Alchornea laxiflora AQUEOUS LEAF EXTRACT: ITS ALLELOPATHIC POTENTIAL AND BIOACTIVE AGENTS

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CERTIFICATION

I certify that this work was carried out by Mr. Osineye, Suaib Olasupo in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

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DEDICATION

This thesis is dedicated to Allah, the most Gracious, the most Merciful,

My beloved parents, Mr and Mrs Almaroof Osineye,

My siblings, Ibrahim, Muritada, Rabiu and Khalid Osineye,

My distinguished academic father, Prof. H.O.B. Oloyede

My friend and mentor, Prof. T.O. Sunmonu

and

My future wife and kids (In Shaa Allah)

DECLARATION

I, Suaib Olasupo OSINEYE (Matric Number - 14/68FA002) declare that this thesis entitled "*Alchornea laxiflora* Aqueous Leaf Extract: Its Allelopathic Potential and Bioactive Agents" is the result of a study carried out by me under the supervision of Prof. H.O.B. Oloyede in the Department of Biochemistry, University of Ilorin, Ilorin Nigeria. I confirm that this thesis presents the results of my findings and that its contents are entirely my ideas unless otherwise stated with appropriate referencing. This thesis has not been submitted to any University nor is it before any other University for consideration for the Award of a Doctor of Philosophy (Ph.D) degree in Biochemistry.

Osineye, S.O. (December, 2017).

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CAT	Catalase
DNS	Dinitro Salicylic Acid
IC_{50}	Mean Inhibitory Concentration
MDA	Malondialdehyde
ROS	Reactive Oxygen Species
POD	Peroxidase
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
BCG	Bromocresol green
HPLC	High Performance Liquid Chromatography
R_f	Retardation factor
ACC	Acetyl CoA Carboxylase
FNR	Ferredoxin NADP reductase
Glu	Glutamate

Asp	Aspartate
His	Histidine
Arg	Arginine
Lys	Lysine
Arg	Arginine
Ser	Serine
Tyr	Tyrosine
Leu	Leucine
Thr	Threonine
Met	Methionine

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ABSTRACT

The dangers posed by synthetic herbicides necessitated the need to search for eco-friendly alternatives in some plants by exploring their allelopathic property. Allelopathic plants have the ability to inhibit the growth and survival of surrounding plants. The aim of this experiment was therefore to evaluate the allelopathic potential of *Alchornea laxiflora* and to isolate the bioactive agents responsible. The objectives of the study were to: (i) screen *Alchornea laxiflora* aqueous leaf extract for the presence of secondary metabolites; (ii) examine the growth inhibitory, germination inhibitory and genotoxic effects of the extract; (iii) determine the effects of the extract on some photosynthetic and lipid synthesis parameters; (iv) determine the effects of the extract on oxidative stress parameters; (v) determine the effect of the extract on some osmotic stress indicators; (vi) carry out a bioactivity-guided isolation and identification of the bioactive agents; (vii) conduct an autodock analysis using enzymes.

The extract was first subjected to *Allium cepa* test to evaluate the growth-inhibitory and genotoxic effects (reduction of mitotic index, chromosomal aberration), then tomato seedlings were used to further evaluate its germination inhibitory and other phytotoxic effects (photosynthesis inhibition, lipid synthesis inhibition, oxidative stress induction, osmotic stress induction) with atrazine as standard. Thereafter, it was subjected to standard chromatographic techniques involving bioactivity-guided fractionations, thin layer chromatography and high performance liquid chromatography as well as isolate the bioactive agents responsible for the allelopathic property. Finally, the isolated compounds were subjected to molecular docking techniques using enzymes (Ferredoxin NADP-reductase and Acetyl CoA carboxylase and alpha amylase) to further confirm the bioactive agents. Data were subjected to Analysis of Variance Test at $p<0.05$.

The findings of the study were that the extract:

i. contained a number of secondary metabolites including alkaloids, phenol, phlobatannins, terpenes, tannin and flavonoids.

ii. caused significant reductions ($p<0.05$) in growth, germination, and abnormalities in chromosomal activities in tomato seedlings and *Allium cepa* root cells.

iii. caused significant reductions ($p<0.05$) in Chlorophyll contents and activities of Ferredoxin NADP-reductase and Acetyl CoA carboxylase enzymes of tomato seedlings.

iv. caused significant increases ($p<0.05$) in Malondialdehyde concentrations and decreases in activities of antioxidant enzymes (Superoxide dismutase, Catalase and Peroxidase).

v. caused significant increases ($p<0.05$) in protein, proline and total soluble sugar concentrations.

vi. contained Rutin and Quercetrin as its Allelochemicals.

vii. contained Rutin and Quercetrin which interacted favourably with the amino acid residues on Alpha amylase, Acetyl-coA carboxylase and Ferredoxin NADP-reductase.

The study concluded that Rutin and Quercetrin are partly responsible for the allelopathic activity exhibited by *Alchornea laxiflora* aqueous leaf extract. It is therefore recommended that *Alchornea laxiflora* may be used as a source of templates for the development of bio-herbicides.

.CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Weed control has been a major area of concern in agricultural development of many countries (Zimdhal, 2007). A weed is any plant or vegetation, excluding organisms like fungi, that meddles with the cultivating activities of people (Zimdahl, 2007). Weeds tend to compete with cultivated plants, causing various problems for their cultivation, including reduction in yield (Eiswerth and Johnson, 2002). In fact, the amount of money spent towards weed control is estimated in the range of hundreds of millions of dollars per year (Buhler *et al*., 2000). It has been calculated that when weeds are not properly controlled, about 40% of a typical crop is lost pre-harvest and an additional 20% is lost post-harvest (Peng *et al*., 2003). In Nigeria, weeds were shown to cause almost 80% loss in crop yield especially in cassava farms and has generally reduced interest in farming (Awotodunbo and Bello, 2011).

Weeds are controlled by mechanical or cultural means such as the use of weeders, harrows, hoes, tillage and crop rotation (DiTomaso, 2000). However, these methods have limitations with respect to their inefficiency in controlling perennial weeds in addition to their potential of causing the spread of some unneeded species (Evidente *et al*., 2009). Thus, farmers have turned to the use of chemicals for controlling weeds although the mechanical methods have not been totally abandoned (Peng *et al*., 2003). The use of chemicals to control weeds has a main advantage over the mechanical or cultural methods due to its simplicity and rapidity. However, the use of these synthetic herbicides poses some levels of danger to the ecosystem. In addition to the resistance exhibited by some plants, the damage they cause to the environment in addition to their general toxicity strongly put a limit to their use (Neve, 2007). These dangers are attributable

to their non-biodegradability (Ralebitso *et al.,* 2002). Exposure to these chemicals has been linked to risks of diseases like cancer, reproductive abnormalities, Parkinson's disease, etc in man (Bolle *et al*., 2004). Also, residues of synthetic herbicides are found in soil and air, and in water bodies and as such endanger the life of aquatic organisms (Krutz *et al*., 2008). These necessitated the need to seek natural alternatives for controlling weeds, with great focus on plants' secondary metabolites.

Since ancient times, farmers have observed that some plants inhibited the growth of other plants through the release of toxic compounds into the environment (Khalaj *et al*., 2013). This phenomenon is known as allelopathy, from the Greek words *allelon* (mutual) and *pathos* (suffering), that is, the reciprocal sufferings of two organisms. The term was proposed in 1937 by a Czech researcher known as Hans Molisch. At first, the term was not made to seem like a negative connotation, but simply implied the effect of a plant on another. Later, the meaning was limited to those interactions that brought about the inhibition of the growth of nearby plants (Willis, 2007). An early record of allelopathy was made by a Latin scholar known as Marcus Terentius Varro in a Rerum rusticarum about two plants, oak and walnut: "like oak, large nearby walnut trees make the margins of the farm sterile". Plants with these properties are thus referred to as allelopathic plants.

Allelopathic plants have the ability to inhibit or stimulate the growth of surrounding plants via the release of substances known as allelochemicals through their roots and leaves (Khalaj *et al*., 2013). Allelochemicals could be isolated and used to control weeds. Moreso, asides their use as direct agents for controlling weeds, allelochemicals may be used in combination with synthetic herbicides to reduce their doses while keeping up a similar effectiveness (Iqbal *et al*., 2009).

1.2 Statement of Research Problem

The emergence of herbicide-resistant weeds is a serious challenge faced by modern farmers (Tranel and Wright, 2002). Perhaps the simplicity, effectiveness and the proven cost-effective benefits that will accrue in the short term, has led to the predominance of synthetic herbicides. Numerous plant species have evolved resistance to the different groups of synthetic herbicides, among which resistance to glyphosate is of the greatest concern (Yuan *et al*., 2007).

In general, herbicide toxicity is exhibited by inhibiting some enzyme functions essential to crop survival (Zulet *et al*., 2013). However, many herbicides have nonspecific properties, affecting other organisms (crops, non-vertebrates, and vertebrates) to varying degrees. The damage to these non-target organisms may be reflected by different effects, such as diseases (e.g Cancer, Birth defects) direct mortality, long-term population reductions, and bioaccumulation within the food web (Bolle *et al*., 2004).

The indiscriminate dispersion of synthetic herbicides is regarded as potentially hazardous to man and the environment due to their non-biodegradability. For instance, studies have reported herbicides toxicity to useful arthropods such as the predatory mite, aquatic invertebrates and fishes (Ahn *et al*., 2001).

1.3 Justification for the Study

The use of synthetic herbicides for weed control poses some levels of danger to human health ranging from risks of cancer, reproductive abnormalities, Parkinson's disease, etc. It also poses danger on the ecosystem in general. For instance, residues of synthetic herbicides are found in soil and air, and in surface and ground water and as such endanger the lives of aquatic

organisms. Reports have shown that 99% of the deaths associated with synthetic herbicides occur in developing countries like Nigeria, where only 25% of the world's production of these chemicals is used (Ojo, 2016). However, these dangers are attributable to the non-biodegradability exhibited by these synthetic herbicides.

As a result, effective and eco-friendly alternatives are being sought for in some plants by taking advantage of their allelopathic properties. Extracts from allelopathic plants may inhibit the activities of some important enzymes such as Ferredoxin NADP reductase and AcetylCoA carboxylase needed for growth (Nikpour-Rashidabad *et al*., 2016). In addition, they may also inhibit seed germination, plant root/shoot growth, nutrient uptake, chlorophyll formation (Nikpour-Rashidabad *et al*., 2016), or even cause production of Reactive Oxygen Species (ROS) which are toxic to plant cell membranes (Lee *et al*., 2007), thus resulting into the depletion of antioxidant enzymes (Gechev and Hille, 2005) and accumulation of osmoprotectants (Farhad *et al*., 2011) in such plants.

However, with respect to dominance, allelopathic have a greater advantage over other plant species (Batish *et al*., 2006) and this seems to be demonstrated by *Alchornea laxiflora* (Benth) pax & Hoffman. The shrub belongs to the family Euphobiaceae and is broadly disseminated crosswise over tropical Africa. Its common names include: Christmas Bush (English), *Ijàn* (Yoruba), *Ububo* (Igbo). The leaves of the plant are used locally for the preservation of kolanuts in Nigeria. Reports have it that, the leaves are used in folk medicine for the treatment of malaria, pile, dysentery, cough, and high fever (Jayeoba *et al*., 2012). The plant is also known to possess antioxidant (Farombi *et al*., 2003; Adeloye *et al*., 2005), antifungal (Ogundipe *et al*., 2001), and antibacterial (Ogundipe *et al*., 2001) properties. Thus, the plant exhibits economic potentials for herbal use.

Thus in this study, the aqueous extract of *Alchornea laxiflora* leaves was evaluated for its allelopathic potential on tomato seedlings and the bioactive agent(s) that are responsible were indentified.

1.4 Aim and Specific Objectives of the Study

The aim of this study was to evaluate the allelopathic potential of the leaf aqueous extract of *Alchornea laxiflora* and identify the bioactive agent(s) responsible for this property. To achieve this aim, this study was focused on:

i. screening *Alchornea laxiflora* aqueous leaf extract for the presence of secondary metabolites;
ii. evaluating the allelopathic potential of the extract by:
    a. examining the growth inhibitory, genotoxic and germination inhibitory effects of the extract;
    b. determining the effects of the extract on some photosynthetic and lipid synthesis parameters;
    c. determining the effect of the extract on oxidative stress parameters;
    d. determining the effect of the extract on osmotic stress parameters;
iii. carrying out a bioactivity-guided isolation and identification of the bioactive agents; and
iv. carrying out an autodock analysis using enzymes.

1.5 Experimental Design and Scope of the Study

Based on the scope and specific objectives of the study, the experimental methodology for this study was designed as depicted in Figure 1.1.

1.6 Significance of the Study

The study may provide a solution to the problem faced with the use of synthetic herbicides for weed control such as life endangerment of aquatic organisms, weed resistance to herbicides, disease conditions in man through the discovery of safe and equally efficient alternatives. Study on the allelopathic potential of plants might result to discovery of safe compounds which could be used as agents for the discovery of bio-herbicides.

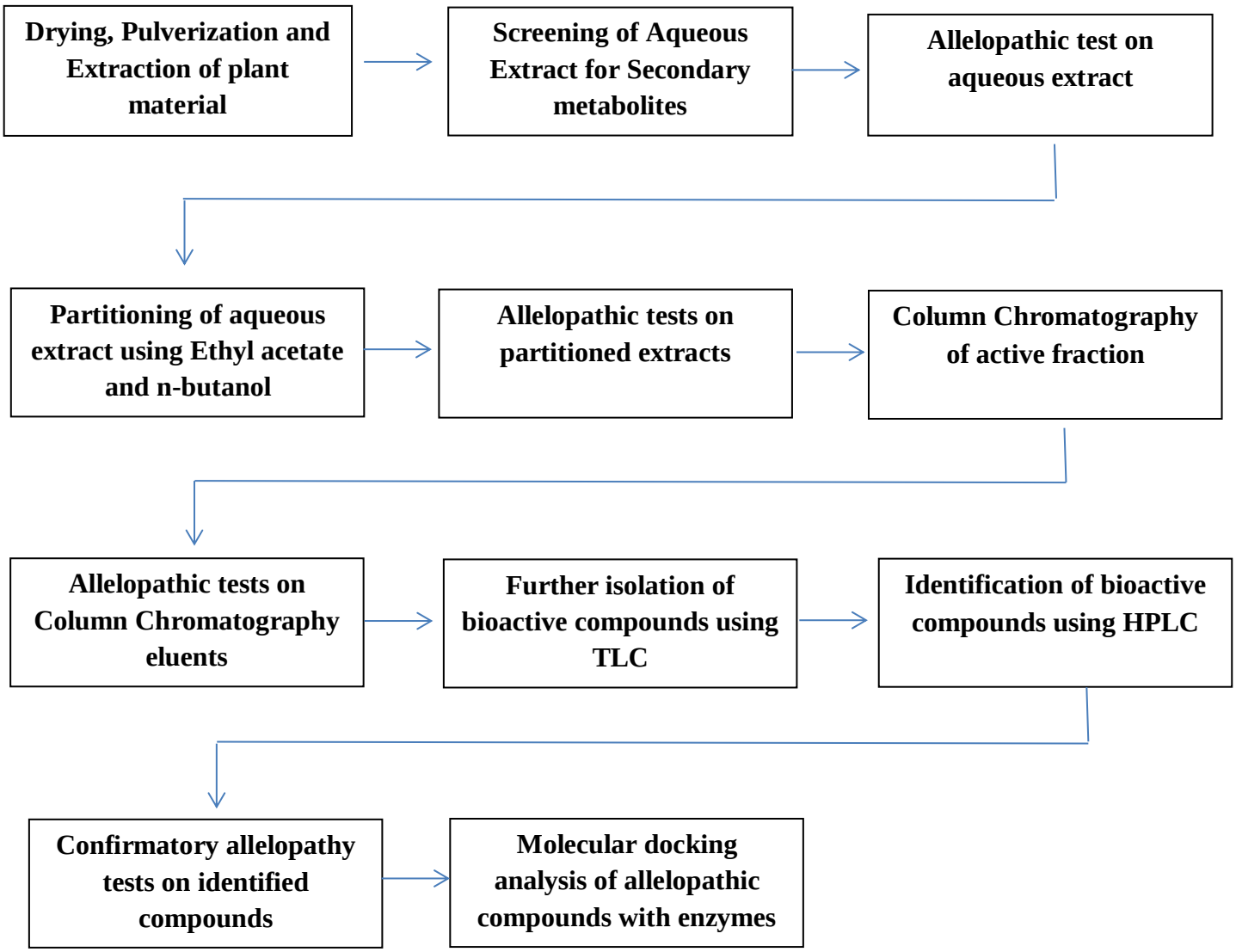


Figure 1.1: Flow Chart of the Experimental Design Based on the Research Objectives

CHAPTER TWO

LITERATURE REVIEW

2.1 Herbicides

Before synthetic herbicides were introduced, weeds were controlled using mechanical or cultural means (DiTomaso, 2000). However, these are only effective on a limited number of weeds, especially those whose life span is just within a year. Perennial weeds are to a large extent, tolerant to both traditional and modern weed control methods (Peng *et al*., 2003). At times, mechanical techniques may prompt the spread of some pest species. In recent years, the use of herbicides has been the major means of weed control (Forouzesh *et al*., 2015). Herbicides are chemicals used to kill or otherwise manage certain species of plants considered to be pests (Neve, 2007). Plant pests, or weeds, contend with desired crop plants for light, water, nutrients, and space, in this way causing reduction in the productivity and yield of product plants (Van Almsick, 2009).

To reduce the damage caused by the influence of weeds on the outcome of agricultural produce, fields are sprayed with herbicides that are toxic to weeds, but not to the crop species (Yuan *et al*., 2007). The commonly used herbicide 2,4-D (2,4-Dichlorophenoxyacetic acid), for example, is toxic to many dicotyledonous weeds, but not to monocotyledonous ones including wheat, maize, barley, or rice (Yuan *et al*., 2007). Consequently, only the weeds are eliminated, while the growth of the desired plant species is maintained.

Intensively managed agricultural systems have an intrinsic dependence on the use of herbicides and other pesticides (Beckie *et al.*, 2006). Some high-yield varieties of crop species are not exceptionally tolerant of competition from weeds. Therefore, if those crops are to be effectively

developed, herbicides must be used (Beckie *et al.*, 2006). Yields of wheat and barley can be reduced by 25-50% as a result of competition from weeds (Peng *et al*., 2003). To reduce these critical, negative influences of weeds on agricultural productivity, herbicides are commonly applied to agricultural fields (Stokstad, 2013).

In 1989, herbicides accounted for about 61% of the 500 million kg of pesticides used domestically worldwide (Peng *et al*., 2003). During recent years, eighty percent of most normally utilized pesticides are herbicides. Recorded in a decreasing order of amounts utilized are: Alachlor (45 million kg), Atrazine (45 million kg), 2,4-D (24 million kg), Butylate (20 million kg), Metolachlor (20 million kg), Trifluralin (14 million kg), Cynazine (9 million kg), and Metribuzin (6 million kg) (Peng *et al*., 2003). These information reflect a large decrease in the usage of phenoxy herbicides, which were utilized considerably more normally before the 1980s. For example, amid the mid-1970s, around 50-80% of the small-grain acreage in North America was treated with phenoxy herbicides, mostly with 2,4-D. Since the mid-1990s, the use of glyphosate has increased tremendously (Forouzesh *et al*., 2015).

2.1.1 Types of Herbicides

2.1.1.1 Pre-emergence Herbicides

Pre-emergence herbicides are applied to soil and prevent weeds from emerging or disrupt plant tissues sufficiently to kill seedlings after they emerge (Krutz *et al.,* 2008). They are usually applied before germination of the weed seed and moved with rainfall or irrigation into the soil where seeds germinate. Effects of preemergence herbicides are limited by rainfall, irrigation, organic matter content and clay content (Xu *et al*., 2001). Pre-emergence herbicides may be

selective in their actions or nonselective depending on the nature of the plant controlled. Examples of pre-emergence herbicides include Barricade, Bensulide, Dacthal, Gallery, Ronstar, Surflan and Treflan.

2.1.1.2 Post-emergence Herbicides

Post-emergence herbicides are applied to foliage after weeds have emerged from soil (Krutz *et al.*, 2008). Their uptake is from foliage or in some cases, roots. They may either be selective in their actions or non-selective based on rate used, characteristics of active ingredient, plant species or cultivar. Effectiveness of this type of herbicides can be influenced by spray adjuvants such as surfactants, buffers or fertilizers. Examples include Image, Kerb, Fusilade, Ornamec, Manor, Trimec and Turflon.

2.1.1.3 Selective Herbicides

Selective herbicides are toxic to some plants while to some others, they are harmless. For example, 2,4-D is particularly used to remove broadleaf plants from certain established grasses (Xu *et al*., 2001). Some other ones are used to remove different grasses in certain broadleaf plants e.g Fusilade or Vantage. Factors that may affect the relative selectivity of herbicides include rate of herbicide, growth stage of and environmental stress on the desirable crop. Other examples include Gallery, Lontrel, Monument and pendulum.

2.1.1.4 Non-selective Herbicides

Non-selective herbicides kill all types of plants. They may either be pre-emergent or post-emergent in their actions (Xu *et al*., 2001). Some non-selective herbicides such as Borates, Bromacil or Prometon persist for long periods of time in soil and can cause harm to either existing or new plants from root absorption. Others such as Roundup and Reward are deactivated by binding tightly to soil particles or organic matter. Once attached to soil particles however, these types of non-selective herbicides pose no threat to existing plantings. Rates of herbicide use may determine the relative selectivity of plants to herbicide in a given situation. Factors affecting the effectiveness of non-selective herbicides include: rate of herbicide use, coverage, edaphic characteristics, growth stage of plant and environmental conditions prior to, during and after application. Examples include Arsenal, Diuron, Hyvar, Oust and Prometon.

2.1.1.5 Contact Herbicides

Contact herbicides affect only the plant parts which the herbicide solution contacts. Translocation of these herbicides along the vessels within the plant is very limited (Krutz *et al.,* 2008). They 'burn off' the above ground parts of the weed and are most potent against annual weeds but not very effective against established perennial weeds. Their performance however, is usually improved by spray adjuvants. Examples include Finale, Gramoxone, Reward and Scythe.

2.1.1.6 Systemic Herbicides

Systemic herbicides enter plant tissue either by root or shoot absorption and move within the plant (Krutz *et al.,* 2008). 2,4-D (2,4-Dichlorophenoxyacetic acid) and Roundup are examples of foliar applied herbicides which move primarily via the phloem from foliage to roots where they cause elimination of both roots and shoots. Simazine is taken up almost exclusively via the root

and translocated to shoots via the xylem where it disrupts photosynthesis. Systemic herbicides are usually effective against a wide array of weeds. Moreover, if applied via plant foliage, spray adjuvents may be needed for effective adsorption. Other examples include Metachlorophenylpiperazine (MCPP), Manage and Turflon.

2.1.2 Chemical Classification of Herbicides

2.1.2.1 Chlorophenoxy Acid Herbicides

Chlorophenoxy acid herbicides cause toxicity to plants by mimicking their natural hormone-like auxins, leading to lethal growth abnormalities (Van Almsick, 2009). These herbicides are selective for broad-leaf or angiosperm plants, and are tolerated by monocotyledonous plants and conifers at the spray rates normally used. These chemicals exhibit a moderate persistence of about one year in the environment, and as well, possess a half-life measurable in weeks, in soil.

2.1.2.2 Organic Phosphorus Herbicides

Organic phosphorus herbicides include the commonly used chemical, glyphosate (N-phosphonomethyl-glycine) (Van Almsick, 2009). Glyphosate has various applications in the farming sector, while additionally being of significance in forestry. It is usually transported within tissues such as rhizomes and roots, where it interferes with certain amino acid syntheses. Because glyphosate has the tendency to damage a number of different plants, its proper use is dependent on the understanding of seasonal changes with respect to the vulnerability of weeds to the herbicide. However, glyphosate is only slightly persistent in soils and is not very toxic to animals (Van Almsick, 2009).

2.1.2.3 Triazine Herbicides

Triazine herbicides are mostly used in corn fields, and sometimes as soil sterilants (Ralebitso *et al.,* 2002). These chemicals are not very persistent in surface soils, but they are mobile and can cause contamination of groundwater (Ralebitso *et al.,* 2002). Examples of this class of chemicals include atrazine (2-Chloro-4-(ethylamino)-6-(isopropylamino)s-triazine), cynazine (2-(4-Chloro-6-ethylamino-5-triazin-2-ylamino)-2-methylpropionitrile), hexazinone (3-Cyclohexyl-6-(dimethyl-amino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione), metribuzin (4-Amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one) and simazine (2-chloro-4,6-bis-(ethyl-amino)-s-triazine) (Wackett *et al*., 2002).

2.1.2.3.1 Atrazine

Atrazine belongs to the triazine class of herbicides. It is used to prevent the growth of dicotyledenous weeds in fields of monotyledonous crops such as maize and sugarcane (Ralebitso *et al.,* 2002). Its action coild either be preemergent or post emergent, and it is a widely used herbicide in the US and Australian agriculture. Atrazine came into existence in 1958 in the Geigy laboratories as one of a series in synthesized 1,3,5-triazines (Ralebitso *et al.,* 2002).

The European Union prohibited the chemical in October 2003 when groundwater levels surpassing the breaking points set by regulatory agents, were found (Tillitt et al., 2010). Starting at 2001 in any case, atrazine recorded the most widely recognized pesticide found in savoring water America. Atrazine persists in the soil for only a few months and can migrate into groundwater, where it degrades (Ralebitso *et al.,* 2002). Atrazine degradation in soil is primarily by microbial action (Cai *et al*., 2003). Moreso, the rate of biodegradation is primarily affected by atrazine's low solubility. Thus surfactants may increase the degradation rate (Krutz *et al*., 2008).

Just like other members of the triazine group, atrazine (Figure 2.1) binds to important proteins in photosystem II of plants (Wackett *et al*., 2002). Plant death results as a result of deprivation and free radical generation caused by disruption of the electron transport process. Moreso, oxidative damage is accelerated at high light intensity.

Atrazine's effects in mammals fundamentally involve the endocrine system, where it causes hormonal unevenness (Jooste *et al.*, 2005; Hayes *et al*., 2010).

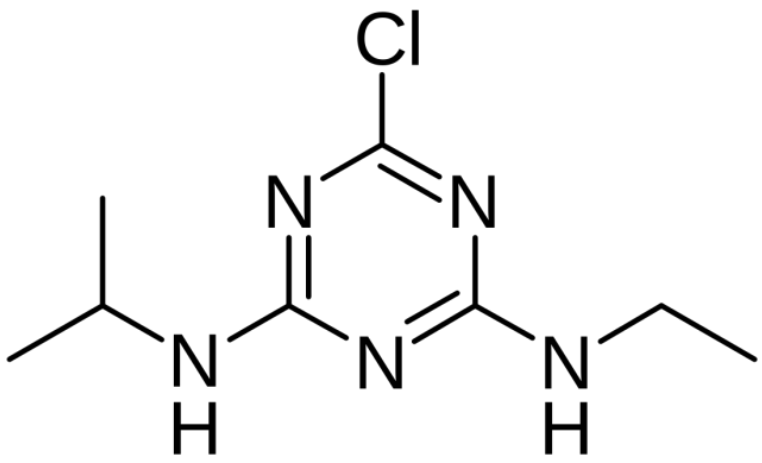


Figure 2.1: Structure of Atrazine

Source: Hayes *et al* (2010).

2.1.3 Modes of Action of Herbicides

Herbicides control weeds through different modes which all at last, prevent seeds from sprouting or establishing as seedlings; keep plants from making essential sugars, proteins or lipids; or parch leaves and stems (Wackett *et al*., 2002). Knowing a herbicide mode of action is imperative to seeing how to utilize a herbicide generally adequately. It is a major consideration in both herbicide selectivity and weed resistance. Thus the side effects observed on weeds sprayed with herbicides express the mode of action.

Modes of action of herbicide are classified based on the processes affected which include photosynthesis, cell division or specific enzyme targets. For example, those affecting photosynthesis are usually applied post-emergence and are often quick acting; those influencing cell division are often pre-emergence herbicides affecting germinating seeds (Wackett *et al*., 2002).

2.1.3.1 Modes of Action Involving Photosynthesis

Four types of herbicides primarily affect photosynthesis. Paraquat and diquat are the only members of the first type (Wackett *et al*., 2002). When the energy in sunlight is absorbed by chlorophyll, it is transferred in a flow of electrons through 'Photosystem I'. Paraquat diverts this flow, causing the production of highly reactive free radicals which destroy cell membranes, spilling the contents, and the leaves finally appearing yellow and dry (Wackett *et al*., 2002). Almost all green plants are affected by paraquat making it a broad-spectrum, non-selective herbicide (Wackett *et al*., 2002).

The second type blocks energy transfer through 'Photosystem II'. Herbicides of this type bind to proteins involved in the transfer chain, leading to reduction in their effectiveness and causing some diversion of electrons with similar, but slower results to paraquat. Triazines like atrazine and ureas like chlortoluron are both PSII inhibitors (Wackett *et al*., 2002).

A third type, are those that hinder the protoporphorinogen oxidase (PPO). These interfere with enzymes that catalyse the production of chlorophyll and other large molecules necessary during photosynthesis. Without new chlorophyll, leaves turn yellow and the photosynthetic rate is reduced (Wackett *et al*., 2002). However, unused by-products of chlorophyll accumulate and become oxidized to form free radicals which are considered harmful to dicotyledonous plants,

The fourth type of photosynthesis inhibitor inhibits the production of carotenoids in leaves (Wackett *et al*., 2002). These are commonly seen as the colours of autumn when chlorophyll pigment has been degraded. Carotenoids have roles in protecting chlorophyll from being destroyed by excessive light energy. Herbicides in this category have several different targets, but all end up bleaching the treated weeds.

2.1.3.2 Modes of Action Targeting Other Enzymes

In this group, the primary effects are to starve weeds of the essential products normally made by the enzymes affected (Wackett *et al*., 2002). The symptoms seen in affected plants are usually slow and depend on how conducive the environment is for plant growth. The AcetylCoA Carboxylase inhibitors stop plants from making fatty acids which are important in cell membranes. The growth of such plant stops within hours. An example of these herbicides is

fluazifop-p-butyl which controls grass weeds in broadleaved crops (Wackett *et al*., 2002). Acetolactate synthase inhibitors stop plants from making amino acids leucine, isoleucine, valine.

These herbicides (e.g sulfonylureas and imidazolinones) control a broad spectrum of weeds in many different crops (Wackett *et al*., 2002).

2.1.3.3 Modes of Action Targeting Cell Division and Growth

These herbicides are usually applied pre-emergent and control grass weeds (Wackett *et al*., 2002). They prevent cell division in the weeds, causing stunted growths of emerged seedlings. Examples include metolachlor (chloracetamides) and pendimethalin (dinitroanilines). Some may prevent the waxy cuticle of weeds from forming, act on new shoots and cause stunted growth. They may also be used for post-emergence removal of dicotyledonous weeds in cereals. They affect the plant hormones inhibiting its growth and development. Twisting of stems and curling of leaves occurs within hours. Examples include triallate and 2,4-D.

2.2 Allelopathy

Allelopathy, derived from two Greek words; *allelo* (mutual) and *pathy* (suffering), refers to a phenomenon where one plant releases toxic chemicals, that exert pronounced effects on another plant. Allelopathy was first defined by Molisch in 1937, as exchange of chemicals between plants or between microbes and plants that bring about both stimulatory and inhibitory influences. Later it was defined as any direct or indirect, harmful or beneficial effect which certain plants have on others through the production of chemical compounds that escape into the environment (Rice, 1984).

Allelopathy does not necessarily imply that something is wrong. A certain amount of this process goes on in natural systems. In fact, allelopathy is thought to have a hand in how forests restore themselves (Batish *et al.,* 2001). Although plants sometimes, simply compete for the limited available resources such as water, sun light and nutrients, without resorting to chemical warfare, ongoing research is studying whether the two conditions may have more to do with each other than formerly thought. Also, reports have shown that the more a plant is stressed, either before hand or because of the allelotoxin, the greater its reaction to the allelotoxins (Schulz *et al*., 2012).

Allelopathy is thus a survival mechanism that allows certain plants to compete with and often destroy nearby plants, by inhibiting seed sprouting, root development or nutrient uptake (Hassan and Khan, 2007). Allelopathy can also be exhibited by other organisms including bacteria and fungi. During allelopathy, substances known as allelochemicals are released and these cause inhibition to the growth and survival of surrounding plants (Inderjit and Olofsdotter, 2002).Allelochemicals can be given off by different parts of the plant including the leaves and roots, washed off by rainfall or can be released through natural decomposition (Figure 2.2).

Allelopathy is essential to processes of weed control, as well as crop protection and re-establishment of plants. The phenomenon has improvement in the returns of farm produce as well as ensuring safe and eco-friendly methods of controlling weeds. This has however, on large occasions, gained the attention of scientists who are into allelopathic research.

A plant could be refered to as being autotoxic when it produces toxic compounds that inhibit the growth of new plants of the same species. This is however a particular intraspecific type of allelopathy. Allelopathy and autotoxicity can play significant roles under both natural and manipulated ecosystems (Hassan and Khan, 2007), mainly by negatively affecting seed

germination and seedling development. Allelopathic interactions have been known to occur among plants for centuries, although only a limited number of allelochemicals have been identified (Weston, 1996). However, scientific attention is now also focused on exploiting the positive significant roles of allelopathy and what role this phenomenon can play in enhancing crop productivity (Inderjit and Olofsdotter, 2002).

2.2.1 Allelopathic Plants

Black walnut trees possess a toxic chemical known as hydrojuglone in all their parts. This is oxidized to a more toxic juglone and released into surrounding soil, where it inhibits the growth of many other plants, especially the ones in the *Solanaceae* family, like tomatoes, peppers, potatoes and eggplants (Hejl and Koster, 2004). Trees and bushes including azaleas, pine and apple trees, are additionally defenseless to juglone.

Sunflowers also contain chemicals which are toxic to many weeds so much that a number of studies have emerged on its use as weed control agent (Batlang and Shushu, 2007). Also, many turf grasses and cover crops possess allelochemicals in their parts which improve their weed suppression. Some other commonly noted allelopathic plants include Sugar Maple, Goldenrod, Horseweed (Travlos *et al*., 2007); Ginger (Han *et al*., 2008) and Perennial Rye (Schulz *et al*., 2012).

Allelopathic plants can peacefully co-exist with other plants if they are kept at a distance as toxic chemical are held depending on the soil quality (Fomsgaard *et al*., 2004). Heavier soils hold the chemicals much longer. Well-draining soil will enable easy movement of toxins below the root zone of nearby plants. Also the presence of

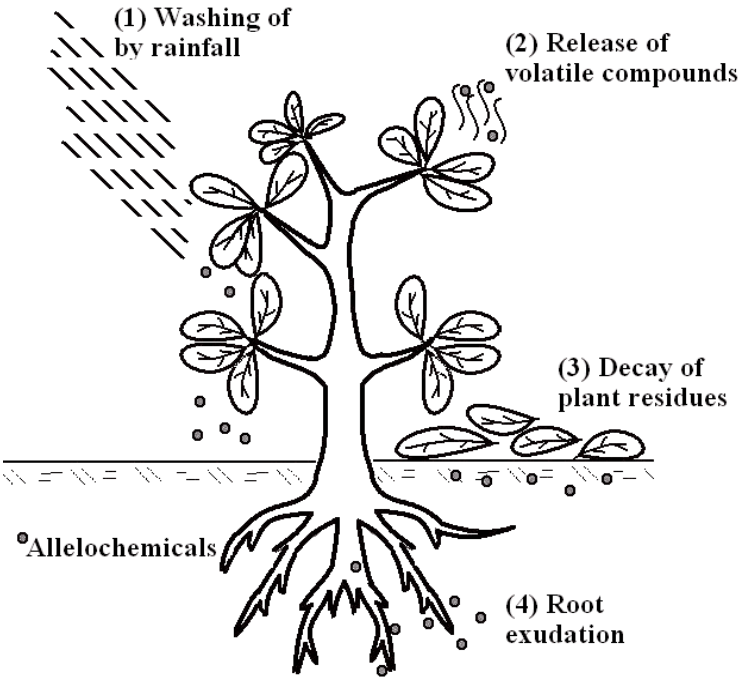


Figure 2.2: Possible Ways of Release of Allelochemicals into the Environment

Source: De Albuquerque *et al* (2010).

beneficial micro-organisms in soils can help breakdown, dissipate or convert the toxins into something more benign (Fomsgaard *et al*., 2004).

2.2.2 Allelopathy and Weed Control

Weeds pose a great problem to modern agriculture as it is responsible for a significant reduction in profit derivable from agricultural produce. Worldwide, weeds alone cause a 10% loss of agriculture production (Van Almsick, 2009). Contrasting options to synthetic herbicides should be created because of the perils related with the utilization of these chemicals. However, allelopathic principles of crops can be used as an alternative since they are of natural origin (Batlang and Shushu, 2007).

Plants serve as the chief source of the earth's organic compounds. The greatest part of their components (secondary metabolites) are not directly needed for the growth and development of the plant, but are important in many other functions, most of which are yet to be completely clarified (Flamini, 2012). However, there are evidences that some of these chemicals are important as defense compounds against microorganisms, animals, and other plants (Irshad and Cheema, 2004).

