**sTOPIC**

**ASSESSMENT OF THE CYTOTOXICITY OF AQUEOUS LEAF EXTRACT OF VERNONIA AMYGDALINA USING THE ALLIUM TEST**

**CHAPTER ONE**

**INTRODUCTION**

**1.0 BACKGROUND TO THE STUDY**

**1.1 EXPERIMENTAL PLANT: VERNONIA AMYGDALINA**

Vernonia amygdalina, a member of the daisy family, is a small shrub that grows in tropical Africa. V. amygdalina typically grows to a height of 2–5 m (6.6–16.4 ft). The leaves are elliptical and up to 20 cm (7.9 in) long. Its bark is rough. *V. amygdalina* is commonly called bitter leaf in English because of its bitter taste. African common names include grawa (Amharic), ewuro (Yoruba), etidot (Ibibio), onugbu (Igbo), ityuna (Tiv), oriwo (Edo), chusar-doki (Hausa) (Burkill, 1985).

Vernonia amygdalina (family of asteraceae) is a valuable medicinal plant that is widespread in East and West Africa2. It is known as bitter leaf and may be used as active anti-cancer3, anti-bacteria, anti-malarial, and anti-parasitic agent4. This plant contains complex active components that are pharmacologically useful. The roots and the leaves are used in ethnomedicine to treat fever, hiccups, kidney problems, and stomach discomfort5. The stem and root divested of the bark are used as chew-sticks in many West Africa countries like Cameroon, Ghana, and Nigeria. *Vernonia amygdalina* (VA) leaves are one of the most widely leaf vegetables consumed by Cameroonians during special occasions such as marriages, baptisms, Christmas, and birthday.

Pharmacological studies have also shown that the leaf extracts have both hypoglycaemic and hypolipidaemic properties in experimental animals and so could be used in managing diabetes mellitus6. Traditional medical practitioners, herbalists, and local healers in West Africa recommend aqueous VA for their patients. The beneficial use of VA in animal nutrition in Nigeria has been well documented (Aregheore et al., 1998) reported that VA leaf extract enhanced the prophylactic and therapeutic efficacy of chloroquine against Plasmodium berghei malaria in mice.

It is popularly called bitter leaves because of its bitter taste and is used as vegetables or as flavour decoction soups. The bitter taste of VA is as a result of its anti-nutritional components such as alkaloids, saponins, glycosides and tannins (Aregheore et al., 1998). In Nigeria, it is known by several local names such as “Ewuro” in Yoruba language, “Onugbu” in Igbo language, “Oriwo” in Bini language, “Ityuna” in Tiv language, “Chusar doki or fatefate” in Hausa language and “Etidot” in Ibiobio. The roots and leaves decoction of VA are commonly used in ethno medicine to treat fevers, hiccups, kidney problems and stomach discomfort among other several uses (Smaka-Kinel et al., 1996). It is also used in the treatment of diarrhea, dysentery hepatitis and cough and as a laxative and fertility inducer (Smaka-Kinel et al., 1996).

The leaves of VA are also commonly used as a treatment against nematodes in humans and chimpanzees as well as other intestinal worms (Okoh et al., 2010). In addition, extracts of the plants have been reported to be used in Nigerian herbal homes as tonic, in the control of tick and treatment of hypertension. The reported activity of VA is attributable to the complex active secondary plant compounds that are pharmacologically active. Besides, this review also discusses the toxicity of this species and it gave a reasonable information that no abnormality or toxicity was caused by the administration of some of the extracts as well as single compounds on the organs of the animal samples. Nevertheless, there is need to carry out further study on chemical constituents and their mechanisms of action in other to fully comprehend its full phytochemical profile and complex pharmacological effects. Furthermore, clinical studies on the toxicity of all the plant parts extracts and the compounds isolated from this plant are required to ensure that they are eligible as sources of drugs.

**1.1.1 SCIENTIFIC CLASSIFICATION**

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Asterids

Order: Asterales

Family: Asteraceae

Genus: Vernonia

Species: V. amygdalina

Binomial name: *Vernonia amygdalina*

**1.1.2 MEDICINAL USE AND PROPERTIES**

The Tongwe use cold concoctions of this plant as a treatment for malaria, intestinal parasites, diarrhea, and stomach upset. For numerous African ethnic groups, a concoction of this plant is also a prescribed treatment for malarial fever, schistosomiasis, amoebic dysentery, and several other intestinal parasites and stomach aches.

The aqueous and alcoholic crude extracts of the leaves, bark, stem and roots are reported to be widely used as antimalarial, for the treatment of eczema and as a purgative. The roots and the leaves of VA are used in traditional medicine to treat fever, stomach discomfort, hiccups and kidney problems. It is known as quinine substitute because it is widely used for the treatment of fevers. The wood, particularly those from the root is a tooth cleaner, an appetizer, fertility inducer and also for gastrointestinal upset. The root infusion is taken in Nigeria for the treatment of intestinal worms as well as for enteritis and rheumatism. Wild Chimpanzees have been observed to eat both the leaves and stems of the plant as a medication for self-deparasitization. Other documented medicinal uses include the treatment of schistosomiasis, amoebic dysentery, treatment of malaria, wound healing, veneral diseases, hepatitis and diabetes. Fresh leaves of VA have been reported to have abortifacient and purgative activities. It is used in some part of Africa to prepare cough remedy. The chopped roots of VA are used for the treatment of sexually transmitted diseases in parts of Zimbabwe. The root of VA is used for its antifertility effect and for the treatment of amenorrhoea .

