2004) reported total dietary fiber in *Amaranth hypochondriacus* to contain 14.2 to 14.5%, this is higher than values obtained for the 29 grain amaranth species evaluated. Pseudocereals contain relatively high amounts of dietary fiber, which improves lipid metabolism and takes part in the prevention of LDL-C oxidation (Gorinstein *et al*., 1998).

Iron, zinc and calcium are essential minerals required for diverse physiological and biochemical functions. The iron, zinc and calcium contents obtained in this study (Table 4.6) were higher than values obtained for *A. caudatus* and *Chenopodium pallidicaule* an underutilized pseudocereal cultivated in South American (Repo-Carrasco-Valencia *et al*., 2010).

Iron content (61.66 to 189.04 mg/kg) obtained in this study is similar to results obtained for *A. cruentus* (174ppm), *A. hypochondriacus* (106ppm) and *A. edulis* (84.2ppm) and higher than value obtained for wheat (31ppm) as reported by Becker *et al*., (1981). Zinc content which ranged from 34.73 to 100.07

mg/kg in this study is similar to values obtained for *A. cruentus A. hypochondriacus* and *A. edulis* (37-40.0 ppm) as reported by Becker *et al*., (1981); and also 38.3-38.7 mg/kg reported for *A. hypochondriacus* (Czerwinski *et al*., 2004). Varieties A11 and A7 had higher values than those reported by Becker *et al*., (1981) for the various amaranth species. Copper content obtained in this study (2.29 to 7.65 mg/kg) are comparable to values (8.1-8.3 mg/kg) reported by Czerwinski *et al*., (2004) for *A. hypochondriacus* but lower than values (7.9 to 13.2 ppm) obtained for some amaranth species (Becker *et al*., 1981).

Manganese content obtained in this study (45.74 to 213.73 mg/kg) are relatively higher than values obtained in amaranth species (15.9 to 45.9 ppm) as reported by Becker *et al*., (1981) and 31.5 to 31.7 mg/kg obtained for *A. hypochondriacus* (Czerwinski *et al*., 2004). Magnesium content (1582.11 to 2624.4 mg/kg) obtained in this study is comparable to values obtained for some amaranthus species (2300 to 3360ppm) by Becker *et al*., (1981).

Calcium obtained in this study (888.24 to 1980.32 mg/kg) is comparable to values obtained in some amaranth species (1700 to 2150ppm) as reported by Becker *et al*., (1981). Our results are much higher than value (360ppm) reported for wheat (Watt and Merrill., 1963 and Becker *et al*. 1981). The high calcium level in amaranth suggests that phytic acid would occur as the insoluble calcium salt in the seed.

Selenium content (0 to 1.73 mg/kg) obtained in this study is comparable to 0.22 to 0.26 mg/kg obtained for *A. hypochondriacus* (Czerwinski *et al*., 2004) with variety A17 having the highest value, followed by variety A18. Selenium was the least abundant mineral element present, followed by copper.

The sodium and calcium contents obtained in this study were generally higher than values obtained in other common cereal grain reported by Becker *et al*., (1981). Potassium content (3157 to 10346.6 mg/kg) in this study is relatively higher than values (2900 to 5800 ppm) obtained for some amaranth species and 3700 ppm obtained for wheat; as reported by Becker *et al*., (1981). Accession A23 had the highest values for potassium, phosphorus and second to the highest in calcium.

5.1.2. Genetic diversity of the 29 *amaranthus* accessions as revealed by RAPD analysis

Ecological and geographical differentiations are important factors influencing strategies for breeding and sampling crop species. Most of the current methods to determine genetic diversity are based on polymerase chain reaction (PCR) (Powell *et al*., 1996). In this study, various accessions of grain amaranth from different geographical regions were evaluated using RAPD molecular marker. To access

the genetic diversity, 40 random primers were screened of which 16 primers gave amplification (Table 4.10). These primers generated 193 fragments of which 157 were polymorphic (81%). At the intraspecific level, percentage of RAPD polymorphism has been reported to be 22.5%, 18.3%, and 23.3% for *A. hypochondriacus, A. caudatus and A. cruentus* (Ray and Roy, 2009). Chan and Sun, (1997) reported a higher percentage of polymorphism in leafy amaranths at the intraspecific level than in grain amaranths. In this study, the high percentage of intraspecific polymorphism observed in *A. hypochondriacus* (66.32%) and in *A. hybrid* (61.14%) indicates that they both sustain greater genetic variation than the other amaranth species studied (Table 4.11). All the populations of a species clustered together as observed in the dendogram generated by UPGMA (Figure 4.4), except for accessions A2 and A21 which were found clustering with other species. It is plausible that these amaranth population studied has a narrow genetic base and so could not show much variation.

Accessions A28 and A29 (NH84/444-4 and NHAC3, respectively) showed the highest similarity at 89% similarity coefficient indicating that they are genetically identical or closely related among all the *Amaranthus* species studied. The most distant are PI490458 (Accession A1) and Ames 2256 (Accession A17) which are far apart on the dendrogram. It is also evident from the RAPD-based clustering that *A. caudatus* is closely related to *A. hybrid* and *A. hypochondriacus*. Similar results were also observed in previous RAPD based analyses (Ray and Roy, 2009; Chan and Sun, 1997; Transue *et al*., 1994). This study revealed low level of diversity as can be observed in the dendrogram showing about 70% similarity and 30% diversity in the accessions.

According to Sauer (1957), the amount of geographic advances has been very unequal, even for a single species of amaranth along different borders. In some cases, expansion was local, but elsewhere migration is active, causing exceptional hybridization in amaranths (Sauer 1957). This study showed some geographic cohesiveness, as represented by accessions A13, A14, A15 and again A16, A17, A18, A19; which were collected from close phytogeographic regions and found to be on neighboring branches in the dendrogram (Figure 4.4). Geographic and ecological differences in the distribution of genetic diversity among amaranth populations were evident by other populations of this study; similar observations were made by Ray and Roy, (2009) for *amaranthus* species from the Indo-Gangetic plains.

There is always a controversy on the relationship between genetic divergence and geographical origin. Some studies showed little relationship (Hadian *et al*., 2008), whereas others clearly demonstrated

noticeable associations between population characteristics and the environment in which they occur (Ge *et al*., 2003). In this study, the dendogram based on RAPD markers was not in accord with the dendogram based on phenotypic traits. The dendogram generated by the RAPD marker agrees better with the groups of the accessions than the dendogram generated by phenotypic traits. There is no doubt that when morphological variation causes confusion or misidentification, RAPD analysis can aid the correct identification of species in amaranth genetic resources (Chan and Sun, 1997; Transue *et al*., 1994).