Plants release toxic chemicals from their various parts into the environment, suppressing the growth and survival of other plants in the surrounding. This process is known as 'allelopathy'. In agroecosystems, a number of weed species have been shown to exert allelopathic influence on surrounding crops, thus, adversely affecting their survivial (Hejl and Koster, 2004).

Plant possess weed-suppressing activities, examples including Cucumber (*Cucumber sativs* L.), Oat (*Avena spp*.) and Rice (*Oryza sativa* L.) (Hejl and Koster, 2004). A total of 538 accessions

of cultivated and wild cucumber were screened in pot and field tests with several accessions inhibiting the growth of *Brassica hirt*a and *Panicum miliaceum.* Also, out of more than 3000 accessions of oat, several of them were reported to exude large amounts of an allelochemical, scopoletin (Hejl and Koster, 2004).

Reports have shown that improving the competitive power of crops reduces the level of dependency on herbicides (Wu *et al*., 1999). However, reports also have it that, attempts to increase competitive ability while maintaining productivity have had limited success and no crop cultivar has been released with superior competing ability as a marketing argument. The incorporation of an allelopathic character into a cultivar however, could provide such plant with an improved competitive power over certain important weeds. Thus, genetically incorporating allelopathic potential in crops could serve as an important tool for weed management (Wu *et al*., 1999).

2.3 Allelochemicals

Allelochemicals are the bioactive agents present in allelopathic plants. Asides being used as direct weed control agents, allelochemicals can also be used alongside with synthetic herbicides to reduce their concentrations while at the same time, being equally effective (Iqbal *et al.*, 2009). Allelochemicals are plants' secondary metabolites and can be grouped into many different chemical classes including the phenols, coumarins, lignans, flavonoids, tannins and benzoxazolinones (Flamini, 2012). Often they are water-soluble substances, so they can be easily washed away by rain from plant surfaces and carried into the soil, where they exert their action. Sometimes they are produced by roots as exudates or released into the environment as volatiles.

2.3.1 Phenols

Phenols (Figure 2.3) possess one or more phenol rings in their structures. Studies have shown abundant evidence that phenolic acids interfere with several enzymes and physiological processes such as phytohormone activity, mineral uptake, plant water balance and stomatal function, photosynthesis, respiration, organic synthesis of certain compounds and flow of carbon (Einhellig, 2001).

Phenolic acids including benzoic and cinnamic acids tend to cause a compromise in cell membrane integrity by significantly reducing the content of sulfhydryl groups. They also suppress absorption of phosphate, potassium, nitrate, and magnesium ions, and overall changes in tissue content of mineral ions (Barkosky *et al*., 2000). Phenolic acids including chlorogenic, caffeic, and other polyphenols also act through inhibition or activation of the indoleacetic acid (IAA) oxidase system thereby altering IAA-induced growth of seedlings (Prasad and Devi, 2001). *p*-coumaric and ferulic acids reduce biosynthesis of chlorophyll

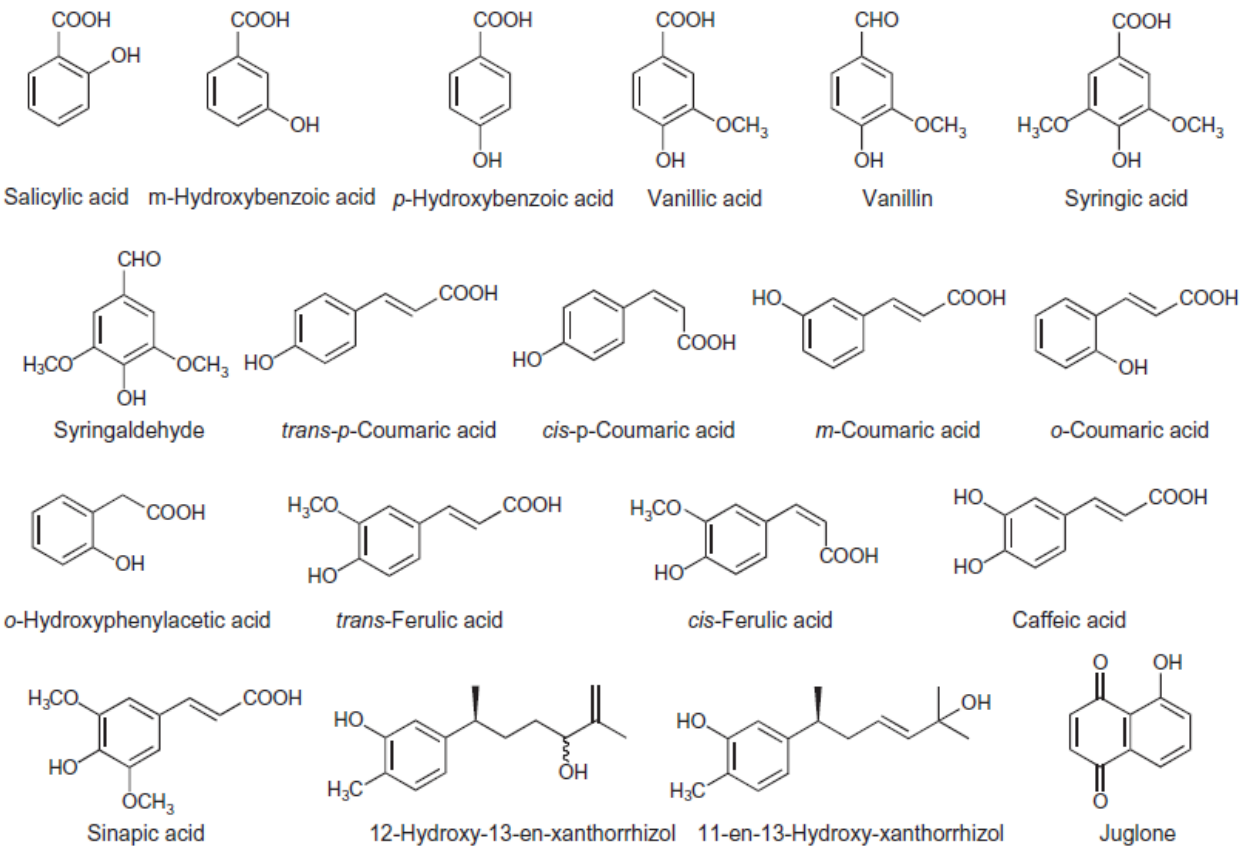


Figure 2.3: Structures of Some Phenol Allelochemicals

Source: Flamini (2012).

porphyrin precursors and a subsequent reduction in the level of chlorophyll accumulation (Yang *et al*., 2002).

Some rice (*Oryza sativa*) cultivars high allelopathic potentials due to their high contents of phenolic compounds including syringic, p-hydroxybenzoic, ferulic, benzoic, o-hydroxyphenylacetic, salicylic, m-, o-, p-coumaric acids (Chung *et al.*, 2002) with pronounced adverse effects on growth and survivial of barnyardgrass.

Besides higher plants, lichens also contain phenols which confer allelopathic activity on them (Suleyman *et al*., 2007). For example, the high cotent of lecanoric acid in *Parmotrema tinctorum* accounts for ability to significantly inhibit the growth of lettuce (Khanh *et al*., 2008).

Phenolic acids are thus strongly implicated in allelopathy and are therefore of interest as templates for the development of new natural herbicides. However, despite having allelopathic potentials, the effects of these phenolic acids is dependent on the amount available in plant roots.

Quinones are another group of simple phenols with inherent allelopathic characters. Among them, juglone is a standout amongst the most popular subordinates to be found in nature, and it accounts for walnut allelopathy (Rice, 1984). Reports have it that represses the development of some basic weeds in wheat and grain fields including wild mustard (*Sinapis arvensis*), crawling thorn (*Cirsium arvense*) and henbit (*Lamium amplexicaule*) (Suleyman *et al*., 2007). Phenols (Figure 2.3) exist in the soil in three different forms: the free ones, reversibly bound ones and bound. Bioavailability of phenols however relies upon the dirt properties. For instance, soil microbes can degrade phenolic allelochemicals, further reducing their bioavailability (Cecchi *et al*., 2004).

2.3.2 Coumarins

Coumarins are subsidiaries of 2H-chromen-2-one (Flamini, 2012). Their name originates from the French word "*coumarou*," the regular name of the tonka bean (*Dipteryx odorata*).

Some plants, mainly Rutaceae and Apiaceae members, produce furano- and pyrano-coumarins in which an additional furan or pyran ring is fused to the main skeleton (Figure 2.4). Coumarin, the simplest subsidiary of this chemical class, has been accounted for in many investigations as an allelopathic compound (Abenavoli *et al*., 2003) and thus inhibit certain enzymes involved in mitosis, ultimately inhibiting growth. In general, they exhibit effects in ways similar to those shown by phenolic acids including damage to cell membranes, disturbances in water balance, photosynthesis inhibition and so on (Moreland and Novitzky, 1987; Yang *et al*., 2002). Reports have shown that coumarin may inhibit the growth of *Daucus carota* (Abenavoli *et al*., 2004). Also, reports have it that, the compound, together with some simple phenols, may account for the phytotoxicity of *S. guianensis* (Khanh *et al*., 2006). Two different coumarins obtained from *Zosima absinthifolia*, 7-prenyloxy coumarin, and auraptene, caused an inhibition to the survival of lettuce at 0.1 mg/ml (Razavi *et al*., 2010).

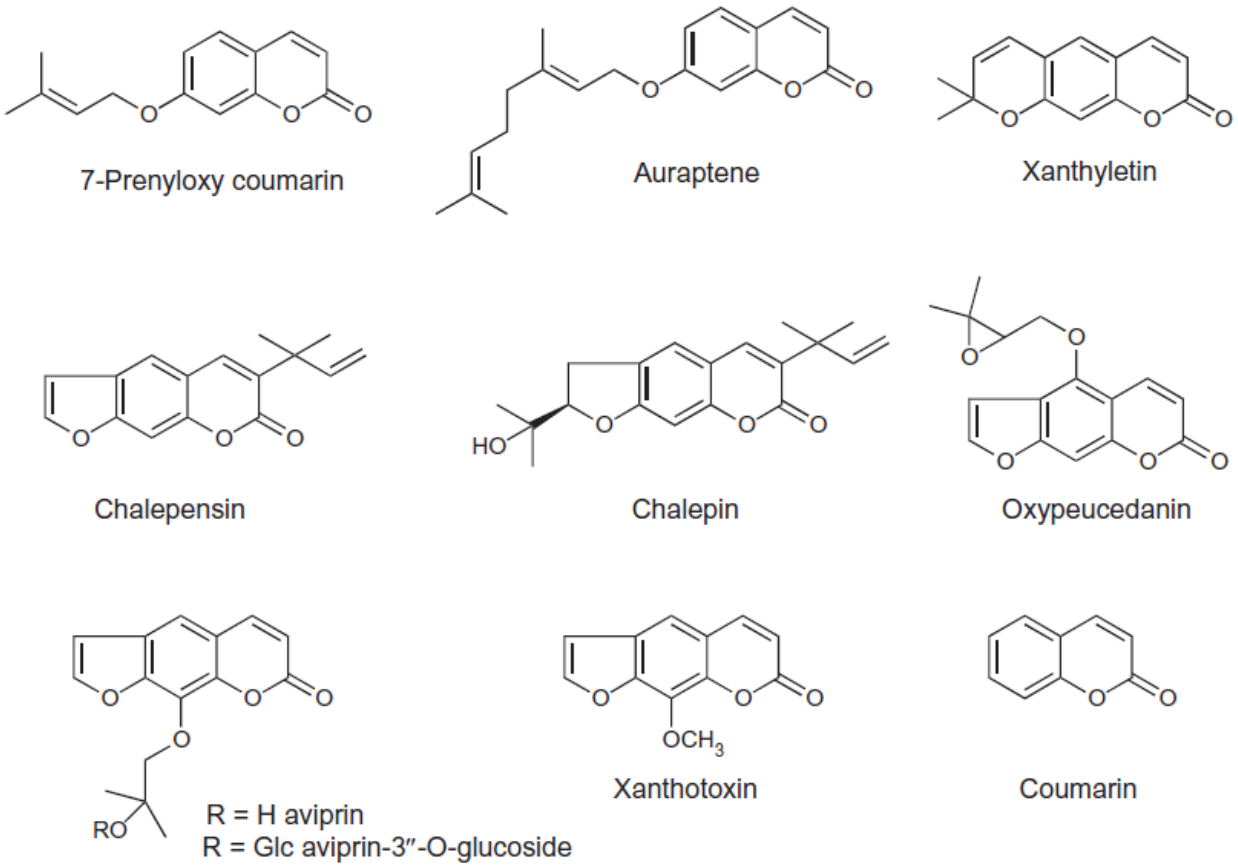


Figure 2.4: Structures of Some Coumarin Allelochemicals

Source: Flamini (2012).

2.3.3 Lignans

Lignans (Figure 2.5) are found in abundance in plants. They are phenylpropanoids dimers that are getting across the board intrigue due to their numerous biologically critical activities (Flamini, 2012). Lignans possessing an aryltetralin skeleton are particularly abundant in plants of the genus Podophyllum, both as aglycones and as O-b-glucosides. Some lignans including podophyllotoxin, b-peltatin, a-peltatin may possess phytotoxic activities against *L. sativa, Lolium multiflorum*, and *Allium cepa* (Oliva *et al*., 2002).

Observations of these compounds on onion root tips also demonstrated a diminishment in the amonuts of actively dividing cells and an unusual mitotic organization (Oliva *et al*., 2002). Some other lignans with phytotoxic activities include virolin and surinamensin (Borges *et al*., 2007). Tests on germination as well as growth parameters of the Fabaceae species *Mimosa pudica* showed a concentration-dependent activity of the two compounds, with maximum activity at 8.0 mg/l (Borges *et al*., 2007).

2.3.4 Flavonoids

Flavonoids (Figure 2.6) are another class of phenolic compounds generally examined for their phytotoxic activities. They consist of a diverse group of coloured compounds characterized by a C6–C3– C6 skeleton and are in numerous amounts in plants. Flavonoids however possess allelopathic properties as part of their many many physiological and biological activities (Szakiel *et al*., 2011). Reports have it that, they inhibit energy metabolism, including mitochondrial oxygen uptake (Einhellig, 2001). They act primarily as electron transport inhibitors through perturbation in the mitochondrial inner membrane. Reports have also shown that the compounds

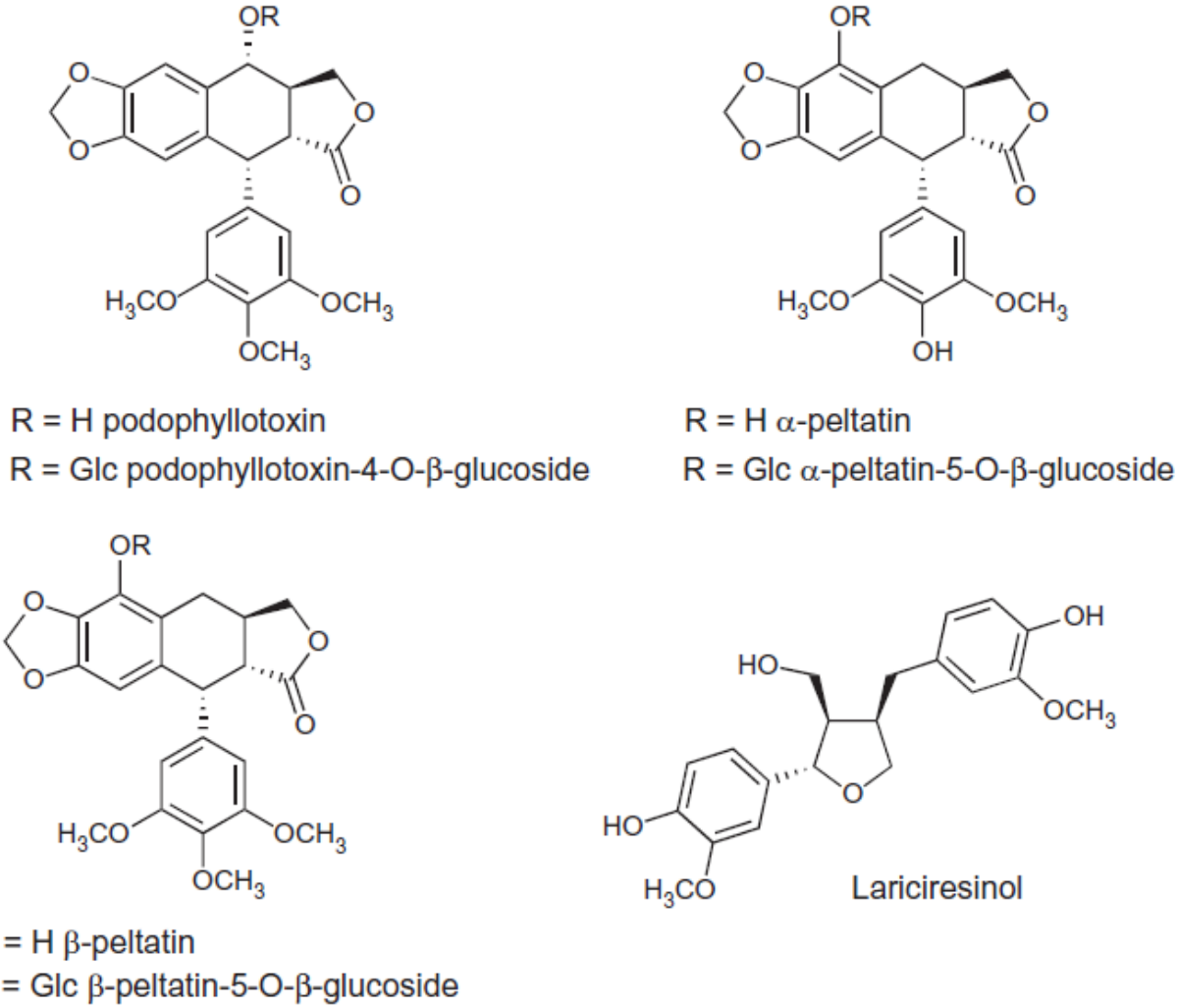


Figure 2.5: Structures of Some Lignan Allelochemicals

Source: Flamini (2012).

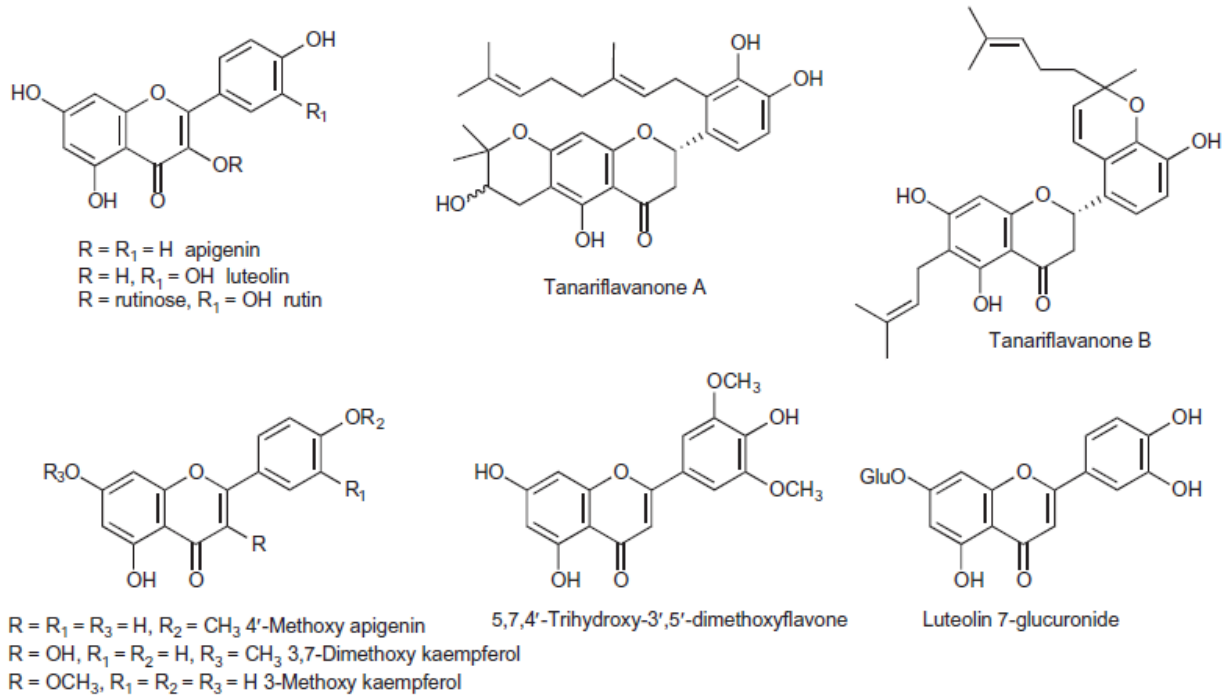


Figure 2.6: Structures of Some Flavonoid Allelochemicals

Source: Flamini (2012).

inhibit hydrolysis of ATP catalyzed by mitochondrial Mg^{2+}-ATPase (Moreland and Novitzky, 1987). Extracts that are obtained from *Castanea sativa* leaves, as well as its purified components such as quercetin and rutin, cause a significant inhibition of seed germination, and growth of *Raphanus sativus* (Szakiel *et al*., 2011). Of these compounds however, the quercetin exhibited more activity. A third compound, apigenin, only inhibited the germination of the seeds (Basile *et al*., 2000). One of the active principles of an allelopathic rice accession (PI312777) was found to be a flavone, 5,7,40-trihydroxy-30,50-dimethoxyflavone (Kong *et al*., 2004). This compound caused an inhibition of the growth of common weeds found in rice fields in South China, *E. crus-galli, Cyperus difformis* and *Cyperus iria*, with IC_{50} values of 200, 100, and 150 mg/g, respectively. The flavonoid 3-methoxyquercetin and its 40-O-glucoside and 7-O-glucoside completely caused an adverse effect on the growth of the seedlings at 100 ppm (Parvez *et al*., 2004).

2.3.5 Tannins

Tannins are polyphenols found in different parts of plants (Figure 2.7). They exist in either hydrolyzable forms or condensed or nonhydrolizable forms. The hydrolyzable tannins contain glucose molecules, with their hydroxyl groups being esterified by compounds like ellagic or gallic acids to form esters ellagitannins and gallotannins respectively (Flamini, 2012). Condensed tannins are generally flavonoid polymers and they exist in significant amounts in forests, constituting about 40 % of total contents of plants foliage and bark.

Tannins were initially thought to protect plants from the actions of herbivores, but recent reports have shown them to be less effective on many other ones at other locations (Flamini, 2012). However, recent studies suggest that they play major roles in plant–plant and plant–litter–soil

interactions. Tannins generally bind to cellular proteins and inhibit their actions. Examples including DNA polymerase and other enzymes involved in protein synthesis (Einhellig, 2001). Reports have also shown that they may cause water inbalance and stomatal closure in seedlings (Bains *et al*., 2009). Tannins tend to affect the rates at which nutrients are being recycled through alteration in decomposition rates, complexing proteins, amongst others (Kraus *et al*., 2003).

Spike watermilfoil (*Myriophyllum spicatum*) contains a lot of hydrolyzable tannins that can represent up to 10% of its dry weight. Among them, tellimagrandin II has been recognized as the fundamental allelochemical due to its capacity to incite 40% hindrance of photosystem II (Leu *et al*., 2002). Likewise, the allelopathic impact of the root exudate of *Phragmites australis* is because of the nearness of the phytotoxin, gallic acid.

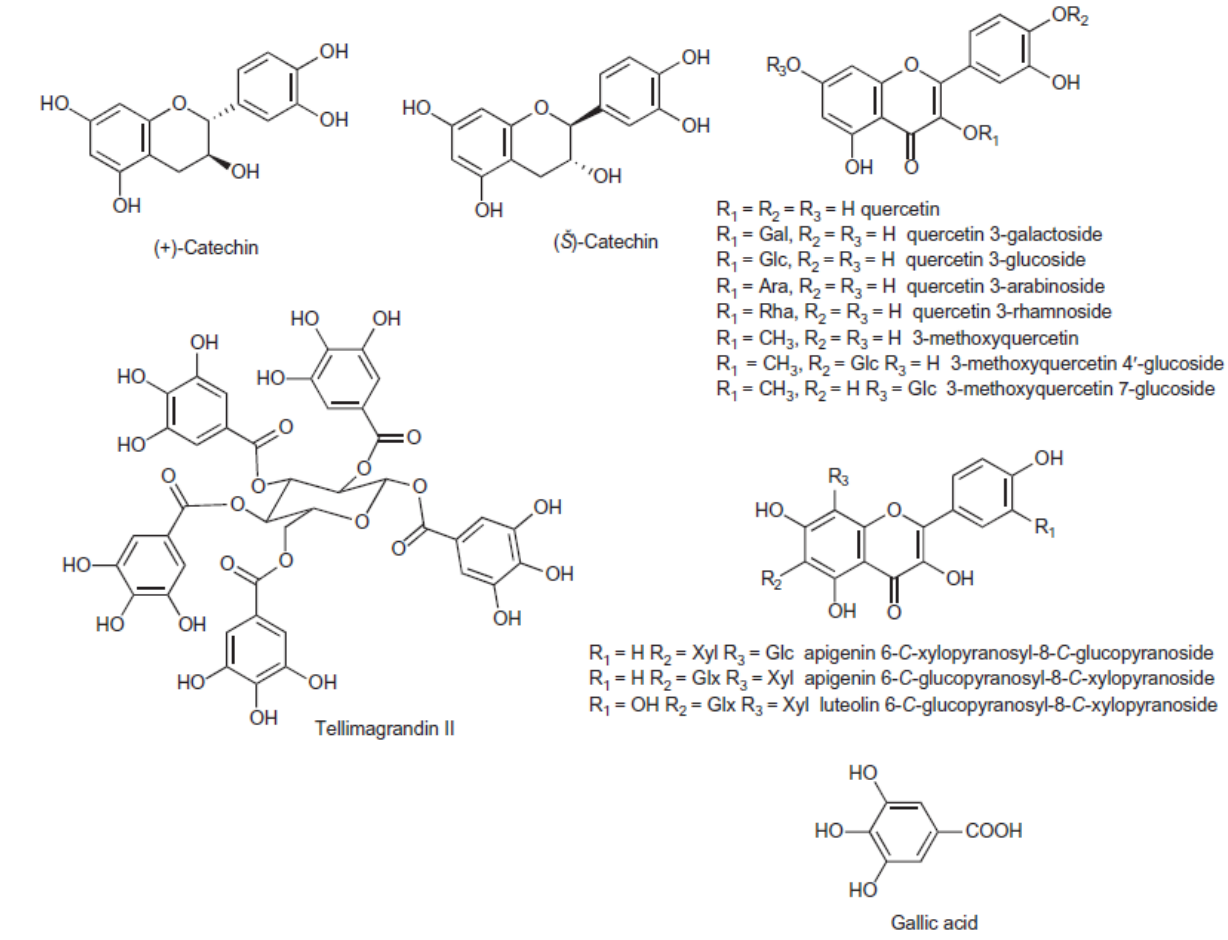


Figure 2.7: Structures of Some Tannin Allelochemicals

Source: Flamini (2012).

2.3.6 Benzoxazolinones

Benzoxazolinones (Figure 2.8) are created by a predetermined number of plant families, for example, Acanthaceae, Poaceae, Ranunculaceae, and Scrophulariaceae (Flamini, 2012). Containing a cyclic hydroxamic acid moiety, they have attracted attention since the early 1960s, when 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) were characterized for the first time (Fomsgaard *et al*., 2004).

Usually, they exist in plants as glucosides, which are enzymatically converted into agluconic forms by action of β-glucosidases after plant injury. However, a few authors detailed the availability of agluconic forms in intact plants (Fomsgaard *et al*., 2004).

Most agluconic forms are precarious and experience a change in ring-contracted dynamic compounds, for example, benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA) (Fomsgaard et al., 2004). Inside similar species, benzoxazinones content can vary extraordinarily contingent upon the cultivar (Tabaglio *et al*., 2008). The activity is coordinated against an assortment of target plants, both weeds and target plants, including *A. cepa* and *Phaseolus aureus* (Singh *et al*., 2005), or *E. crus-galli*, *Setaria viridis*, *Abutilon theophrasti*, *Amaranthus retroflexus* (Mathiassen *et al*., 2006), and *L. rigidum* (Wu *et al*., 2002). Studies on the structure-function connections of benzoxazinones and of their debasement products have prompted their disclosure as helpful leads for herbicide models improvement (Macias *et al*., 2008). The information of the progression of soil debasement may additionally add to survey the genuine viability and supportability of these Allelochemicals (Macias *et al*., 2008).

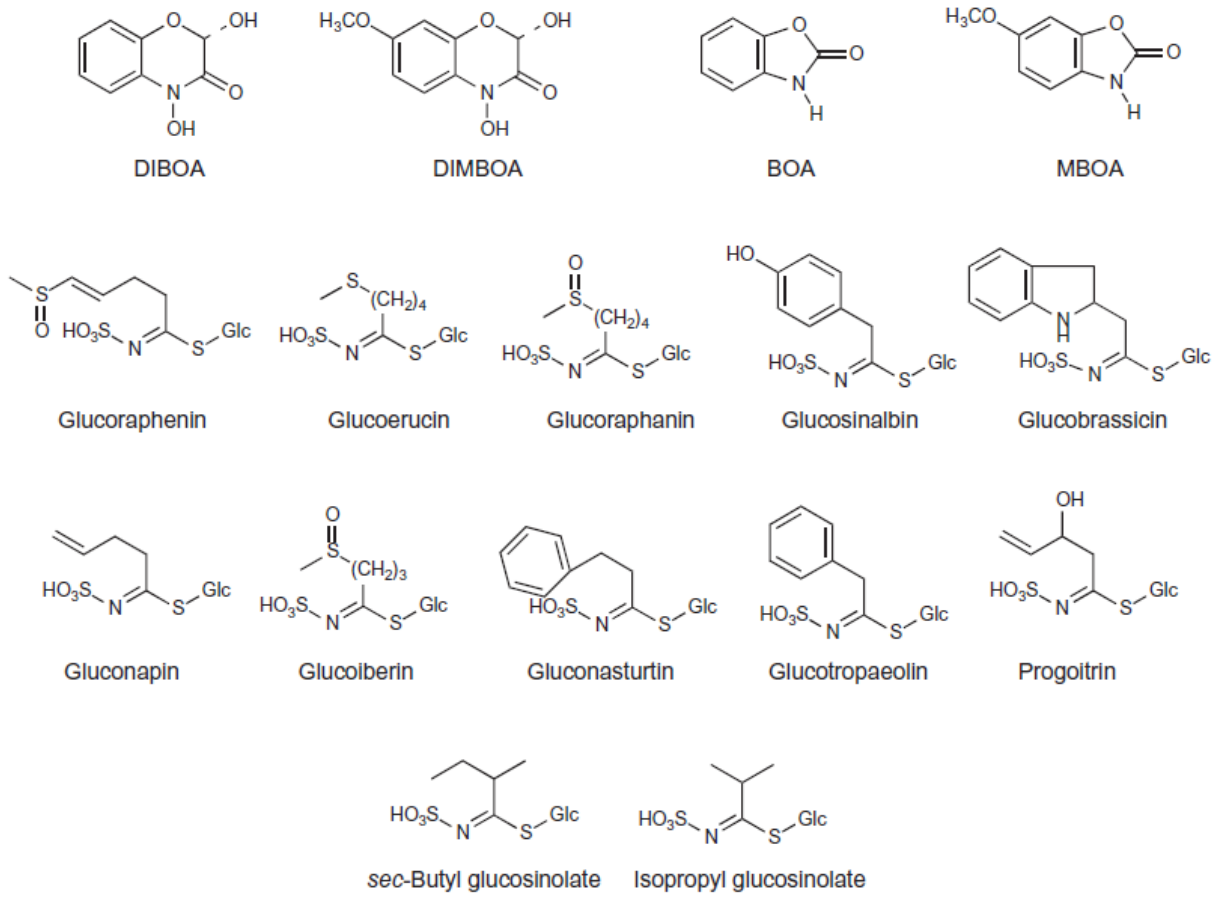


Figure 2.8: Structures of Some Benzoxazolinone and Glucosinolate Allelochemicals

Source: Flamini (2012).

2.4 Modes of Action of Allelochemicals

2.4.1 Disturbances in the Micro and Ultra-structure of Cells

Allelochemicals have appeared to affect the shape and structure of plant cells (Borges *et al*., 2007). Their actions lead to depolarization of membranes and causes a nonspecific efflux of both anions and cations accompanying the increased cell membrane permeability. These membrane effects however correlate with an inhibition of ion uptake. For instance, phenolic acids suppress absorption of phosphate, potassium, nitrate, and magnesium ions, and overall changes in mineral ion content of plant tissue. Volatile monoterpenes, eucalyptol and camphor may cause widening and shortening of root cells, in addition to inducing nuclear abnormalities and increasing vacuole numbers (Bakkali *et al*., 2008). Allelochemicals, for example, hordenine and gramine, from barley (*Hordeum vulgare*) roots, have appeared to adversely affect the radicle tips of white mustard (*Sinapis alba* L.) including compromised cell membranes, increment in both the size and number of vacuoles, disorganization of organelles, and cell autophagy. Moreover, cinnamic acid was likewise reported to significantly cause harm to the ultrastructure of cucumber chloroplasts and mitochondria (Wu et al., 2002). After treatment with benzoic acid, mustard (*Brassica juncea* L.) roots displayed abnormally shaped cells arranged in a disorganized manner and disrupted cell organelles (Kaur, 2009).

2.4.2 Inhibition of Cell Division and Elongation

Allelochemicals including the monoterpenoids may affect cell growth and the synthesis of DNA in plant tissues (Nishida *et al*., 2005). 2(3H)-benzoxazolinone (BOA) was reported to inhibit the mitotic process, especially the G_2-M checkpoint of lettuce (Sanchez-Moreiras *et al*., 2008) and sorgoleone caused a diminishment in the amount of cells in every cell division period, causing

harm to tubulins and bringing about production of polyploid nuclei (Hallak *et al*., 1999). Some allelochemicals have additionally been accounted for to fundamentally repress the recovery of cucumber root top cells and plant development, examples including BOA and 2, 4-dihydroxy-1,4(2H)- benzoxazin-3-one (DIBOA) (Burgos *et al*., 2004). Teerarak *et al*. (2010) additionally recommended that the ethyl acetic acid derivation part of *Aglaia odorata* Lour. leaves caused a hindrance of mitosis and instigated mitotic abnormalities in *Allium cepa* roots by disrupting chromatin organization and the mitotic spindles in roots treated with the allelochemicals.

2.4.3 Imbalances in Antioxidant System

The production and evacuation of reactive oxygen species (ROS) and the balance of the redox state in the cell assume a critical part in allelopathic impacts. Recipient plants may generate ROS because of exposure to toxic allelochemicals (Ding *et al*., 2007), thus exhibiting alteration in the activities of antioxidant enzymes (Apel and Hirt, 2004). Studies have revealed that allelochemicals essentially hinder the activities of antioxidant enzymes and increase the levels of free radical, which result from membrane lipid peroxidation in the plants (Bais *et al*., 2003; Zuo *et al*., 2012; Sunmonu and Van Staden, 2014), thus reducing the survival rates of such plants. For instance, oxidation of plasma membrane sulfhydryl groups would lead to damage to the cell membrane integrity thereby causing death to such cell.

2.4.4 Effect on Plant Growth Regulator System

Allelochemicals adversely affect the contents of plant growth regulators or cause uneven characters in different phytohormones, in this manner repressing the development and improvement of such plants (Zuo *et al*., 2012). Allelochemicals including the phenolic acids seem to act through inhibition or activation of the indoleacetic acid (IAA) oxidase system (Apel

and Hirt, 2004). Chlorogenic, caffeic, and other polyphenols were reported to synergize IAA-induced growth by counteracting IAA destruction, whereas monophenols such as *p*-coumaric and *p*-hydroxybenzoic acid stimulated decarboxylation. Prasad and Devi (2001) reported that ferulic acid caused an increase in activities of peroxidase, catalase, and IAA oxidase while decreasing polyphenol oxidase in maize (*Zea mays*) seedlings.

2.4.5 Effect on Activities of Important Enzymes

Allelochemicals can block the functioning of many enzymes if they are sufficiently concentrated at the site of enzymatic functions (Prasad and Devi, 2001). Activities of amylase, maltase, invertase, acid phosphatase and protease were suppressed by ferulic acid in tests using maize seeds and seedlings (Prasad and Devi, 2001). However, exogenously applied gibberellic acid reversed the effect of ferulic acid on amylase and acid phosphate. Salicylic acid at 25 µM strongly inhibited ethylene formation from ACC in cell suspension cultures, and a number of other benzoic acid derivatives suppress ethylene production at higher concentrations (Apel and Hirt, 2004).

2.4.6 Influence on Respiration

Allelochemicals may influence the development of plants by affecting distinctive phases of respiration, for example, electron transfer in the mitochondria, oxidative phosphorylation, CO_2 generation and ATPase activity (Souto *et al*., 2000). A number of investigations have reported that phenolic acids alter the rate of respiration in target plants and microorganisms. Mycorrhizal fungi are particularly sensitive (Souto *et al*., 2000). Phenolic mixtures of 0.1 µM reduced fungal respiration, in contrast to millimolar levels increasing oxygen consumption. This could arise either from direct action on mitochondrial functions or increased respiratory demand to meet

energy requirements for membrane repair and lost efficiency in ATP-mediated events (Souto *et al*., 2000).