**Pharmacology**

The Pharmacological properties of VA have been investigated with a view to validate the wide traditional uses of the plant as a therapeutic agent. Several research has shown that VA possesses the following activities; antidiabetic, antiplasmodial, cathartic, hepatoprotective, antimicrobial, antioxidant, chemoprotective and cytotoxic, antihelmintic, hypolipidaemic, anti-platelet and abortifacient activities.

**1.1.3 CYTOTOXICITY**

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are an immune cell or some types of venom, e.g. from the puff adder (*Bitis arietans*) or brown recluse spider (*Loxosceles reclusa*).

**Cell physiology**

Treating cells with the cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis).

Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shut down metabolism and release their contents into the environment. Cells that undergo rapid necrosis in vitro do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers. Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments. Cells in culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, lose membrane integrity and lyse (Yedjou et al., 2008).

**Measurement**

Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, if they are interested in developing a therapeutic that targets rapidly dividing cancer cells, for instance; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical.

Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components (Yedjou et al., 2008). Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside. One molecule, lactate dehydrogenase (LDH), is commonly measured using LDH assay. LDH reduces NAD to NADH which elicits a color change by interaction with a specific probe (Burkill, 1985). Protease biomarkers have been identified that allow researchers to measure relative numbers of live and dead cells within the same cell population. The live-cell protease is only active in cells that have a healthy cell membrane, and loses activity once the cell is compromised and the protease is exposed to the external environment. The dead-cell protease cannot cross the cell membrane, and can only be measured in culture media after cells have lost their membrane integrity (Izevbigie, 2003)

Cytotoxicity can also be monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or with 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), which yields a water-soluble product, or the MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use ATP content as a marker of viability (Yedjou et al., 2008). Such ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction.[4]

Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and clonogenic assay.

**1.1.4 ALLIUM CEPA TEST**

The *Allium cepa* test has been used by many researchers mainly as a bioindicator of environmental pollution (Bagatini *et al.,* 2009; Leme & Marin-Morales, 2009), testing crude extracts of cyanobacteria (Laughinghouse, 2007), as well as to evaluate the genotoxic potential of medicinal plants (Camparoto *et al.,* 2003; Knoll *et al.,* 2006; Fachinetto *et al.,* 2007;Lubini *et al.,* 2008; Fachinetto *et al.,* 2009; Fachinetto & Tedesco, 2009; Dalla Nora *et al.,* 2010),because this test uses a model that is adequately sensitive to detect innumerous substances that cause chromosomal alterations. The *Allium cepa* test is important since it is an excellent model *in vivo*, where the roots grow in direct contact with the substance of interest (i.e. effluent or complex medicinal mix being tested) enabling possible damage to the DNA of eukaryotes to be predicted. Therefore, the data can be extrapolated for all animal and plant biodiversity. The analysis of chromosomal alterations can be equal to the test of mutagenicity mainly for the detection of structural alterations; however, it is possible to observe numerical chromosomal alterations, as well. The *Allium cepa* test is one of the few direct methods for measuring damage in systems that are exposed to mutagens or potential carcinogens, and enables the evaluation of the effects of these damages through the observation of chromosomal alterations.

For this undertaking, it is necessary that the sample remain in constant mitotic division, seeking to identify the toxic effects and alterations over a cell cycle; and the *Allium cepa* test has been widely used for this purpose. It is advantageous to use the *Allium cepa* test system since its main component is a vascular plant, making it an excellent genetic model for evaluating environmental pollutants, detecting mutagens in different environments and evaluating many genetic endpoints (point mutations to chromosomal alterations). *Allium cepa* is distinctive in regards to its efficiency in detecting genetic damage and was introduced by Levan, in 1938, for helping observe disturbances in the mitotic fuse due to colchicine action. Relevant studies by Fiskesjö (1985), showed the importance of the *Allium cepa* test system for evaluating genotoxicity, demonstrating that *Allium cepa* cells contain an oxidase enzyme system capable of metabolizing polycyclic hydrocarbonates.

Even though other test systems have been shown to be sensitive for this detection, the results of the *Allium cepa* test should be considered as an alert for other organisms (i.e. bioindicators). Furthermore, Rank & Nielsen (1993) performed adaptations for evaluating complex mixtures and Ma *et al.,* (1995) adjusted the test for assessing mutagenicity and micronucleus analyses (MN) in F1 cells. Some researchers show certain restriction in regards to using plant test systems for evaluating certain classes of carcinogens, which require complex metabolization systems for the activation of its genotoxic action.