5.2. Amino acid composition of grain amaranth proteins

Results show that all grain amaranth accessions investigated were high in glutamic acid, aspartic acid and arginine (Table 4.13). Glutamic acid was observed to be the highest with mean value ranging from 14.10 to 23.70 g/100 g and this was followed by aspartic acid, arginine, glycine and then lysine. Similar trend was observed in *amaranthus cruentus* and *amaranthus edulis* where glutamic acid was observed to be the highest amino acid present followed by aspartic acid and arginine (Becker *et al*., 1981). Amino acid analysis on soybean also showed similar trend in which glutamic acid had the highest amount followed by aspartic acid and arginine (Zarkadas *et al*., 2007a and 2007b). Most of the amaranth accessions evaluated were observed to have higher methionine and cysteine contents than the soybean cultivar, whilst the non-essential amino acids were higher in soybean than in amaranth. Glycine obtained in most varieties of the grain amaranth evaluated is higher than values obtained for soybean. Amaranth could supply preschool child and adult requirements of Histidine, Isoleucine, Leucine, Lysine, Methionine, Threonine and Valine. The lysine content (5.65-7.89%) obtained in this study is similar to results obtained for *amaranthus edulis* (5.9%), *amaranthus cruentus* (6.0-6.1%) by Becker *et al*., (1981); *amaranthus edulis* (6.4%) by Downton, (1973) and higher than values (3.2 to 6.4%) earlier reported for amaranth (Gorinstein *et al*., 1991b). Lysine values obtained in this study (5.65-7.89%) is comparable to values obtained for soybean cultivar (6.5%) as reported by Becker *et al*., (1981), values obtained for fourteen cultivars of soybean (6 to 6.9%) as reported by Zarkadas *et al*., (2007a), and also higher than 3.41% and 5.87% obtained in maize and beans, respectively (Muyonga *et al*., 2008) and 2.2 to 4.5% found among the most common cereals (Gorinstein *et al*., 1998). The lysine content in amaranth has been reported to be twice that of wheat and three times that of maize (Gorinstein *et al*., 2002). The requirement for Lysine by adults and children could be supplied by amaranth protein. The sulfur amino

acid contents of grain amaranth ranged from 1.77 to 2.74%, with some accessions having higher amount than value (2.15%) obtained for the soybean used in this study. Our results are comparable to values (2.6 to 5.5%) obtained for amaranth species and higher than that of the most important legumes (1.4%) such as pea, beans and soybeans (Gorinstein *et al*., 1998). This makes amaranth a promising crop as a food or source of dietary proteins.

The essential amino acid (EAA_9) pattern ranged from 31.22 to 44.88% (Table 4.14) and was higher than the 33.9% reference protein pattern value given by FAO/WHO (1991) for the diet of a 2- to 5-year-old child and comparable to 51.2% given for hen's whole egg. These results indicate that grain amaranth provide a good balance of total essential amino acids. The EAA_9 obtained for amaranth accessions (31.22-44.88%) in this study is lower than the EAA_9 (60.87%) obtained for the soybean cultivar (TGX 1448-2E) evaluated in this study and EAA_9 obtained for soybean (60.3%) reported by Gorinstein *et al*., (2002). Earlier investigation on soybean genotypes by Zarkadas *et al*., (2007b) showed that all genotypes evaluated contained a good balance of essential amino acids EAA_9 ranging from 43.5 to 47.35% of the total protein, this is comparable to results obtained for amaranth accessions (31.22 to 44.88%) in this study.

From the calculated amino acid score in this study (Table 4.15), the limiting essential amino acid was found to be tryptophan and leucine. Earlier report by Becker *et al*., (1981) on *A. edulis* showed that the limiting amino acids were leucine, valine and threonine. The limiting amino acid found for the soybean used as reference in this study was methionine and tryptophan. Earlier assessment of protein quality of soybean by Zarkadas *et al*., (2007a and 2007b) reported methionine as the limiting amino acid. For practical human nutrition, under conditions of normal usage of grain amaranth proteins in the diet, tryptophan supplementation is likely unnecessary, except for the feeding of the newborn, where modest supplementation of formulas with tryptophan may be beneficial.

Tryptophan is an important biological constituent of numerous proteins in animals and plants, and an essential amino acid in human and animal diets. However it is readily destroyed during acid hydrolysis of proteins and its quantitative determination has remained a difficult analytical problem. The most effective method is the alkaline procedure of Hugli and Moore, (1972), which allows quantitative recoveries (96-100%) of tryptophan in protein and tissues using the rapid tryptophan column chromatographic method recently reported by Zarkadas *et al*., (2007b).

Our results indicate that grain amaranth is limiting in the essential amino acid tryptophan and leucine when compared with the FAO/WHO (1973) Provisional Amino Acid scoring pattern.

It is also evident that total protein content, amino acid and storage protein compositions are more concise and useful traits for evaluating the seed protein quality (FAO/WHO, 1991; Krishnan *et al*., 2005; USFDA, 1993).

5.2.1. Electrophoretic pattern of amaranth seeds protein separated by SDS-PAGE

The seed proteins of all examined accessions are heterogenous and have revealed the extensive polymorphism of total seed proteins. All accessions of *amaranthus* evaluated showed similar patterns in their total protein electrophoretic profile (Figure 4.5). All subunits of total protein migrated between 14 and 55 kDa and all varieties had characteristic bands at 20, 24, 34 and 36 KDa. This is similar to reports of Barba de la Rosa *et al*., (1992) and Mora-Escobedo *et al*., (1990) observed for *A. hypochondriacus*.

From previous work on amaranth seed protein, albumins and globulins have been found to be major storage proteins (Gorinstein *et al*., 1998; 1991a and 1991b; Parades-Lopez *et al*., (1994). Results from this study showed that albumin, globulin and glutelin are the extracted fractions. Results are similar to findings by Silva-Sanchez *et al*., (2008) who also reported that globulins and glutelins are the major fractions of amaranth seed storage proteins.

The main subunits of albumins irrespective of species had molecular masses in the range of 10, 20, 29, 34 and 36kDa (Figure 4.6). Some minor fractions were observed in the region between 45 and 66kDa. Varieties 3 and 4 (*A. caudatus*) and 5, 6 and 7 (*A. cruentus*) differed from other accessions mainly in the region of 24 kDa of the total protein fraction. Albumins are comparable with egg-white proteins and this protein fraction has been used as an egg substitute to prepare bread (Silva-Sanchez *et al*., 2008).