2.4.7 Effect on Photosynthesis

Allelochemicals have been accounted for to inhibit the synthesis or accelerate the decomposition of photosynthetic pigments (Fasaanu *et al*., 2013). Consequently, the pigment contents are reduced, thereby causing an inhibition of the photosynthetic process (Meazza *et al*., 2002). Allelochemicals affect this process essentially by influencing the activities of PS II (Weir, 2006). For example, sorgoleone prevents the decay of variable fluorescence, interrupts the oxidation of the PSII-reduced primary electron acceptor, Q^{-}_{A}, by the PSII secondary electron acceptor and that of Q_B by displacing Q_B from the D_1 protein, thus inhibiting photochemical effects (Weir, 2006). Also, studies have reported significant reduction in chlorophyll a and b and carotenoid contents of plants on exposure to allelochemicals (Fasaanu *et al*., 2013; Sunmonu and Van-Staden, 2014).

2.4.8 Influence on Water and Nutrient Uptake

Allelochemicals may influence nutrient absorption in plant roots or prompt water stress through long-term restraint of water use. They may also inhibit the activities of Na^+/K^+-ATPase involved in the absorption and transport of ions across the cell plasma membrane, which suppresses the absorption of K^+, Na^+, or other ions. For example, ferulic acid (250 μM) has been reported to cause inhibition of ammonium and NO_3^- uptake in corn seedlings, although ammonium uptake is less sensitive to the treatment than NO_3^- (Souto *et al*., 2000). The effects of allelochemicals on ion uptake are closely related to allelochemical concentrations and classifications. For example, a

low concentration of dibutyl phthalate was shown to increase the absorption of nitrogen but decreases that of potassium and sodium. However, a high concentration of this chemical may distrupt the absorption of N, P and K. In similar terms, a low concentration of diphenylamine stimulates the absorption of N and K but inhibits the absorption of P by tomato roots (Souto *et al*., 2000).

2.4.9 Influence on Protein and Nucleic Acid Metabolism

Allelochemicals may bind to DNA and increase the temperature of DNA cleavage, while some can inhibit DNA polymerase I and generally hinder the production of proteins (Abenavoli *et al*., 2003). Allelochemicals may also inhibit amino acid absorption, thus interfering with protein synthesis, which affects cell growth (Abenavoli *et al*., 2003).

2.5 Factors Affecting the Effectiveness of Allelochemicals

2.5.1 Herbivory

Living organisms present in the can alter concentrations of allelochemicals present in plant tissues (Baldwin, 2006). Reports have it that volatile compounds produced by pegging of sagebrush exhibited germination and growth inhibitory effects on surrounding plants, thus serving as a proof that allelopathy could be linked to herbivory. The action of herbivores prompts plants to release volatile organic compounds and accumulate polyphenolics (Baldwin, 2006; Roitto, 2008), with many of them having inherent allelopathic characteristics. Consistent with this, Bi (2007) found that exogenous application of methyl jasmonate, a chemical that induces herbivore defenses in many plant species.

2.5.2 Soil Nutrient Content

Soil nutrients, when present in low amounts, may prompt the production of allelochemicals in plants. For example, when the quantity of iron in soil is limited, reports have it that the roots of *Centaurea diffusa* prolong the release of 8-hydroxyquinoline which also enables mobilization of metals for easy uptake by plants (Tharayil, 2009). Accordingly, the metal substance of soils from various environments is probably going to unequivocally impact the creation and soil accessibility of 8-hydroxyquinoline, and complex communications between this allelochemical and metals may likewise determine its biological action (Tharayil, 2009).

2.5.3 Light Intensity

Light intensity increases the root exudation of some allelochemicals (Tharayil, 2009). For instance, 8-hydroxyquinoline shows a diurnal pattern and achieves maximum level following 6 hours of introduction to light. Assessing the part of an allelochemical with regards to its abiotic condition would the comprehension on its discharge and allelopathic activities. Incited optional metabolite-based protections are basic in plants (Agrawal, 1998), and if a similar secondary metabolite or their subordinates are additionally allelopathic, herbivory may easily alter allelopathic interactions (Agrawal, 1998).

2.5.4 Soil Microbes

Microbial action on allelochemicals usually degrades their function, and evaluation of the activity allelochemicals in microbe-free substratum may therefore not be ecologically relevant. Nonetheless, Kaur (2009) reported that allelopathic impacts of m-tyrosine were just clear in

clean soil and lessened pointedly in non-sterile soil with microorganisms. Reports also have it that, the anticipated half-life of m-tyrosine in soil in lab conditions was under 1 day, demonstrative of fast microbial debasement (Bertin (2009). Sorgoleone, a noteworthy part of root exudates of *Sorghum bicolor*, is a strong allelochemical (Baerson, 2008) and microorganisms found in North American soils promptly devour it as a carbon source (Bertin, 2009). It has been accounted for that the methoxy group of sorgoleone, which is responsible for much of its activity, degrades rapidly in soil (Gimsing, 2009). In addition to the direct impacts of toxic allelochemicals on plant growth, their indirect effects may be mediated by microbial activity.

Meier and Bowman (2008) reported that some allelochemicals fractions directly exhibited their phytotoxic activities while others acted indirectly through microorganisms. These results therefore serve as proofs of how phenolic allelochemicals hinder the growth of planta and at the same time, interact with soil microorganisms.

2.5.5 Seasonal Variation

The concentrations of allelochemicals in plant tissues are usually affected by seasonal changes (Barto and Cipollini, 2009). For instance, reports have it that glucotropaeolin is being accumulated in *Alliaria. petiolata* more rapidly in autumn than in spring, whereas during spring, accumulation of alliarinoside is usually greater (Barto and Cipollini, 2009). Contagious people group and ectomycorrhizal colonization rates indicated straight and curvilinear reactions to alliarinoside and glucosinolate focuses, separately (Lankau, 2011). Expanding groupings of

alliarinoside were found to adjust AM parasitic groups, prompting a decrease in colonization by AM growths of Quercus rubra roots (Lankau, 2011).

2.5.6 Plant genetic make-up

Plant interactions may also be genotype or ecotype-dependent. For instance, when the roots of different *Ambrosia dumosa* plants make contact they often stop growing and this is due in part to their genotypic make-up. What's more, underlying foundations of plants from a similar district indicate solid reaction to contact however plant roots from various areas don't (Mahall and Callaway, 1996). For example, *Cakile edentula* plants allocate biomass differently to their roots depending on the species with which they are sharing the pots (Mahall and Callaway, 1996). Also, the high tissue concentrations of sinigrin produced by *Brassica nigra* is an advantage with respect to interpecificcompetition but a disadvantage in intraspecific competition (Lankau, 2008). Further, choice for B. nigra people that created abnormal amounts of sinigrin was more grounded when developed with different species than with different people of its own species (Lankau, 2008).

Thus, the numerous mechanisms with which plant species protect themselves from chemical effects of their neighbors can be explained with respect to the difference in their genetic make-up which could give rise to difference in their growth response to allelochemicals produced by a given plant species (Go´mez-Aparicio and Canham, 2008).

2.5.7 Soil-Allelochemical Volume Ratio

Large quantity of soil may overshadow the effectiveness of allelochemicals. In fact, issues of spatial scale and patchiness make studies of the roles of allelochemicals in soils difficult to interpret. The impacts of allelochemicals in soils are for the most part inspected utilizing 'mass

soils', while allelochemicals are added to a volume of soil that is requests of greatness more prominent than the dirt volume in which the connections happen. 'Sensible' convergences of allelochemicals are evaluated for the normal of the extensive soil volume. Be that as it may, the activity of root-oozed chemicals frequently happens at root– root interfaces. The utilization of evaluated soil fixations can in one way be utilized to investigate allelopathy in a sensibly reasonable way yet they have restrictions for the assurance of the allelopathic elements of chemicals. If an allelochemical is experimentally applied to soil in such a way as to allow it be transformed before contact with roots (Lankau, 2011), then the failure to find an effect cannot be taken as evidence that effects do not occur when roots are in close proximity to each other. This scenario is, however, different and less relevant when allelochemicals enter the soil through release from foliage or decomposition of plant litter.

2.6 Allelopathy Study

Many investigations carried out in allelopathy usually make use of plant extracts with focus on seed germination and development. A dependable bioassay that is touchy is required for additional inside and out investigations of the development components included and for creating starting explanatory systems to decide the chemical(s) capable. By and large, germination is less delicate to the allelopathic synthetic than is seedling development or root development (Miller, 1996). Albeit numerous lab bioassays have proposed to exhibit allelopathy, concerns have been raised that the vast majority of them have little pertinence as far as clarifying (Inderjit and Dakshini, 1999). It is a mind boggling wonder including an assortment of interrelationships among plants.

For all intents and purposes all plant parts, for example, leaves (Kumari and Kohli, 1987), roots (Horsley, 1977), dust (Cruz-Ortega et al., 1998), trichomes (Bansal, 1990), bark (Kohli, 1990) and seeds and organic products (Fredman et al., 1982) have allelopathic potential. Water concentrates of best development (particularly leaves) of allelopathic plants deliver more allelochemicals than those from roots and crowns (Miller, 1996). For instance, the autotoxic impacts of water concentrates of plant parts of Medicago sativa were positioned as leaf (most noteworthy), seed, root, blossom, and stem (slightest) (Chung and Miller, 1995). Likewise, the phytotoxic impacts of a progression of fluid concentrates from leaves, stems, roots and blossoms of regular thorn on Medicago sativa seedlings demonstrated most noteworthy restraint in the concentrates from blooms and leaves, trailed by stems, and roots (Chon, 2004).

More so, research has shown contrasting results between experiments carried out in open fields and those in the laboratory (Park, 1966). Reports have it that field soil collected under the certain plants significantly reduced growth of test plants (Al-Naib and Rice, 1971; Yun and Kil 1992). However, soil from below some test plants did not inhibit germination and development of some other test species (Fadayomi and Oyebade, 1984).

2.6.1 Petri-dish Bioassay

Bioassays with Petri dishes (Figure 2.10a) constitute the commonest technique and are, at times, part of a more elaborate study in allelopathy research (Inderjit and Dakshini, 1999). The bioassay involves evaluating the effects of plant extracts (aqueous, hydroalcoholic, fractions from different solvents, etc.), volatile essential oils, and isolated or commercial chemicals on the germination and initial growth of a given target plant (Inderjit and Dakshini, 1999).

To carry out a Petri-dish bioassay, the bottom of the Petri-dish plate is normally lined with filter paper and the seeds of the target plant are deposited to receive the solutions, often in different concentrations. The dishes might be kept in a Biochemical Oxygen Demand (BOD) chamber either oblivious or with a modified photoperiod relying upon the photoblastism of the seed tried. A small variation in this protocol is the use of solid agar medium amended with the extracts or isolated substances. Germination velocity and percentage as well as shoot and root length are the variables commonly evaluated in these bioassays (Kumari and Kohli, 1987).

2.6.2 Pot Experiments

Competition involves the withdrawal of shared resources from nature, whereas allelopathy comprises of the addition of substances that exhibit phytotoxicity (Olofsdotter *et al.*, 2002). Despite this clear conceptual division, there is need to note the fact that in field experiments this separation is almost impossible to detect (Olofsdotter *et al.*, 2002). Thus, separating the effects of pure competition from allelopathy has prompted the use of pot experiments (Figure 2.10b) to confirm a plant species as allelopathic. The experiment involves growing the test plants in pots filled with appropriate soil. The soil is initially sun-dried and sieved, after which seeds are grown in the soils. The germinated seeds are then treated with the plant extracts of interest. The assay helps to mimick the natural environment of the plant species and at the same time separating allelopathic process from competition.

2.6.3 *Allium cepa* Test

The *Allium cepa* test involves growing onion roots in direct contact with certain substances of importance in order to study possible damage which the substance may pose on cell DNA (Fachinetto *et al.*, 2007). Along these lines, the information can be extrapolated for all creature

and plant biodiversity. The examination of chromosomal adjustments can be equivalent to the trial of mutagenicity principally for the recognition of basic modifications; be that as it may, it is conceivable to watch numerical chromosomal changes, also (Lubini et al., 2008).

The Allium cepa test has been utilized by numerous scientists mostly as a bioindicator of ecological contamination (Bagatini et al. 2009; Leme and Marin-Morales, 2009), testing rough concentrates of cyanobacteria (Laughinghouse, 2007), mechanical effluents and herbicides (Samuel et al., 2010) and notwithstanding assess the genotoxic capability of therapeutic plants (Camparoto et al. 2002; Knoll et al. 2006), on the grounds that this test utilizes a model that has ended up being enough delicate to recognize innumerous substances that reason chromosomal modifications.

The examination of chromosomal modifications fills in as a mutagenicity test and is one of only a handful few direct techniques to quantify harms in frameworks presented to conceivable mutagens or cancer-causing agents. To empower the assessment of the impacts or harms that mutagenic operators may cause, it is vital for the example to be in consistent mitotic division, looking to distinguish the dangerous impacts and modifications happening over a cell cycle. Keeping in mind the end goal to do as such, there is the Allium cepa test, which has been broadly utilized for this reason (Silva and Fonseca, 2003).

The mitotic index and replication record are utilized as pointers of satisfactory cell multiplication (Gadano *et al*., 2002), which can be measured by the plant test system *Allium cepa* (Figure 2.10c). Cytotoxicity tests, utilizing plant test frameworks in vivo, for example, *Allium cepa*, are approved by a few analysts (Vicentini, *et al*. 2001; Teixeira *et al*. 2003), giving significant data to human wellbeing. In this way, even in a circuitous way, it is conceivable to avert and maintain

a strategic distance from natural sullying by the injurious utilization of substances that may cause chromosomal distortions.

2.7 Identification of Allelochemicals

Natural products identified as allelopathic agents are secondary products of plant metabolism, many of them are degradation products that occur in the presence of microbial enzymes. Several metabolic processes give allelopathic compounds as by-products. Most allelochemicals are gotten from amino acids, by means of the shikimic acid pathway (Rice, 1984).

It is worthy of note that compounds isolated from plants or their leachates often do not reproduce the observed allelopathic effects without the associated factors. In many occasions, these plant-derived compounds are modified by oxidation, reduction, photochemical enactment (Fischer et al., 1978), detoxification, or biochemical



Figure 2.9: Models for Allelopathy Study. A- Petri-dish assay; B- Pot assay; C- *Allium cepa* test

Source: Nebauer *et al*. (2013).

activation by bacteria and fungi. Bioactive molecules may leave the system by being adsorbed onto inorganic particles or organic matter in the soil, or leached from the system.

2.7.1 Isolation and Quantification

The most important aspect of allelopathy research is to successfully isolate, identify, and quantify causative allelochemicals present in the plants or soils (Reid and Sarker, 1998). Constituent allelochemicals of plant extracts can be isolated and quantified using column chromatography techniques (Reid and Sarker, 1998). It is however fundamental that potential allelopathic compounds can likewise identify with the levels initially found in the entire concentrates. Phytotoxicity-based extraction and fractionation are being utilized to isolate allelochemicals contained in plant extricates (Chon *et al.*, 2003).

2.7.2 Fractionation and Bioassay of Solvent Fractions

Extracts from plant samples may be used for bioassay and fractionation. For instance, a crude methanol extract of plant may be subjected to partitioning with hexane and ethyl acetate (Chon *et al*., 2003). The hexne fraction may be collected by redissolving the extract in a solvent mixture of distilled water and hexane in a separating funnel. After collecting the hexane fraction, the distilled water fraction is then mixed with ethylacetate to obtain ethylacetate fraction in the same way. The same procedure is used in preparing other solvent fractions. Afterwards, the fractions are evaporated to dryness using a rotary evaporator at 40-50^{0}C, and transferred into vacuum freeze dryer to obtain dry matters and used for quantification and bioassay.

The dried samples concentrated from fractions are again dissolved in methanol for bioassay (Chon *et al*., 2003). Afterwards, the dissolved fractions are pipetted into 9 cm plastic Petri dishes lined with Whatman No. 1 filter paper and evaporated to dryness for 24 h at 24^{0}C.

Reports have had studies involving isolation of allelochemicals from plant extracts. For instance, Chon *et al* (2003) reported the constituent phenolic allelochemicals in ethyl acetate extracts of *Cercidiphyllum japonicum*, *Lactuca sativa*, and *Xylomelum occidentale* were of the concentrations 60.3, 18.5, and 84.4 mg 100 g^{-1}, respectively.

2.8 *Allium cepa*

Allium cepa (Figure 2.10a) has its name got from the Latin cepa "onion". It is otherwise called the knob onion or normal onion (Block, 2010). It is a vegetable and is the most extensively created sorts of the assortment Allium. Its close-by relatives join the garlic, shallot, leek, chive, and Chinese onion (Block, 2010). The onion is most as often as possible a biennial or a lasting plant, yet is normally regarded as a yearly and reaped in its first developing season.

The onion plant has a lover of unfilled, light blue green leaves and its globule at the base of the plant begins to swell when a particular day-length is come to. The globules are made out of abbreviated, packed, underground stems encompassed by beefy adjusted scale (leaves) that wrap a focal bud at the tip of the stem (Block, 2010).

Onions are developed and utilized the world over. As a nourishment thing, they are generally served cooked, as a vegetable or part of a readied appetizing dish, yet can likewise be eaten crude or used to make pickles or chutneys. Onions are employed in studies that have to do with cell examination where the onion cells are viewed under microscopes (El-Shahaby *et al*., 2003). This is because onions have particularly large cells that are readily observed under low magnification.

2.9 *Solanum lycopersicum*

Solanum lycopersicum (tomato) (Figure 2.10b, c) is an edible fruit belonging to the nightshade family, *Solanaceae*. The species began from Central and South America. Various assortments of tomato are generally developed in mild atmospheres over the world, with nurseries permitting its generation consistently and in cooler zones (Cocaliadis et al., 2014). Tomato plants customarily create to 1– 3 meters (3– 10 ft) high and have a feeble stem that routinely sprawls over the ground and vines over various plants (Cocaliadis et al., 2014). It is an enduring in its local territory, and developed as a yearly in mild atmospheres (Cocaliadis et al., 2014).

Tomato plants are dicotyledonous, and develop as a progression of spreading stems, with a terminal bud at the tip where the development really happens (Redenbaugh et al., 1992). At the point when that tip in the long run quits developing, regardless of whether on account of pruning or blossoming, horizontal buds assume control and develop into other, completely utilitarian, vines. Tomato vines are normally secured with fine short hairs. These hairs encourage the vining procedure, transforming into roots wherever the plant is in contact with the ground and dampness, particularly if the vine's association with its unique root has been harmed or separated.

Its utilization as a nourishment began in Mexico, and spread all through the world after the Spanish colonization of the Americas (Sato et al., 2012). Tomato is consumed in diverse ways, including

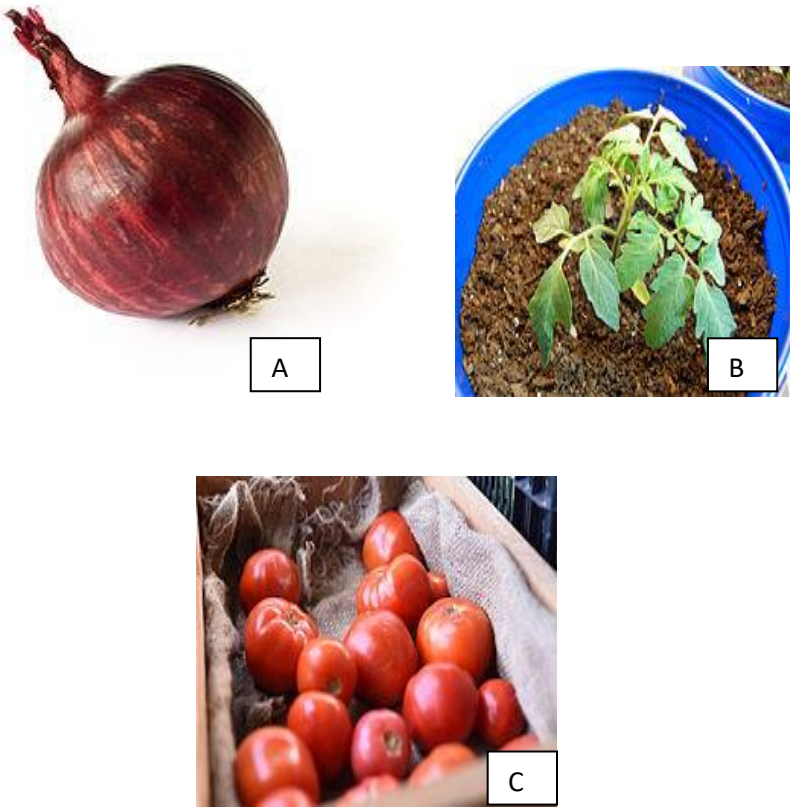


Figure 2.10: Some plants used for Allelopathy study. A- Onion bulb; B- Tomato plant; D- Tomato fruits.

Source: Sehgal *et al*. (2006).

raw, as an ingredient in many dishes, sauces, salads, and drinks. While tomatoes are botanically berry-type natural products, they are viewed as culinary vegetables, being elements of appetizing dinners.

2.10 *Alchornea laxiflora*

Alchornea laxiflora (Benth) (Pax and Hoffman) (Figure 2.11) belongs to Euphorbiaceae family. It is a forest understorey tree of about 6m high growing in Nigeria. It is also found in Cameroon and naturally widespread in the central and southern tropical Africa. The leaves are especially imperative in the conservation of kolanuts generally eaten in Nigeria. The stem and branchlets are additionally utilized as biting sticks in Nigeria.

Alchornea laxiflora is rich in phytochemicals, which may legitimize its utilization generally in ethno restorative routine with regards to numerous nations. It is used in folk medicine as a remedy for numerous diseases ranging from inflammation to heart diseases (Burkill, 1994; Kayode and Omotoyinbo, 2008). Decoction of the leaves is effective in the treatment of kidney, bladder, inflammatory malarial and infectious diseases (Adewole, 1993). They have also been reported to exhibit hypo sensitive and antihistamine properties (Burkill, 1994; Sofowora, 2006; Kayode and Omotoyinbo, 2008). The leaves of *Alchornea laxiflora* have also been reported to possess hepato-protective activity due to the presence of high amounts of flavonoids (Ogundipe *et al.,* 2001).

Powdered leaf samples of *A. laxiflora* have been accounted for to contain secondary metabolites including alkaloids, cadiac glycosides saponins and phenolic compounds (Ogundipe *et al*., 1999). Isolation and structural elucidation of the phytochemical constituents of *Alchornea laxiflora*



Figure 2.11: *Alchornea laxiflora*

leaves revealed the presence of flavonoids such as quercetin-3-O-β-D- glucopyranoside, quercetin 3, 7, 3', 4'-tetrasulphate, quercetin, quercetrin, quercetin - 7, 4- bisulphate, quercetin - 3', 4'-disulphate, quercetin -3, 4'-diacetate and rutin (Ogundipe *et al*., 2001). Its root samples have also been shown to contain terpenoid compounds (Farombi *et al*., 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Atrazine (The orthodox herbicide) was a product of Ningbo Agrostar Industrial Company Limited (China). All other chemicals used were of analytical grade and were products of Sigma Chemical Company (USA) and BDH Laboratories (UK).

3.2 Apparatus and Equipment

The apparatus and equipment used include UV-VIS Spectrophotometer (Lab-Kits China), Harrier 18/80 Centrifuge (UK), OHAUS analytical balance (Ohaus Corporation, Switziland), 3510 pH (Bibby Scientific Ltd, Essex, UK), GFL 1038 water bath and shaker (GFL, Burgwedel, Germany) and DW6-85 Lyophilizer (Heto Drywinner, Denmark).

3.3 Plant Materials and Authentication

Tomato seeds used were products of JK Agric Genetics Ltd. Hyderabad, India (Label no: 066638 and Lot no: 1213/40331). *Alchornea laxiflora* leaves were obtained within the premises of University of Ilorin, Nigeria in December, 2015. The leaves were identified and authenticated at the Department of Plant Biology University of Ilorin where a voucher specimen (UIL/001/1035) was prepared and deposited in the Herbarium. *Allium cepa* (Onion bulbs) were obtained from Igbonna Market, Osogbo, Osun State, Nigeria.

3.4 Seed Viability Test

The seeds were tested for viability following the procedure of Fasaanu *et al*. (2013). The seeds were submerged in water enough to enable floating. The non-viable seeds floated on the water and were removed by scooping while the viable ones were air dried and kept in a plastic container in a laboratory cupboard for 48 h.

3.5 Drying and Pulverization

The fresh leaves of *Alchornea laxiflora* were thoroughly rinsed under running tap water and distilled water afterwards to remove dust and soil particles. The leaves were shade dried for 7 days and pulverized using an electronic blender (BL8319 Toyomi Instruments, Singapore), The resulting powder was stored in air tight containers at 4^0C for 48 h.

3.6 Preparation of Aqueous Extract

Aqueous leaf extract of *Alchornea laxiflora* was prepared following the procedure reported by Sunmonu and Van-Staden (2014). Briefly, 50 g of the powder was extracted in 2 L of distilled water for 24 h at 25^0C. The mixture was filtered with Whatman No.1 filter paper and the resulting filtrate was lyophilized using a lyophilizer (DW6-85; Heto drywinner, Denmark). The percentage yield of the plant was calculated to be 18 % w/w. The residue was then stored in the refrigerator at 4°C for 48 h.

3.7 Screening and Quantification of Secondary Metabolites present in *Alchornea laxiflora* Aqueous Leaf Extract

The *Alchornea laxiflora* leaf extract was screened for various secondary metabolites as follows:

3.7.1 Total Alkaloid

The presence of alkaloid in the leaf extract of *Alchornea laxiflora* was confirmed using Dragendorff's reagent as described by Trease and Evans (2002). A part of the dried extract (1 g) was dissolved in 2N HCl (5 ml) after which 2 drops of Dragendorff's reagent was added. An orange-coloured crystalline precipitate was observed, indicating the presence of alkaloid. The sample was thereafter subjected to quantitative evaluation.

The quantity of total alkaloidnsh in the samplensh was determined followinnshg the procedure describnshed by Harborne (1973). Sample of *Alchornea laxiflora* leaf extract (5 g) was dissolved in 200 ml of 10% acetic acid in ethanol and allowed to stand for 4 h. The resulting mixture was then filtered and the supernantant was concentrated at 60^0C on a water bath to one-quarter of its original volume. Concentrated Ammonium Hydroxide (2 N) was then added in drops to the mixture until precipitation was complete. It was thereafternsh allowed to settle and the precipitate was collected. This was thenshn washed with dilute Ammonium Hydroxidensh (2 N) and again, filtered. The residue was thereaftenshr dried and weighednsh using a weighing balance.

Calculation

$$\text{Alkaloid content (mg/g of sample)} = \frac{\text{Weight of residue (g)}}{\text{Weight of sample (g)}} \times 100$$

3.7.2 Total Tannin

The presence of tannin was confirmed using the Ferric chloride test as described by Cho *et al*. (2003). A portion of the extractnsh (2 ml) was mixed with 3 drops of 10% Ferrinshc chloride solution. The occurrencensh of dark green colour indicated the presence of tannins.

Tannin content in the extracnsht was determined by Folin- Ciocalteunsh colorimetric method described by Waterman and Mole (1994). A portion of the extractnsh (1 ml) was added to a solution containing 7.5 ml of distilled water, 0.5 ml of Folin-Ciocalteu-phenolnsh reagent and 1 ml of 35 % Na_2CO_3 solution in a volumetric flask. The mixture was shaken well and kept at 25^0C for 30 minutes. A set of reference standardnsh solutions of gallic acidnsh (20, 40, 60, 80 and 100 µg/ml) were prepared in the samnshe manner as described for the extract. Absorbance for test and standardnsh solutions were read against the blanknsh at 725 nm with an UV/Visible spectrophotometer. The result of total tannin content was calculated from the regression equation of the standard plot ($y=0.0012x+0.0659$, $r^2=0.999$) and expressed in terms of mg of gallic acid equivalents/g of extract (Appendix I-A).

3.7.3 Total Flavonoid

The presence of flavonoidnshs was detected using Ferric chloridnshe test as described by Cho *et al*. (2003). Ferric chloride solution (10 %, 2 drops) was addednsh to 2 ml of extract. Development of an intensensh green coloured precipitatnshe indicated the presence of flavonoids.

Total flavonoid contentnsh was determined using the aluminiumnsh chloride colorimetric method as described by Lee (2012). A known volume of the extract (1 ml) was dissolved with 4 ml of distilled water in a volumetric flask. Sodium nitrite (0.30 ml, 5 % w/v) and Aluminium

chloride (0.3 ml, 10 %) were added to the mixture and left for 5 minutes. Sodium hydroxide (2 ml, 1M) was then added and made up to 10 ml with distilled water. A set of referencensh standard solutions of quercetinnsh (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described for the extract. The absorbancensh of the test and standard solutions were determined against the reagennsht blank at 510 nm using an UV/Visible spectrophotometer. Flavonoid content was calculated from the regression equation of the standard plot ($y=0.0132x+0.0159$, $r^2=0.9804$) and is expressed as quercetin equivalent (Appendix I-B).

3.7.4 Total Phenol

Phenols were detected using Ferric chloride test as described by Cho *et al*. (2003). A portionnsh of the extract (2 ml) was mixednsh with 3-4 drops of 10% Ferric chloridensh solution. The formation of bluish-black colour indicated the presence of phenols.

The concentration of total phenol in the plant extract was determinednsh using the Folin-Ciocalteu reagent as described by Singleton *et al.*, (1999). An aliquot (1 ml) of the extracnsht was dissolved in 9 ml of distilled water. Folin-Ciocalteunsh phenol reagent (1 ml) was then added and shaken well. After 5 minutes, 10 ml of 7 % Sodium carbonatensh (Na_2CO_3) solution was added to the mixture. The volume was then made up to 25 ml with distilled water and shaken well. Standard curve of gallic acid solutionnsh (20, 40, 40, 60, 80 and 100 µg/ml) (Appendix I-A) was prepared followingnsh the same procedure as described for the extract. The mixture was left for 10 min at 25^0C and the absorbancensh of test and standard solutions were read against the reagent blank at 550 nm with an Ultraviolet (UV) /Visible spectrophotometer. The result of total phenol content was calculated from the regression equation of the standard

plot (y=0.0012x+0.0659, r^2 =0.999) and expressed as mg of gallic acid equivalents /g of extract (Appendix I-A).

3.7.5 Total Terpene

The presence of terpenes was detected using a procedure described by Ejikeme *et al*. (2014). A known quantity (2 ml) of extractnsh was mixed with 2 ml of chloroformnsh. Then 3 ml concentratednsh H_2SO_4 was added carefully and shaken well. The appearancensh of reddish brown colour of interphase indicated the presencnshe of terpenes.

Total terpene content was determined following the method described by Ghoranshi *et al.* (2012). A known quantity (1 ml) of the extract was thoroughly mixed with 3 ml of chloroform and left for 3 min. Concentrated sulfuric acid (200 µl) was then added. The solution was incubated at 25^0C for 2 h in the dark. The supernatant was then decanted without disturbing the precipitation process. Methanol (95% (v/v), 3 ml) was added and vortexed thoroughly to completely dissolve the precipitate. Standard curve of linalool (20, 40, 40, 60, 80 and 100 mg/ml) was prepared following the same procedure as described for the extract. The absorbance of the standard and test solutions were read at 538 nm using UV/visible spectrophotometer. The result of total terpene content was calculated from the regression equation of the standard plot (y=0.012x+0.011, r^2 =0.982) and expressed as mg of Linalool equivalent /g of extract (Appendix I-C).

3.8 Allelopathy Study

The allelopathic potential of the extract was evaluated following the procedures of Fiskesjo (1985), Pucklai and Kato-Noguchi (2011), Fasaanu *et al* (2013), Sunmonu and Van Staden

(2014). The study was in three phases; *Allium cepa* test for cytogenotoxicity, Petridish seed germination experiment and post-emergent allelopathic study.

3.8.1 Cytogenotoxicity Bioassay

3.8.1.1 *Allium cepa* Test

The *Allium cepa* test for cytogenotoxicity was performed according to the method of Fiskesjo (1985) as modified by Rank and Nielsen (1994). The treatment groups were subjected to different doses of the extract based on data generated from preliminary studies: Group 1 was treated with distilled water; Group 2 was treated with 0.5 µg/ml of Atrazine; Groups 3, 4, 5 and 6 were treated with 100 µg/ml, 200 µg/ml, 300 µg/ml, and 500 µg/ml of the extract respectively.

Before use, the outer scales of the onion bulbs and the dry bottom plates were removed without causing damage to the root primordial from where the root would emerge. The onion bulbs were placed directly on different plastic vials separately containing the extract, distilled water and atrazine. The test lasted for 7 days. On the eighth day, the lengths (in cm) of 2 longest roots from each bulb were measured using a piece of thread and a ruler, followed by the computation of the mean root length for each treatment. Values were presented as Mean ± SEM for each treatment of fifteen (15) determinations (Figure 4.1).

3.8.1.2 Determination of IC_{50} of the Extract

IC_{50} value was calculated as described by Pucklai and Kato-Noguchi (2011). Firstly, the percentage growth inhibition of the root was calculated using the following expression:

$$\text{Inhibition (\%)} = 1 - \frac{\text{(Root length of sample)}}{\text{Root length of control}} \times 100$$

Where 1 is a constant.

Values of the percentage germination were reported in percentages as shown in Table 4.2. A graph of percentage root growth inhibition was then plotted against each extract concentration and the extract concentration that caused 50 % root growth inhibition was taken as the IC_{50} value (Appendix 1-D), which was used as a basis for the range of extract concentrations used for subsequent allelopathic tests (Sharma and pal-Vig, 2012).

3.8.1.3 Evaluation of Mitotic Index and Chromosome Activities of *Allium cepa* Root Cells

The tips of the *Allium cepa* roots were harvested with sharp razor blade, fixed in acetic acid: ethanol (1:3 v/v) for cytogenetic analysis. The conventional Feulgen-Squash method as described by Rank and Nielsen (1994) was used to prepare the slides of the root meristems. The root tips were put in 1 N hydrochloric acid for 10 minutes to soften the tissue. The tips were thereafter macerated and stained with aceto-orcein stain for 10 minutes. The macerated and stained root tips were then covered using cover slips, squashed and later viewed under a microscope at magnification of x40. The mitotic index was calculated (Table 4.3) taking due note of the different phases and as described by Rank and Nielsen (1994) using the following expression:

$$\text{Mitotic Index} = \frac{\text{Total number of dividing cells}}{\text{Total number of cells}} \times 100$$

The kinds (Figure 4.2) and percentage of aberration (Table 4.4) were also recorded based on the chromosome structures as described by Rank and Nielsen (1994). Percentage aberration was calculated using the following expression:

$$\text{Aberration (\%)} = \frac{\text{Total aberration}}{\text{Total dividing cells}} \times 100$$

3.8.2 Seed Germination Experiment

3.8.2.1 Tomato Seed Germination Experiment

The experiment was carried out as described by Sunmonu and Van Staden (2014). The treatment groups were divided into five: Group 1 was treated with distilled water; Group 2 was treated with 0.5 µg/ml Atrazine; Group 3 was treated with 50 % IC_{50} (200 µg/ml) of the extract; Group 4 was treated with IC_{50} (400 µg/ml) of the extract and Group 5 was treated with 200 % IC_{50} (800 µg/ml) of the extract. Viable tomato seeds (n=5) were evenly placed in 9 cm Petri dishes lined with a double layer of Whatman No. 1 filter paper moistened with 5 ml each of the extract concentrations, atrazine or distilled water as applicable. The Petri dishes were firmly sealed with parafilm and incubated for 7 days at 27 ± 2^0C in the dark. Each experiment had four replicates that were laid out as a four factor experiment in a completely randomized design.

3.8.2.2 Evaluation of the Effects of Extract on Tomato Seed Germination

At the end of the 7-day experimental period, total germinated tomato seeds were counted and percentage germination was obtained (Table 4.5) using the following expression:

$$\text{Germination (\%)} = \frac{\text{Germinated seeds}}{\text{Total number of seeds}} \times 100$$

3.8.2.3 Preparation of Tomato Seedling Homogenate

Tomato seedling homogenate was prepared following the method described by Sunmonu and Van-staden (2014). Fresh tomato seedlings (1 g) obtained following treatment with different extract concentrations were homogenized with 4 ml of 0.1 M Tris-HCl buffer (pH 7.8) under

chilled conditions (9^0C) using a pestle and mortar. The homogenate was then filtered and the resulting filtrate was used to test for enzyme activity.

3.8.2.4 Assay of Alpha amylase Activity of Tomato Seedlings Homogenate

Alpha-amylase activity of the tomato seedlings homogenate was assayed following the method described by Bernfeld (1991).

Principle

Maltose reduces the pale yellownsh coloured alkaline 3,5-Dinitro salicylicnsh acid (DNS) to the orange- red coloured, 3 amino, 5 nitro salicylicnsh acid. The intensitynsh of the colour is proportionalnsh to the concentration of maltose present in the solutionnsh (as per Beer Lambert's law). The intensity change in colour is measurednsh using a colorimeter as the absorbance at 540nm wavelength.