Vincentini *et al.,* (2001) reported that the *Allium cepa* test system is well accepted for the analysis of cytotoxicity and genotoxicity because the roots are in direct contact with the tested substance, allowing evaluation of different concentrations and times. The *in vivo* test of *Allium cepa* are necessary for contributing to their safe and efficient use. The plant test system of *Allium cepa* is as an ideal bioindicator for the first *screening* of genotoxicity, helping with studies that prevent damages to human health (Bagatini *et al.,*, 2007).

1.1.5 **STATEMENT OF THE PROBLEM**

Vernonia amygdalina is one of the most effective medicinal plant known worldwide. It has been well known for its ethnobotanical and therapeutical properties and the bio-active compounds that it contained. Despite these qualities, we all know that things with great advantages are also faced with several disadvantages (problems). It has been identified by some researchers that bitter leaf is having some side effects apart from the good qualities that its offers especially on the cell. Therefore cytotoxicity test has been carried out to look into this problem

Mitotic studies were carried out on Allium cepa (onion) root cells treated with aqueous extract of Vernonia amygdalina (bitter leaf). The onion cells showed reduced mitotic indices with corresponding increase in the bitter leaf concentration. Chromosomal aberrations such as endopolyploidization, lagging of chromosomes and cells with giant chromosomes were observed. Others include scattering of chromosome at anaphase, anaphase bridge, chromosome fragmentation etc. These observations indicate that abnormal use of this medicinal herb could cause genetic damage. By analyzing these cytotoxic effects of Vernonia amygdalina, observations, conclusions and proper recommendations can be derived successfully.

1.1.6 **RESEARCH QUESTION**

* What is the effect of the leaf extract on the different mitotic stages of the onion root tip cell at different concentrations of the aqueous leaf extract?
* What is the percentage of genetically damaged cell to the normal cell?
* What is the intensity and frequency of chromosomal aberration present in the onion root cell as a result of the increasing leaf extract concentration?
* What is the relationship between the leaf extract concentration and the effects it has on the onion root tip cells?

1.2 **OBJECTIVES OF THE STUDY**

1.2.1 BROAD OBJECTIVE

* To assess the cytotoxicity of Vernonia amygdalina using Allium cepa test

1.2.2 SPECIFIC OBJECTIVES

* To check for the normal mitotic division that is taking place in the onion root cells outside the influence of the leaf extract so that comparisons can be made.
* To check the effects of the medicinal herb on the different mitotic stages at different concentration of the aqueous extract of Vernonia amygdalina.
* To determine the extent of genetic damage the leaf extract of Vernonia amygdalina has on the onion root cell.
* To analyze the intensity and frequency of chromosomal aberration present in the onion root cell as a result of the increasing leaf extract concentration.
* To determine the relationship between the leaf extract concentration and the effects it has on the onion root tip cells.
* To make use of the observations to give a well defined recommendation on the intake of the medicinal herb and the use of it for treatment and cures.

1.3 **JUSTIFICATION**

There is a wide use and wide information on the economic importance, impacts, usefulness and diverse advantages of Vernonia amygdalina (bitter leaf) ranging from consumption to it medicinal, ethnobotanical, therapeutical and pharmaceutical properties and qualities. Therefore conducting a test to check on the effects it could have on the genetic make-up and structure of it users is very important to assess and make recommendation on the concentration at which it is advisable to make use of it after considering the effect it has over different and varying concentrations. By so doing, the general public will be well educated on the effects of this widely used medicinal herbs when used abnormally especially when considering the effects it has at different concentrations on the genetic make-up over time.

1.4 **HYPOTHESIS**

1.4.1 EXPERIMENTAL HYPOTHESIS

Mitosis consists of four distinct phases which are prophase, metaphase, anaphase and telophase. Mitosis occurs most frequently in apical meristematic tissue of the plant. The size of cell undergoing mitosis is greater than the size of cell that are not undergoing mitosis.

1.4.2 NULL HPOTHESIS

There are no distinct phases in mitosis. There is no distinct difference between the size of cell undergoing mitosis and the one that is not. Mitosis occurs averagely throughout the plant tissues.

**CHAPTER TWO**

**2.0 LITERATURE REVIEW**

2.1 MITOSIS AND ITS STAGES

The best characteristic of organisms is the ability to reproduce itself that best differentiate the living things from the nonliving matter. This continuity of life reproduction is based on the cell cycle. Cell cycle occurs in three stages or periods which are the interphase, karyokinesis and cytokinesis. Interphase is a long period which the cell grows and it is a period in which the cell is carrying an intense activity. During this phase, the proteins and the cytoplasmic organelles such as mitochondria, centrosome and endoplasmic reticulum are synthesized and the chromosomes are replicated and duplicated in preparation for mitosis. The chromosomes are in thin, long thread-like form which is called as chromatin. The chromatin is not so visible as they are not in a condensed form yet.

Cell cycle continues with cell division after going through interphase. Cell division consists of two stages which are karyokinesis and cytokinesis. Karyokinesis is a process where the nucleus divides either through mitosis or meiosis. If the cell undergoes mitosis, the each daughter cell will have the same number of chromosome as to their parent cell and both of the cells are genetically identical to one another. If the cell undergoes meiosis, four daughter cells are produced at the end of the process. Each of them have half number of chromosome of their parent cell and all of them are genetically varied from each other. Cytokinesis is the continuation process after the karyokinesis where during this process, the cytoplasm divides.