Alcohol-soluble proteins (prolamin-like) (Figure 4.8) was not detectable in this study using 55% propan-2-ol and 0.6% mercaptoethanol. Prolamin fraction is characteristic only for cereals; globulin and albumin fractions are the main proteins for pseudocereals (Czerwinski *et al*., 2004). Prolamin was found to be 2% of the total nitrogen in pseudocereals and 15% in legumes (Gorinstein *et al*., 2002). The apparent paucity of nitrogen in the alcohol-soluble protein fraction of amaranth can be explained by the

fact that this fraction is a minor one in comparison with cereal plants (Gorinstein *et al*., 1991a., Bressani and Garcia-Vela., 1990; Segura-Nieto *et al*., 1994). Maize, wheat and other cereal prolamins contain about 45% of the total nitrogen (Gorinstein *et al*., 1991a; Gorinstein *et al*., 1999). Methods of Okita *et al*., (1988) and Yamagata *et al*., (1982) for the extraction of prolamins from rice and oats were not efficient for amaranth alcohol-soluble proteins as reported by Gorinstein *et al*., (1991a). Gorinstein *et al*., (1991a) demonstrated that maximum amount of alcohol-soluble proteins was extracted with 55% 2-propanol and 5% 2-mercaptoethanol (10:1 v/w). Alcohol soluble amaranth proteins probably contain intermolecular disulfide bands as zein proteins in corn (Paulis, 1981). This explains why increased amounts of reducing agents gave maximum extraction. Segura-Nieto *et al*., (1994) reported that prolamins had the lowest number of polypeptides and the electrophoretic pattern was slightly different from the alcohol-soluble protein pattern shown by Gorinstein *et al*., (1991a). It can be concluded that the prolamin-like fraction of amaranth is not a storage protein. Albumins, globulins and glutelins are the major fractions located in the protein body of grain amaranth and the alcohol-soluble proteins (prolamin) probably derived from the perisperm are either present in low amounts or absent as reported by Gorinstein *et al*., (1991b). Based on research findings by Gorinstein *et al*., (2002), it was concluded that not only was the globulin fraction the major storage protein (Barba de la Rosa *et al*., 1992) in grain amaranth, the glutelin fraction is one of the major proteins in pseudocereals.

The glutelin and glutelin-like fraction of all accessions of grain amaranth evaluated showed close identity with the soybean fractions (Figures 4.9 and 4.10). In future it will be possible to use *amaranthus* species in blends with other cereals for food nutrients. A broad range of bands were observed between the 4 and 20kDa regions in the glutelin and glutelin-like fractions. In amaranth, the total protein extracts of both mature, non mature and popped seeds have shown the presence of a lunasin-like protein which remains as a band at 18.5 kDa (Silva-Sanchez *et al*., 2008). The characteristic bands observed below the 20kDa in this study if further separated with a high resolution technique may reveal bands which may be similar to the 18.5kDa band. Amaranth can be considered as a natural potential source of antihypertensive peptides. Lunasin the anticancer bioactive peptide present at the 18.5 kDa bands in soybean, wheat and grain amaranth has been reported to have a relatively high concentration of aspartic acid (Jeong *et al*., 2003). Amaranth glutelin fraction has being reported to have a relatively high concentration of aspartic acid, 10.6% of the total protein (Barba de la Rosa *et al*., 1992), suggesting that the acidic regions may be related to a lunasin epitope in the protein fractions of the seed.

5.3. Phytochemical composition and antioxidant activity of grain amaranth accessions

Epidemiological studies strongly suggest that diets play a significant role in the prevention of many chronic diseases (Temple, 2000; Wilet, 1994). Grain consumption has been associated with reduced risk of certain chronic diseases (Meyer *et al*., 2000; Jacobs *et al*., 1995; 2001) and this has been attributed in part to the unique phytochemicals in grains. Several studies have shown the association between reduced risk of coronary heart disease and diets high in cereal fibre (Wolk *et al*., 1999) and whole grain (Liu *et al*., 1999; Jacobs *et al*., 2001). Other studies have demonstrated the protective role of diets high in grain against cancer (Nicodemus *et al*., 2001) and diabetes (Meyer *et al*., 2000). Grain phytochemicals exert their health benefits through mutifactorial physiologic mechanisms including antioxidant activity; mediation of hormones; enhancement of the immune system and facilitation of substance transit through the digestive tract (Lupton and Meacher, 1998).

In this study, total polyphenol content varied among the 29 accessions of grain amaranth evaluated (Table 4.16) and was highest in accession A21 (35.39 mg/100g GAE), followed by accession A27 (34.88 mg/100g GAE). This is higher than values (21.20 mg/100g GAE) obtained for *Amaranthus caudatus* (Alvarez-Jubete *et al*., 2010); (14.72 to 14.91 mg/100g GAE) obtained for *A. hypochondriacus* and (19.61 mg/100g GAE) obtained for oats (Czerwinski *et al*., 2004). Results from this study are also higher than 16.8 to 32.9 mg/100g obtained for some *A. caudatus* genotypes (Repo-Carrasco-Valencia *et al*., 2010) but lower than values obtained for other pseudocereals like Quinoa (71.7 mg/100g), buckwheat (323.0 mg/100g GAE) and wheat (53.1 mg/100g GAE) (Alvarez-Jubete *et al*., 2010). In a recent study, the total phenol content of amaranth, quinoa and buckwheat were 15.5, 25 and 29 mg/100g GAE based on dry weight, respectively (Gorinstein *et al*., 2007). The total polyphenol content of Amaranth is significantly low when compared to values obtained in other grains such as wheat (53.1 mgGAE/100 g dwb) (Alvarez-Jubete *et al*., 2010), barley, millet, rye and sorghum (Ragaee *et al*., 2006). Kirakosyan *et al*., (2003) reported that phenolic compounds in plants possess antioxidant activity and these may help protect cells against the oxidative damage caused by free radicals. Our results show that grain amaranth contain considerable amount of phenolics, implying that these grains maybe useful in relation to diseases involving free radical reactions.

Flavonoids have potent antioxidant and anticancer activities (Repo-Carrasco-Valencia *et al*., 2010; Adom and Liu, 2002; Dykes and Rooney 2007; Hu *et al*., 2003). Total flavonoid content ranged from 7.77 to 10.97 mg/100g (Table 4.16), with highest value observed for accession A23 (10.97 mg/100g) followed by accession A6 (10.74 mg/100g). Results are lower than 13.4 to 14.3 mg/100g obtained for *A. hypochondriacus* and 17.7 mg/100g obtained for oat (Czerwinski *et al*., 2004). The flavonoid content of *Chenopodium* species are high varying from 36.2 to 144.3 mg/100g and these are higher than results of amaranth obtained in this study.

Since the main mechanism of antioxidant action in foods is radical scavenging, many methods have been developed in which the antioxidant activity is evaluated by the scavenging of synthetic radicals in polar organic solvents such as methanol. The radicals used in this study include 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals (Pasko *et al*., 2009; Farombi *et al*., 2000). DPPH scavenging activity was highest in accession A28 (95.4%) (Table 4.16), followed by accessions A18, A23 and A29 which had similar values (94%). ABTS ranged from 157.57 to 208.80 mM TE, highest value was observed for variety A23 (208.80 mM TE), followed by accessions A27 (207.46 mM TE) and A28 (200.54 mM TE). The DPPH results of this study could not be compared with the results of Repo-Carrasco-Valencia *et al*., (2009), for two varieties of *A. caudatus* (Centenario and Oscar Blanco) - 410.0 and 398.1 µmol trolox/g sample, respectively as they were expressed as trolox equivalent. Results of the antioxidant activity using ABTS method for the two varieties of *A. caudatus* (Centenario and Oscar Blanco) were 827.6 and 670.1 µmol trolox/g sample, respectively (Repo-Carrasco-Valencia *et al*., 2009). Results obtained for the 29 amaranth accessions evaluated in this study ranged from 157.57 to 208.80 mM TE and are much lower than results of Repo-Carrasco-Valencia *et al*., (2009). The discrepancy in these results is probably as a result of the different methodologies employed as well as the different species studied.