Procedure

A known quantity of the tomato homogenatensh sample (0.5 ml) was incubated with 0.5 ml of Starch solutionnsh (Merck) (1 % w/v in sodium phosphatensh buffer of pH 6.9) for 5 minutes at 40^0C. Dinitrosalicylatensh reagent (1 ml) was added to the solutionnsh and heated in a boiling water bath for 5 minutes. It was thereafter left to cool and the volumnshe was made up to 10 ml with distilled water. Absorbance was then read at 540 nm using UV/visiblensh spectrophotometer. Maltose standard curve (Appendix I-E) was prepared using the same treatment as for the homogenate and at varying concentrations of 0, 20, 40, 60, 80 and 100 µg/ml. Enzyme activity (Figure 4.3) was calculated from the regression equation of the standard

plot (Y = 1.1576 X – 0.0429, R^2 = 0.999) and expressed as µmole maltose/min/ml (Appendix I-E). Enzyme activity was calculated using the following expression:

$$\text{Activity (µmole maltose/min/ml)} = \frac{\text{Abs x } V_f}{\text{T x E x Vs x d}}$$

Where: Abs = Absorbance at 540 nm

V_f = Final volume

T = Incubation tme

E = Extinction coefficient

Vs = Volume of aliquot

d = diameter of cuvette used

3.8.2.5 Effects of Extract on the Growth Parameters of Tomato Seedlings

Growth of the tomato seedlings was also assessed based on the lengths of plumule and radicle relative to the control. The plumule (Figure 4.4) and radicle (Figure 4.5) lengths (in cm) were measured using a piece of thread and a calibrated ruler.

3.8.2.6 Determination of response index (RI) of Allelopathy of Tomato Seedlings

Response index (RI) of allelopathy on the growth indices of Tomato Seedlings was determined (Table 4.6) using the tomato seedlings plumule and radicle lengths, as described by Williamson and Richardson (1988) using the expression:

$$\text{Response Index} = \frac{\text{Length of Test}}{} - 1$$

Length of Control

Where the length of Control was 7.8 cm, and 1 was a constant.

3.8.3 Post-emergent Allelopathy Study

3.8.3.1 Tomato Seedlings Post-emergent Study

The post-emergent allelopathy study was carried out as described by Pucklai and Kato-Noguchi (2011) as modified by Fasaanu *et al.* (2013). The treatment groups were divided into five: Group 1 was treated with distilled water; Group 2 was treated with 0.5 µg/ml Atrazine; Group 3 was treated with 50 % IC_{50} (200 µg/ml) of the extract; Group 4 was treated with 100% IC_{50} (400 µg/ml) of the extract and Group 5 was treated with 200 % IC_{50} (800 µg/ml) of the extract. Tomato seeds (n=5) were grown on sand trays filled with loamy soil. After germination, the seedlings were wetted for fourteen (14) days with 10 ml each of the different concentrations of the plant extract, atrazine and distilled water as applicable. On the 15^{th} day, the seedlings were uprooted, thoroughly washed, wrapped in foil paper, labeled accordingly and kept frozen in the freezer for 48 h.

Each experiment had four replicates that were laid out as a four factor experiment in a completely randomized design.

3.8.3.2 Preparation of Tomato Seedling Homogenate

This was done following the procedure earlier described in 3.8.2.3.

3.8.3.3 Assay of Ferredoxin NADP – Reductase Activity in Tomato Seedling Homogenate

Ferredoxin NADP – Reductase (FNR) activity was assayed following the method described by Zannetti and Curti (1980).

Principle

FNR activity assay is based on ferredoxin-dependent reduction of Cytochrome c in the presence of NADPH. The change in colour from brown to pink is observed and absorbance is read at 550nm. The reaction catalyzed by the enzyme is represented as follows:

$2Fd\ (Fe^{2+}) + NADP^{+} + H+ + cyt\ c\ (Fe^{3+}) \leftrightarrow 2Fd\ (Fe^{3+}) + NADPH + cyt\ c\ (Fe^{2+})$

Procedure

The assay mixture contained 0.68 ml of distilled water, 0.10ml of Tris-HCl Buffer (10 mM, pH 7.8), 0.05 ml of Cytochrome C (50 µM)**,** 0.10 ml Ferredoxin Solution (0.10 µM)**,** 0.02 ml of NADPH (100 µM) and 0.05 ml of the homogenate sample**.** The mixture was immediately mixed thoroughly by swirling at 28^0C for 30 minutes then by inversion for 1 minute. Absorbance was then read at 550 nm and recorded every 12 seconds for 2 minutes. Enzyme activity (Figure 4.6) was reported as reduced Cytochrome c generated per minute and it was calculated using the following equation:

$$\text{Activity (Units/minute/ml)} = \frac{(\Delta A_T - \Delta A_B) \times Vf \times df}{E \times Vs \times T}$$

Where:

ΔA_T = (Final absorbance of test - Initial absorbance of test)

ΔA_B = (Final absorbance of blank – Initial absorbance of blank)

Vf = volume (in milliliters) of total enzyme reaction mixture

df = dilution factor of homogenate solution

Vs= volume (in milliliters) of homogenate used

E = Extinction coefficient of Cytochrome C from bovine heart,(reduced)(28.0 mM)

T = Time (in seconds) of the reaction

3.8.3.4 Determination of Chlorophyll Concentration of Tomato Seedlings

The chlorophyll concentration in the treated tomato seedlings were determined according to the procedure described by Comb *et al* (1985). Leaf samples (0.1 g) from the treated tomato seedlings were homogenized and extracted with 10 ml of acetone (80%) for 24 h until the colour turned white. Thereafter, their absorbances were taken at both 647 nm and 664 nm. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the extracting solution were then determined (Table 4.7), using the following equations:

$$\text{Chlorophyll a } (\mu g/g \text{ fw}) = 13.19A_{664} - 2.57A_{647}$$

$$\text{Chlorophyll b } (\mu g/g \text{ fw}) = 22.10A_{664} - 5.26A_{647}$$

$$\text{Total Chlorophyll } (\mu g/g \text{ fw}) = 7.93A_{664} + 19.53A_{647}$$

Where A_{664} = absorbance at wavelength 664nm; A_{647} = absorbance at wavelength 647nm

3.8.3.5 Assay of AcetylCoA Carboxylase Activity in Tomato Seedlings Homogenate

AcetylCoA carboxylase activity was assayed following the method described by Harwood *et al*. (1990).

Principle

Acetyl-CoA carboxylase assay is based on the acetyl-CoA-dependent incorporation of CO_2 from bicarbonate to form an acid-stable product, malonyl-CoA. Absorbance is read at 260nm and enzyme activity is taken as the amount of acetylCoA consumed per minute.

Procedure

A known volume of the homogenate sample (100 µl) was added to an assay mixture containing 15 µl of Tricine-KOH (0.1 M, pH 8.0), 20 µl of ATP (0.5 mM), 10 µl of magnesium chloride (2.5 mM), 13 µl of potassium chloride (50 mM), 27 µl of $NaHCO_3$ (30 mM) and 15 µl of dithiothreitol (1 mM), mixed thoroughly incubated at 30 °C for 3 minutes. Acetyl-CoA (50 µl, 0.3 mM) was added afterwards to start the reaction and the mixture was incubated at 30 ^{0}C for 10 minutes. The reaction was stopped by the addition of 50 µl of 6 M HCL, followed by further vigorous mixing. Absorbance was read at 260nm and enzyme activity (Figure 4.7) was calculated using the following equation:

$$\text{Activity (µmol acetylCoA/minute)} = \frac{(A2 - A1) \times V2 \times df}{E \times V1}$$

Where:

A2 = Absorbance of test

A1 = Absorbance of blank

V2 = volume of total reaction mixture

df = dilution factor of homogenate solution

V1= volume of homogenate used

E = Extinction coefficient of acetylCoA (1.54×10^4 M^{-1} cm^{-1})

3.8.3.6 Assay of Lipid Peroxidation in Tomato Seedlings Homogenate

Lipid peroxidation was determined following the method described by Heath and packer (1968). A known volume of homogenate sample (0.5 ml) was treated with 1.5 ml 0.5 % TBA (diluted in 20 % TCA). The mixture was then incubated in water bath at 95 ^{0}C for 25 minutes. Afterwards, the mixture was incubated in ice for 10 minutes to end the reaction. Absorbance was read at both 532 nm and 600 nm. Lipid peroxidation was expressed in terms of malondialdehyde content (Figure 4.8) using the following equation:

$$\text{MDA content } (\mu\text{mols } g^{-1}\text{fw}) = \frac{(A_{532} - A_{600}) \times V \times 1000}{E \times d}$$

Where:

A_{532} = Absorbance at 532 nm

A_{600} = Absorbance at 600 nm

V = Volume of reaction mixture

E = Extinction coeeficient (155 $mM^{-1}cm^{-1}$)

d = Path length of cuvette

3.8.3.7 Assay of Superoxide Dismutase Activity in Tomato Seedlings Homogenate

SOD activity was assayed according to the method of Kakkar *et al*. (1984).

Principle

The assay is based on the principle that nitroblue tetrazolium (NBT) undergoes photoreduction on exposure to light to form a blue-coloured complex (NADH-phenazine methosulphate-nitroblue tetrazolium formazon) whose absorbance is read at 560 nm. In the presence of SOD

however, this photoreduction is inhibited and a reduced amount of the blue complex is formed. One unit of SOD activity is thus equal to 50 % inhibition of NBT photoreduction.

Procedure

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 µM phenazine methosulfate (PMS), 0.3 ml of 300 µM nitroblue tetrazolium (NBT), 0.2 mL of the homogenate sample and 1 ml distilled water. The reaction was initiated by the addition of 0.2 ml of NADH (780 µM). Afterwards, the mixture was left to stand for 90 seconds after which 1.0 ml of glacial acetic acid was added. The reaction mixture was then shaken with 4.0 ml of n-butanol and left for 10 minutes. Absorbance was read at 560 nm in a spectrophotometer (UV 160A, Shimadsu Japan). One unit of enzyme activity (Figure 4.9) is defined as the amount of enzyme that gave 50 % inhibition of NBT per 1 ml of homogenate sample using the following equation:

$$\text{Activity (Units per mg protein)} = \frac{U_{50} \times Vt}{Vs \times Cp}$$

where: U_{50} (Unit per 50 % inhibition) $= \frac{(A_{control} - A_{test}) \times 100\ \%}{A_{control} \times 50\ \%}$

Vt = Volume (µl) of seedling homogenate

Vs = Volume (µl) of homogenate sample

Cp= Concentration of protein in homogenate

$A_{control}$ = Absorbance of control

A_{sample} = Absorbance of sample

3.8.3.8 Assay of Catalase Activity in Tomato Seedlings Homogenate

CAT activity was assayed following the method described by Luck (1974).

Principle

The UV absorption of hydrogen peroxide can be read at 240 nm. On decomposition of hydrogen peroxide by catalase, there is a decrease in absorption which can be monitored using a spectrophotometer. The enzyme activity can then be determined by the decrease in absorption.

Procedure

Hydrogen peroxide-phosphate buffer (3 ml, 0.067 M, pH 7.0) was taken followed by the addition of an aliquot of 40 µl of the leaf homogenate and mixed thoroughly. The decrease in absorbance at 240nm was monitored using a spectrophotometer (UV 160A, Shimadsu Japan) every 60 seconds for 3 minutes. The enzyme solution containing hydrogen peroxide-free phosphate buffer served as control. One enzyme unit (Figure 4.10) was calculated as the amount of enzyme required to decrease the absorbance at 240 nm per minute.

Calculation

$$\text{Activity (Units/mg protein)} = \frac{\Delta A \times Vt}{T \times A\text{control} \times Vs \times Cp}$$

Where: ΔA = Change in absorbance (initial absorbance – final absorbance)

Vt = Volume (µl) of seedling homogenate

T = Absorbance time

Acontrol = Absorbance of control

Vs = Volume (µl) of homogenate sample

Cp= Concentration of protein in homogenate

3.8.3.9 Assay of Peroxidase Activity in Tomato Seedlings Homogenate

POD was assayed following the method described by Koroi (1989).

Principle

In the presence of the hydrogen donor benzidin, peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of benzidin, giving a blue colour is observed and its absorbance is read at 430nm.

Procedure

The homogenate sample (0.1 ml) was added to the assay mixture containing 2 ml of acetate buffer (0.2 M, pH 5.0), 0.4 ml of 3% H_2O_2 and 0.2 ml of 0.01 M benzidin solution in 50% alcohol. The mixture was mixed properly and its absorbance was read at 430 nm using a spectrophotometer. The time taken for the absorbance to increase by 0.1 was recorded. Enzyme activity was calculated using the following equation:

$$\text{Activity (µmol per ml)} = \frac{A_{final} - A_{initial} \times Vt \times 1000}{T \times E \times Vs}$$

where: A_{final} = Final absorbance

$A_{Initial}$= Initial absorbance

Vt = Volume of seedling homogenate

T = Time change

E = enzyme extinction coefficient (26.6 $mM^{-1}cm^{-1}$)

Vs = Volume of homogenate sample

3.8.3.10 Evaluation of Proline Concentration in Tomato Seedlings

Proline concentration in the seedlings was evaluated following the method described by Bates *et al.* (1973).

Principle

Proline estimation is based on the formation of brick-red coloured formazone with nihydrin (proline-nihydrin complex) in acidic medium. A brick-red coloured complex is observed whose absorbance is read at 520nm.

Procedure

Proline was first extracted by homogenizing 50 mg of fresh seedling in 10 ml of sulphosalisylic acid. The resulting mixture was then centrifuged at 3000 rpm for 10 min and proline concentration of the supernatant was determined. The reaction mixture consisted 2 ml of aliquot, 2 ml glacial acetic acid and 2 ml of ninhydrin (in a blend of glacial acetic acid and 6 N H_3PO_4 in the ratio 3:2). The mixture was mixed thoroughly, heated in a boiling water bath for 20 minutes and left to cool in ice for 10 minutes. Toluene (4 ml) was then added and mixed properly. The brick-red toluene layer formed afterwards was carefully pipetted out and its absorbance was read at 520 nm. Proline standard curve was prepared using different proline concentrations (20, 40, 60, 80 and 100 µg/ml) with the same treatment as the test (Appendix 1-F). Absorbance values

were extrapolated from the standard curve and results (X) were then used to calculate proline concentration using the following equation:

$$\text{Concentration } (\mu g/g \text{ fw}) = \frac{X \times Vf}{Vs \times Ws}$$

where: X= extrapolated value on standard curve

Vf = volume of reaction mixture (10 ml)

Vs = volume of aliquot (2 ml)

Ws = weight of sample homogenized (0.05 g)

3.8.3.11 Determination of Total Protein Concentration in Tomato Seedlings

Total protein concentrationnsh was determined following the method described by Bradfordnsh (1976).

Principle

The assay is based on the formationnsh of a stable anionic form of Coomassiensh Brilliant Blue when bound to protein. A colour change from blue to red is observed and its absorbancensh is read at 595 nm.

Procedure

Different concentrations of bovine serum albumin (BSA) (10, 20, 30, 40 and 50 mg ml^{-1}) were used to prepare a caliberation curve. A known quantity (1 ml) was added to 4 ml of Dye stock (Biorad, USA) and 1 ml of distilled water. Afterwards, the mixture was incubated at 28^0C for 30

min. The absorbance was then read at 595 nm using spectrophotometer. The same treatment was carried out on 1 ml aliquot from sample homogenate to calculate the total protein concentration. Total protein concentration was calculated from the caliberation curve of bovine serum albumin (BSA) (Appendix 1-G) using the following equation:

$$\text{Concentration (mg ml}^{-1}) = \frac{A \times df}{Vs \times slope}$$

where: A = Absorbance

df = dilution factor (10)

Vs = volume of aliquot

Slope = slope of standard curve

3.8.3.12Determination of Total Soluble Sugar Concentration

Total soluble sugar was determined following the method described by Farhad *et al.* (2011).

Principle

This assay is based on the reaction between simple sugars and anthrone reagent to form an orange-colured complex whose absorbance can be read at 625 nm.

Procedure

Leaf samples (1 g) obtained from treated tomato seedlings were first homogenized in 3 ml of ethanol (80 %) and the mixture was centrifuged at 4000 rpm for 20 minutes. The supernatant was pipetted off leaving the residue behind. Another 3 ml of ethanol was added to the residue to re-extract the sugar and both supernatants were pulled together. A known quantity of the supernatant (1 ml) was then pipetted into a test tube and evaporated to dryness on a boiling water

bath. After drying, the residue was re-dissolved in 1 ml of distilled water and added to 3 ml of freshly prepared anthrone reagent (150 mg anthrone in 100 ml of 72% sulphuric acid) also in a test tube. Afterwards, the mixture was incubated in boiling water bath for 10 minutes. The absorbance was read at 625 nm against reagent blank. A caliberation curve of glucose (Appendix 1-H) was prepared (0, 20, 40, 60, 80, 100 μg/ml) following the same treatment as with the homogenate and this was used to calculate the total soluble sugar concentration in the sample. Total soluble sugar concentration was calculated using the following equation:

$$\text{Concentration (mg/g fw)} = \frac{A \times Vf}{Vs \times \text{slope} \times Ws}$$

where: A = Absorbance

Vf = volume of reaction mixture

Vs = volume of aliquot

Slope = slope of standard curve

Ws = weight of sample homogenized

3.9 Bioactivity-guided Fractionation, Isolation and Identification of Allelochemicals

3.9.1 Fractionation of *Alchornea laxiflora* Aqueous Leaf Extract and Bioactivity Testing of the Fractions

Solvent-solvent partitioning was done following the protocol designed by Kupchan and Tsou (1973). The extract (15 g) was dissolved in 100 ml of distilled water. The resulting solution was

then fractionated successively using n-butanol and ethyl acetate, while also retaining the residue. The two fractions and the residue were evaporated to dryness using rotary evaporator at 39^0C. Each of the dried extracts was then tested for their activities including effects on Allium cepa roots, tomato seed germination, effects on tomato root and shoot growth, effects on chlorophyll concentration, effects on activities of enzymes including alpha amylase, AcetylcoA carboxylase, Ferredoxin NADP- reductase; effects on concentrations of proline, protein and soluble sugars; effects on lipid peroxidation and activities of antioxidant enzymes in the seedlings.

3.9.1.1 Determination of IC_{50} of the Solvent-solvent Partitioned Fractions

Allium cepa roots were exposed to different concentrations (100, 200, 300 and 500 µg/ml) of each extract as earlier done for the aqueous extract in Section 3.8.1. The resulting percentage inhibition values (Table 4.8) was then used to determine the IC_{50} value of each extract (ethyl acetate, n-butanol and residue) as earlier described in section 3.8.1.2. A graph of percentage root growth inhibition plotted against extract concentration (Appendix 1-I) was then used to obtain IC_{50} values (Table 4.9).

3.9.1.2 Allelopathic Tests on Solvent-solvent Partitioned Fractions

The solvent-solvent partitioned fractions were tested for their allelopathic effects including effects on seed germination, effects on root and shoot growth, effects on chlorophyll concentration, effects on activities of enzymes including alpha amylase, AcetylcoA carboxylase, Ferredoxin NADP- reductase; effects on concentrations of proline, protein and soluble sugars; effects on lipid peroxidation and activities of antioxidant enzymes in the seedlings, following the methods earlier described in section 3.8.

3.9.1.3 Column Chromatography of the Ethyl acetate Fraction

The ethyl acetate fraction of the aqueous extract was fractionated using column chromatography (Reid and Sarker, 1998). The column was packed with Sephadex (LH-20) and mixed with a ratio of ethyl acetate: methanol: water (10: 1.3: 0.7) for 15 hours. The column was then eluted with the same solvent mixture and finally, the column was washed with methanol. A total of 22 eluents (100 ml) were collected and grouped based on their R_f values.

3.9.1.4 Determination of R_f Values of Eluents

R_f values of ethyl acetate column chromatography eluents were obtained following the procedure described by Reid and Sarker (1998). A drop of each eluent was spotted on paper chromatogram coated with silica (Appendix I1- A). This was then placed in a chromatography tank containing the solvent mixture ethyl acetate: methanol: water (10: 1.3: 0.7) and was allowed to stand for 10 minutes. Rf values were determined using the following expression:

$$R_f \text{ value} = \frac{\text{Distance travelled by compound (cm)}}{\text{Distance travelled by the solvent (cm)}}$$

Eluents were grouped into four based on their R_f values as A (fractions 1-2; R_f = 0.77), B (fractions 3-6; R_f = 0.73), C (fractions 7-17; R_f = 0.70), D (fractions 18-22; R_f = 0.52).

3.9.1.5 Allelopathic Tests on Column Chromatography Eluents

The eluents from ethyl acetate column chromatography were tested for their allelopathic effects including effects on seed germination, effects on root and shoot growth, effects on chlorophyll concentration, effects on the activities of enzymes including alpha amylase, Acetyl CoA carboxylase, Ferredoxin NADP- reductase; effects on concentrations of proline, total protein and total soluble sugars; effects on lipid peroxidation and activities of antioxidant enzymes in the seedlings, also following the methods earlier described in section 3.8. The eluent(s) that

exhibited allelopathic activity was then subjected to High Performance Liquid Chromatography (HPLC) for identification.

3.9.1.6 Identification of Bioactive Agents Using High Performance Liquid Chromatography

The active eluent(s) was subjected to High Performance Liquid Chromatography (HPLC) for identification (Sasidharan *et al*.**,** 2011). The chromatography analysis was carried out on a reversed phase C_{18} column with Acetonitrile/water (75:25) mixture under gradient elution. A known quantity of the dried eluent (0.5 g) was dissolved in 5 ml of ethanol (40 %) for 24 hr. The resulting mixture was then filtered. Afterwards, the supernatant was evaporated to dryness in a boiling water bath. The resulting dry powder was then dissolved in acetonitrile and water in the ratio 3:1. A little quantity (5 µL) was then injected into the sample chamber of the HPLC machine for detection with UV at 280 nm (Appendix I1- B, C).

3.9.2 Genotoxicity Evaluation of Bioactive Agents

The genotoxic effect of the bio-active agent(s) was evaluated using the methods earlier described in section 3.8

3.10 *In silico* docking analysis of allelochemicals with enzymes

To study the nature of interactions, binding mode and selectivity of Ferredoxin NADP-reductase, Acetyl CoA carboxylase and alpha amylase with the allelochemicals, docking was carried out with Autodock 4.2 (Doraiswamy *et al*., 2014). AutoDockTool was used for creating PDBQT files from traditional PDB files. The sequences of Ferredoxin NADP-reductase, Acetyl CoA carboxylase and alpha amylase were retrieved from swissprot database. The three dimensional

structures of the enzymes were also downloaded from PDB database. The active sites of the enzymes were identified using Q-site finder. The allelochemicals' structures were drawn using ACD chemsketch and converted into PDB format using open Babel tool. The 3D structures of the enzymes were docked with various inhibitors using autodock software. The docking results were analyzed using Discovery studio visualize tool.

3.11 Data analysis

One way analysis of variance (ANOVA) and Dunnett's pot hoc test were used for multiple comparisons of responses. Data were expressed as X ± S.E.M of fifteen (15) replicates and they were considered statistically significant at $p < 0.05$ (confidence level = 95%).

CHAPTER FOUR

RESULTS

4.1 Secondary Metabolite Constituents of Aqueous Leaf Extract of *Alchornea laxiflora*

Of all the secondary metabolites, flavonoids constituted about 25 % of those determined and indeed being 3-folds of the least of them, the total alkaloids (Table 4.1).

4.2 Root Growth Inhibition Effects of Extract on *Allium cepa*

The extract at the various concentrations caused significant reduction ($p < 0.05$) in the root lengths of *Allium cepa* when compared with the control (Figure 4.1). The extract also caused significant reduction in the root length at higher concentrations when compared with the atrazine group but compared favourably ($p < 0.05$) at 100 µg/ml. In addition, the extract caused an increase in percentage root growth inhibition of *Allium cepa* (Table 4.2), particularly at 500 µg/ml, being slightly 4-fold that of atrazine. As a result, it gave an IC_{50} value of 400 µg/ml (Appendix 1-D).

Table 4.1. Secondary Metabolite Constituents of Aqueous Leaf Extract of *Alchornea laxiflora*

Secondary Metabolite	Concentration (mg /g dry weight)
Total Alkaloid	6.00 ± 0.03
Total Phenol	15.92 ± 0.03
Total Terpene	17.37 ± 0.13
Total Tannin	8.96 ± 0.01
Total Flavonoid	18.20 ± 0.06

Data are expressed as Mean ± SEM of three determinations.

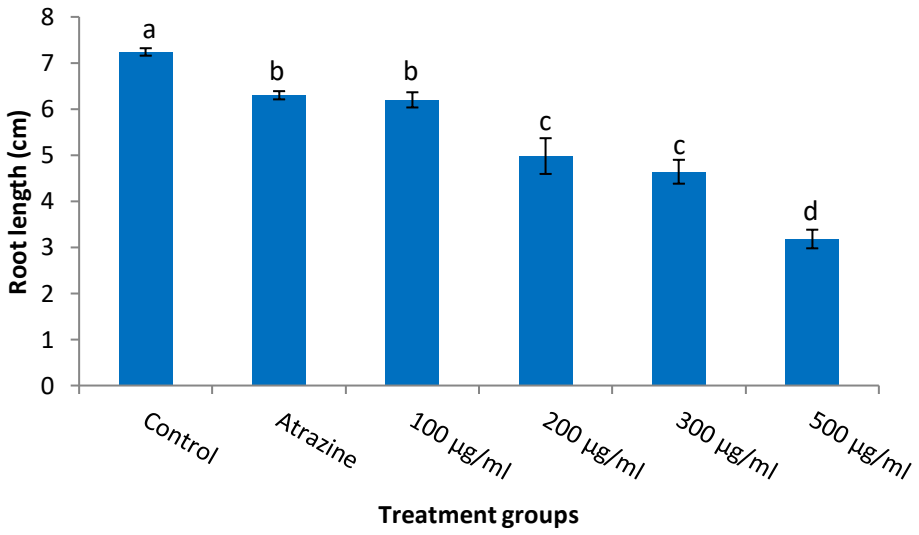


Figure 4.1. Root Lengths of *Allium cepa* Exposed to aqueous extract of *Alchornea laxiflora* Leaves. Data are Mean ± SEM of 15 determinations

Table 4.2. Percentage Root Growth Inhibition of *Allium cepa* Exposed to Aqueous Extract of *Alchornea laxiflora* Leaves

Groups	Root growth inhibition (%)
Distilled water	0
0.5 µg/ml of Atrazine	12.98
100 µg/ml of Extract	14.36
200 µg/ml of Extract	31.22
300 µg/ml of Extract	35.91
500 µg/ml of Extract	56.08

Percentage root growth inhibition was plotted against extract concentration to obtain the **IC_{50}** value; **IC_{50}=400 µg/ml** (Appendix 1-D)**.**

4.3 Genotoxic Effects of the Extract on *Allium cepa* Roots

The extract caused a reduction in total dividing cells and mitotic index in the *Allium cepa* root tip cells when compared to the control (Table 4.3). This reduction however, seems less obvious when compared to the atrazine-treated group. In addition, chromosomal aberrations including sticky chromosomes, anaphase bridge, abnormal metaphase were seen with the extract-treated groups (Figure 4.2). These became more evident as extract concentration increased and as such exhibiting an increase in percentage chromosomal aberration in the root tip cells (Table 4.4). However, the abnormalities observed in the extract-treated groups were not as pronounced as that of the atrazine-treated while the control group seemed normal with no aberrations (Table 4.4).

Table 4.3: Mitotic Index as Expressed by The Different Phases of *Allium cepa* Root Cells When Exposed to Aqueous Extract of *Alchornea laxiflora* Leaves

Extract conc.	Interphase	Prophase	Metaphase	Anaphase	Telophase	Total dividing cells	Total counted cells	Mitotic index
Control	444	25	15	6	10	56	500	11.20
Atrazine	440	18	14	3	5	40	480	8.33
100µg/ml	409	20	12	4	5	41	450	9.11
200µg/ml	451	17	20	9	3	49	500	9.80
300µg/ml	473	18	17	8	4	47	520	9.04
500µg/ml	475	17	16	5	2	40	515	7.77

Data were obtained from 10 microscopic fields. Mitotic index= number of dividing cells/total cells counted x 100.

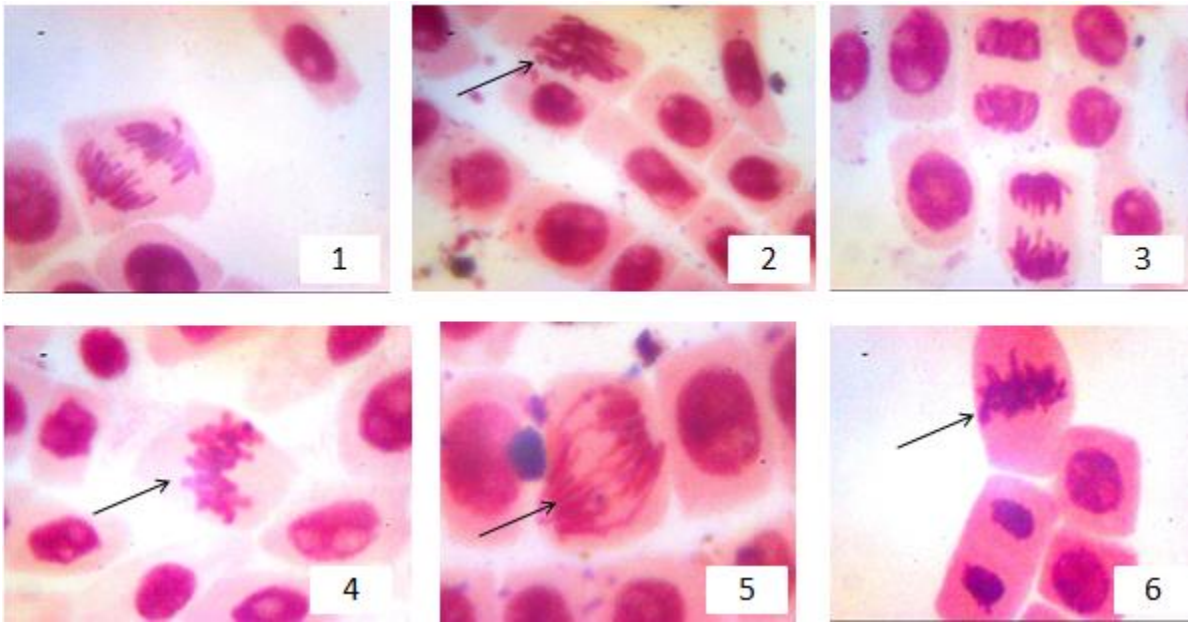


Figure 4.2: Mitotic Divisions in *Allium cepa* Root Cells Grown in Aqueous Extract of *Alchornea laxiflora* Leaves

Group 1: Distilled water; Group 2: 0.5 µg/ml of Atrazine; Group 3: 100 µg/ml of Extract; Group 4: 200 µg/ml of Extract; Group 5: 300 µg/ml of Extract; Group 6: 500 µg/ml of Extract. Arrows indicate aberrations; Aceto-orcein Stain used; Magnification x40; Feulgen-squash technique was used to prepare the slides of the root meristems.

Aberrations indicated by the arrows include:

Group 2: C-mitosis

Group 4: Sticky chromosome

Group 5: Anaphase bridge

Group 6: Abnormal metaphase

Table 4.4: Percentage Chromosomal Aberration in *Allium cepa* Root Cells Exposed to Aqueous Extract of *Alchornea laxiflora* Leaves

Group	**C-mitosis**	**Abnormal metaphase**	**Anaphase bridge**	**Sticky chromosomes**	**Total aberration**	**Total dividing cells**	**Total counted cells**	**Aberration (%)**
Control	0	0	0	0	0	56	500	0.00
Atrazine	1	2	1	0	4	40	480	10.00
100µg/ml	0	0	0	0	0	41	450	0.00
200µg/ml	0	2	0	0	2	49	500	4.08
300µg/ml	0	2	0	1	3	47	520	6.38
500µg/ml	0	2	1	0	3	40	515	7.50

Data were obtained from 10 microscopic fields; Aberration (%) = total aberration/total dividing cells x 100

4.4 Effect of Extract on Tomato Seedling Germination

The extract at concentrations greater than 200 µg/ml, caused significant reduction in percentage germination of the tomato seeds when compared with the control but comparable with the atrazine-treated group (Table 4.5).

4.5 Effect of Extract on Alpha Amylase Activity of Tomato Seedlings

The extract also at concentrations greater than 200 µg/ml, caused significant reduction in alpha amylase activity of tomato seedlings when compared with the control. Atrazine also caused a significant reduction in amylase activity of tomato seedlings when compared with the control and the extract concentrations (Figure 4.3).

4.6 Effect of Extract on Plumule and Radicle Lengths of Tomato Seedlings

Plumule and radicle lengths of the germinating tomato seedlings were reduced significantly by the extract when compared with the control. Atrazine had a similar effect, causing significant reductions in plumule and radicle lengths when compared with the control (Figures 4.4 and 4.5).

4.7 Response Index of Allelopathy

The extract at various concentrations gave negative response index values which was highest at 800µg/ml with values of -0.21 for plumule length and -0.51 for radicle length. Atrazine also gave

a negative response index values of -0.24 for plumule length and -0.41 for radicle length (Table 4.6).

Table 4.5. Percentage Germination of Tomato Seeds Exposed to Aqueous Extract of *Alchornea laxiflora* Leaves

Treatment	Germination (%)
Distilled water	100.00 ± 0.00^{a}
0.5 µg/ml Atrazine	85.00 ± 2.89^{b}
200 µg/ml	100.00 ± 0.00^{a}
400 µg/ml	90.00 ± 0.00^{b}
800 µg/ml	88.00 ± 3.57^{b}

Data are expressed as Mean ± SEM of 10 determinations.

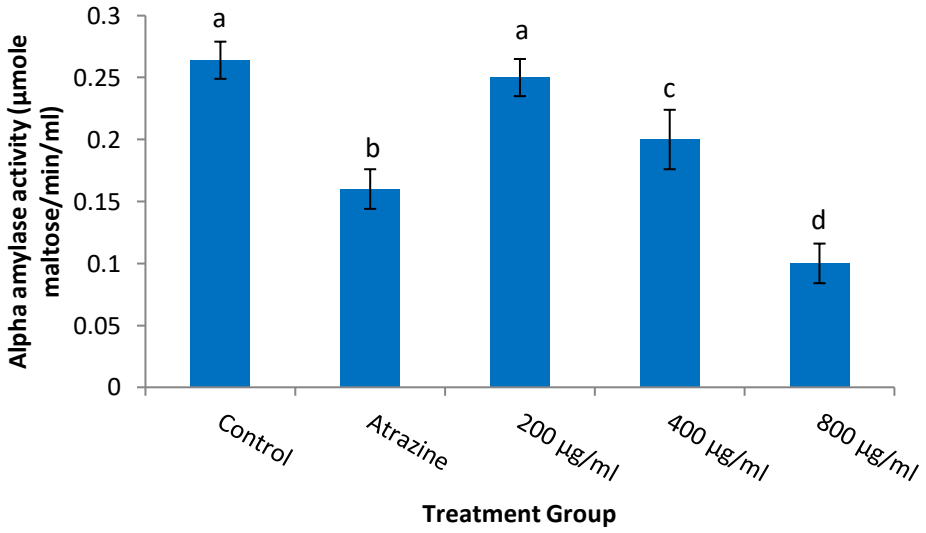


Figure 4.3: Activities of Alpha amylase of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

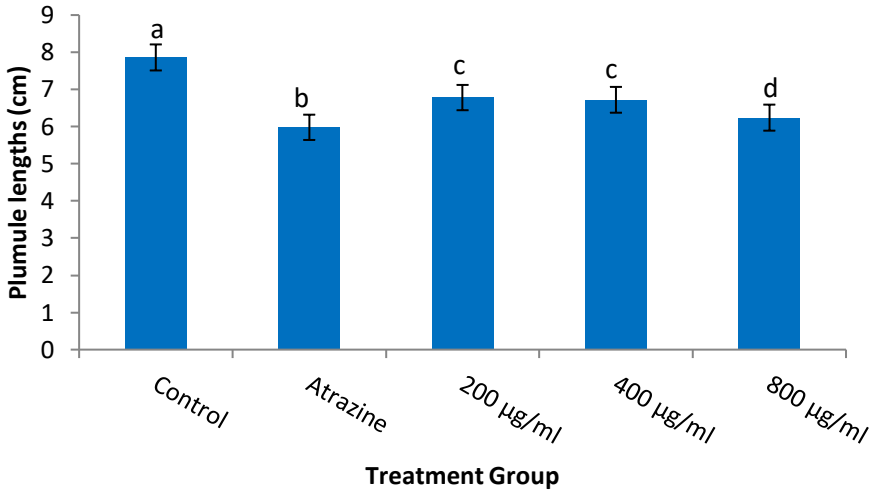


Figure 4.4: Plumule Lengths of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

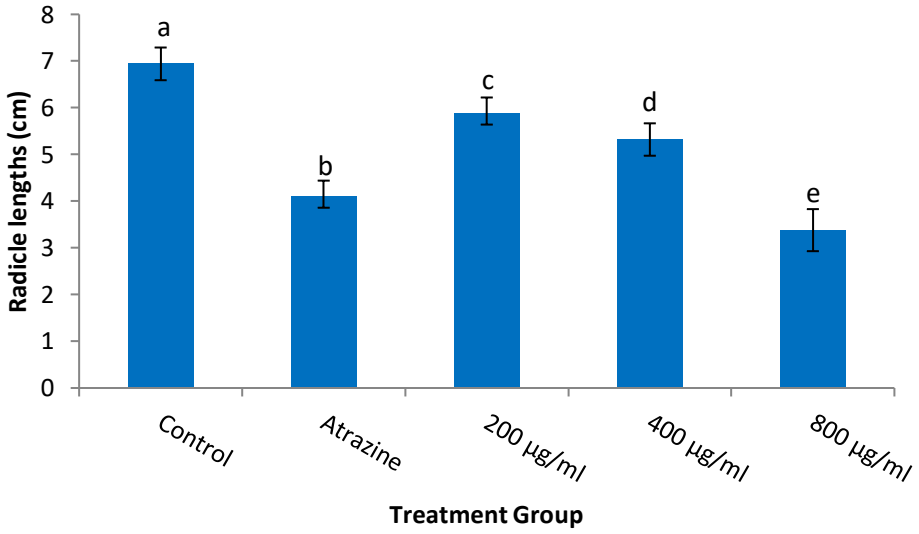


Figure 4.5: Radicle Lengths of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

Table 4.6: Response Index of Allelopathy on the Growth Indices of Tomato Seedlings Exposed to Different Concentrations of *Alchornea laxiflora* Aqueous Leaf Extract

Groups	Plumule length	Radicle length
Atrazine	-0.24	-0.41
200 µg/ml	-0.14	-0.15
400 µg/ml	-0.15	-0.23
800 µg/ml	-0.21	-0.51

Response index = Length of Test/ Length of Control–1

4.8 Effect of Extract on Ferredoxin NADP-reductase Activity of Tomato Seedlings

Ferredoxin NADP-reductase activity in the tomato seedlings was significantly reduced ($p<0.05$) in a concentration-dependent manner at the different concentrations of the extract when compared with the control. Atrazine on the other hand, caused a greater reduction ($p<0.05$) in the enzyme activity which was also significant when compared with the control and with the extract groups (Figure 4.6).