2.1.1 THE MITOTIC STAGES

In karyokinesis, the mitosis is conventionally broken down into several distinct phases. There are four main phases which are prophase, metaphase, anaphase and telophase. During the first mitotic phase, the prophase, the chromatin has become more tightly coiled and condensed which is now visible under the microscope. The nucleoli disappear and the nuclear membrane disintegrates. Each duplicated chromosome are now in pair of sister chromatids, joined together at the centromere. The spindle fibre starts to form and extend from the centrosome. The centrosome move away from each other towards the opposite poles.

Prophase is followed by metaphase. During metaphase, the centrosomes are now at opposite ends of the cell. Each pair of sister chromatids arranged and aligned themselves at the metaphase plate, which is at the equator of the cell. For each chromosome, the kinetochores at the centromere of the sister chromatids is attached with the spindle fibre coming from the opposite poles.

The next stage is anaphase. Anaphase begins when the centromere divide, separating the sister chromatids. Each sister chromatid is now has completely become a fully-fledged daughter chromosome. As the spindle fibre shorten itself, the two separated chromosomes begin moving toward opposite poles of the cell as their kinetochore at the centromere attached to the spindle fibres.

Telophase is the final stage of mitosis. During telophase, the daughter chromosomes are now arrived at the corresponding opposite poles. The nuclear membrane begins to reform and the nucleoli now reappear in the two new nuclei. The chromosomes start to uncoil, returning to thin, long thread-like chromatin and less condensed then before.

After karyokinesis complete, the cell cycle continue and entering into cytokinesis. Although mitosis is the same in animal and plant cells, cytokinesis is different. In animal cells, the cytoplasm divides by constricting inward in a process called furrowing. In the furrow region, the protein actin encircles the cell. This contractile ring gradually pinches the cell in half, forming two daughter cells. In plant cells, there is no constriction process. Instead, membrane vesicles containing cell wall components and derived from the golgi apparatus migrate to the center of the cell. These vesicle fuses with each other and the plasma membrane to form the end membranes of two new daughter cells. Hence, the phrase cytokinesis by cell plate formation is used.

Mitosis is best to be observed in region of cells that are growing at a rapid pace, such as the lateral and the apical meristematic tissue. Mitosis occurs most frequently at the apical meristems such as the root tip. The root of a plant continually grow in order to search for nutrients and supply the plant to keep them growing and survive. The tissue region at the root needs to have very active dividing cells, especially at the very root tip, as to enable the root to grow in length. Therefore, various stages of mitosis can be observed at this region.

2.2 VERNONIA AMYGDALINA : Medicinal plant

In spite of the efficacy of the medicinal herbs in the treatment of various kinds of ailment, the unrefined nature of the preparations and the lack of standard prescriptions on dosage constitute a major setback in the use of herbs in medicare. These two weak points on the medicinal herbs can lead to complications in human system resulting from bioaccumulation of plant ingredients due to over consumption of the herbs (Okafor, 1987).Other causes of complications include uptake of toxic plant ingredients, and possible herb/herb and herb/drug interactions (Okafor, 1987; Mathews et al., 1999).

*Vernonia amygdalina* Del. belongs to the family Asteraceae, tribe Vernonieae and it is widely distributed in Nigeria (Hutchinson and Dalziel, 1963). The plant is a perennial shrub or small tree, usually cultivated for its leaf as vegetable, medicinal, traditional and domestic uses. The leaves have a very bitter taste, due to its chemical contents (Belitz and Grosch, 1987; Lasekan et al., 1998), which are responsible for its medicinal and anti-microbial properties (Rice et al., 1987; Okoh et al., 1995). Herbal preparations made from leaves of *V. amygdalina* are used in curing ailments such as malaria, measles, dysentery, onchocerciasis, yellow fever, constipation, stomach pain, etc, while slender roots and stem branches of the plant are used as chewing stick that are very effective in dental care (Oluwalana and Adekunle, 1998).

Herbal preparations of various forms are prepared from different parts of bitter leaf plant, but the easiest form is the fresh leaf extract, which is prepared by squeezing the leaves in water. The leaf extract is usually taken raw by people at an unregulated rate, depending on the severity of the ailment (Eisenberg et al., 1993).

The unrefined nature of the herbal preparations, coupled with the apparent lack of specificity or precision in the application of the plant in traditional medicine could lead to over dosage of the herbal medicine, which can result in accumulation of essential and non-essential plant ingredients in the human system. The accumulation can reach a toxic level, especially in the systems of people who rely heavily on unrefined herbal products, with severe consequences on their biochemical and genetic systems (Adebowale, Personal Communication).

2.3 ECONOMIC IMPORTANCE OF BITTER LEAF

**Bitter leaf** has numerous medicinal values and benefits to human health and lifestyle. The leaf exhibit some anti-bacterial and antifungal properties that make it a good home remedy to several health issues such as dysentery, diarrhoea, high blood pressure and many others.

Some people may not find bitter leaf so fantastic due to bitter taste but the health benefits of bitter leaf highlighted in the sections below will go a long way to change some people's perspectives about the leaf.