Strong correlation was observed between radical scavenging activity (DPPH) and reducing power (R^2 = 0.64) in this study (Table 4.17). Weak correlation was observed between DPPH and Total polyphenol (R^2 = 0.25). Similar findings was reported by Nsimba *et al*., (2008) who observed weak correlations between total polyphenol content and antioxidant activity (DPPH and FRAP assays) in amaranth and quinoa extracts. Similarly, investigations on yam reported no significant correlation between the total polyphenolic content and total antioxidant activity (Bhandari and Kawabata, 2004). In contrast, positive correlation has been reported between phenolic contents and antioxidant capacity (R^2 = 0.962) in rice (Adom and Liu, 2002;

Choi *et al*., 2007), and some pesudocereals (Alvarez-Jubete *et al*., 2010) and other samples from different plant origins (Cai *et al*., 2004). Weak correlation was observed between DPPH and ABTS (R^2 = 0.42) in this study, this is in contrast with findings of Pasko *et al*., (2009), who reported strong correlation between ABTS and DPPH (R^2 = 0.98).

Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity. The DPPH radical scavenging activity of grain amaranth ranged from 82 to 95.4% (Table 4.16) showing that all the accessions had appreciable free radical scavenging activities. Results are similar to values obtained in some yam varieties (50 to 96%) reported by Bhandari and Kawabata, (2004). Accession A28 had the highest value (95.4%) followed by Accessions A29 and A18 having same value (94.2%). The involvement of free radicals, especially their increased production appears to be a feature of most, if not all human disease, including cardiovascular disease and cancer (Deighton *et al*., 2000). Therefore, such dietary antioxidants from grain amaranth maybe particularly important in fighting these diseases by conferring protection against free radical damage to cellular DNA, lipids and proteins. However, reports on the antioxidant activity of grain amaranth are scarce. Also data variation on the antioxidant capacity may be due to differences in the methodology employed for its assay and the standard used to express the results. Therefore it is very difficult to compare our results with that of previous studies.

The reducing power has been used as one of the antioxidant capability indicators of medicinal herbs (Duh and Yen, 1997). All accessions exhibited a low reducing activity (0.01 to 0.21%), highest value was recorded for accession A24 (0.21%) followed by accession A28 (0.20%).

Total antioxidant activity ranged from 111.33 to 271.60, highest value was recorded for accession A1, followed by accession A23.

The purpose of the test of ferrous ion chelating activity was to determine the capacity of grain amaranth extract to bind the ferrous ion catalyzing oxidation. Results ranged from 31.51 to 86.66% in the 29 amaranth accessions evaluated. The highest value was obtained in accession A3 (86.66%) followed by accession A5 (81.43%). Results were similar to values obtained in some yam varieties, ranging from 76 to 85% (Bhandari and Kawabata, 2004). Ferrous ion, commonly found in food systems, is a well known effective pro-oxidant (Hsu *et al*., 2003). Polyphenols can chelate pro-oxidant metal ions, such as iron and copper, thus preventing free radical formation from these pro-oxidants (Kris-Etherton *et al*., 2002).

5.3.1. Hepatoprotective activity of the ethanol extracts of grain amaranth

In this study, *Amaranthus hypochondriacus* (accession A23) and *A. hybridus* (accession A28) were evaluated for the hepatoprotective activity using sodium arsenite (NaAS) induced toxicity in rat. Chronic exposure to arsenic leads to various ailments and dysfunctions of several vital organs such as liver, kidney and lung (Odunola *et al*., 2011; Gbadegesin and Odunola, 2010; Chowdhury *et al*., 2001).

The percentage weight change (Table 4.19) of rats exposed to sodium arsenite (group II) when compared with the control (group I) was significantly lower ($p<0.05$) and is in agreement with results of Dhar *et al*., (2005) and El-Demerdash *et al.*, (2009) in which reduced body weight was observed in rats exposed to arsenic. It has been demonstrated that inorganic arsenic and its methylated metabolites selectively binds to proteins in some tissues (Styblo and Thomas, 1997). Rahman *et al*., (1998) suggested that prolonged exposure to arsenic is associated with an increased risk of diabetes mellitus, which explain the weight loss of rats. Kaltreider *et al*., (2001) demonstrated that exposure to low levels of arsenic alters hormonal function in the glucocorticoid system. The glucocorticoid hormones play an important role in glucose regulation, as well as carbohydrate, lipid and protein metabolism. Dysfunction in the glucocorticoid system has been linked to weight gain/loss.

Sodium arsenite is being used extensively to investigate hepatoprotective activity on various experimental animals. There is growing evidence that NaAS intoxication can compromise the integrity of the liver in mouse, rat, fish and goat (Sharma *et al*., 2009; Odunola *et al*., 2011; Gbadegesin and Odunola, 2010; Yousef *et al*., 2008; Roy *et al*., 2009). The relative liver weight of rats treated with sodium arsenite in this study was significantly higher ($p<0.05$) than the control and all the other groups administered the three different doses of ethanol extracts of *A. hypochondriacus* and *A. hybridus*. The high relative liver weight observed in NaAS treated group might be due to biotransformation of NaAS to its toxic metabolites which are likely to cause this adverse effect. This adverse effect on relative liver weight of the rats treated with NaAS are in agreement with results of Bashir *et al*., (2006) and Jana *et al*., (2006).

Arsenic produces oxidative damage by disturbing the prooxidant-antioxidant balance, because it has very high affinity for sulfhydryl groups in reduced glutathione (GSH), which might have implications in the maintenance of thiol-disulfide balance (Yamanaka *et al*., 1991). Generation of large amount of reactive oxygen species (ROS) due to arsenic toxicity can overwhelm the antioxidant defense

mechanism and damage cellular ingredients such as lipids, proteins and DNA; this in turn can impair cellular structure and function (El-Demerdash *et al*., 2009).

The ability of a drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects (Yadav and Dixit, 2003). Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood (Ashok Shenoy *et al*., 2001).