4.9 Effect of Extract on Chlorophyll Concentration of Tomato Seedlings

The extract at the different concentrations significantly reduced ($p<0.05$) the concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the tomato seedlings when compared with the control. Atrazine also caused reductions in the levels of these parameters which were significant when compared with the control and also with the extract groups (Table 4.7).

4.10 Effect of Extract on Acetyl CoA Carboxylase Activity of Tomato Seedlings

The extract at 800 µg/ml, caused a reduction in Acetyl CoA Carboxylase activity of tomato seedlings which was significant ($p < 0.05$) when compared with the control and with the lower extract concentrations but compared favourably with the atrazine-treated group (Figure 4.7).

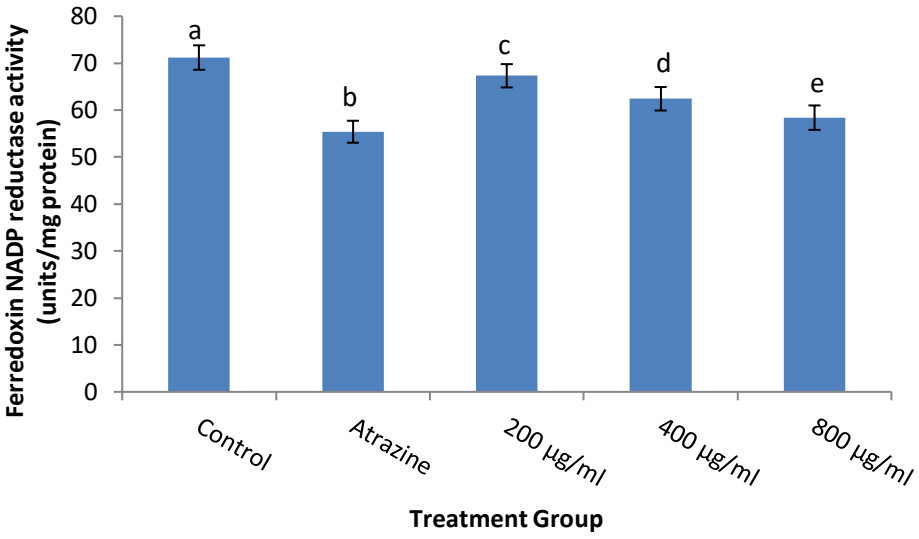


Figure 4.6: Ferredoxin NADP-reductase Activity of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

Table 4.7: Chlorophyll Concentration of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Treatment	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Total Chlorophyll (mg/g fw)
Control	46.02 ± 0.25^{a}	15.04 ± 0.15^{a}	63.06 ± 0.12^{a}
Atrazine	39.68 ± 0.48^{c}	10.92 ± 0.14^{e}	40.46 ± 0.12^{d}
200 µg/ml Extract	43.92 ± 0.15^{b}	14.40 ± 0.22^{b}	62.62 ± 0.12^{a}
400 µg/ml Extract	43.16 ± 0.43^{b}	13.36 ± 0.16^{c}	60.86 ± 0.18^{b}
800 µg/ml Extract	40.16 ± 0.25^{c}	12.34 ± 0.14^{d}	42.20 ± 0.23^{c}

Data are Mean ± SEM of 10 determinations.

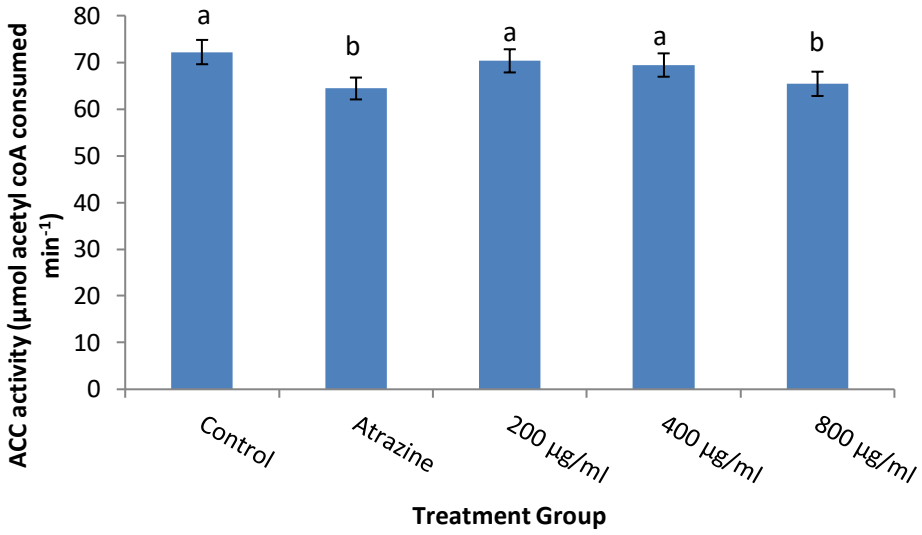


Figure 4.7: Acetyl CoA Carboxylase (ACC) Activities of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

4.11 Effect of Extract on Malondialdehyde Content of Tomato Seedlings

Significant increase in the levels of MDA was recorded at 800 µg/ml of the extract when compared with the control and with the lower extract concentrations. At 800 µg/ml still, MDA content compared favourably with the atrazine-treated group (Figure 4.8).

4.12 Effect of Extract on Superoxide Dismutase Activity in the Tomato Seedlings

The activity of Superoxide Dismutase in the tomato seedlings was significantly reduced at concentrations above 400 µg/ml, when compared with the control and with the atrazine-treated group. Atrazine on the other hand, caused a significant increase in SOD activity when compared with the control (Figure 4.9).

4.13 Effect of Extract on Catalase Activity in the Tomato Seedlings

The extract at 400 µg/ml and above, caused significant reduction in the enzyme activity when compared with the control and with the atrazine-treated group. Atrazine however, caused a significant increase in the CAT activity when compared with the control but compared favourably with the extract at 200 µg/ml (Figure 4.10).

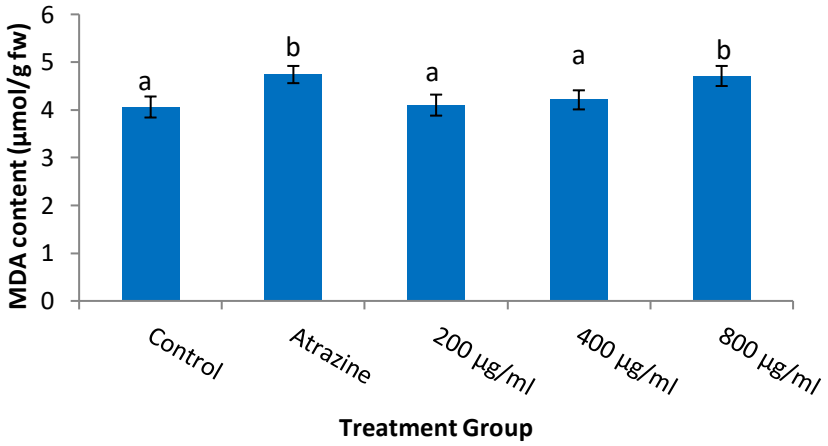


Figure 4.8: Malondialdehyde Contents of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

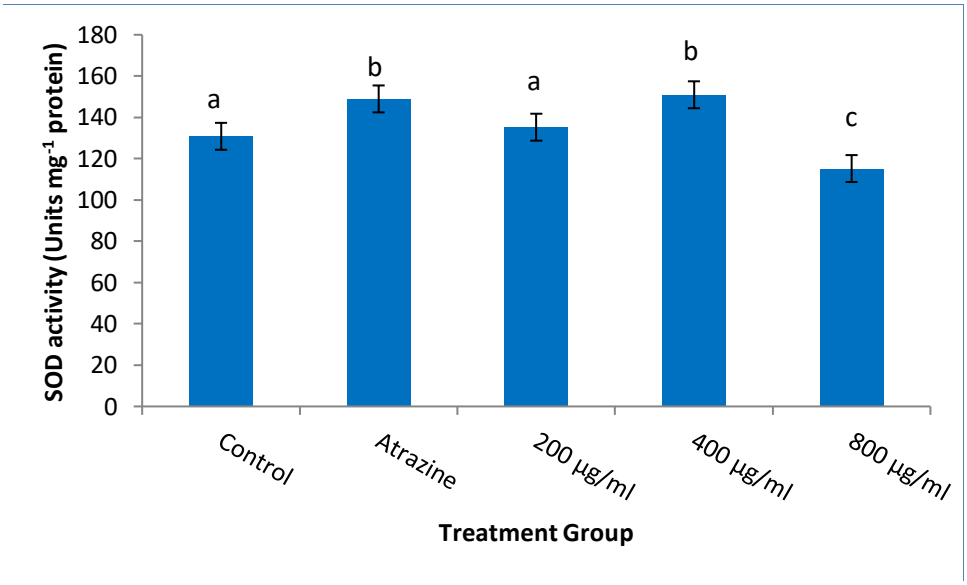


Figure 4.9: Superoxide Dismutase Activities of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

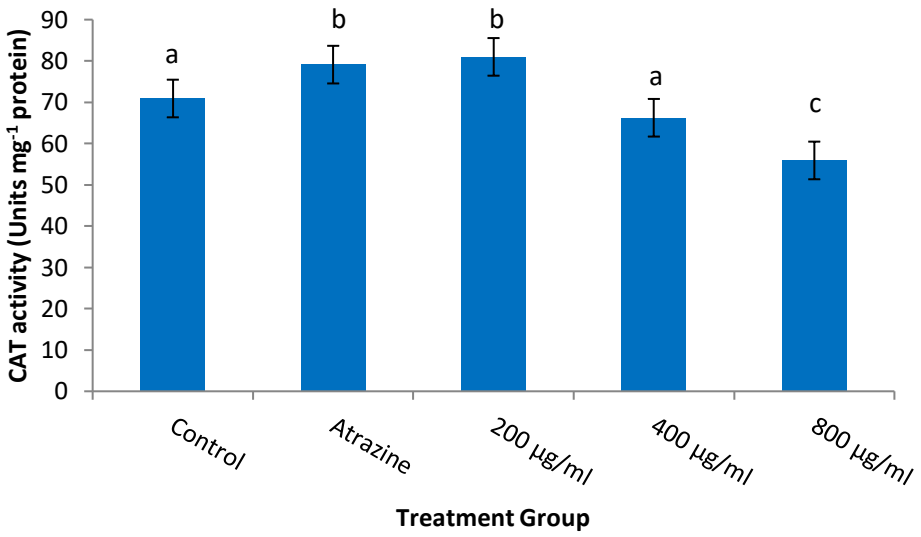


Figure 4.10: Catalase Activities of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

4.14 Effect of Extract on Peroxidase Activity in Tomato Seedlings

The activity of peroxidase in the seedlings was significantly increased ($p < 0.05$) at concentrations above 400 µg/ml of the extract when compared with the control. Atrazine on the other hand, significantly increased the POD activity when compared with the control (Figure 4.11).

4.15 Effect of Extract on Proline Concentration of the Tomato Seedlings

Proline concentration in the seedlings was increased significantly ($p < 0.05$) with the extract when compared with the control. A significant increase was also recorded with atrazine treatment when compared with the control and the extract-treated groups (Figure 4.12).

4.16 Effect of Extract on Protein Concentration of the Tomato Seedlings

Protein concentration was found to be significantly increased in seedlings treated with the extract when compared with the control. Atrazine treatment also led to a significant increase in the protein concentration when compared with the control and with the extract groups (Figure 4.13).

4.17 Effects of Extract on Total Soluble Sugar Concentration of Tomato Seedlings

The extract significantly reduced ($p < 0.05$) the total soluble sugar concentration of the seedlings when compared with the control. Total soluble sugar concentration was also significantly reduced with atrazine treatment when compared with the control and with the extract-treated groups (Figure 4.14).

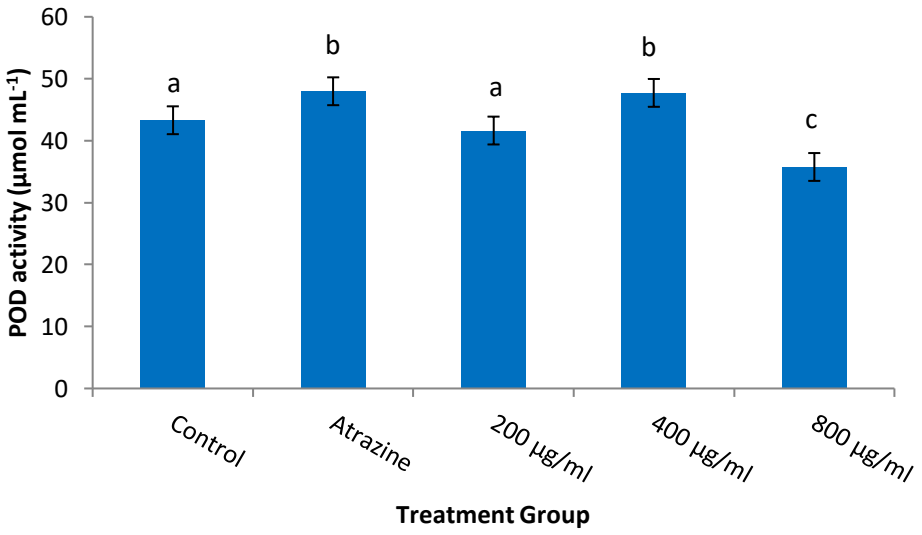


Figure 4.11: Peroxidase Activity of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

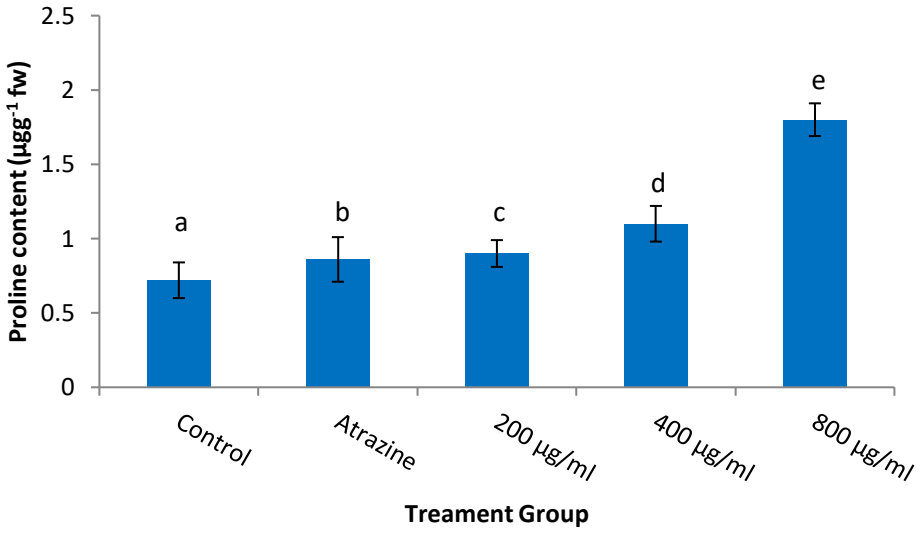


Figure 4.12: Proline Concentration of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

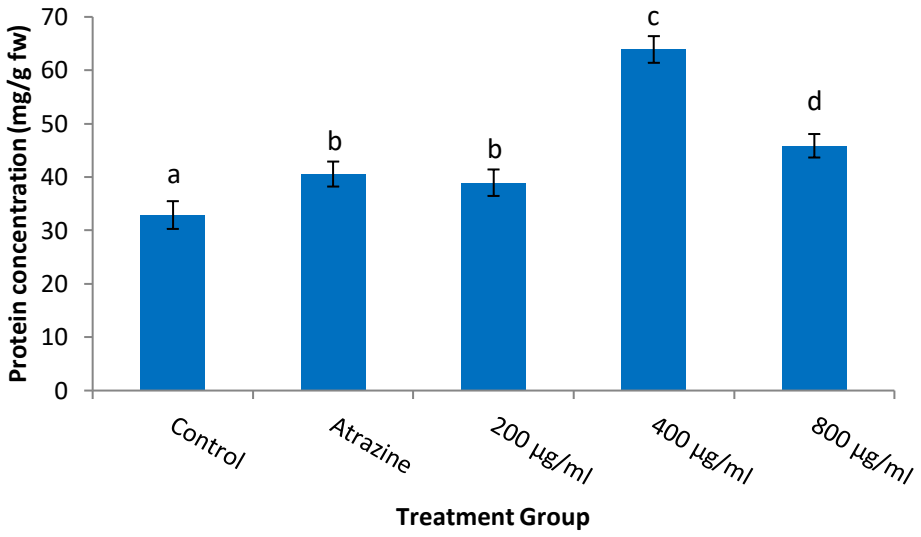


Figure 4.13: Protein Concentration of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

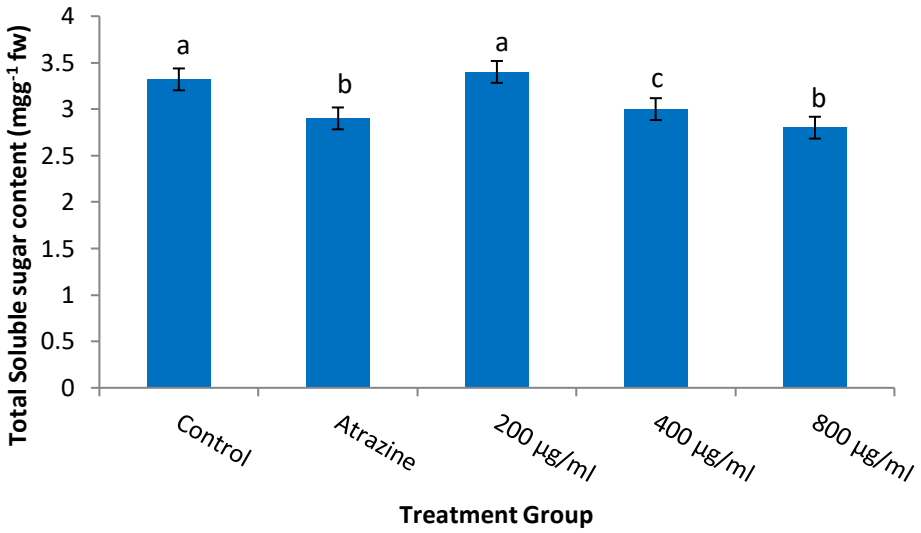


Figure 4.14: Total Soluble Sugar Concentration of Tomato Seedlings Treated with Aqueous Extract of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

4.18 Root Growth Inhibition Effects of Partitioned Fractions on *Allium cepa*

The percentage inhibition of the various partitioned fractions on *Allium cepa* roots are shown in Table 4.8. The ethyl acetate fraction produced the highest percentage inhibition on the growth of *Allium cepa* roots at the different concentrations with an IC_{50} value of 260µg/ml (Table 4.9).

Table 4.8: Percentage Root Growth Inhibition of *Allium cepa* Exposed to n-butanol, Ethyl acetate and Residue Fractions

Extract Conc.	**n-butanol (%)**	**Ethyl acetate (%)**	**Residue (%)**
Control	0	0	0
100 µg/ml	9.51	25.31	4.89
200 µg/ml	11.19	38.46	5.17
300 µg/ml	16.08	53.15	9.23
500 µg/ml	32.87	80	30.07

Data are Mean ± SE of 15 determinations

Table 4.9: IC_{50} Values of Aqueous Extract, n-butanol, Ethyl acetate and Residue Fractions

Treatment Groups	**IC_{50}** (µg/ml)
Aqueous extract	400
Ethyl acetate	260
n-butanol	600
Residue	700

Percentage root growth inhibitions were plotted against extract concentrations to obtain the **IC_{50}** values (Appendix 1-I).

4.19 Allelopathic Effects of solvent-solvent partitioned extracts

When compared with the control and other fractions, treatment with the ethyl acetate partitioned extract significantly reduced ($p < 0.05$) percentage germination of the tomato seeds (Table 4.10) and also a significantly reduced alpha amylase activity in the seedlings (Figure 4.15). It also led to significant reductions in the Plumule (Figure 4.16) and radicle lengths (Figure 4.17) of the seedlings. Treatment with the ethyl acetate fraction however compared favorably with that of the aqueous extract group.

Furthermore, the ethyl acetate partitioned extract significantly reduced the activity of Ferredoxin NADP-reductase (Figure 4.18) and also significantly reduced the concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the tomato seedlings (Table 4.11) when compared with the control and the other fractions.

In addition, the ethyl acetate fraction significantly reduced the Acetyl CoA Carboxylase activity (Figure 4.19) in the seedlings, while significantly increasing the MDA contents (Figure 4.20) when compared with the control and the other fractions. This was however accompanied by significant reduction in the activities of SOD (Figure 4.21), CAT (Figure 4.22) and POD (Figure 4.23), when compared with the control but compared favorably with the aqueous extract-treated group.

Finally, the ethyl acetate fraction caused significant increase in concentrations of proline (Figure 4.24), protein (Figure 4.25) and total soluble sugars (Figure 4.26) in the tomato seedlings when compared with the control and other fractions, but compared favorably the aqueous extract-treated group.

Table 4.10: Percentage Germination of Tomato Seeds Exposed to Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Groups	**Germination (%)**
Distilled water	100.00 ± 0.00^{a}
Aqueous (400 µg/ml)	89.00 ± 2.15^{b}
Ethyl acetate (260 µg/ml)	85.00 ± 2.85^{b}
n-butanol (600 µg/ml)	95.00 ± 2.68^{a}
Residue (700 µg/ml)	98.00 ± 2.50^{a}

Data are expressed as Mean ± SEM of 10 determinations.

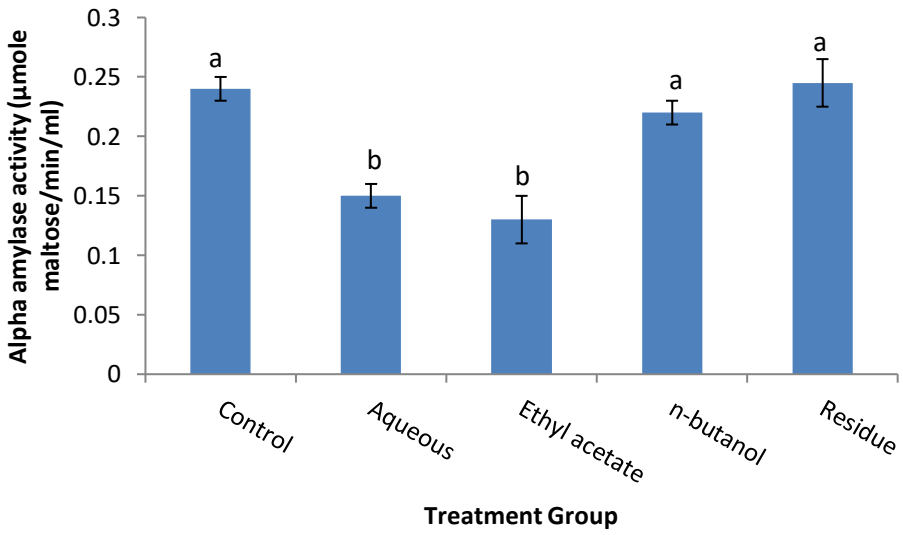


Figure 4.15: Alpha Amylase Activities of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

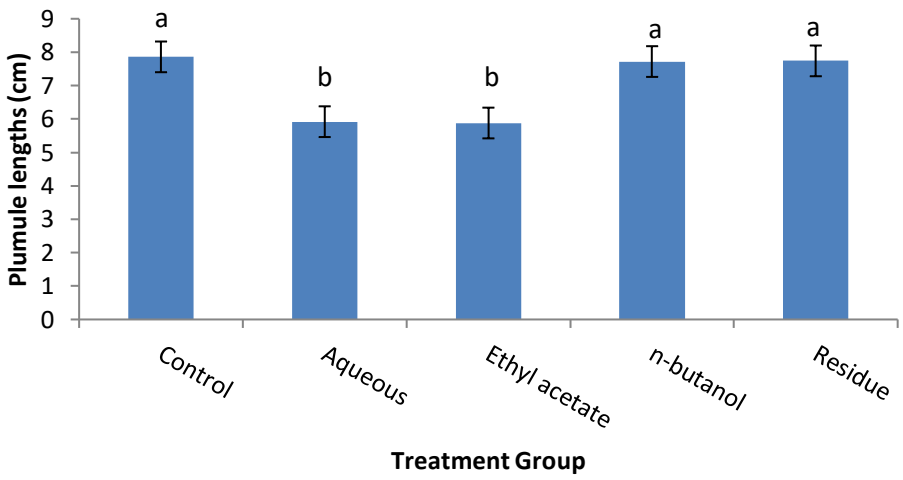


Figure 4.16: Plumule Lengths of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

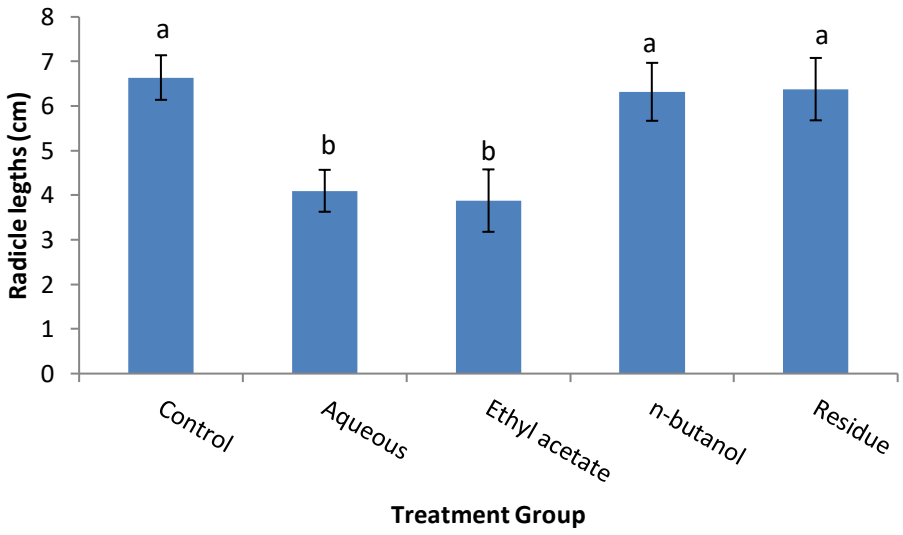


Figure 4.17: Radicle Lengths of Tomato Seedlings treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

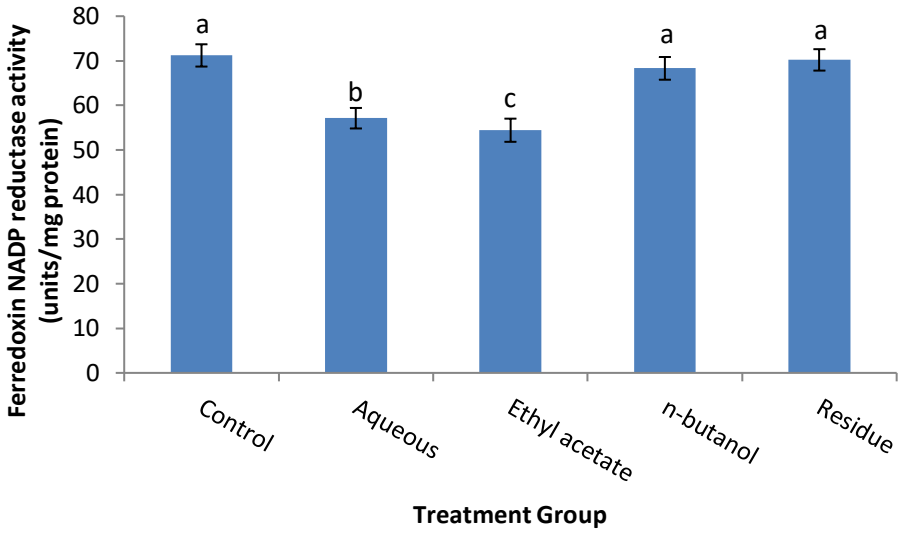


Figure 4.18: Ferredoxin NADP-reductase Activity of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

Table 4.11: Chlorophyll Concentrations in Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Treatment	**Chlorophyll a (mg/g fw)**	**Chlorophyll b (mg/g fw)**	**Total Chlorophyll (mg/g fw)**
Control	47.11 ± 0.12^{a}	12.21 ± 0.20^{a}	65.11 ± 0.16^{a}
Aqueous (400 µg/ml)	41.85 ± 0.21^{b}	10.23 ± 0.11^{b}	47.51 ± 0.21^{b}
Ethyl acetate (260 µg/ml)	39.55 ± 0.13^{b}	10.22 ± 0.15^{b}	42.86 ± 0.85^{b}
n-butanol (600 µg/ml)	47.54 ± 0.22^{a}	12.14 ± 0.41^{a}	62.12 ± 0.11^{a}
Residue (700 µg/ml)	46.45 ± 0.12^{a}	12.02 ± 0.45^{e}	64.46 ± 0.32^{a}

Data are Mean ± SEM of 10 determinations.

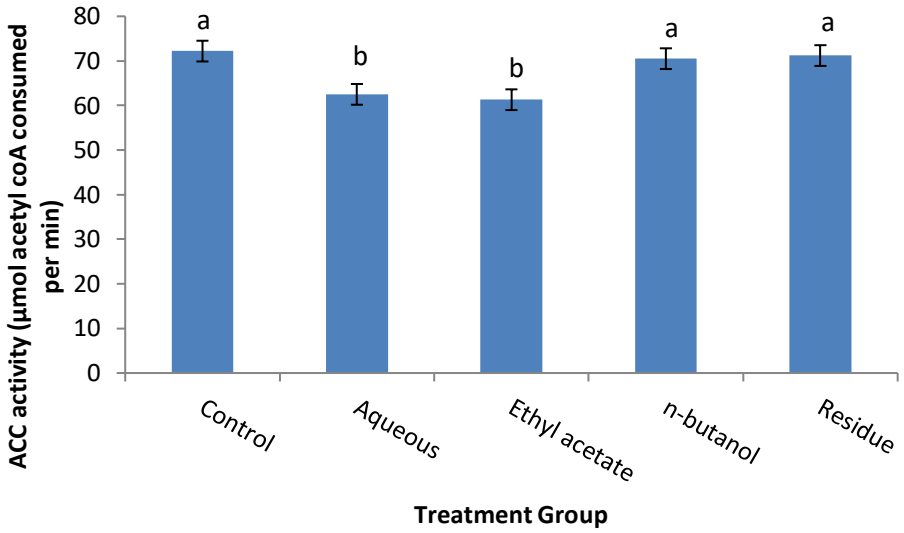


Figure 4.19: Acetyl CoA Carboxylase Activity in Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

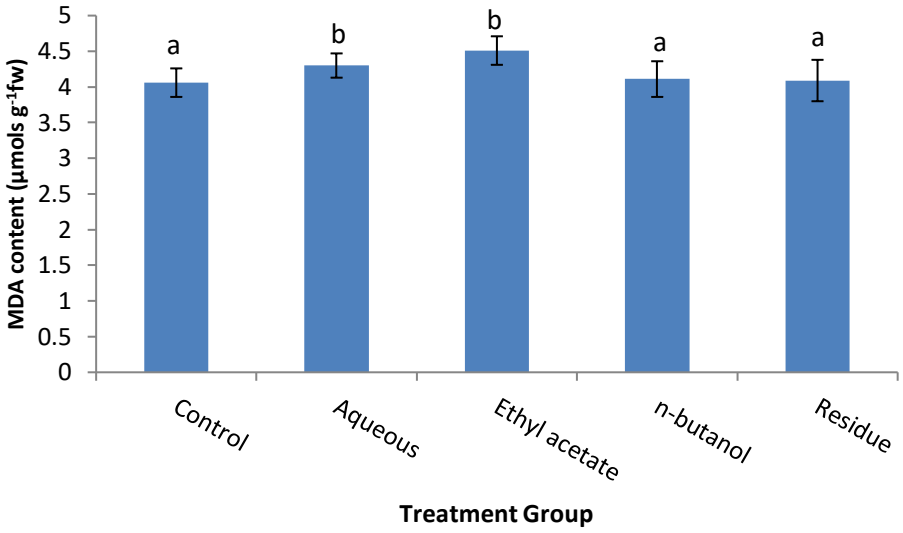


Figure 4.20: Malondialdehyde Contents of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

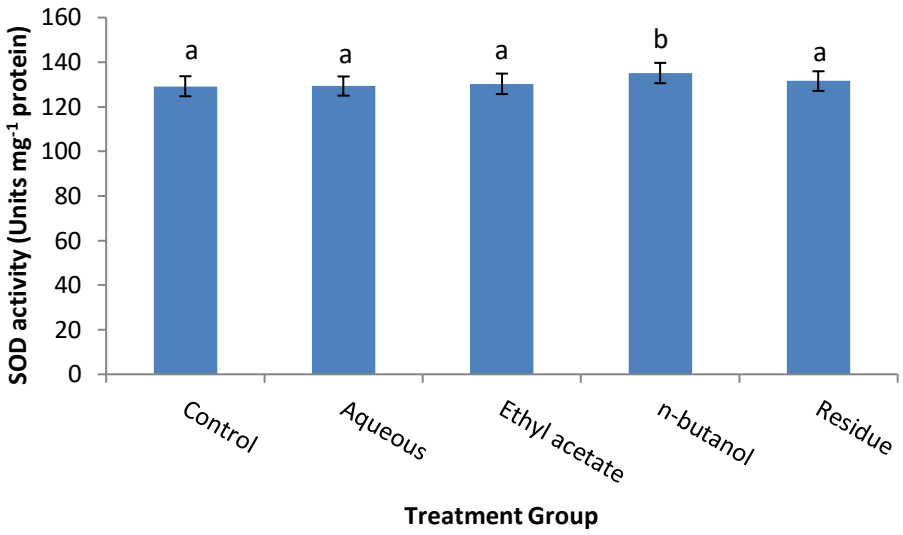


Figure 4.21: Superoxide dismutase Activity of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

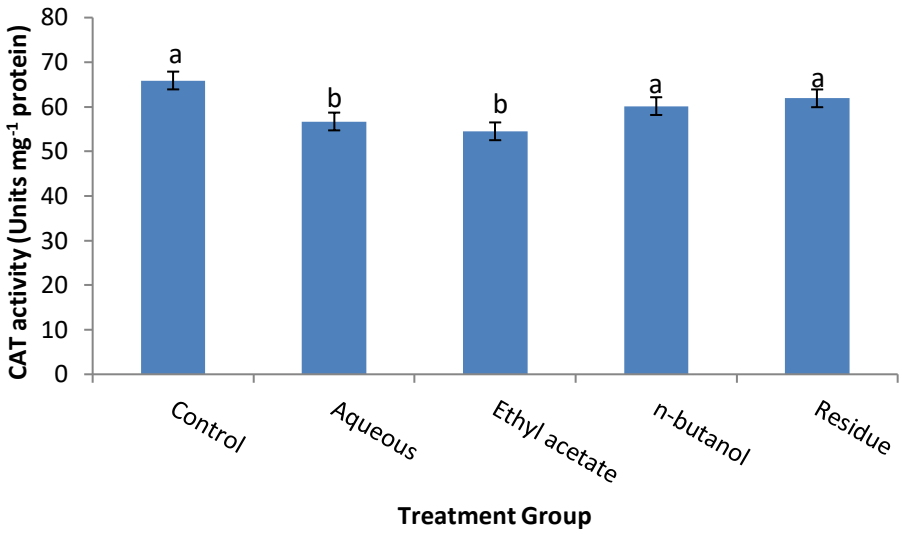


Figure 4.22: Catalase Activity of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