Bitter leaf contains **flavonoid** and other plant compounds that have nutritional values which promote overall health.

**1.  Helps To Clear Fever**: **Bitter leaves contain flavonoids**which have powerful antioxidant effects in treating several health issues such as **feverish conditions.** Other elements like andrographolide lactones, glucosides, diterpene are also present in the leaves and work together to treat and trim down fever and its symptoms. A glass of bitter leaf juice is a strong herbal medicine that helps to combat malarial symptoms and reduce fever effects.

**2.  Lowers High Blood Pressure:** Chewing fresh bitter leaves or drinking the juice extracted from the leaves are commonly known to reduce the level of sugar in the blood and control blood pressure due to the bitterness. This is true because bitter leave has **andrographolide** content that contributes to sugar reduction in the blood and as reducing the risk of resulting diabetes. There is also a trace of potassium in bitter leaf which is another good remedy for **hypertension.**

This mineral helps to flush out the accumulation of salt which spikes the amount of sodium in the bloodstream and causes delicate blood pressure (high blood pressure).

**3.  Treats Stomach Upset**: They are eaten raw or mashed to get the juice which is used in treating abdominal difficulties such as diarrhea, stomach upset and gastrointestinal tract diseases like dysentery and related other issues. Bitter leaves are the major agents that are recommended as a natural remedy for intestinal problems and stomach cramps.

**4.  Good for the Bones & Teeth**: **Vitamin C** is a strong antioxidant mineral found in bitter leaves that has a special role in the body which is the maintenance of bones and teeth as well as prevention of deficiencies associated with this essential vitamin. It also contains a trace of **vitamin K**. The functions of this vitamin extend outside blood clotting as it includes maintaining healthy bones and prevention of bone tissues weakness called **osteoporosis.**

**5.  Improves Body Metabolism**: **Vitamin B1 called thiamine,** in other words, plays important role in the metabolism of lipids, amino acids and glucose in the human body. **Thiamine** is an important dietary supplement occurring naturally in a bitter leaf that helps to oxidize fatty acids in other to produce the synthesis of lipids. Metabolism of lipids is commonly associated with that of carbohydrate which can be converted into fats by known processes. Bitter leaf is very effective in this chemical synthesis known as metabolism because it contains the mineral that plays the key role.

**6.  Fights Free Radicals**

Another nutrient in bitter leaf, which is **vitamins E** serves as an antioxidant that fights against free radicals which have harmful effects on the body system. Bitter leaf contains also **anti-bacterial compound** known as **sesquiterpenoids** that give the property of bitter taste. The major function of this plant compound is to scavenge free radicals from the body system as well as terminate their activities.

**7.  Prevents Cancer Risks**: Presence of anti-cancer properties in bitter leaves makes it effective in preventing and management of **hydatidiform mole, trophoblastic** tumor**and lung**tumor which are signs of cancer. Bitter leaf has also been mixed with other herbal preparations in an alternative medicine experiment on breast cancer and was found effective. **Andrographolide**, labdanediterpenoid present in bitter leaf is a plant compound and a **powerful cancer curative agent** that has been studied scientifically and found effective in the treatment of gastric cancer, colon and prostate cancers.

**8.  Treats Diabetes**

In fact, bitter leaves have versatile curative and medicinal properties which can treat and manage certain health conditions. Scientifically, it has been proven that bitter leaf contains plant compounds which contribute to its bitter taste and play a pivotal role in moderating the blood sugar level and promoting normal insulin function. This compound in bitter leaf hinders the rising of glucose in the blood which leads to the risks of diabetes.

**9.  Improve Fertility in Women**: Bitter leaf has the ability to improve a very important female sex hormone that contributes to reproductive development and regulation. It helps to **improve quality hormone profile** and as well prevents the toxification of**immunoglobulin** that fights an important female sex hormone, estrogen and reproduction. Bitter leaf is very effective in women reproductive life for its capability of providing balance in the genital hormone.

**10.   Detoxifies The Body**: The juice extracted from bitter leaf has **detoxifying properties** that help in mopping off impurities from the body. This effect also **helps to get rid of toxins in the liver, kidney**as well as the lungs and entire human body. In such a manner prevents liver and kidney problems such as stone development in such areas.

**11.    Helps in Weight Loss**: Most of the properties of the bitter leaf have a direct association with weight loss. To start with, incorporating bitter leaf as a vegetable in daily meals or eating just a few fresh leaves a day or drinking the juice helps to **reduce excess calories** which can cause weight gain. The fibre content in it and all the properties associated with weight loss that is found in bitter leaf make it a good weight loss diet.

**12.    Helps in Treating Several Infections**: Aside all the health benefits pin-pointed above, bitter leaf has the properties to treat skin wounds, it can also help in **treating mouth inflammations, skin infections, toothache, typhoid, ear inflammation, tuberculosis and respiratory tract diseases.**

**Bitter Leaf Tea**

Bitter leaf tea is an extract from the bitter leaf itself, used for medicinal purpose in **treating gastrointestinal problems** and generally protects the body from damages to cells, tissues and organs.