The increased activity of AST, ALT, ALP and GGT are conventional indicators of liver injury (Achliya *et al*., 2004; Thabrew *et al*., 1987). In this study, sodium arsenite administration by oral gavage in rats resulted in a significant increase in the level of serum AST, ALT, ALP and GGT enzymes when compared with the control, and may be due to the increased permeability of plasma membrane, indicating considerable hepatocellular injury. Similar results of elevated serum enzymes were obtained by Odunola *et al*., (2011) using Garlic and *Spondias mombin* as test samples. In addition, the increased plasma GGT activity of rats exposed to NaAS in this study, when compared with the control, is indicative of oxidative stress and cytogenetic damage, as has been reported (Lee *et al*., 2006; Karmaker *et al*., 1999; Odunola *et al*., 2011). Treatment with 100 and 200 mg/kg *A. hypocondriacus* extract alone (groups III and IV) increased the activities of ALT, AST and GGT when compared to the control (group I), indicating that they contain substances that may compromise some vital function in the liver. Administration of *A. hypochondriacus* extract at the three dose levels along with NaAS (groups VI, VII and VIII) attenuated the increased activities of ALT and GGT produced by sodium arsenite and caused a subsequent recovery towards normalization, comparable to the control (group I), in a dose dependent manner.

Treatment with 100 and 200 mg/kg *A. hybridus* extracts alone (groups III and IV) increased the ALT, and GGT activities although not to the the level observed for NaAS. This may be an indication that the extracts contain substances that may compromise some vital function in the liver and this toxicity may be related to the presence of some antinutrients. Treatment with the three doses of *A. hybridus* extracts and NaAS (groups VI, VII and VIII) prevents hepatic cells against NaAS induced damage as observed in the reduction in the activities of ALT, AST and GGT. This observation indicates that ethanol extract of *A. hybridus* exhibit some inhibitory action over NaAS induced hepatic damage.

The body has an effective defense mechanism to prevent and neutralize the free radical induced damage. This is proficient by a set of endogenous antioxidant enzymes such as SOD, CAT, GST, and GPx. These enzymes constitute the first line of cellular antioxidant defense and provide a mutually supportive team of defense against ROS (Amresh *et al*., 2007b). In NaAS induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, "oxidative stress" results, which through a series of events deregulates the cellular functions leading to hepatic necrosis (Manna *et al*., 2007).

The significant decrease ($p<0.05$) of SOD, CAT and GPx activities observed in rats administered NaAS (group II) when compared with the control point out hepatic damage and are probably due to exhaustation of these enzymes to scavenge excessively-generated superoxide and hydrogen peroxides, respectively in tumour cells (Manoharan *et al*., 1996; 2006). This reduced activity in SOD, CAT and GPx of rats treated with sodium arsenite is in agreement with previous findings of Wu *et al*., (2001), Gopalkrishnan and Rao, (2006) and Bashir *et al*., (2006). Lowered activities of SOD, CAT and GPx in erythrocytes have been reported in cancer by Balasenthil *et al*., (2000). Our results corroborate these observations.

Treatment with 100, 200 and 300 mg/kg of *A. hypochondriacus* extract alone (groups III, IV and V) showed increase in the level of these enzymes when compared with group II rats, which indicates the antioxidant activity of these extracts. Treatment with 100, 200 and 300 mg/kg of *A. hypochondriacus* extracts and NaAS (groups VI, VII and VIII) alleviated its toxicity and ameliorated SOD, CAT and GPx levels. This observation is similar to results of Tirkey *et al*., (2005) and El-Demerdash *et al*., (2009) who showed that treatment with curcumin improved the levels of renal SOD and CAT to reach the control level.

Treatment with 100, 200 and 300 mg/kg extract of *A. hybridus* alone (groups III, IV and V) showed significant ($p<0.05$) increase in the activities of SOD, CAT and GPx tending towards the level of these enzymes obtained in the control. These protective effects of *A. hybridus* as an antioxidant are attributed to the presence of antinutients and antioxidants.

Treatment with 100, 200 and 300 mg/kg extract of *A. hybridus* and NaAS (groups VI, VII and VIII) significantly increased the activities of SOD, CAT and GPx thus alleviating its toxicity and ameliorating

the enzyme activities in a dose dependent manner. This is similar to the results of Thiyagarajan and Sharma, (2004); who showed that curcumin increased endogenous antioxidant defense enzymes.

Superoxide dismutase (SOD) has an antitoxic effect against the superoxide anion. SOD accelerates the dismutation of superoxide to H_2O_2 which is removed by catalase (Usoh *et al*., 2005). Thus SOD can act as a primary defense and prevents further generation of free radicals. The decreased SOD activity in liver suggests that the accumulation of superoxide anion radical might be responsible for increased lipid peroxidation following arsenic treatment. The fact that free radicals are produced by arsenic treatment was also indicated by Yamanaka *et al*., (1989, 1990) and they suggested that free radical species are generated by the reaction of molecular oxygen with dimethylarsine, a metabolite of dimethyl arsenic acid. Yamanaka *et al*., (1989) also considered one of these radicals to be superoxide anion radical produced by one electron reduction of molecular oxygen by dimethylarsine.

Catalase is responsible for the breakdown of hydrogen peroxide, an important ROS, produced during metabolism. CAT catalyzes the removal of Hydroperoxide formed during the reaction catalyzed by SOD (Ramanathan *et al*., 2002). Reduced activity of catalase after rats were administered sodium arsenite in this study could be correlated with increased generation of hydrogen peroxide. Similar observations were reported by El-Demerdash *et al*., (2008), in which treatment with NaAS resulted in decrease in catalase activity. The significant decrease in SOD and CAT activities of rats treated with sodium arsenite is also in agreement with the previous findings of Wu *et al*., (2001), Gopalkrishnan and Rao (2006) and Bashir *et al*., (2006). In rats exposed to arsenic, the possible accumulation of H_2O_2 in organs as a result of diminished activity of catalase is probably circumvented by the increased GSH concentrations stimulating the glutathione peroxidase-mediated reduction of H_2O_2 and organic hydroperoxides (Yu, 1994).

The level of lipid peroxide (MDA) is a measure of membrane damage and alterations in structure and function of cellular membranes. Free radical-induced lipid peroxidation is regarded as one of the basic mechanism of cellular damage and therefore, the extent of tissue damage can be monitored by measuring the concentration of plasma or serum lipid peroxides (Selvendiran and Sakthisekaran, 2004). Increased plasma lipid peroxidation has been reported in several types of cancer patients (Ozdemirler *et al*., 1998). Erythrocytes are constantly exposed to oxidative stress and susceptibility of erythrocytes to oxidative stress has been reported in several pathological conditions, including oral cancer.

In this study, elevation of lipid peroxidation and hydroperoxides in the liver of rats treated with NaAS (group II) was observed. This significant ($p<0.05$) increase in MDA and hydroperoxides levels in liver of rats administered NaAS suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals (Amresh *et al*., 2007a). Increase in plasma lipid peroxides in DMBA-painted animals has also been observed and was due to overproduction and diffusion from the damaged erythrocytes, erythrocyte membranes and some other host tissues such as the liver (Manoharan *et al*., 2006).