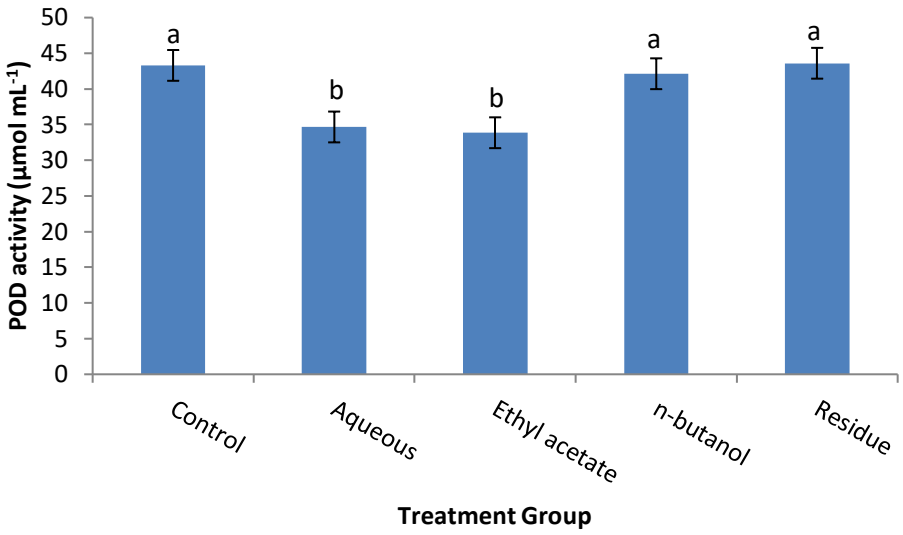


Figure 4.23: Peroxidase Activity of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

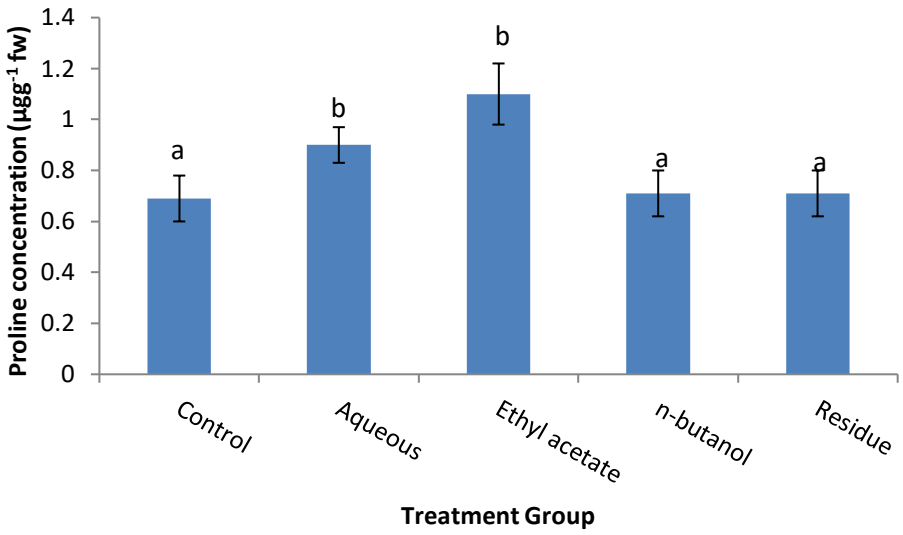


Figure 4.24: Proline Concentrations of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

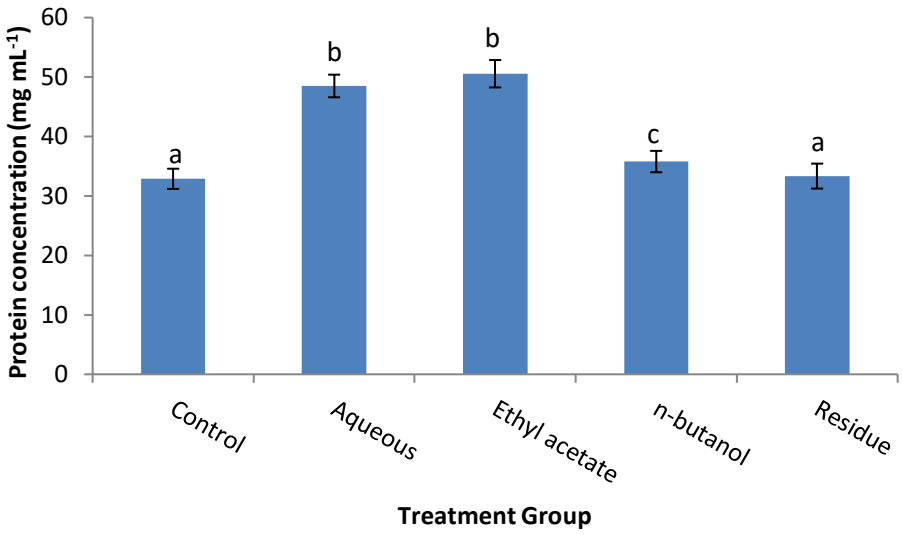


Figure 4.25: Protein Concentration of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

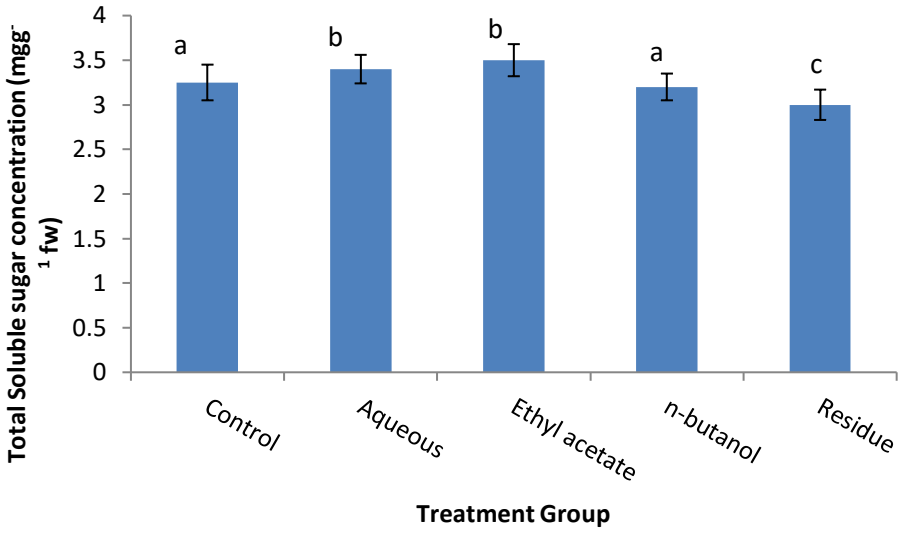


Figure 4.26: Total Soluble Sugar Concentration of Tomato Seedlings Treated with Solvent-solvent Partitioned Fractions of *Alchornea laxiflora* Leaves

Data are Mean ± SEM of 10 determinations.

4.20 Column chromatography on Ethyl acetate fraction

Column chromatography of the ethyl acetate fraction gave a total of 22 eluents which were then grouped into four based on their R_f values (Table 4.12) as calculated from the thin layer chromatography plate (Appendix II-A).

Table 4.12: R_f Values of Chromatographic Eluents

Fractions	R_f values
A	0.77
B	0.73
C	0.70
D	0.52

Fractions represent spots with similar R_f values pulled together

R_f value = Distance travelled by compound (cm)/Distance travelled by solvent (cm)

Calculations were done on the TLC plate (Appendix II-A)

4.21 Allelopathic Effects of the chromatography eluents

Column Chromatography eluents C and D caused allelopathic effects which were similar to those observed with the ethyl acetate fraction and the aqueous extract groups. Eluents A and B on the other hand, had effects that compared favourably with the control. The allelopathic effects caused by eluents C and D include significant reduction in the percentage germination of the tomato seeds (Table 4.13), significant reduction in alpha amylase activity in the tomato seedlings (Figure 4.27), and also significant reduction in the plumule (Figure 4.28) and radicle lengths (Figure 4.29) of the tomato seedlings when compared with the control and also with other chromatography eluents (A and B).

Treatment with eluents C and D also led to significant reduction in activities of Ferredoxin NADP-reductase (Figure 4.30) and significant reductions in the concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the tomato seedlings (Table 4.14) when compared with the control and also with other eluents (A and B).

C and D further caused a significant reduction in Acetyl CoA Carboxylase activity (Figure 4.31) and significant increase in concentrations of MDA in the seedlings (Figure 4.32) when compared with the control. This was also accompanied by significant reduction in activities of SOD (Figure 4.33), CAT (Figure 4.34), POD (Figure 4.35) when compared with the control and also with other eluents (A and B).

Finally, C and D caused significant increases in concentrations of proline (Figure 4.36), protein (Figure 4.37) and total soluble sugars (Figure 4.38) in the tomato seedlings when compared with the control and also with other eluents (A and B).

Table 4.13: Percentage Germination of Tomato Seeds Exposed to Different Chromatography Eluents of the Ethyl acetate Fraction.

Chromatographic eluents (260 µg/ml)	**Germination (%)**
Control	98.00 ± 2.75^{a}
Fraction A	97.00 ± 2.17^{a}
Fraction B	95.00 ± 2.89^{a}
Fraction C	87.00 ± 2.85^{b}
Fraction D	85.00 ± 2.50^{b}

Data are Mean ± SEM of 10 determinations.

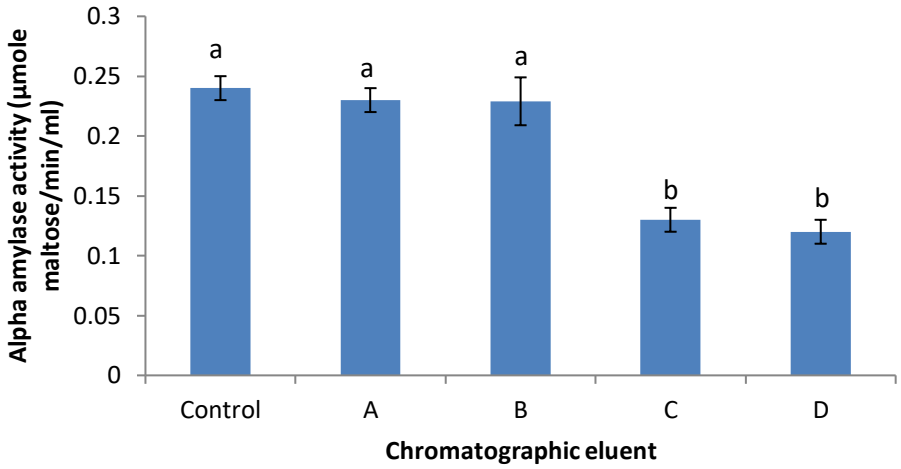


Figure 4.27: Alpha Amylase Activity of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

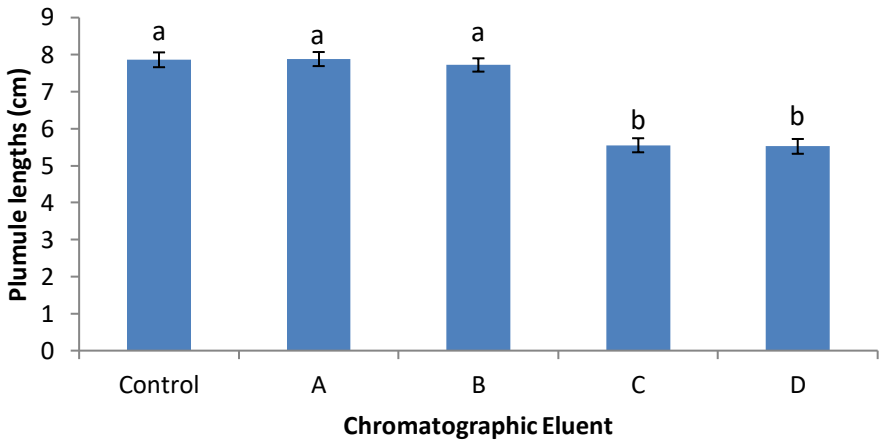


Figure 4.28: Plumule Lengths of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents.

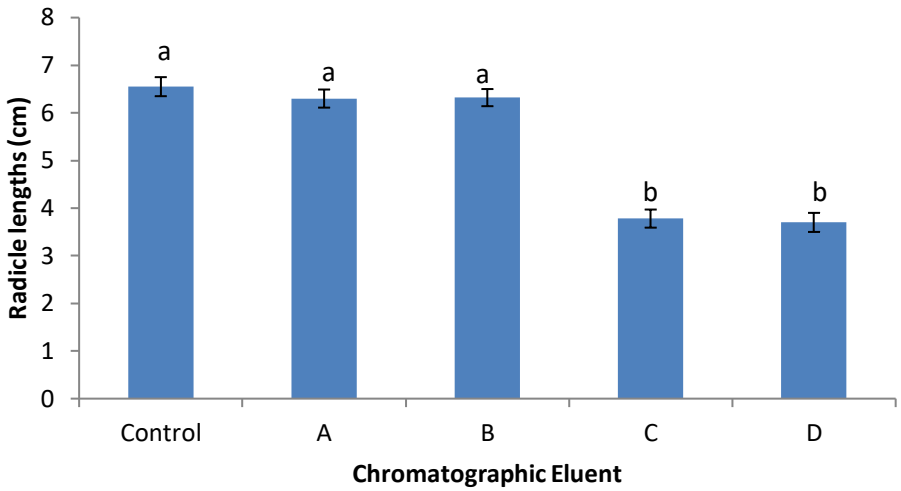


Figure 4.29: Radicle Lengths of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

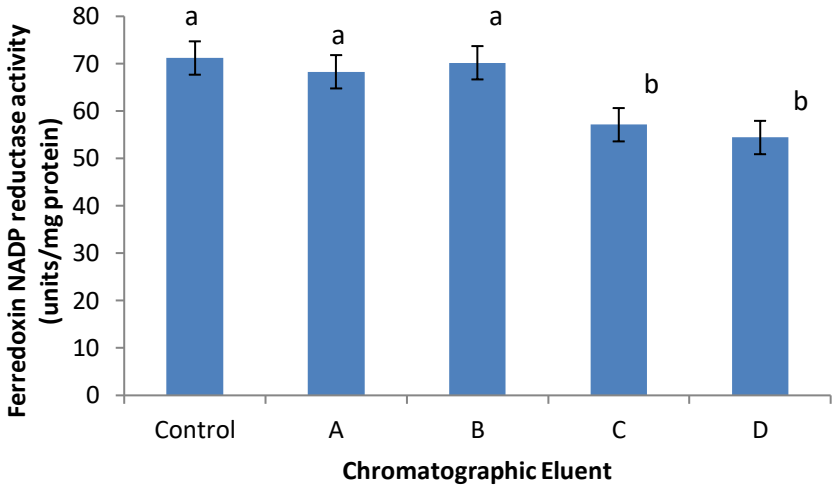


Figure 4.30: Ferredoxin NADP-reductase Activity of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

Table 4.14: Chlorophyll Concentration of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

Treatment	Chlorophyll A (mg/g fw)	Chlorophyll B (mg/g fw)	Total Chlorophyll (mg/g fw)
Control	46.02 ± 0.52^{a}	14.86 ± 0.58^{a}	63.09 ± 0.11^{a}
A	45.32 ± 0.12^{a}	13.42 ± 0.91^{a}	61.76 ± 0.51^{a}
B	46.15 ± 0.15^{a}	12.96 ± 0.97^{e}	62.51 ± 0.29^{a}
C	40.85 ± 0.17^{b}	11.97 ± 0.86^{b}	45.91 ± 0.92^{b}
D	39.55 ± 0.72^{b}	11.21 ± 0.25^{b}	44.42 ± 0.35^{b}

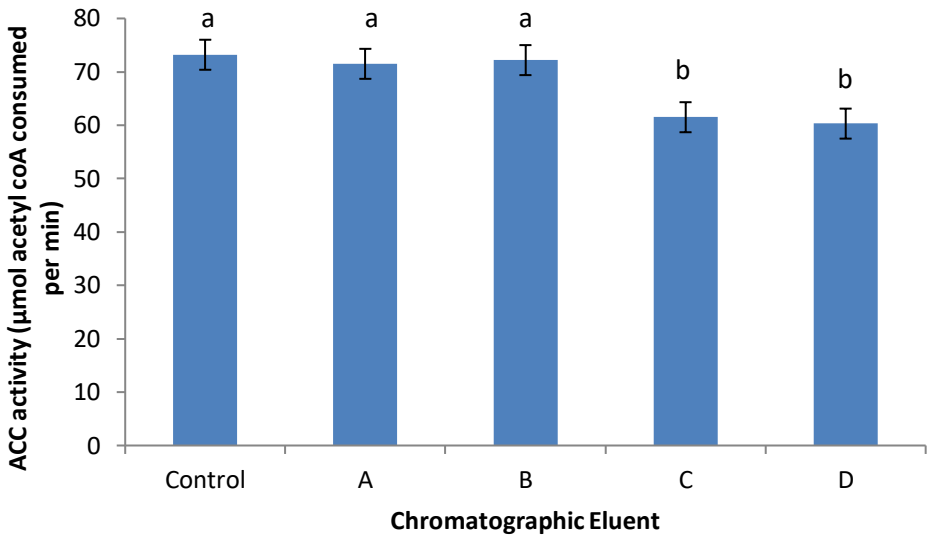


Figure 4.31: Acetyl CoA Carboxylase Activity of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

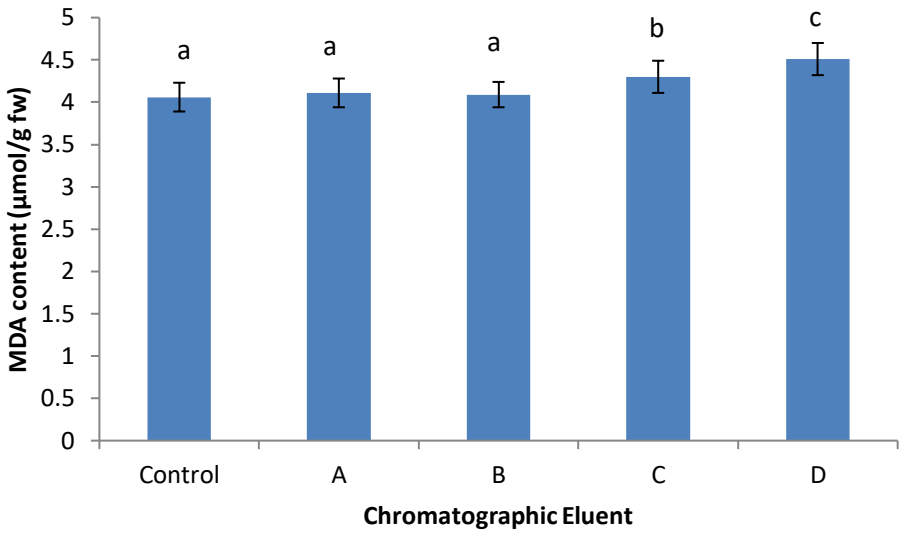


Figure 4.32: Malondialdehyde Content of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

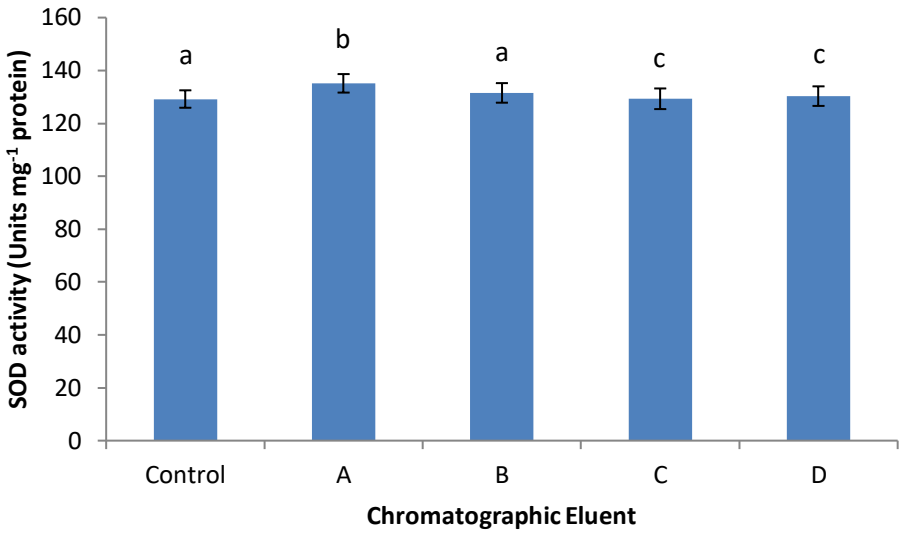


Figure 4.33: Superoxide dismutase Activities of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

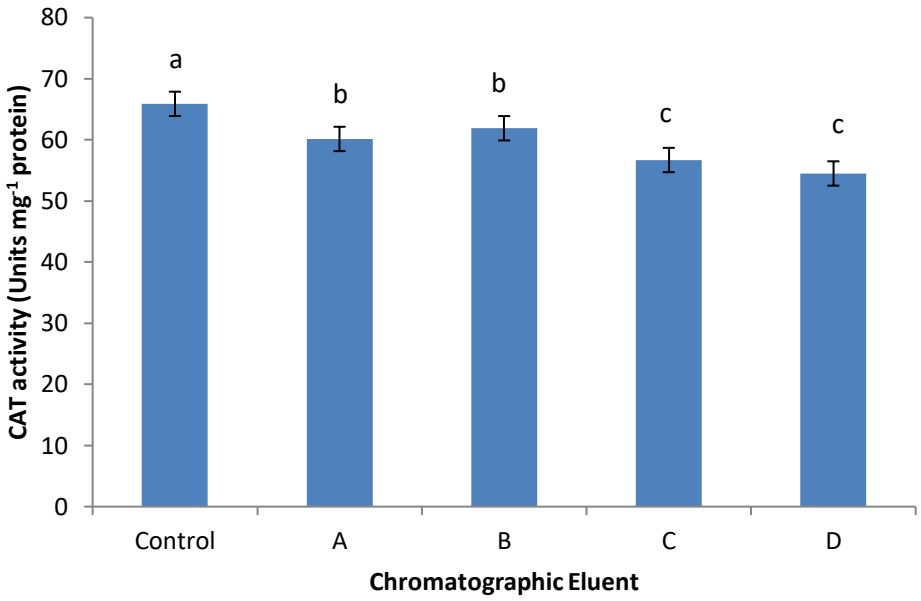


Figure 4.34: Catalase Activity of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

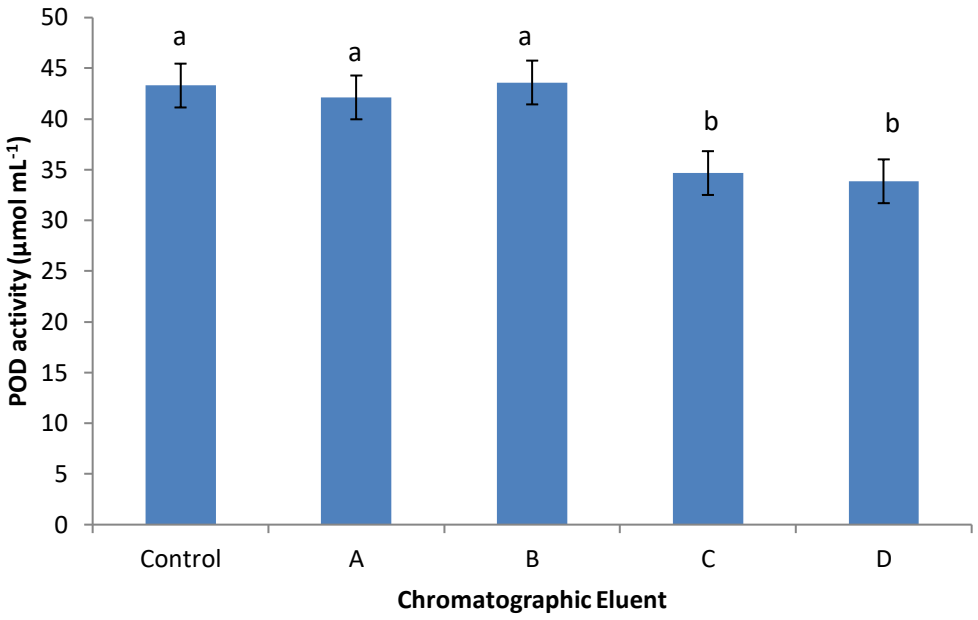


Figure 4.35: Peroxidase Activity of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

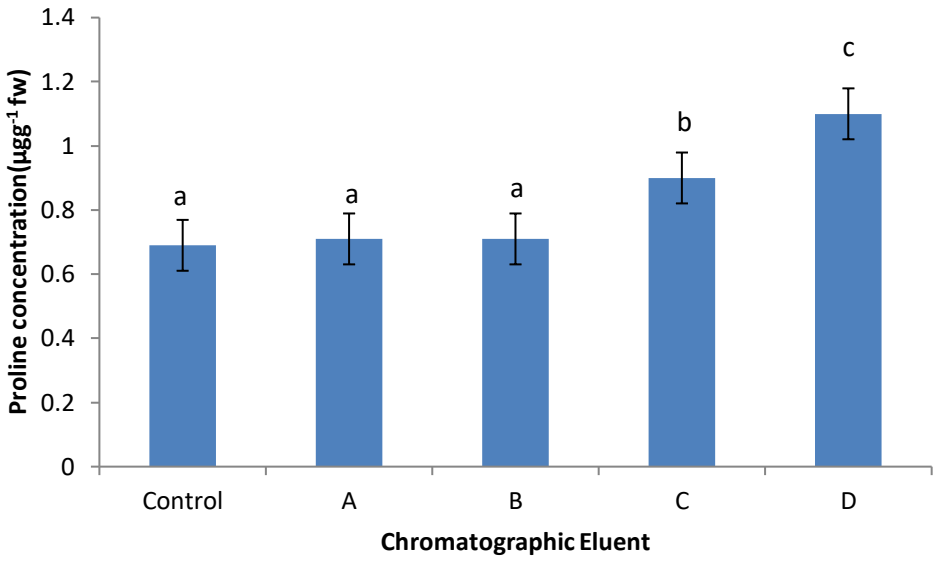


Figure 4.36: Proline Concentrations of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

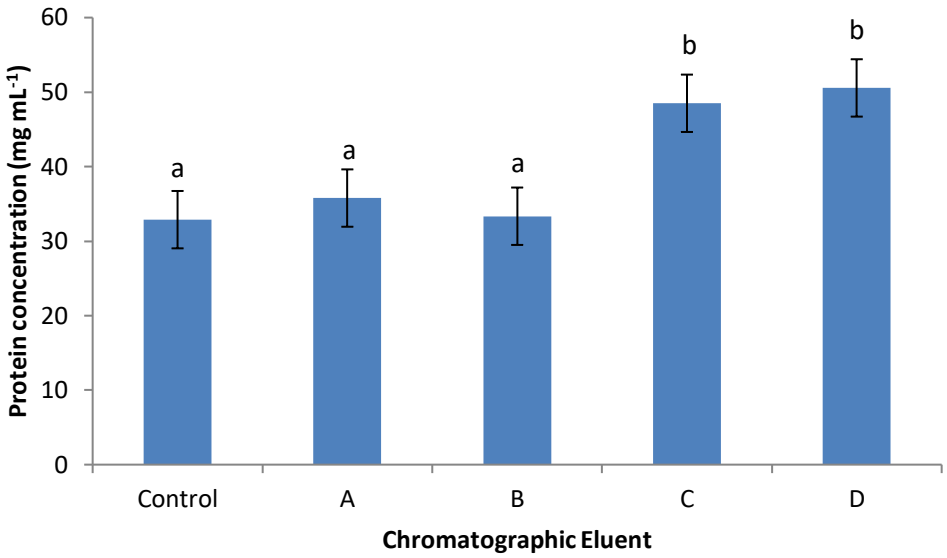


Figure 4.37: Protein Concentrations of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

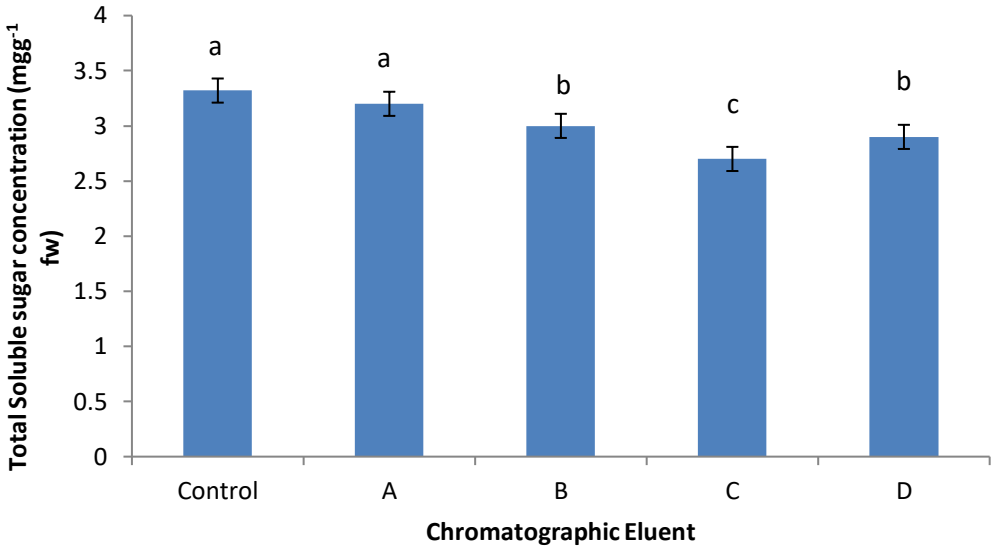


Figure 4.38: Total Soluble Sugar Concentrations of Tomato Seedlings Treated with the Ethyl acetate Chromatographic Eluents

4.23 High Performance Liquid Chromatography Identification of Bioactive Chromatography Eluents

The active eluents, when analysed using HPLC were identified as Quercetrin and Rutin.

4.24 Allelopathic Effects of Quercetrin and Rutin Isolates

The seedlings, when treated with the Quercetrin and Rutin isolates, exhibited effects which were similar to those produced by the seedlings that were treated with the bioactive chromatography eluents and atrazine. These include significant reduction in the percentage germination of seeds (Table 4.15), significant reduction in alpha amylase activities (Figure 4.39), and also significant reduction in the Plumule (Figure 4.40) and radicle lengths (Figure 4.41) when compared with the control.

Quercetrin and Rutin also caused significant reduction in activity of Ferredoxin NADP-reductase (Figure 4.42) and significant reduction in the concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the tomato seedlings (Table 4.16) when compared with the control.

Quercetrin and Rutin further caused significant reductions in Acetyl CoA Carboxylase activities (Figure 4.43) and significant increases in concentrations of MDA in the seedlings (Figure 4.44) when compared with the control and atrazine. This was also accompanied by significant reductions in activities of SOD (Figure 4.45), CAT (Figure 4.46), POD (Figure 4.47) when compared with the control.

Finally, Quercetrin and Rutin significantly increased the concentrations of proline (Figure 4.48), protein (Figure 4.49) and total soluble sugars (Figure 4.50) in the tomato seedlings when compared with the control.

Table 4.15: Percentage Germination of Tomato Seeds Exposed to Quercetrin and Rutin

Groups	Germination (%)
Distilled water	96.00 ± 1.55^{a}
Atrazine (0.5 µg/ml)	81.00 ± 2.34^{b}
Rutin (260 µg/ml)	79.00 ± 2.85^{b}
Quercetrin (260 µg/ml)	83.00 ± 1.76^{b}

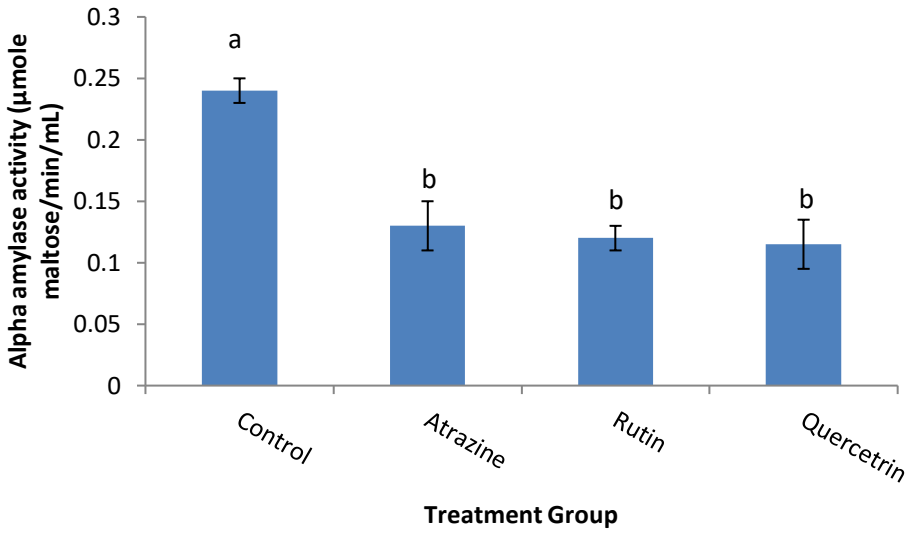


Figure 4.39: Alpha amylase Activity of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

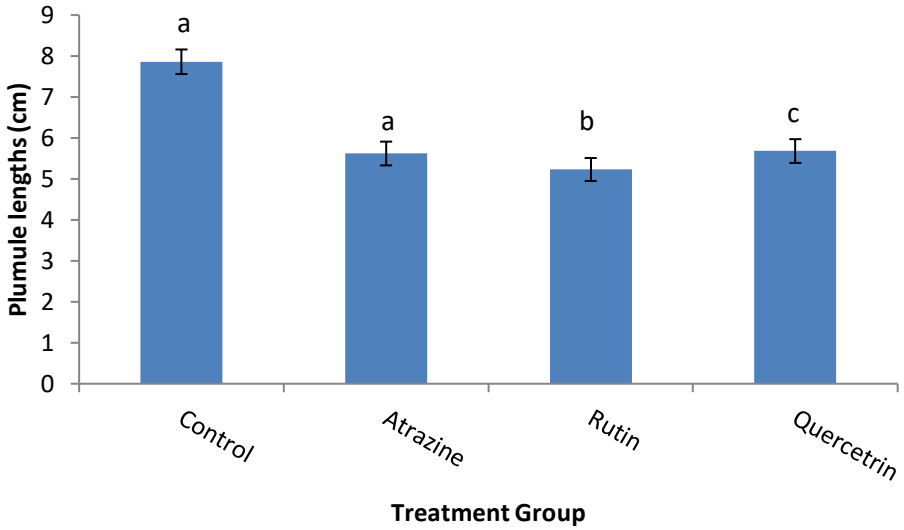


Figure 4.40: Plumule Lengths of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

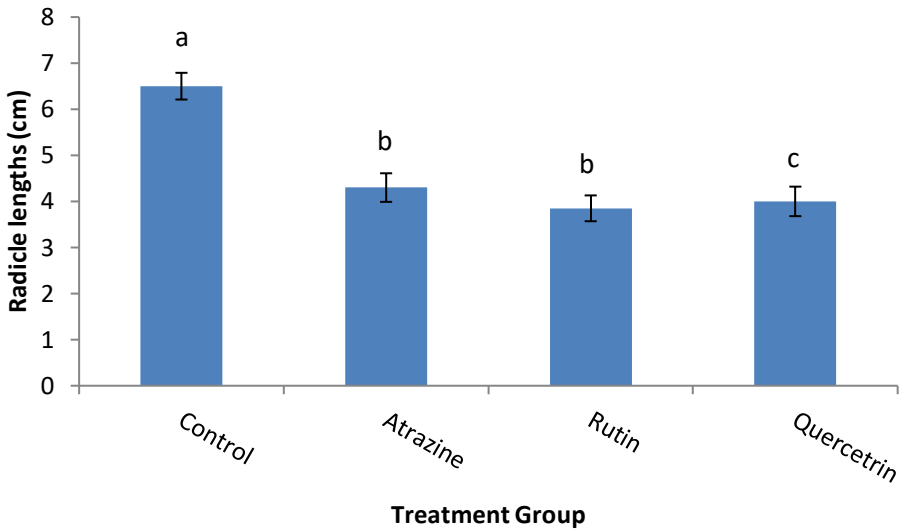


Figure 4.41: Radicle Lengths of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

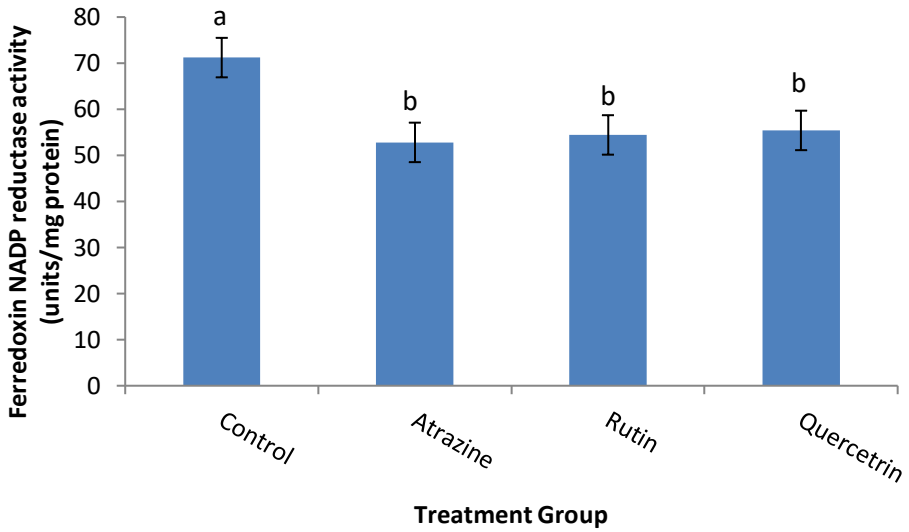


Figure 4.42: Ferredoxin NADP-reductase Activities of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