**2.3.1 NUTRITIONAL QUALITIES OF BITTER LEAF**

Bitter leaf though bitter in taste contains essential vitamins such as vitamin A, C and E, vitamin B1 and B2. Other important nutrients found in bitter leaf are nutrients are fibre, proteins and minerals such as manganese, zinc, iron, calcium, potassium, phosphorus, selenium and selenium in traceable amount. There is also a presence of plant compounds such as andrographolide, sesquiterpenoids, polimetoksiflavon, apigenin, labdanediterpenoid and andragrafin in traceable amounts.

Phytochemicals such as alkaloids, steroid, tannins, terpenes and saponins, phylate, oxalate, flavonoids, cyanogenic glycosides, anthraquinone and phenol are also found in bitter leaf.

2.4 ADVANTAGE OF ALLIUM TEST

*Allium* test is a sensitive test that has often been used for the determination of cytotoxic and/or genotoxic effects of various substances (Grant, 1982; Smaka-Kinel et al., 1996). The test has been shown to have a good correlation with tests in other living systems; hence, results obtained from *Allium* test are usually handled with care, because it could serve as an indicator of toxicity of the test materials (Fiskesjo, 1997). The usefulness of root tips of *A. cepa* as a test system for monitoring the genotoxic effects of test materials was demonstrated by Fiskesjo (1985). Different concentrations of aqueous leaf extract of *V.* *amygdalina* were subjected to *Allium* test to check their mutagenic effects on the chromosome activities of *A. cepa*.

2.4.1ALLIUMCEPA: ‘WARNING’ BIOINDICATOR IN DETECTING GENOTOXICITY OF MEDICINAL PLANTS

Various species of medicinal plants are used in popular medicine for the treatment of illnesses. However, the presence of cytotoxic and mutagenic substances in their composition or resulting from their metabolism can cause damage to human health. The mutagenic effects result in chromosomal alterations detected during the cell cycle through cytogenetic analysis. What does genotoxicity mean? It refers to the capacity of clastogenic agents causing lesions in the genetic material. Genotoxic agents can be defined functionally for possessing the ability to alter DNA replication and genetic transmission. The evaluations of genotoxicity include, mainly, damage in the DNA, mutations and chromosomal alterations. The observation of cells in interphase and cell division is used as an indicator of adequate proliferation of the cells, which can be measured through the *Allium cepa* test system.

The studies by various authors, such as Vicentini *et al.,*, (2001) and Camparoto *et al.,*, (2002) were performed with the *Allium cepa* test to test the genotoxicity of complex mixtures, in reality known as teas or extracts. The evaluation of the genotoxic effects of the plant extracts has been studied by Chauan *et al.,*, (1999), who indicated the sensitivity of the *A.* *cepa* system and correlated it with the mammal test system, validating its use as an alternative test for monitoring the potential genotoxicity of environmental chemicals and pesticides.

Meristematic onion cells and rat cells were used as test systems to verify the effects of genotoxicity of extracts (infusions) of medicinal plants such as *Maytenus ilicifolia* Mart and *Bauhinia candicans* Benth. by Camparoto *et al.,*, (2002) demonstrating that there was not a significant difference in the decrease of the mitotic index in both cases studied; there was only a decrease in the mitotic index of the meristematic cells in the onion, whose bulbs were treated with a higher concentration (10 x higher than the one used by the population in the form of medicinal tea) of *Bauhinia candicans*. These studies indicated that the use of these plants could be continued, as long as they are always used in the recommended dosage. Lubini *et al.,* (2008) analyzed the genotoxicity of two species of *Psychotria* (*P. leiocarpa* and *P.* *myriantha*) through the *Allium cepa* test and the results indicated that both species possess capacity to inhibit cell division and *P. myriantha* possesses genotoxic activity.

Çelik *et al.,* (2006) studied extracts of *Plantago lanceolata* L. and their results showed that aqueous extracts reduced mitotic index and chromosome aberrations in treatment groups compared to controls. The results of the presented study are therefore important since they suggested the anti-genotoxic effect of the *P. lanceolata* leaf extract. In order to reach certain conclusions about this subject, however, further research should be performed with different test systems.

Extracts are most commonly prepared by infusion or decoction, depending on the part of the plant used. In infusion, extraction is carried out when the plant material is maintained in boiling water, in a covered container, for a certain period of time. Infusions can be applied to plant parts of soft structure, which should be beaten, cut or pulverized roughly according to their nature so that they can be easily penetrated and extracted with water. However, decoction consists of maintaining the plant material in contact, during a certain period of time, with a boiling solvent (usually water). It is a technique of restricted use, since many active substances are altered by a prolonged period of heating and it is customary to employ it with rigid/woody plants (Simões *et al.,*, 2011).

Worldwide, many species of medicinal plants are used to treat illnesses. Most of these species are not thoroughly studied, especially regarding the presence of toxic/mutagenic substances in their composition or arising from their own metabolism, thus damaging the health of the population. The presence of mutagenic substances in the plant species that might cause chromosomal alterations can be detected during the cell cycle of a species. The *Allium cepa* test system is frequently used for evaluating the potential genotoxicity of medicinal plant extracts through the analysis of meristematic cells from root-tips treated with medicinal infusions (teas).