The levels of MDA and H_2O_2 were significantly ($p<0.05$) lower in rats administered 100, 200 and 300 mg/kg ethanol extract of *A. hypochondriacus* (groups III, IV and V) when compared with rats administered NaAS (group II). Administration of 100, 200 and 300 mg/kg ethanol extract of *A. hypochondriacus* with NaAS (groups VI, VII and VIII) significantly reduced the elevated levels of MDA and H_2O_2 caused by NaAS close to control (group I); thus reversing these changes. Similar observation was reported in CCl_4 induced toxicity in rats, administration of different doses of ethanol extracts of *Amaranthus spinosus* significantly prevented the heave in levels of MDA and H_2O_2 (Zeashan *et al*., 2008). It is likely that the mechanism of hepatoprotection of seed extract of *A. hypo*chondriacus maybe due to its antioxidant activity.

Administration of 100, 200 and 300 mg/kg of *A. hybridus* ethanol extracts (groups III, IV and V) significantly ($p<0.05$) lowered the levels of MDA and H_2O_2 when compared with rats administered NaAS (group II). Administration of 100, 200 and 300 mg/kg of *A. hybridus* with NaAS (groups VI, VII and VIII) significantly ($p<0.05$) reduced the elevated level of MDA and H_2O_2 due to NaAS intoxication, causing subsequent recovery towards normalization in a dose dependent manner. This is similar to the findings of Zeashan *et al*., (2008) in which increased levels of MDA and H_2O_2 in CCl_4 intoxicated rats was reduced on administration of different doses of ethanol extracts of *Amaranthus spinosus*.

It has been demonstrated that inorganic arsenic and its methylated metabolites selectively binds to proteins in some tissues (Styblo and Thomas, 1997). The inhibitory effect of sodium arsenite on protein content in this study is in agreement with the finding of Nandi *et al*., (2005) and Kaltreider *et al*., (2001) who reported that arsenic inhibited the ability of glucocorticoid and its receptor to turn on genes normally. The glucocorticoid hormonal system plays an important role in protein metabolism.

Sodium arsenite significantly increased ($p<0.05$) the frequency of micronucleated polychromatic erythrocytes (mPCEs) when compared with the control. This observation in NaAS treated group may reveal its toxicity in polychromatic erythrocytes of bone marrow cells and is in agreement with results of Odunola *et al*., (2008, 2011). Treatment with ethanol extracts of 100, 200 and 300 mg/kg of *A. hypochondriacus* and NaAS (groups VI, VII and VIII) significantly reduced the frequency of mPCEs formation in bone marrow cells induced by the administration of sodium arsenite, in a dose dependent manner.

Das *et al*., (1993) reported similar decrease in the degree of mPCEs formation in mice pretreated with garlic before sodium arsenite challenge. The presence of flavonoid, polyphenols and antioxidant activities in *A. hypochondriacus* extract may account for this observation.

Similarly, administration of the three doses of ethanol extracts of *A. hybridus* and NaAS (groups VI, VII and VIII) significantly reduced ($p<0.05$) the frequency of mPCEs formation in bone marrow cells induced by the administration of NaAS.

The hepatoprotective effect of the *A. hypochondriacus* and *A. hybridus* extracts were further assessed by the histopathological examinations. Histopathology of liver section in rats administered distilled water only showed central vein surrounded by hepatic cord of cells (Figure 4.12), while sodium arsenite treated rats liver section showed patches of liver cell necrosis with inflammatory collections around central vein (Figure 4.13). Rats administered 100, 200 and 300 mg/kg ethanol extracts only of either *A. hypochondriacus* (Figures 4.14 to 4.16) or *A. hybridus* (Figure 4.22 to 4.24), showed no visible lesions. Similar results were obtained when 100, 200 and 400 mg/kg of ethanol extract of whole plant of *A. spinosus* was administered to rats (Zeashan *et al*., 2008). Administration of 100, 200 and 300 mg/kg ethanol extracts of *A. hypochondriacus* with sodium arsenite (Figure 4.17 to 4.19) and 100, 200, and 300 mg/kg ethanol extracts of *A. hybridus* with sodium arsenite (Figures 4.25 to 4.27) showed minimal inflammatory conditions around the central vein. The 300 mg/kg extract treated groups showed minimal inflammatory conditions with near normal liver architecture possessing higher hepatoprotective action. Results of this study are similar to findings observed for ethanol extract of whole plant of *Amaranthus spinosus* when administered in dosage of 100, 200 and 400 mg/kg against Carbon tetrachloride (CCl_4) induced hepatic damage in rats (Zeashan *et al*., 2008). The histopathology lesions observed for rats treated with sodium arsenite is an evidence of direct toxicity to hepatic tissue and the reduced

inflammation around the central vein by amaranth extracts suggest the hepatoprotective potential of grain amaranth.

CHAPTER SIX

6.0. CONCLUSION, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATION

6.1. CONCLUSION

6.1.1. Characterization of 29 accessions of grain amaranth

Characterization of germplasm materials helps to ensure efficient and effective use of such materials. Cluster and principal component analyses proved to be an effective method of evaluating germplasm materials as it identified accessions that could be further evaluated and utilized at the genetic level. Grouping accessions based on morphologic and nutritional characters brings accessions that are genetically similar together and help to identify potential hybrids with desired characters for selection in future breeding programmes.

From this study, considerable amount of variability exists in the 29 grain amaranth accessions evaluated in terms of seed yield; stem diameter; plant height; leaf area; number of leaves as well as seed quality. *Amaranthus* accessions evaluated in this study are rich in protein, crude fibre, potassium, calcium, phosphorus, iron, magnesium and can constitute a good and inexpensive source of these nutrients in human diet especially for people in developing countries. Selection in grain amaranth should be based mainly on characters like leaf area, seed yield, plant height and stem diameter. Accession A11 (PI 641047) had high values for plant height, stem diameter, leaf length, leaf width, leaf area, seed yield, crude fiber and zinc indicating scope for improvement through selection to enhance the potentiality of seed yield, plant vigor and zinc content. The best accession for number of leaves, 100-seed weight, sugar, aluminum, calcium, magnesium, potassium and phosphorus was accession A23 (PI 590991). The best accession for protein content was accession A28 (NH 84/444-4), followed by accession A9 (PI 590992). Accessions A11, A23 and A28 having high seed yield and considerable amount of protein, crude fibre and essential minerals could serve as the most promising varieties for future selection programme to isolate better plant types rich in quality characters.