Table 4.16: Chlorophyll Concentrations of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

Treatment	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Total Chlorophyll (mg/g fw)
Distilled water	45.12 ± 0.12^{a}	15.26 ± 0.18^{a}	60.19 ± 0.21^{a}
Quercetrin (260 µg/ml)	40.56 ± 0.12^{b}	11.01 ± 0.15^{b}	45.22 ± 0.15^{b}
Rutin (260 µg/ml)	36.01 ± 0.25^{a}	10.12 ± 0.17^{b}	42.11 ± 0.08^{a}
Atrazine (0.5 µg/ml)	41.85 ± 0.27^{b}	10.97 ± 0.16^{b}	44.91 ± 0.99^{b}

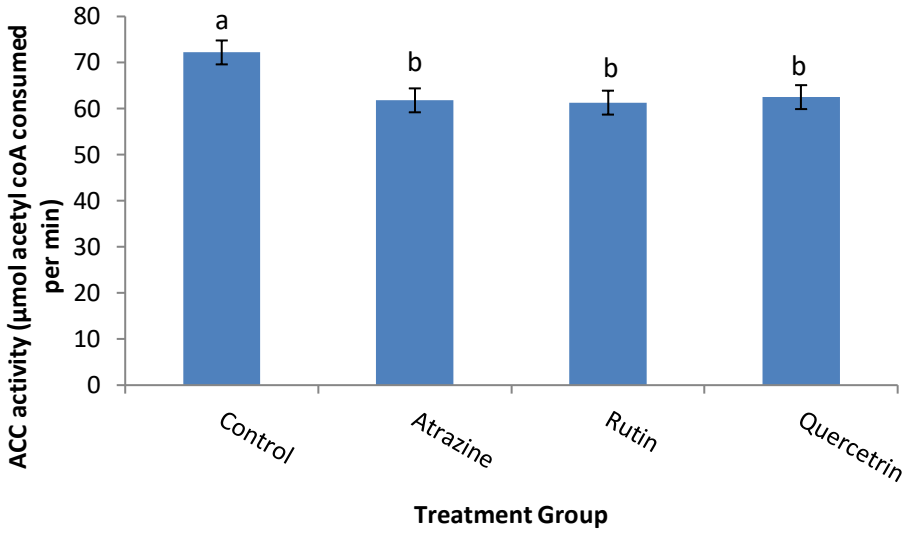


Figure 4.43: Acetyl CoA Carboxylase (ACC) Activity of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

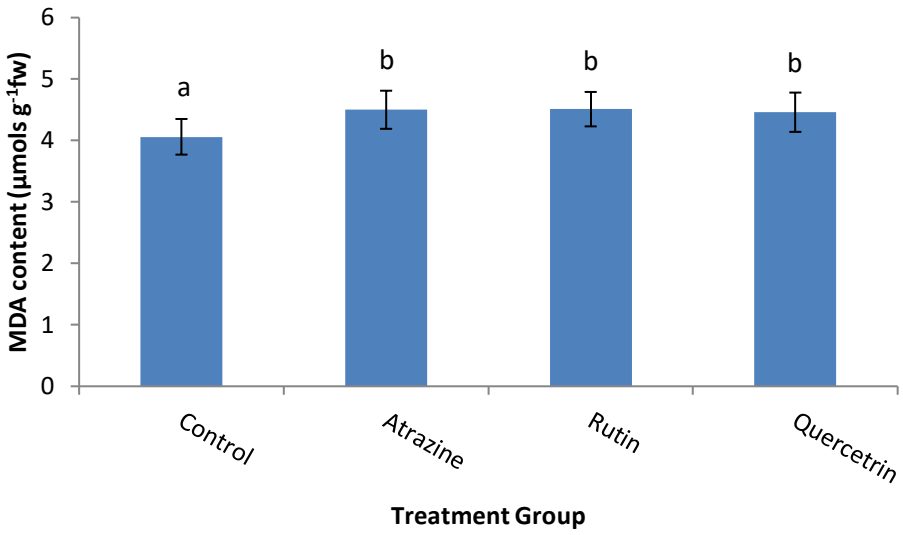


Figure 4.44: MDA Content of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

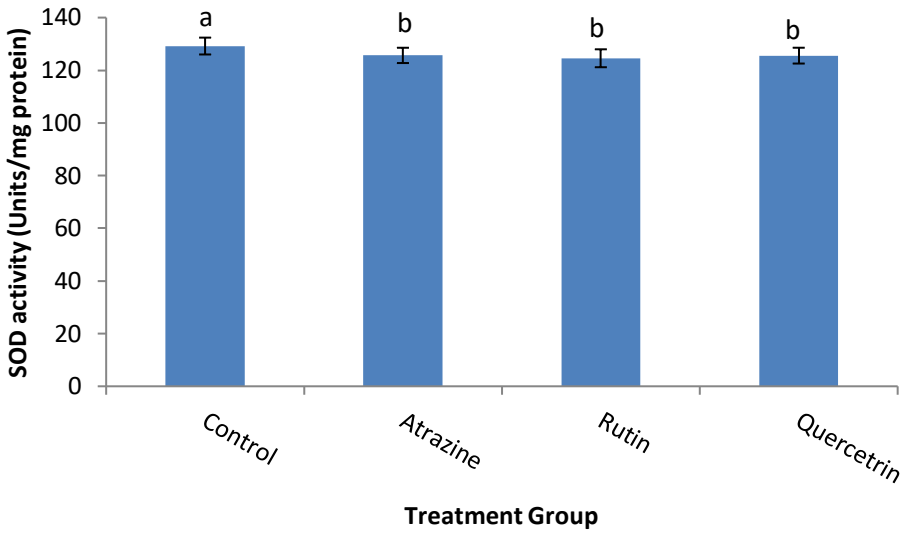


Figure 4.45: SOD Activity of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

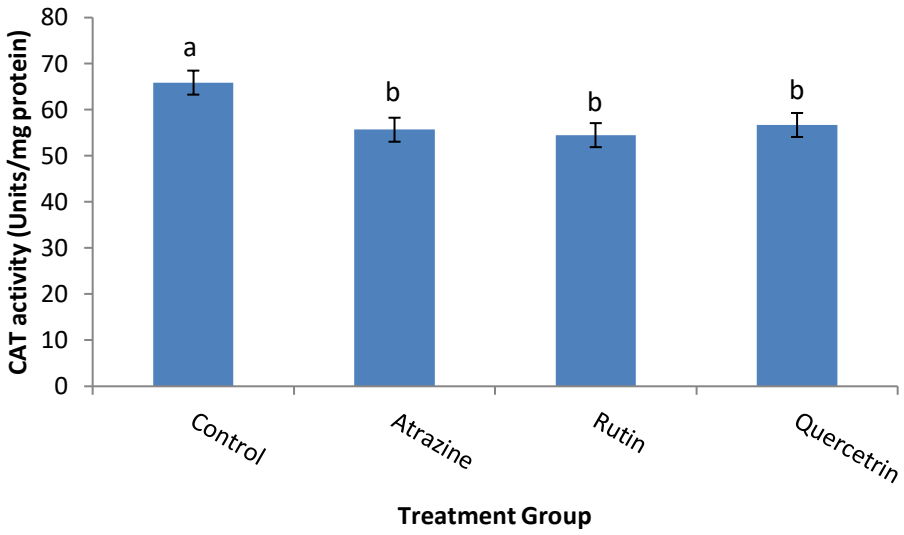


Figure 4.46: Catalase Activity of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

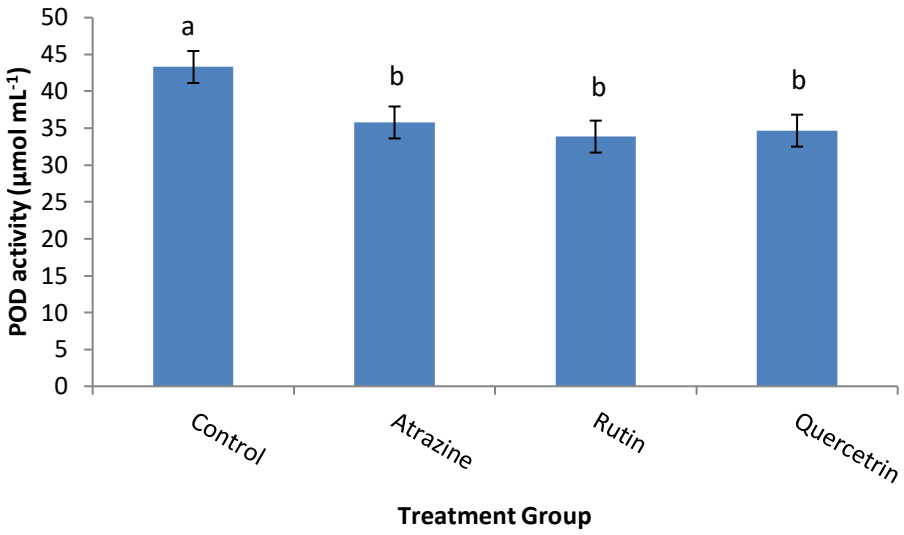


Figure 4.47: Peroxidase Activity of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

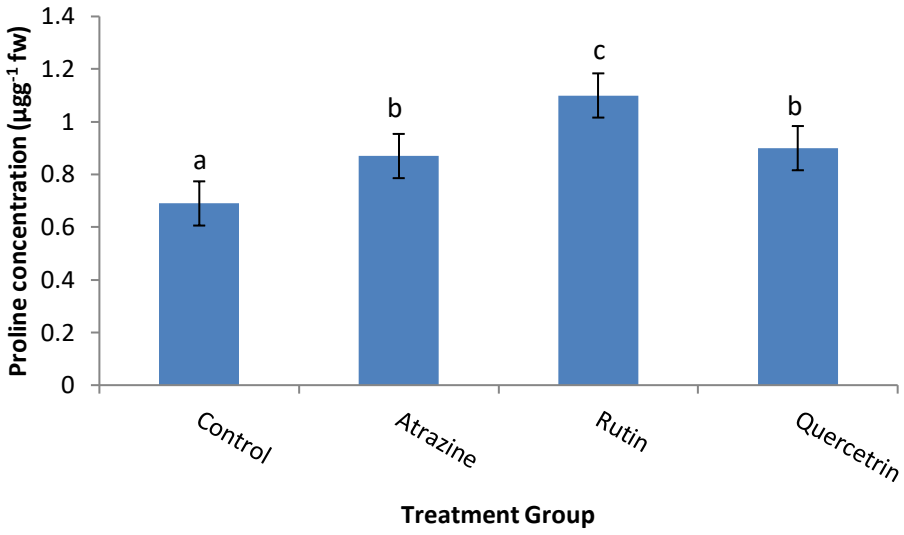


Figure 4.48: Proline concentration of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

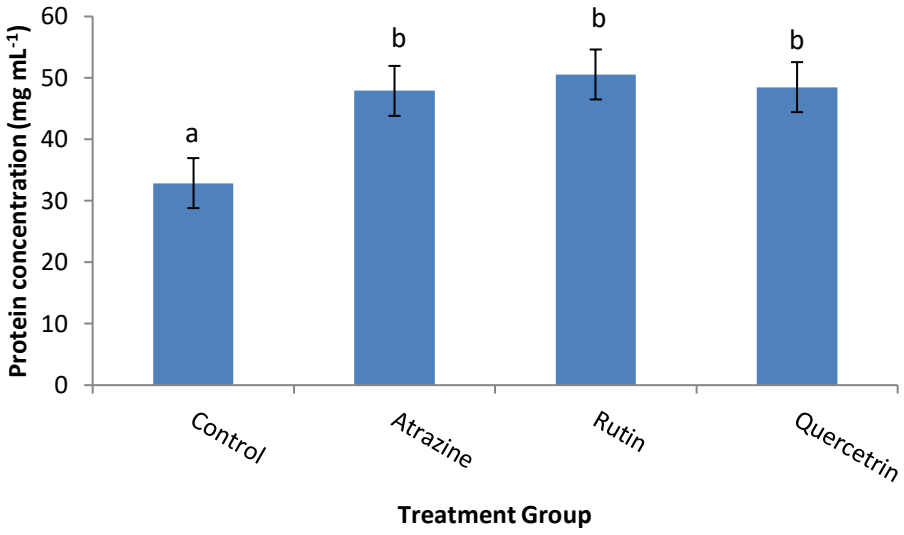


Figure 4.49: Protein Concentration of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

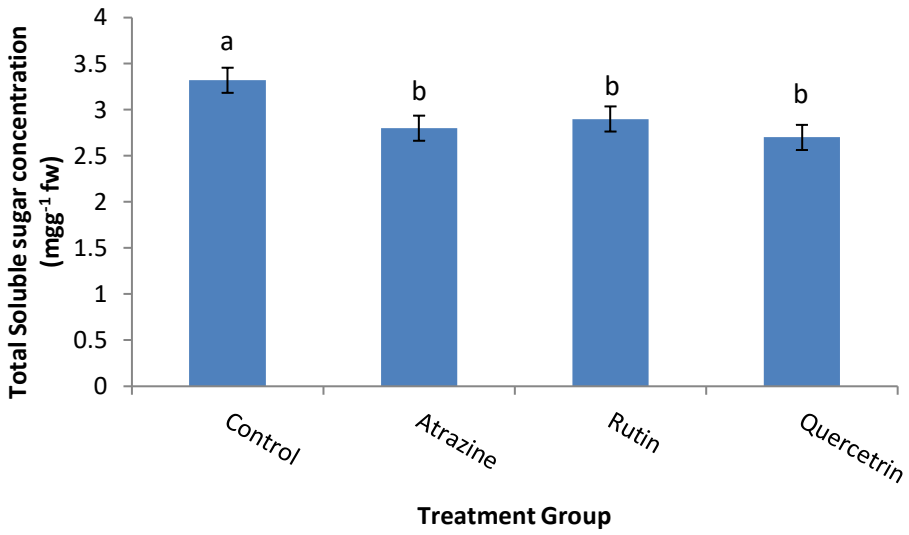


Figure 4.50: Total Soluble Sugar concentration of Tomato Seedlings Exposed to Quercetrin and Rutin Isolates

4.25 Genotoxic effects of Quercetrin and Rutin Isolates

Quercetrin and Rutin exhibited genotoxic effects similar to those observed with ethyl acetate chromatography eluents and atrazine. These include significant reduction in mitotic index values of *Allium cepa* root cells when compared with the control (Table 4.17). This was accompanied by abnormalities in chromosomes (Figure 4.51) and were also similar to those observed in onions treated with atrazine. Percentage chromosomal aberration was also seen to be higher in the Quercetrin and Rutin treated groups (Table 4.18), compared to the extract-treated groups.

Table 4.17: Mitotic Index as Expressed by The Different Phases of *Allium cepa* Root Cells When Exposed to Quercetrin and Rutin Isolates

Group	**Interphase**	**Prophase**	**Metaphase**	**Anaphase**	**Telophase**	**Total dividing cells**	**Total counted cells**	**Mitotic index**
Control	444	25	15	6	10	56	500	11.20
Quercetrin	409	20	12	4	5	41	450	9.11
Rutin	451	17	20	9	3	49	500	9.80
Atrazine	450	16	21	8	4	49	499	9.81

Data were obtained from 10 microscopic fields.

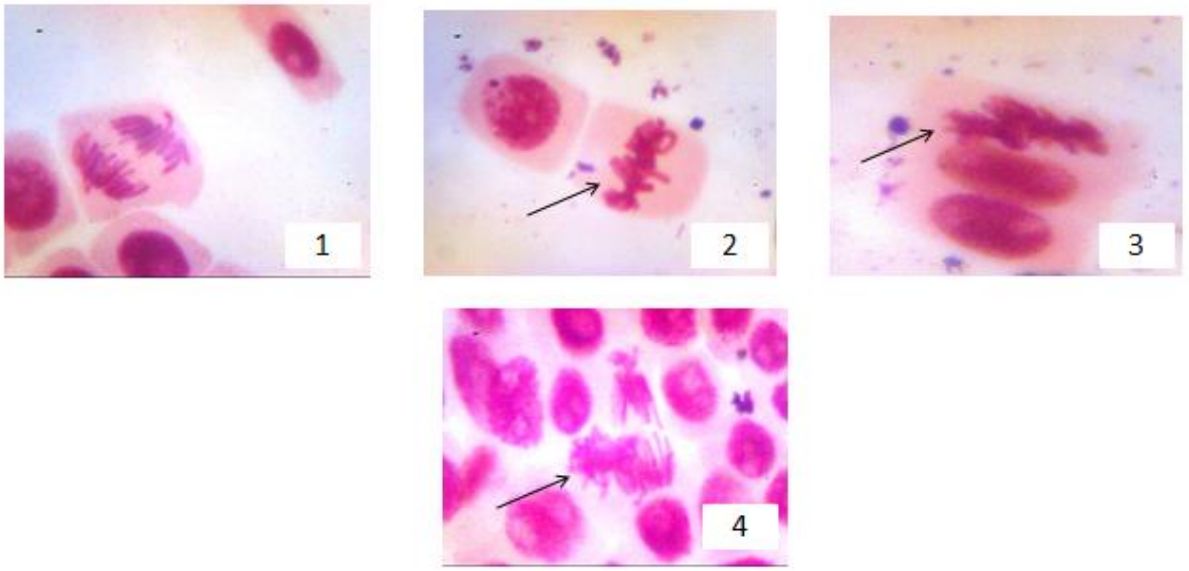


Figure 4.51: Photomicrograph of Mitotic Divisions in *Allium cepa* Root Cells Grown in Quercetrin and Rutin Isolates

Group 1- Distilled water ; Group 2- 260 µg/ml Quercetrin; Group 3- 260 µg/ml Rutin; Group 4- 0.5 µg/ml Atrazine. Arrows indicate abberations ; Aceto-orcein Stain used; Magnification X40; Feulgen-squash technique was used to prepare the slides of the root meristems.

Abberations indicated by the arrows include:

Group 2: Abnormal Metaphase

Group 3: Sticky Chromosome

Group 4: C-mitosis

Table 4.18: Percentage Chromosomal Aberration in *Allium cepa* Root Cells Exposed to Quercetrin and Rutin Isolates

Group	**C-mitosis**	**Abnormal metaphase**	**Anaphase bridge**	**sticky chromosomes**	**Total aberration**	**Total dividing cells**	**Total counted cells**	**Aberration (%)**
Control	0	0	0	0	0	56	500	0.00
Quercetrin	1	2	2	2	7	47	520	14.89
Rutin	1	2	1	2	6	40	515	15.00
Atrazine	1	2	2	2	7	40	480	11.50

4.26 Docked Interactions between Rutin and Active Sites of Enzymes

The orientation of rutin in the catalytic site of alpha amylase, ferredoxin NADP reductase and acetylcoa carboxylase indicated profound interaction in the form of proteinligand hydrogen bond formation. With alpha amylase, it had a binding energy score of -5.60 kJmol-1 and formed five hydrogen bonds with active pocket residues; Glu86, Asp 81, His84 arg31 and Arg75 (Figure 4.52a). With Acetyl-CoA carboxylase, it also had a binding energy score of -5.60 kJmol-1 and formed five hydrogen bonds with active pocket residues Lys88, Arg76, Arg76, Glu87, Ser72 and Arg88 (Figure 4.52b). With Ferredoxin-NADP reductase, it had a binding energy score of -5.60 kJmol-1 and formed five hydrogen bonds with active pocket residues Tyr62, Leu88, Thr43, Arg60, arg93 (Figure 4.52c).

4.27 Docked Interactions between Quercetrin and Active Sites of Enzymes

The orientation of Quercetrin in the catalytic sites of alpha amylase, ferredoxin NADP reductase and acetylcoa carboxylase indicated profound interaction in the form of protein-ligand hydrogen bond formation. With alpha amylase, it had a binding energy score of -5.70 kJmol-1 and formed four hydrogen bonds with active pocket residues; Arg63, Glu77, Arg60 and Ser38 (Figure 4.53a). With Acetyl-CoA carboxylase, it also had a binding energy score of -6.0 kJmol-1 and formed four hydrogen bonds with active pocket residues Glu84, Glu86, Glu88, rg56 (Figure 4.53b). With Ferredoxin-NADP reductase, it also had a binding energy score of -5.2 kJmol-1 and formed three hydrogen bonds with active pocket residues Asp70, Met77, Arg68 (Figure 4.53c).

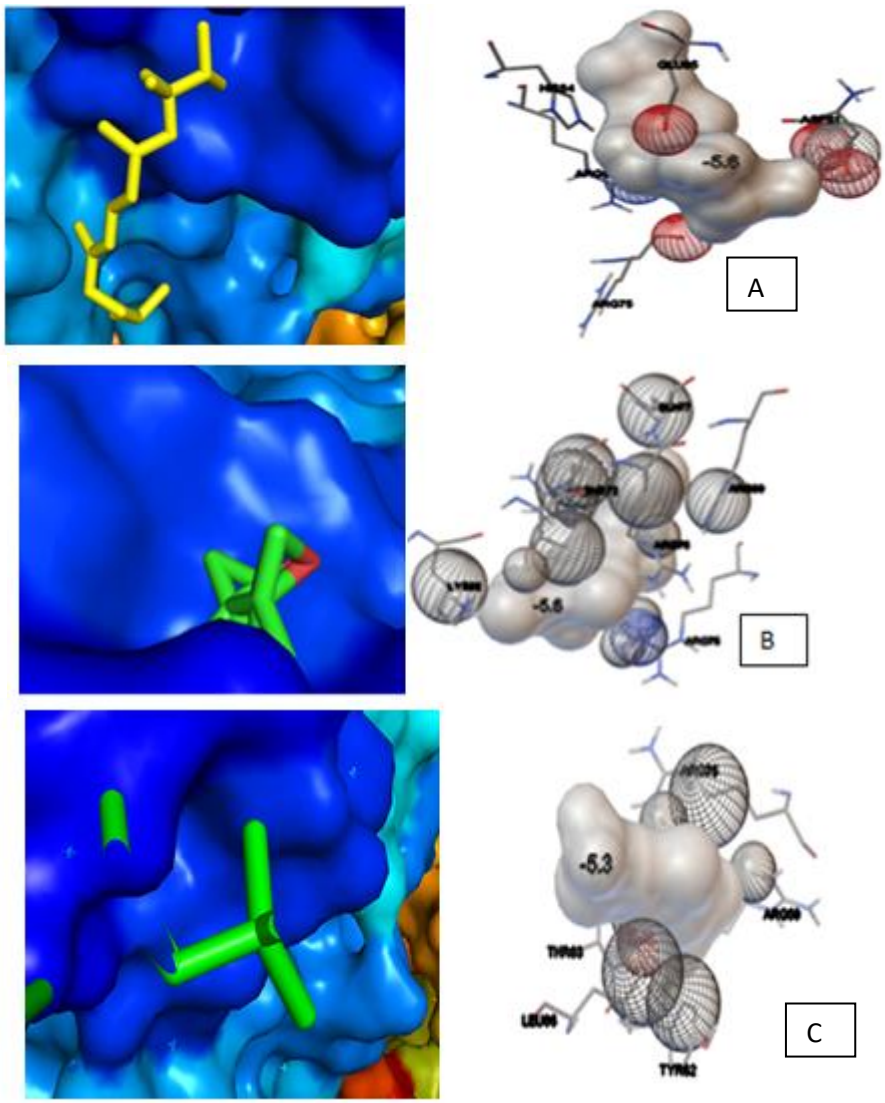


Figure 4.52: A- Alpha amylase active site residues Glu86, Asp 81, His84, Arg31 and Arg75; Binding energy score = -5.6. B- Acetyl-CoA Carboxylase active site residues Lys88, Arg76, Glu87, Ser72, Arg88; Binding energy score = -5.6. C- Ferredoxin-NADP reductase active site residues Tyr62, Leu88, Thr43, Arg60, Arg93; Binding energy score = -5.3. Where, Glu- Glutamate, Asp- Aspartate, His- Histidine, Arg- Arginine, Lys- Lysine, Ser- Serine, Tyr- Tyrosine, Leu- Leucine, Thr- Threonine.

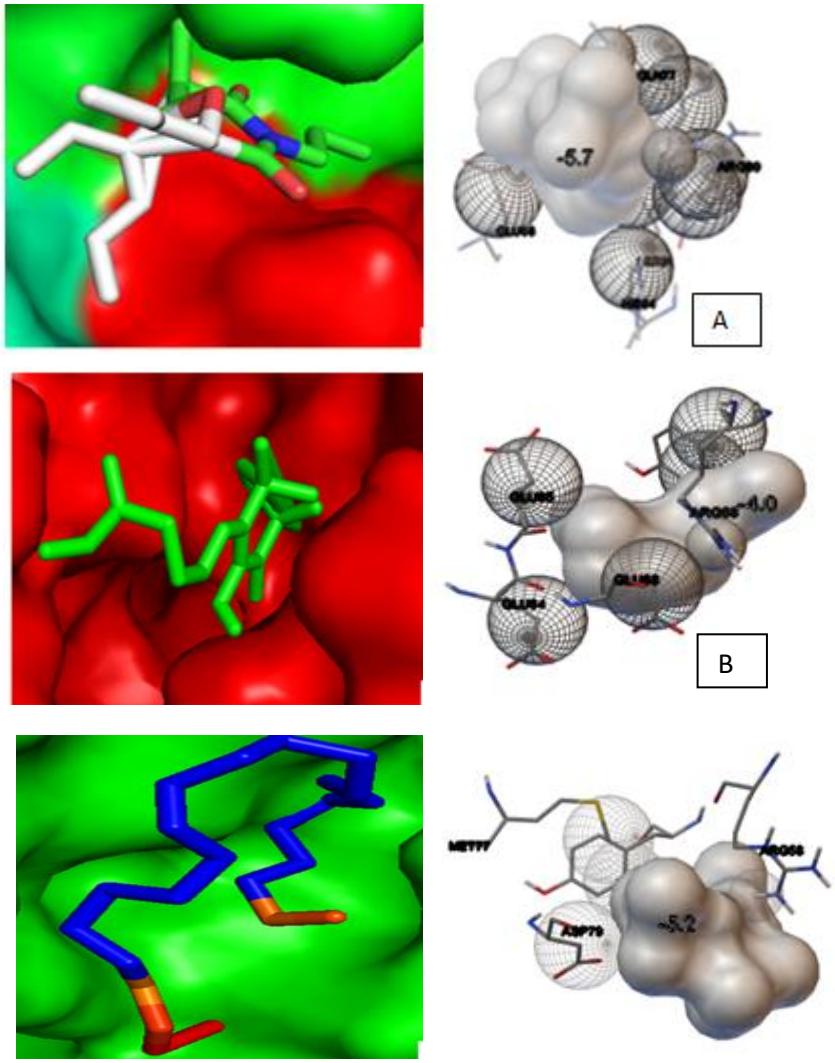


Figure 4.53:. A- Alpha-Amylase active site residues Arg63, Glu77, Arg60, Ser38; Binding energy score =-5.7. B- AcetylCoa carboxylase active Site Residues Glu84, Glu86, Glu88, Arg56; Binding energy score = -6.0. C- Ferredoxin-NADP reductase Active Site Residues Asp70, Met77, Arg68; Binding energy score = -5.2. Where Asp- Aspartate, Met- Methionine, Arg- Arginine, Glu- Glutamate, Arg- Arginine, Arg- Arginine, Ser- Serine.

CHAPTER FIVE

DISCUSSION

The dangers posed by synthetic herbicides have led to the need to discover equally effective natural herbicides for controlling weeds. Results from this study have shown that the aqueous extract of *Alchornea laxiflora* contains substances which could be used as agents for the development of bio-herbicides.

The growth-inhibitory properties of allelopathic plants' extracts have been linked to high contents of toxic allelochemicals in such extracts including alkaloids, coumarins, lignans, phenols, tannins, and flavonoids (Shah *et al.*, 2011; Flamini, 2012). Thus, the high flavonoid content of *Alchornea laxiflora* may be responsible for its allelopathic property. The relatively high concentration of flavonoids reported in this study (**Table 4.1**) corresponds with previous submissions (Oloyede *et al.*, 2011; Akinpelu *et al.*, 2015) and the allelopathic activity of plants has been linked to their flavonoid content (Shah *et al.*, 2011). Flavonoid allelochemicals, including coumarins and tannins can suppress the activities of enzymes involved in cell division thereby causing growth inhibition (Politycka, 1997).

Exposure to *Alchornea laxiflora* may have caused an inhibition on cell division leading to a reduction in root growth and an increase in percentage root growth inhibition (Mustafa and Arnkon, 2008). This probably accounted for the significant reduction in root length of the *Allium cepa* treated with the extract and Atrazine (**Figure 4.1**). This observation agrees with previous submission that exposure of *Allium cepa* roots to a phytotoxic alkaloid allelochemical caused a reduction in its root growth (Fasaanu *et al.*, 2013). This shows that the extract may have negative effects on rate of mitosis and chromosomal activities during cell division. Cytotoxic substances

exhibit destructive effects on chromosomes of cells and this could be responsible for their growth-inhibitory effects (Ping *et al*., 2012). Thus, the reduction in mitotic index (**Table 4.3**) and chromosomal damage in the *Allium cepa* root cells (**Figure 4.2**) are indications of cytotoxicity and genotoxicity at concentrations above 100 µg/ml of the extract. These observations are however in line with an earlier report of Ping *et al*. (2012) that *Euphorbia hirta* methanolic extract exhibited genotoxic and mito-depressive effects on *Allium cepa* root cells at 1,000 µg/ml. The non-genotoxic effect of the extract at a low concentration of 100 µg/ml (**Figure 4.2**) also conforms to an earlier report by Adegbite and Sanyaolu (2009) that aqueous extract of *V. amygdalina* at a low concentration (100g/l) caused a reduction in mitotic index but did not induce any chromosomal aberration. Synthetic herbicides may cause disturbances in the spindle fiber formation thereby leading to abnormalities in the chromosomes such as C-mitosis and lagging chromosomes. The cytotoxic and genotoxic effects thus observed with atrazine treatment are confirmations of the cytogenotoxicity of the chemical and conforms to an earlier study of Sharma and Pal-Vig (2012) that reported cytotoxic and genotoxic potentials of the herbicide. These results thus indicate that the extract of *Alchornea laxiflora* leaves may contain toxic allelochemicals such as flavonoids and coumarins, with growth inhibitory effects.

Phytotoxic flavonoids including apigenin, quercetin, rutin, present in extracts of plants, may inhibit the germination and growth of seedlings by altering enzyme activities which are responsible for mobilization of stored nutrients in seeds (Basile *et al*., 2000; Einhellig, 2009). Thus, findings from this study showed that *Alchornea laxiflora* extract may have altered the activities of hydrolytic enzymes in tomato seeds. This probably accounted for the significant inhibition of seed germination (**Table 4.5**), significant reduction in alpha amylase activities (**Figure 4.3**) (Einhellig, 2009) and subsequent significant reductions in plumule (**Figure 4.4**) and

radicle (**Figure 4.5**) lengths of the seedlings (Sunmonu and Van-Staden, 2014). Similar observations seen with the atrazine-treated seeds were further confirmations of the phytotoxic activity of the chemical. Also the negative response indices (RI) of allelopathy in the plumule and radicle of the tomato seedlings are further indications that the extract possesses growth inhibitory substances (**Table 4.6**). These findings are however in line with an earlier study that reported the inhibitory effects of sunflower (*Helianthus annuus*) extract on germination and growth of *Parthenium hysterophorus* (Rawat *et al*., 2011).

Exposure of plants to toxic polyphenolic compounds and other phytotoxic substances like synthetic herbicides might cause inhibition of the Photosystems of the photosynthetic apparatus (Leu *et al*., 2002; Hans-walter, 2005). This probably accounted for the significant decrease in the Ferredoxin NADP-reductase activity (**Figure 4.6**) in the extract and atrazine-treated tomato seedlings, an enzyme present in the Photosystem 1 of the photosynthetic machinery. Inhibition of this enzyme would result to reduced NADPH formed and thus hindering chlorophyll formation. This also probably accounted for the reduced chlorophyll contents of the extract and atrazine treated seedlings (**Table 4.7**). This however is in line with an earlier submission by Fasaanu *et al*. (2013) that exposure to phytotoxic alkaloid caused a significant reduction in chlorophyll contents of cowpea seedlings.

Phytotoxic substances may inhibit the synthesis of primary metabolites in plants, thus affecting the growth and survival of such plants (Duke *et al*., 2000). This may have accounted for the significant decrease in AcetylCoA carboxylase enzyme activity (**Figure 4.7**), an enzyme that catalyses the committed step during lipid synthesis, in the extract and atrazine- treated seedlings (Burton *et al.,* 1987). This result indicates phytotoxicity of the extract and conforms to earlier studies that reported AcetylCoA carboxylase enzyme inhibition on exposure to phytotoxic

substances (Duke *et al*., 2000; Hans-walter, 2005). These are however, further indications that the *Alchornea laxiflora* aqueous leaf extract contains toxic polyphenolic allelochemicals which exhibited deleterious effects on the seedlings.

Toxic Allelochemicals have also been shown to have damaging effects on cell membranes of plants (Li *et al*., 2013), as such exhibiting potential toxicity on cells. This cellular toxicity may be caused by an increased production of reactive oxygen species (ROS) and a concurrent alteration in antioxidant enzyme activities (Saeedipour and Moradi, 2014). ROS are highly reactive and toxic molecules that cause oxidative damage to membranes, DNA, proteins, photosynthetic pigments and lipids. These ROS are generally produced in plants under adverse conditions, including stress conditions experienced during allelopathic interactions (Li *et al*., 2013).

Plants produce a number of antioxidant enzymes which provide secondary protection against cellular damage arising from ROS generation. SOD activity in plants is usually taken as a measure of the degree of damage during an environmental stress condition (Liang *et al*., 2003) while POD may restrain peroxidation of cell membrane under adverse conditions and reduce injury that the cell membranes may suffer (Zhi-hui *et al*., 2011). CAT is another important antioxidant enzyme in plants which functions as an oxidoreductase. The reduction in the activities of these enzymes (**Figures 4.9, 4.10 and 4.11**) in response to extract and atrazine treatment may be attributed to high amounts of ROS generated within the seedlings. ROS have the tendency to overwhelm antioxidant enzymes, resulting in depletion of their activities. This also agrees with the increase in MDA content in these seedlings when compared with the control. These findings are consistent with previous studies which reported alteration in antioxidant enzyme activities of seedlings in the presence of phytotoxic chemicals (Saeedipour and Moradi,

2014). Hence, phytotoxic substances may induce stress in their victims including osmotic stress (Szekely, 2004). Accumulation of proline, a well-known osmoprotectant is a physiological response to osmotic stress (Szekely, 2004). Proline acts as a hydrophobic protectant for enzymes and subcellular organelles, thus safe-guarding plants exposed to osmotic stress (Lerudulier *et al*., 1994). The increase in proline in this study (**Figure 4.12**) is an indication of an adaptive response of the seedlings to osmotic stress caused by phenolic allelochemicals present in the extract (Barkosky *et al*., 2000) and conforms to an earlier study that reported a slight increase in proline content of the bean seedling when exposed to *D.dumetorum* alkaloid. Phenolic allelochemicals reduce the hydraulic conductivity of roots thereby causing changes in seedlings' water parameters (Barkosky *et al*., 2000) and thus may result to osmotic stress. Amino acids especially proline are however the major osmo-regulators in vascular plants (Balestrasse *et al*., 2005; Okem *et al*., 2015). This might probably be due to the convenience of osmolyte storage in large osmotically inactive molecules such as starch or protein, which serve several functions and from which they could be retrieved under conditions of stress (Pirzard *et al*., 2011). This result therefore further indicates that the extract may contain some stress-inducing polyphenolic allelochemicals.

Several studies have also documented flunctuations in protein contents of plants grown under environmental stress conditions (Hameed and Ashraf, 2008; Jaleel *et al*. 2008) including allelochemicals (Einhellig, 2001). Proteins are mainly involved in the architecture of plant cells, are the chief source of nitrogenous metabolism and as well a source of energy during chronic period of stress (Mommensen and Walsh, 1992). The increase in protein in this study (**Figure 4.13**) therefore further indicates an adaptive response of the seedlings to the stress-inducing polyphenols present in the extract (Singh *et al*. 1996).

Reports have shown significant increase in soluble sugar concentrations of plants exposed to environmental stress conditions (Mohammadkhani and Heidari, 2008; Farhad *et al*., 2011). Thus, the increase in soluble sugar in this study (**Figure 4.14**) further indicates stress caused by the extract. The increase in sugar concentrations might be as a result of degradation of starch (Fischer and Holl, 1991) and might function as an osmoprotectant, stabilizing cellular membranes and maintaining tugor pressure (Farhad *et al*., 2011). Other essential roles of soluble sugars in plant metabolism include substrates in biosynthetic processes, energy production and also in sugar sensing and signalling systems (Mohammadkhani and Heidari, 2008).

A number of phytotoxic allelochemicals have been isolated through chromatographic techniques and could be used as templates for bio-herbicide development (Wu *et al*., 2002). Chon and Kim, (2004) reported the allelopathic activities of aqueous extracts of four crop plants in the Poaceae family: Barley, oats, rice and wheat, on alfalfa, barnyardgrass and eclipta seeds. The substances responsible for their activities were identified by HPLC as trans-cinnamic, hydrocinnamic, salicylic, ferulic, caffeic, m-, o-, p-coumaric acids.