The knowledge of the potential genotoxicity of these medicinal species, through the analysis of the *Allium cepa* cell cycle serves as an indicator of safety for the population, which uses medicinal teas as their only medical treatment. Bagatini *et al.,*, (2007) reviewed this subject and indicated the importance of the *Allium cepa* test as a preliminary screening of genotoxicity in medicinal plant information

He conclude that the *Allium cepa* test is an excellent bioindicator of chromosomal alterations that serve as an alert for the population that uses medicinal teas indiscriminately, and that its constant use in the analysis of the treatment of industrial and hospital effluents is extremely adequate. Currently, due to major concern with environmental pollution, the *Allium cepa* test has occupied an important place for the prevention and prediction of environmental impact that will be caused by the use and disposal of substances including drugs and herbicides. Although the test is merely a first assessment of genotoxicity, it always shows important scientific discoveries, and new adaptations of the test might reveal innumerous possibilities of its use, avoiding the use of animals for testing

2.5 CYTOTOXICITY OF BITTER LEAF

In the work done byAdegbite and Sanyaolu, (2009),mitotic studies were carried out on *Allium cepa* (onion) root cells treated with different concentrations of aqueous leaf extract of *Vernonia amygdalina* (bitter leaf). The onion root cells showed reduced mitotic indices with corresponding increase in concentration of the bitter leaf extract [200 g/L (C2), 400g/L (C3) and 500 g/L (C4)]. Chromosomal aberrations, such as endopolyploidization, lagging of chromosomes and cells with giant chromosomes, were also observed in onion roots treated with same. No chromosomal aberration was observed in the control and in onion roots treated with 100 g/L (C1) of the leaf extract. These observations indicate that abnormal use of this medicinal herb could cause genetic damage. Low concentration and wide spacing of dosage are, therefore, suggested for its dietary intake or use in herbal medicine.

Highly toxic substances have been shown to induce such aberrations in the Allium .These two substances were mito depressive and the effect was concentration dependent.

However, there was correlation in the frequency of chromosomal aberration and concentration of test substances. There was a decrease in the frequency of chromosomal aberrations at higher concentrations. Explanation for this could be that, within creasing concentration and consequently increasing toxicity, there was an inhibitory effect on cell division. These results obtained are indicative of a probable risk of these test substances; which are widely used for therapeutic purposes (VA) or for environmental/household use (Sniper); to the Environment and human health.

Relatively, Allium cepa is one of the many methods for detecting and measuring the degree of alterations in the system subjected to carcinogens/mutagens or chemical causing damage and allow to describe the effects of these damages by observing chromosomal Aberrations (Tedesco and Laughinghouse, 2012).

In addition, Allium test, generally used for analyzing the quality of drinking water and water causing pollution(Rank,2003). Indeed, it is an efficient approach for chemical screening and in situ monitoring of the genotoxicity effect of environmental contaminants (Siddiqui *et al.,*,2011; Nunes *et al.*, 2011).

This test widely used to study the toxicity and genotoxicity of many dangerous contaminants, such as pesticides, azodyes, food preservatives and hydrocarbons (Turkoglu,2007;Leme and Marin-Morales, 2009; Ashraf and Husain, 2010), where all tests have shown that A.Cepa is more sensitive for detecting toxicity and genotoxicity than other tests. Furthermore, this mechanism has been known to identify the presence of pesticides in foods as well as in the environments (Abusalamaetal,2014 and Bakadir *et al.,*,2016).This application plays an Important role in bio-monitoring since roots of onions were sensitive for any toxic materials.

Furthermore, plant roots are extremely useful in biological testing because root tips are the first To be exposed to toxicants dispersed in soil or in water (Fiskesjo,1988). Moreover, the root tip chromosomal aberration assays constitute rapid and sensitive methods for bio-monitoring from the extent of pollution and to evaluate the effects of toxic and mutagenic substances in the natural environment (Matsumoto,*et al.,*,2006;Rodriguez-Ruiz *et al.,*,2014).

In this study, two chemicals H2O2 (Hydrogen Peroxide) and CH2O (Formalin) were used as a Potential transporter of chromosomal aberration while the water samples collected from the

Mining are as of Sorex Barobo, Surigaod el Sur and Rosario,Agus and elSur, were also tested for genotoxicity. At present the areas are undertaking large and small scale mining activities where their runoffs directly contained into the water system.

The cyanide is one of the genotoxic substances being used for cyanidation to extract gold from the ore. In this way, frequent using of the chemical likely affects the condition of the waterways and to the health of the community. This study aims to describe various chromosomal aberrations in the root cells of the onion (Allium cepa L.) which functions as the biomarker for the several types of environmental contaminants.