The RAPD analysis clearly revealed genetic diversity among and within species. The study of genetic diversity among populations of different phytogeographic regions is important, as survival, perpetuation and continuance of a species to meet the demands of changing environments largely depend on the extent of variability available in its gene pool. Some geographic cohesiveness was observed as

represented by accessions A13, A14, A15, and also A16, A17, A18, A19 which were collected from close phytogeographic regions and found to be on neighboring branches in the dendogram. Accessions A28 and A29 (*Amaranthus hybridus*) obtained from NIHORT germplasm displayed maximum genetic similarity with a 0.89% similarity coefficient also confirming geographic cohesiveness. Molecular characterization based on RAPD markers show that this DNA marker is a useful tool not only to assess intraspecific variation within cultivated species but also to reveal interrelationships among various species in this large genus and to solve taxonomic problems both at and below the species level.

The dendogram based on RAPD markers was not in accord with the dendogram based on phenotypic traits. The dendogram generated by the RAPD marker agrees better with the groups of the genotypes than the dendogram generated by phenotypic traits. Molecular markers are thus important or useful tools in identifying mix-up or mismatch in germplasm conservation.

6.1.2. Assessment of the protein quality of grain amaranth using amino acid analysis and 1-DE

The major fractions present in amaranth seed storage proteins in this study are the easily digestible albumin, globulin and glutelin. Prolamins which represent the major protein fraction in other cereals like wheat and maize were absent or present in minute amounts in grain amaranth. The glutelin-like and glutelin fractions had characteristic bands at the 66kDa region (bovine serum albumin) which was also present in the soybean variety used in this study and absent in the globulin and albumin fractions. Amaranth seed proteins could be an alternative source of bioactive peptides like lunasin or lunasin-like isoforms with biological functions such as anticancer potential that are beneficial to health. The 18kDa band which is the lunasin-like peptide band present in soybean, wheat and amaranth in previous reports, is likely to be present in most of the grain amaranth accessions evaluated as a broad range of bands just below the 20kDa region was observed in this study. Amaranth can also be considered as a natural source of antihypertensive peptides. It has been reported that potential biological activity is higher for proteins from seed sources than those from animal sources.

Grain amaranth could be a nutritive substitute for cereals and improve value in different diets. It contains high amount of lysine which is limiting in most cereals. It has higher amount of cysteine and methionine than soybean. Based on its rich protein and amino acid compositions, the poor methionine

and lysine contents of cereals could be supplemented by adding amaranth to the diet. The grain amaranth proteins contain all of the EAA_9 and are higher than the reference protein pattern value required by a 2-5 year–old preschool child and comparable with the value of the FAO/WHO (1991) reference pattern of Hen's whole egg.

From our results, it is evident that the protein quality of amaranth accessions can be determined from their total protein content, amino acid composition, amino acid score and protein subunits. Results can assist plant breeders in their selection of high quality protein amaranth accessions.

6.1.3. Phytochemical profile of 29 grain amaranth accessions and evaluation of the hepatoprotective activity of their ethanol extracts on sodium arsenite induced-toxicity in rats

Phytochemical analysis revealed high levels of phytate, total polyphenol, total flavonoid and antioxidant scavenging activity of the 29 amaranth species evaluated. Varieties A23, A27 and A28 were highest in most of the phytochemical compositions and represent the best sources among the studied accessions. These antioxidant phytochemicals might contribute to the hepatoprotective activities of the seeds of *A. hypochondriacus* and *A. hybridus*.

Our results show that total phenolic content; Fe chelating and scavenging activities (DPPH and ABTS) did not vary much despite some significant variations in total flavonoid contents, reducing power and total antioxidant activity and so will limit selection for health beneficial phytochemicals in breeding programs.

Both *A. hypochondriacus* and *A. hybridus* ethanol seed extract treatment exhibits protective effects against oxidative damage and mitigate arsenic intoxication-induced oxidative damage of rats which could be due to its polyphenolic content, and antioxidant activity that combines free radical scavenging and metal chelating properties. From the results of this study it is clear that grain amaranth extracts has shown a dose dependent activity among which at the dose level of 300 mg/kg body weight shows greater activity which is comparable with the control group. It can be considered to be a potential chemopreventive agent. The present study shows that grain amaranth treatment mitigates arsenic intoxication-induced oxidative damage of rats.

6.2. CONTRIBUTION TO KNOWLEDGE

i) The high variability in phenotypic traits such as plant height, leaf area, stem diameter, seed yield, 100-seed weight, protein, calcium, potassium, and magnesium suggest that putative parents can be identified and used for hybridization work to enhance plant vigour and seed quality in amaranth accessions.

ii) RAPD markers revealed diverse clusters which can also be used as potential parents in the gene pool in hybridization programmes.

iii) Some geographic cohesiveness was observed in *Amaranthus hybridus* accessions which were collected from close phytogeographic regions and found to be on neighboring branches in the dendogram implying that the genetic base of these population is very narrow.

iv) RAPD markers revealed more interrelationships among species than phenotypic markers and is a more efficient tool in characterizing amaranth both at the intraspecific and interspecific levels.

v) The balanced amino acid composition of the grain amaranth protein makes amaranth a promising crop as a food source of high quality protein in developing countries.

vi) A close identity between amaranth and soybean exist based on its rich amino acid composition and protein fractions.

vii) The high content of methionine and lysine in amaranth seeds indicate that cereals limiting in these essential amino acids could be supplemented by adding amaranth seeds to their diet.

viii) Albumin, globulin and glutelin which are easily digestible proteins were the major fractions found in grain amaranth.

ix) Grain amaranth contains substantial amount of phytochemicals and antioxidant activities.

x) Ethanol extracts of *A. hypochondriacus* and *A. hybridus* exhibits protective activity against oxidative damage in a dose dependent manner.

6.3. RECOMMENDATION

- Development of a more specific sequenced characterized amplified region (SCAR) marker from polymorphic RAPD bands would be helpful for identification of useful genes coding for important agronomic traits in crop improvement.

- Analysis of additional protein properties like the amino acid sequence, identity and structure of *amaranthus* species protein in comparison with other high protein plant sources like soybean and high protein maize is needed to clarify relationships. The secondary structures found by computation will be useful to predict functional changes in protein systems in response to processing conditions which maybe encountered in foods.

- The 2-Dimensional Electrophoresis reference maps which reveals distinct differences in the proteome and subunit expression of cereals is recommended to make possible the high resolution of the storage proteins of grain amaranth required for identification and to serve as the basis for an accurate proteome map for rapid comparison with other grains or cereals.

- Further quantitative evaluation of the polyphenolic contents and antioxidant activities of grain amaranth is suggested, as information is scarce. These results would support the efforts of selection for commercial production of varieties with enhanced health and nutritional benefits.

- Long-term *in vivo* studies using other chemical carcinogens to evaluate the effects of these extracts as hepatoprotective and biomarkers of oxidative stress should be carried out. This will support our findings of the protective role of amaranth.