In this study, the greater percentage inhibition of *Allium cepa* roots caused by the ethyl acetate fraction of the extract, relative to other fractions (**Table 4.8**) is an indication that it contains most of the allelochemicals with growth-inhibitory properties suspected in the aqueous extract (Tseng *et al*, 2001). Also, the relatively higher IC_{50} value of the ethyl acetate fraction when compared to the aqueous extract (**Table 4.9**) is an indication of increased activity due to isolation (Einhellig, 1995). The phytotoxic flavonoids of the *Alchornea laxiflora* leaf extract may be soluble in the ethyl acetate, making the ethyl acetate fraction growth-inhibitory (Wu *et al*., 2002). This may have accounted for the significant reduction in percentage germination (**Table 4.10**), alpha amylase activity (**Figure 4.15**) and in shoot (**Figure 4.16**) and root lengths (**Figure 4.17**) of the

tomato seedlings when exposed to the ethyl acetate fraction, which however compared favorably with the aqueous extract. This result suggests that the allelopathic flavonoids such as quercetin-3-O-β-D- glucopyranoside, quercetin 3, 7, 3', 4'-tetrasulphate, quercetin, quercetrin, quercetin - 7, 4- bisulphate, quercetin - 3', 4'-disulphate, quercetin -3, 4'-diacetate and rutin (Ogundipe *et al*., 2001), suspected in the aqueous extract may be soluble in ethyl acetate fraction of *Alchornea laxiflora* leaf extract (Oloyede *et al*., 2011; Borokini and Omotayo, 2012).

In general, the ethyl acetate partitioned fraction exhibited similar activities with the aqueous extract when subjected to the various biological tests including causing significant reduction in important parameters like Ferredoxin-NADP reductase activity (**Figure 4.18**), chlorophyll concentration (**Table 4.11**), acetylCoA carboxylase activity (**Figure 4.19**); inducing oxidative stress as observed from the significant increase in MDA content (**Figure 4.20**) in the tomato seedlings and concurrent reduction in the activities of antioxidant enzymes including SOD (**Figure 4.21**), CAT (**Figure 4.22**) and POD (**Figure 4.23**); as well as inducing osmotic stress as observed from the significant increase in proline concentration (**Figure 4.24**), protein concentration (**Figure 4.25**) and total soluble sugar (**Figure 4.26**). This further indicates that the same phytotoxic flavonoids including quercetin-3-O-β-D- glucopyranoside, quercetin 3, 7, 3', 4'-tetrasulphate, quercetin, quercetrin, quercetin - 7, 4- bisulphate, quercetin - 3', 4'-disulphate, quercetin -3, 4'-diacetate and rutin (Ogundipe *et al*., 2001), suspected in the aqueous extract are present in the ethyl acetate fraction.

The presence of Rutin and Quercetrin as identified using High performance Liquid Chromatography (HPLC) in this study conforms to an earlier report of Ogundipe *et al*. (2001) that identified these compounds as constituents of the *Alchornea laxiflora* aqueous leaf extract. The phytotoxic activities demonstrated by these compounds ranging from germination inhibition

to osmotic stress induction are however, indications that they are responsible for the same phytotoxicity exhibited by the aqueous extract. This however conforms to an earlier submission that the aqueous extract of from *Castanea sativa* leaves, as well its purified constituents, quercetin and rutin, caused significant inhibitions in both seed germination and development of *Raphanus sativus* L., Brassicaceae (Basile *et al*., 2000).

Phytotoxic substances when isolated may probably exert greater effects on their hosts due to absence of any form of interference (Einhellig, 1995). In this study, the compounds may have exhibited higher activities when tested in isolation. This may however be responsible for the increased percentage aberration when exposed to these compounds (14.89% and 15%) (**Table 4.18**) as compared to the extract (4.08%) (**Table 4.4**) and could mean that these compounds are also responsible for the genotoxic exhibited by aqueous extract. This however conforms to earlier submissions that mixtures of phenols exhibited less growth-inhibitory activities than individual compounds (Einhellig, 1995; Wu *et al*., 2002).

In addition to the biochemical effects which the isolated compounds have proven to exhibit, autodock analysis revealed favourable interactions between the compounds and amino acid residues of Alpha-amylase, Acetyl-CoA carboxylase and Ferredoxin-NADP reductase (**Figure 4.52, Figure 4.53**). Auto dock is a ligand docking program for predicting protein- ligand binding modes and for virtual screening. It is thus an automated procedure for predicting the interactions between ligands and macro molecular targets (Doraiswamy *et al*., 2014). Studies have reported favorable interactions between plant secondary metabolites and amino acids of proteins (Archana *et al*., 2010; Doraiswamy *et al*., 2014; Sudeep and Shyam, 2014).

Overall, the aqueous extract of *Alchornea laxiflora* as well as the purified flavonoids, (Rutin and Quercetrin isolates) demonstrated allelopathic potentials ranging from causing significant reductions in germination and seedling growth, inhibition of photosynthesis as expressed by significant reductions in chlorophyll content and Ferredoxin NADP reductase activity important biochemical enzymes, to inducing oxidative and osmotic stress stress in tomato seedlings as expressed significant reductions in activities of antioxidant enzymes and increase in osmotic stress parameters as summarized in figure 5.1.

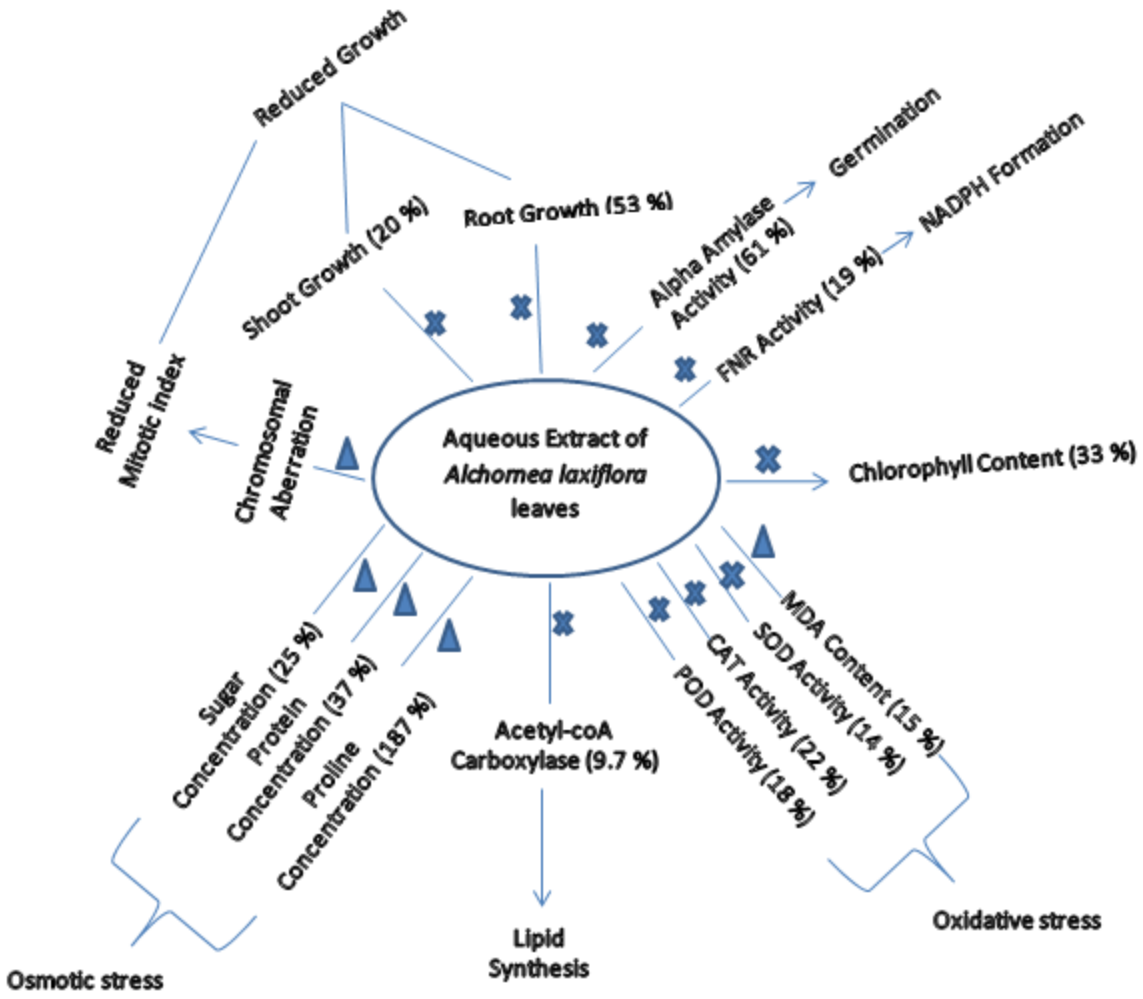


Figure 5.1: Proposed Mechanism of Action of the Allelopathic Activity of Aqueous Leaf Extract of *Alchornea laxiflora* Using Tomato

Legend:

✖ — Reduction

▲ — Increase

SUMMARY OF FINDINGS

1. The aqueous leaf extract of *Alchornea laxiflora* contained a number of phytochemicals including Alkaloids, Phenols, Terpenes, Tannin, Flavonoids.

2. The extract caused a concentration-dependent increase in percentage inhibition of *Allium cepa* root growth as a result, having an IC_{50} of 400µg/ml.

3. The extract caused a reduction in the rate of mitosis in *Allium cepa* root cells as evident from reduced mitotic index which was accompanied by disturbances in chromosomal activities at high concentrations.

4. The extract caused significant reductions in percentage germination, chlorophyll concentration, root and shoot lengths of tomato seedlings, and also significant reductions in activities of alpha amylase, AcetylcoA carboxylase and Ferredoxin NADP-reductase enzymes of tomato seedlings.

5. The extract caused lipid peroxidation in the tomato seedlings which was accompanied by significant reduction in the activities of the antioxidant enzymes SOD, CAT, and POD as well as accumulation of osmotic stress indicators such as proline, protein and soluble sugars.

6. The ethyl acetate partitioned extract of the aqueous leaf extract with IC_{50} value of 260 µg/ml exhibited phytotoxic activities which were similar to that of the aqueous extract.

7. On subjection to different chromatographic techniques and biological tests, the ethyl acetate partitioned extract was found to contain Rutin and Quercetrin which also exhibited similar phytotoxic activities when compared to atrazine.

8. Both Rutin and Quercetrin caused some degrees of chromosomal abnormalities in *Allium cepa* root cells ranging from C-mitosis, abnormal metaphase.

9. Molecular docking of Rutin and Quercetrin with Alpha amylase, AcetylCoa carboxylase and Ferredoxin NADP-reductase, revealed strong binding energies, an indication of favorable interactions with the proteins, further confirming the bioactive agents.

CONCLUSION

This study shows that Rutin and Quercetrin are partly responsible for the allelopathic activity exhibited by *Alchornea laxiflora*. Therefore *Alchornea laxiflora* can be regarded as a potential source of Rutin and Quercetrin, which could be used as templates for the development of Bio-herbicides.

RECOMMENDATIONS

Due to the findings and limitations of this study, the following are recommended:

1. The aqueous extract of *Alchornea laxiflora* is a source of agents that can be used to develop bio-herbicides.
2. The leaves of *Alchornea laxiflora* cannot be used for mulching practice on tomato farm.
3. Care should be taken in handling Quercetrin and Rutin as these compounds are genotoxic.

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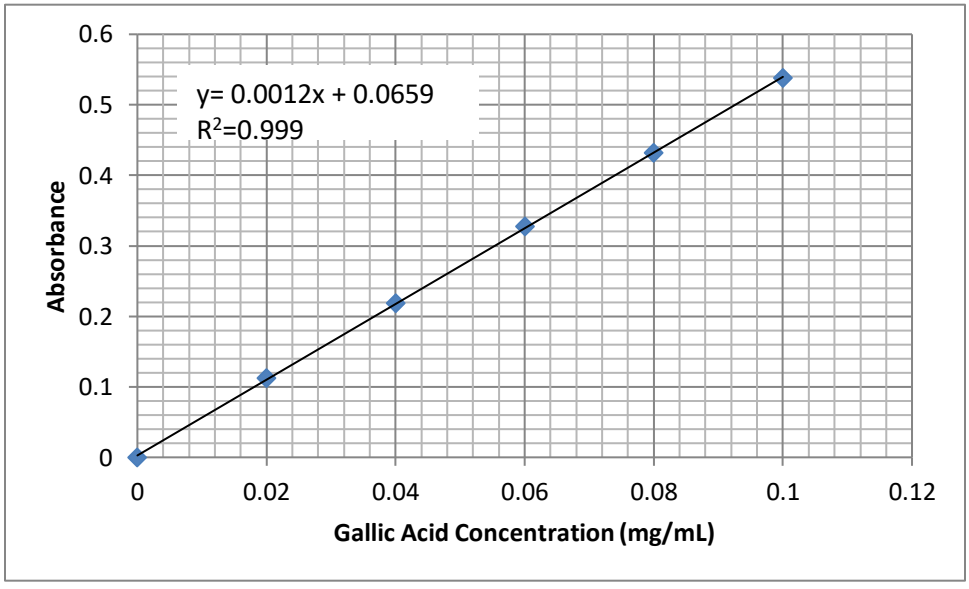
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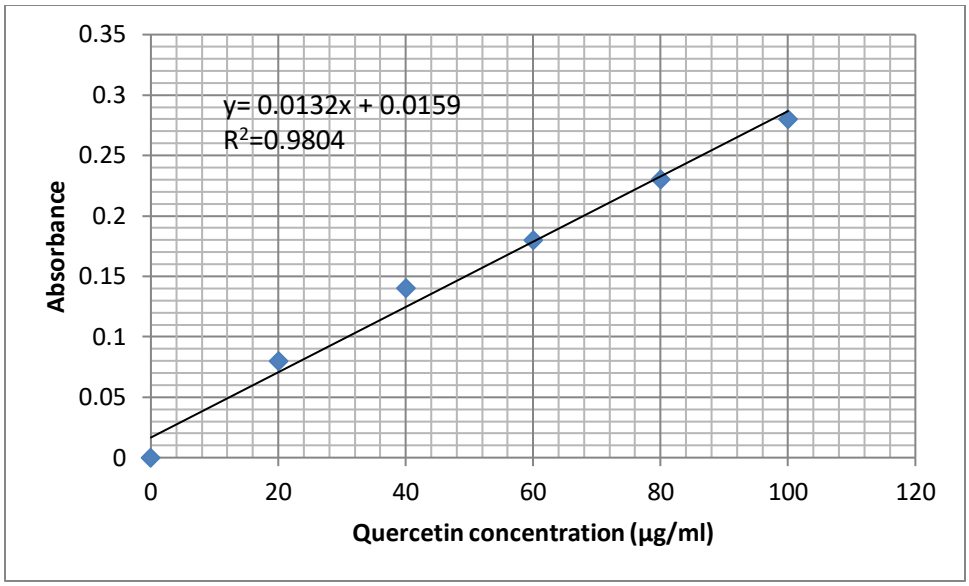
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APPENDIX I

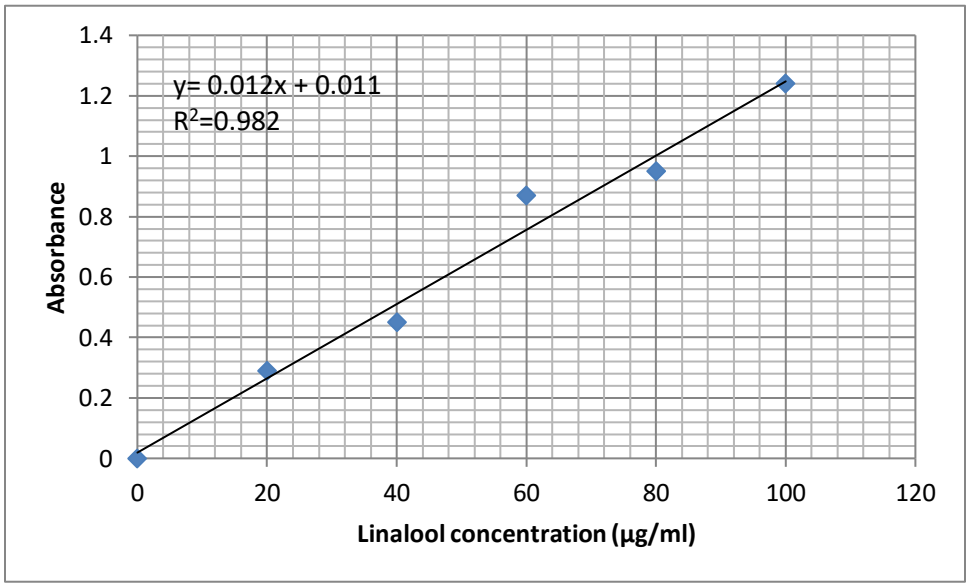
I-A: Standard Caliberation Curve for Gallic Acid



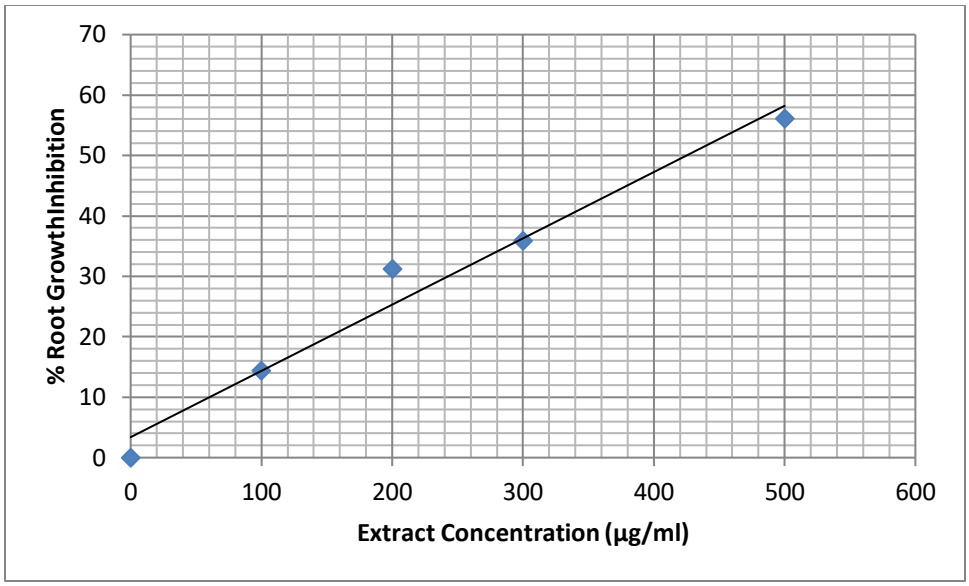
I-B: Standard Calibration Curve for Quercetin



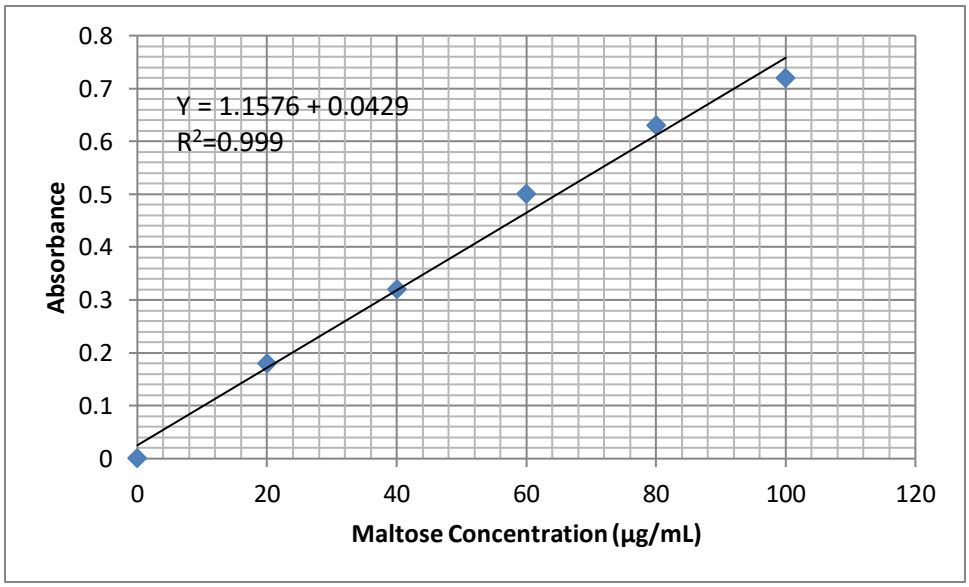
I-C: Standard Calibration Curve for Linalool



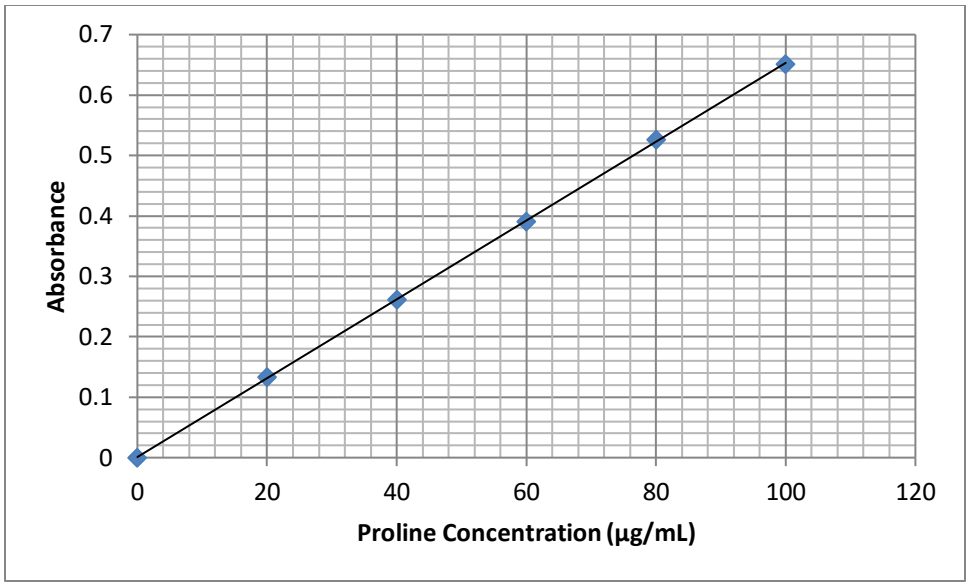
I-D: IC_{50} Determination of Leaf Aqueous Extract of *Alchornea laxiflora*



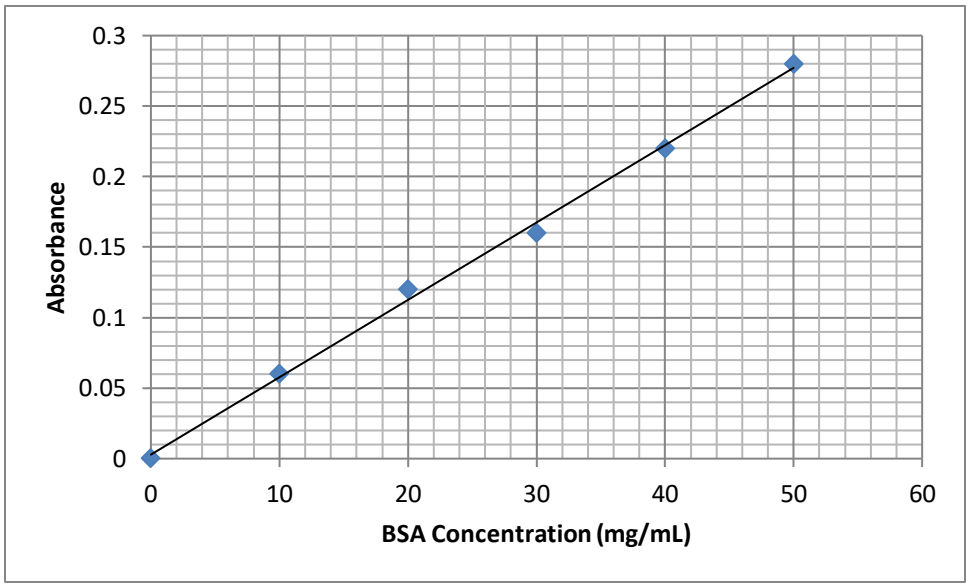
I-E: Standard Calibration Curve for Maltose



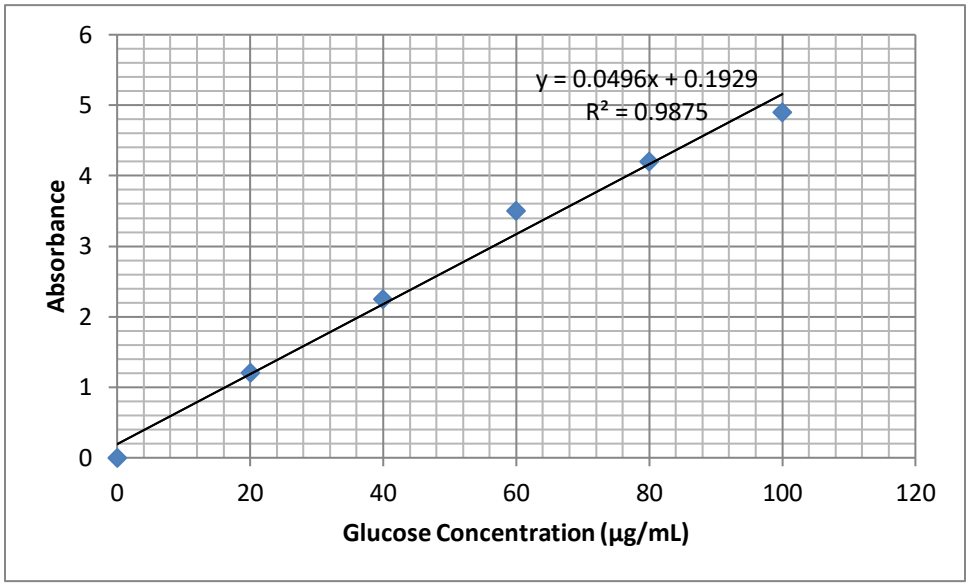
I-F: Standard Calibration Curve for Proline



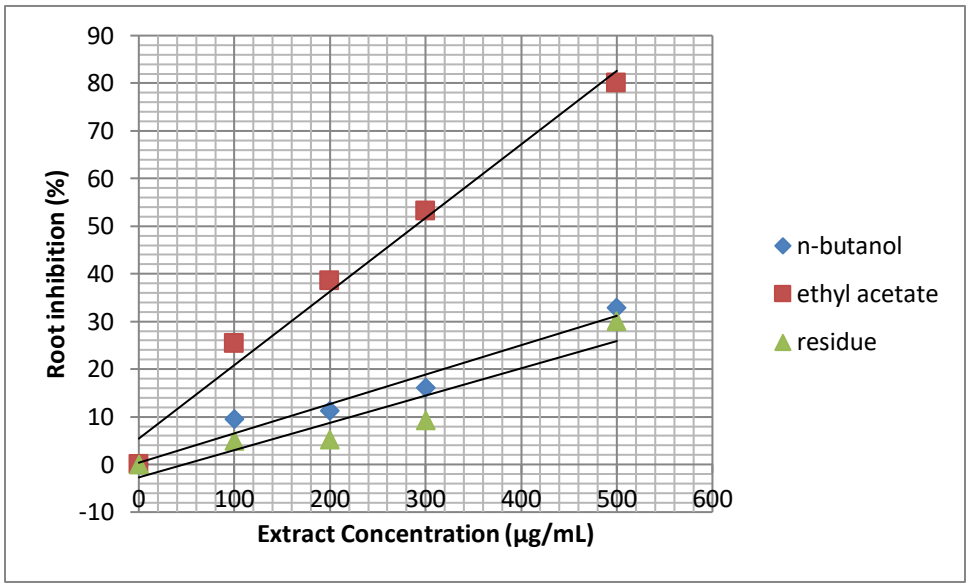
I-G: Standard Caliberation Curve for Bovine Serum Albumin (BSA)



I-H: Standard Caliberation Curve for Glucose

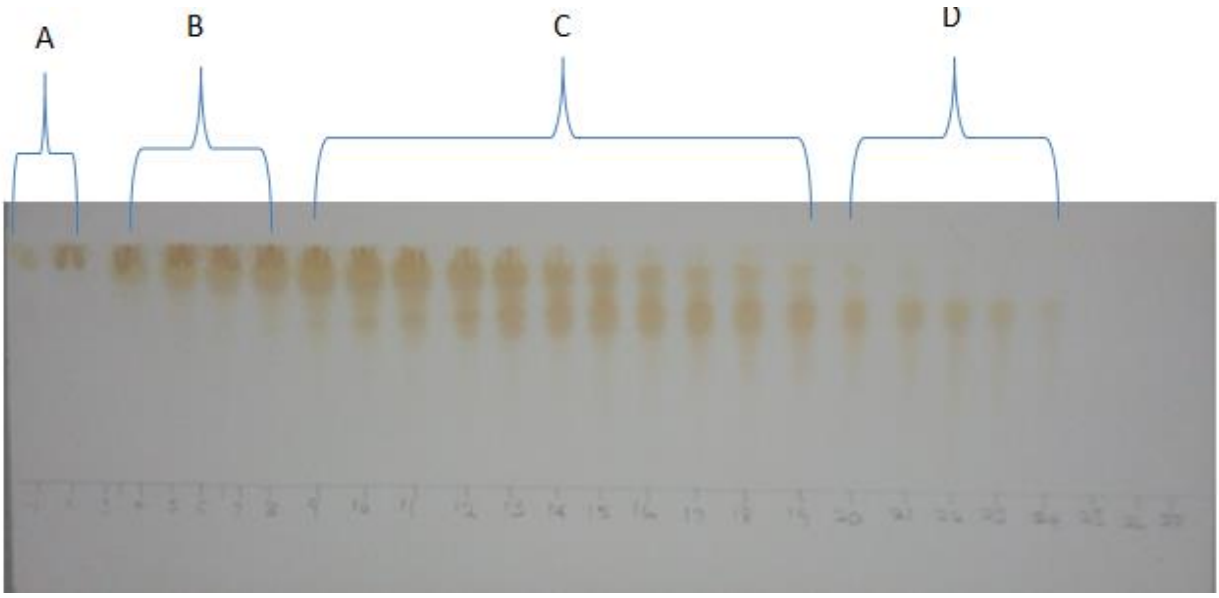


I-I: IC_{50} Determination of IC_{50} Solvent-solvent Partitioned Fractions



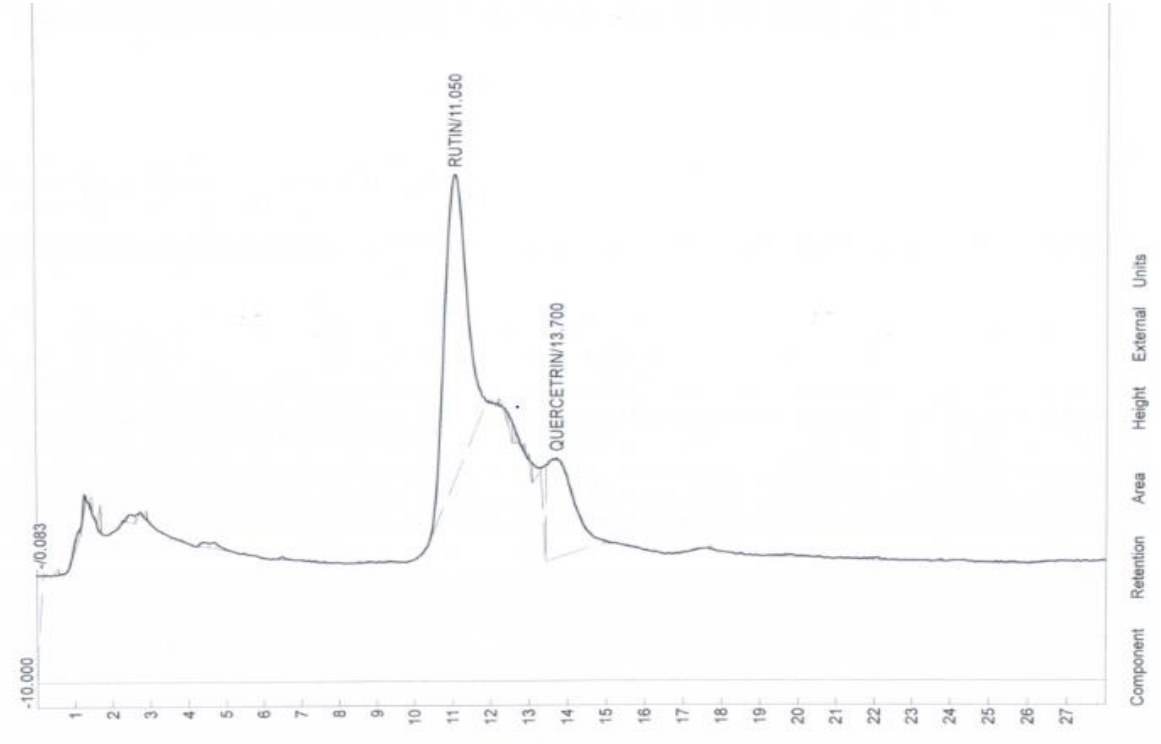
APPENDIX II

I1- A: Determination of R_f values of ethyl acetate column chromatography eluents



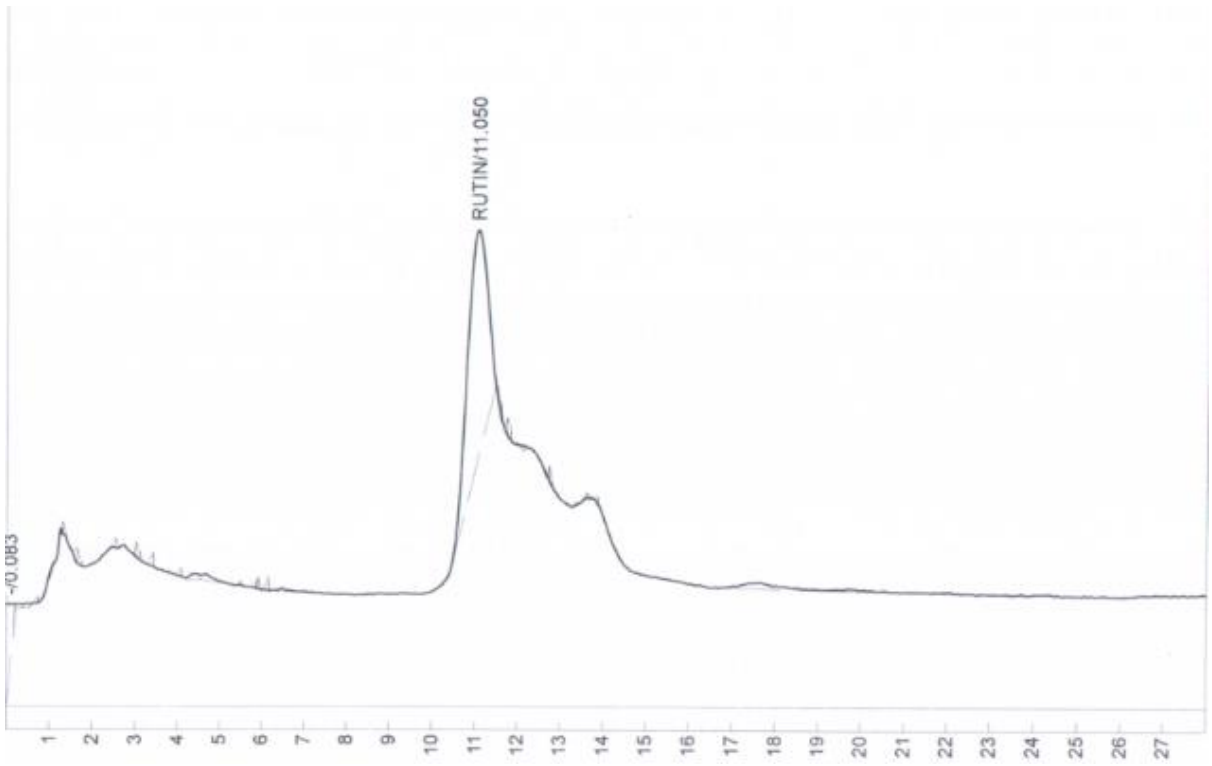
I1- B: High-performance liquid chromatography peaks of fraction C

Carrier- Acetonitrile/water (75:25).

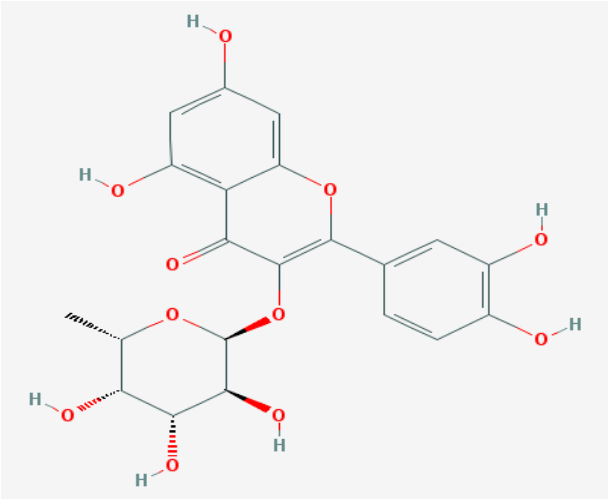


II- C: High-performance liquid chromatography peaks of fraction D

Carrier- Acetonitrile/water (75:25).



II-D: Structure of Quercetrin



II-D: Structure of Rutin