In the work done by Mohd *et al.,,* 2016, The concentration of H2O2 (Hydrogen Peroxide) shows statistically significant (P<0.05) when compared to CH2O (Formalin). Maximum root growth was observed in the control (1.367 ± 1.072) and there were no morphological deformities found. The roots were whitish in color, unbroken and straight. However, at tested concentrations, 1% obtained the highest root growth from Hydrogen Peroxide (0.547 ± 0.012) and (0.167 ± 0.017) in Formalin. It also recorded the highest dividing cells and fewer in a number of aberrant cells. On the other hand, the 2% concentration attained the second highest root growth from Hydrogen Peroxide (0.413 ± 0.085) and Formalin (0.139 ± 0.001).

It results to the second highest number of dividing cells and fewer numbers of aberrant cells. While the 3% tested concentrations show the poorest root growth from Hydrogen Peroxide (0.167 ± 0.033) and Formalin (0.1 ± 9.813) also displays the greatest number of aberrant cells with a lesser number of dividing cells. Thus, the root growth is manifestations of an arrest of a cell division. This indicates that root growth inhibition relatively due to the action of apical meristematic activity and cell elongation in the process of differentiation (Webster and Mcleod, 1996). Indeed, the suppression of mitotic activity is constantly caused by genotoxicity and cytotoxicity (Bianchi, 2016). Consequently, mitotic index is also a result of mito depression during cell division (Mesi and Koplikua, 2013). The response of root growth identifies genotoxic substances as possibly the factor affecting chromosomal aberration.

**CHAPTER THREE**

**3.0 MATERIALS AND METHODS**

* 1. MATERIALS

The materials used for this research include bitter leaf, grinder to grind the dried leaf, beaker, bijou bottle, refrigerator, cheese cloth basically for sieving, onion bulb, disposable cups for growing the onions, microscopic slides, pair of fine forceps, cover slip, scalpel, dropper, stopwatch, and the light microscope for viewing the different samples of the onion root tip cells. The reagents used also include tap and distilled water, 0.1N Hydrochloric acid, Carnoy’s solution 1:3 (acetic acid and absolute ethanol) and lactopropionic orcein (stain). The purpose of the hydrochloric acid is to destroy the substances that unites the cells (usually pectin), but it will not destroy the cell walls. It also has the ability to kill the cells and halt the process of mitosis. Root tips are grown and preserved in the carnoy’s solution. Fixed root tips can be stored at least two weeks prior to staining. Treatment with acid and heat is used to break up the cellulose cell wall allowing stain to permeate the tissues and makes it easier to squash the tissue on the microscopic slide. The carnoy solution is a fixative that rapidly kills the cells by denaturing proteins and extracting lipids and the tissue is usually stained with the colored dye in order to make the chromosome visible under the microscope while viewing.

* + 1. REAGENT PREPARATION

3.1.1.1 CARNOY SOLUTION

Carnoy solution is basically composed of the mixrture of ethanol and acetic acid in the ratio 3:1. For ci, Ekiti state

3.3 ASSESSMENT OF CYTOTOXICITY USING ALLIUM CEPA TEST

A filtrate was obtained by passing the powder/water mixture through a cheese cloth. This filtrate served as the leaf extract stock solution. The filtrate was reconstituted in tap water in appropriate concentration before administration. The dilution of the 150 ml stock of leaf extract, to its lower concentration required for treatments was made by measuring the required millimeters of stock solution into a container and making it up to 150 ml with tap water. For 25 % concentration, 37.5 ml of the stock was added to 112.5 ml of distilled water, while for 50 % and 75 % concentrations, 75ml and 112.5 ml of the stock were added to 75 ml and 37.5 ml of tap water respectively, while 150 ml of the stock solution formed the 100 % concentration. The other concentrations that were considered were 5%, 10% and 15% also. The mixture is 7.5ml, 15ml and 22.5ml of the aqueous leaf extract diluted with 142.5ml, 135ml and 127.5ml of distilled water respectively.

Fresh bulbs of *Allium cepa* (onion) were purchased from the Oye main market. These were grown in disposable cups containing tap water for about 6 days to ensure proper root formation. The bulbs of  *A. cepa* were then transferred into other disposable cups containing different concentrations of leaf extract (5%, 10%, 15%, 25 %, 50 %, 75 % and 100 %). A bulb of *A. cepa* was left in the tap water serving as the control.

After 48 hours, 4 to 6 roots were chopped off from each treated bulb including the control. They were washed three times in tap water and fixed in Carnoy’s solution (a mixture of acetic acid and absolute ethanol) in the ratio 1:3. The fixed materials were stored in the refrigerator for 24 hours. Furthermore, the roots were rinsed in tap water three times before hydrolysing in 0.1N hydrochloric acid for about 7 minutes at 60 0C. The milky portion of the root tips were subsequently, cut, squashed and stained with lacto-propionic orcein. The prepared slides were examined under the light microscope.

3.4 METHOD OF DATA ANALYSIS

The 8 treatments (including control) were laid out in a complete randomized design (CRD) with three replications. A total number of 500 cells were counted for each treatment and various cells undergoing different stages of cell division- Prophase, metaphase, anaphase and telophase were observed and recorded for each treatment including the control. Phase indices of mitotic stages were recorded; Aberrations induced by each treatment at various stages were also recorded. Data pertaining to all dependable variables studied were analyzed using one-way ANOVA. Means were separated using the Least Square Difference at α=0.05.