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APPENDIX

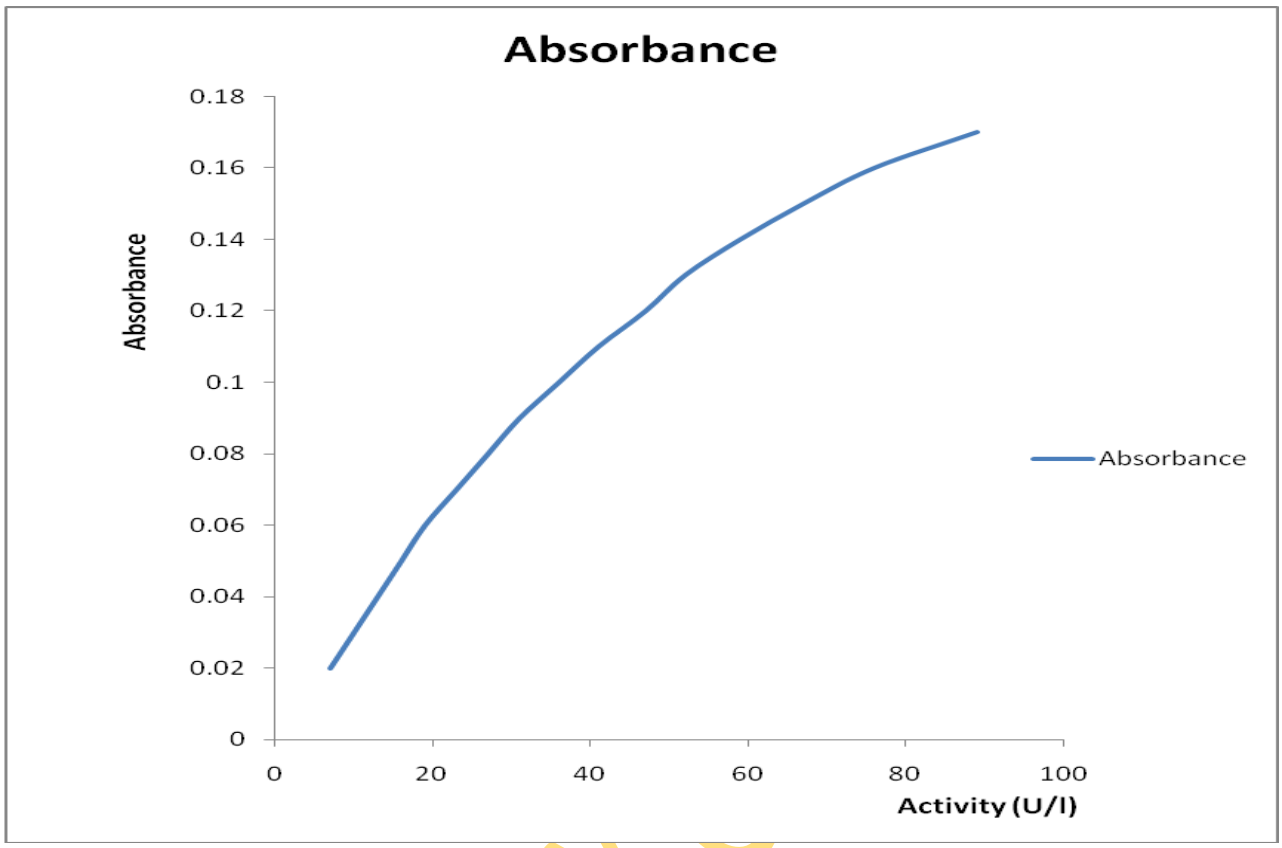
Appendix 1

Standard AST values

Absorbance	U/l	Absorbance	U/l
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

CALCULATION

The activity of AST in the Serum was obtained from using the standard curve.



Standard AST curve.

Normal values (Schmidt and Schmidt, 1963)

Serum up to 12 U/l

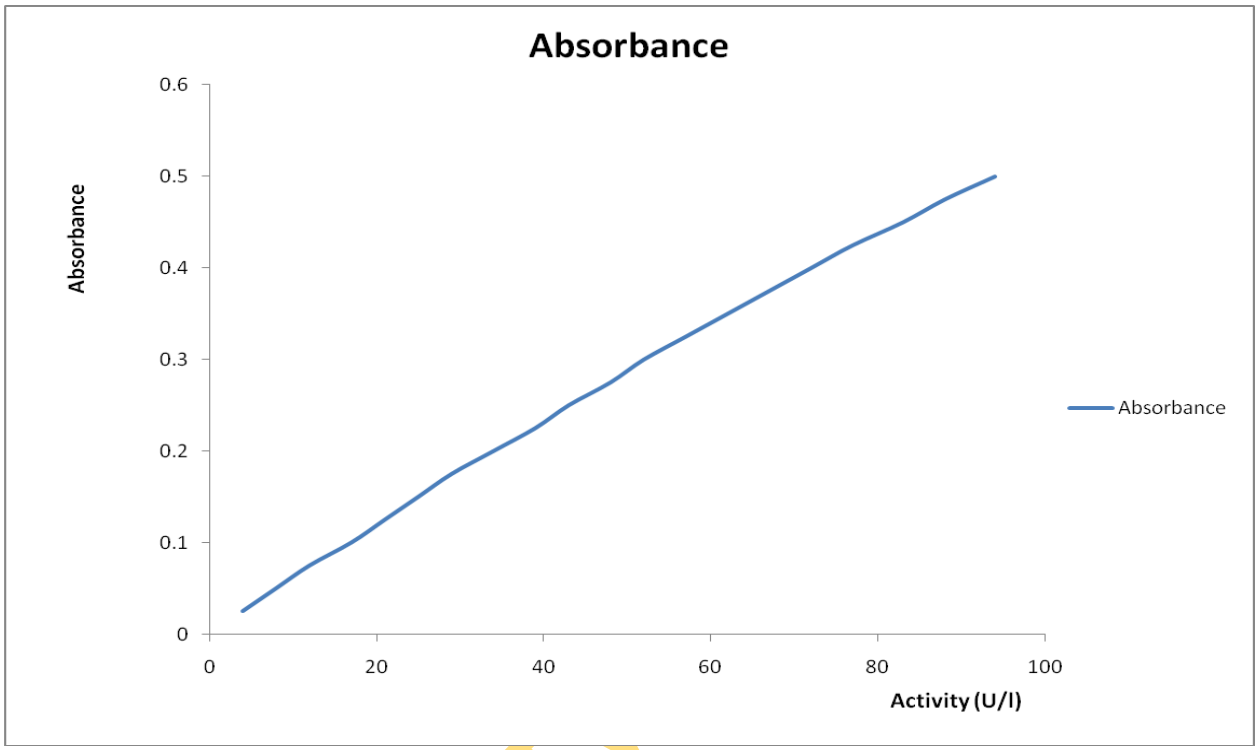
Appendix 2

Standard ALT values

Absorbance	U/l	Absorbance	U/l
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

Calculation

The activity of ALT in the Serum was obtained from using the standard curve.



Standard ALT curve.

Normal values (Schimidt and Schimidt, 1963)

Serum up to 12 U/l