ASSESSMENT OF THE GENOTOXICITY AND MUTAGENIC POTENTIAL OF TWO MEDICINAL PLANTS, *PARINARI CURATELLIFOLIA* (PLANCH. EX BENTH.) KUNTZE AND *AZADIRACHTA INDICA* A. JUSS, USING THE *ALLIUM CEPA* ASSAY AND THE AMES TEST

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university**.**

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DEDICATION

I dedicate this work to my parents for investing in me and also my siblings for their moral support and encouragement.

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May the Dear Lord bless you abundantly.

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ABBREVIATIONS AND ACCRONYMS

A: Adenine

AAI: Aristolochic acid I

AAII: Aristolochic acid II

AIL: *Azadirachta indica* leaves

AILM: *Azadirachta indica* leaves methanol extracts

AILW: *Azadirachta indica* leaves water extracts

C: Cytosine

CERMETRA: Centre d'Etude et de Recherche en Médecine Traditionnelle Appliquée (Centre for Study and Research in Traditional Applied Medicine)

CHO: Chinese Hamster ovary cell line

DNA: Deoxyribonucleic acid

EC50: Half maximal effect concentration

EFSA: European Food Safety Authority

***et al.*:** And others

IARC: International Agency for Research on Cancer

G: Guanine

GIT: Gastro-intestinal tract

HPLC-MS: High Performance Liquid Chromatography- Mass Spectrometry

KEPHIS: Kenya Plant Health Inspectorate Service

NF:	Nitrofluorene
NS:	Non-significant
NQO:	Nitroquinoline-N-Oxide
NTP:	National Toxicological Program
OD:	Optical density
PA:	Pyrrolizidine alkaloid
PCL:	*Parinari curatellifolia* leaves
PCLM:	*Parinari curatellifolia* leaves methanol extracts
PCLW:	*Parinari curatellifolia* leaves water extracts
PCSB:	*Parinari curatellifolia* stem bark
PCSBM:	*Parinari curatellifolia* stem bark methanol extracts
PCSBW:	*Parinari curatellifolia* stem bark water extracts
RG:	Root growth
ROS:	Reactive Oxygen Species
SOD:	Superoxide dismutase
T:	Thymine
TH:	Traditional Healers
TK:	Thymidine Kinase
WHO:	World Health Organization

ABSTRACT

In this study, two medicinal plants, *Parinari curatellifolia* (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) were collected in Sokode town, in Central Region of Togo. Their leaves and stem bark were screened for phytochemical composition, genotoxic and mutagenic potential. Qualitative analysis of the methanol and water extracts of both plants showed presence of saponins, tannins, glycosides, alkaloids, flavonoids, phytosterols and terpenoids. *Azadirachta indica* leaf registered the highest quantity of flavonoids at 25.46 % (g/100g) for the methanol extract and 23.87 % w/w for the aqueous extracts. Total phenols in *Azadirachta indica* leaf was 8.58 % w/w for the methanol extract and 7.33 % w/w in aqueous extracts. The amount of flavonoids in *Parinari curatellifolia* stem bark was 23.63 % w/w and 18.16 % w/w in the methanol and aqueous extracts respectively. Aqueous *Parinari curatellifolia* stem bark extract, had the highest concentration of alkaloids at 1.75 % w/w. which may be a cause of the high genotoxic activity in *A. cepa*. The *Allium cepa* assay was used to test the potential genotoxicity of the extracts from both plants. Four concentrations of each extract were used in the test: 0.125, 0.25, 0.5 and 1 g/l. The cytotoxic effects of the two plant extracts were evaluated regarding their ability to inhibit the root growth of onion seedlings. The EC_{50} values have shown that the extracts displayed an inhibitory property on the root growth of *A. cepa* seedlings. However, *A. indica* leaf aqueous extract was the most cytotoxic with an EC_{50} value of 0.26 g/l, followed by *P. curatellifolia* leaf methanol extract (EC_{50} = 0.46 g/l). The aqueous extract of *P. curatellifolia* stem barks and leaves exhibited a low cytotoxicity with EC_{50} values of 1.12 g/l each. The change in mitotic index was used to evaluate genotoxic potential of the extracts. After exposing *Allium cepa* cells to *P. curatellifolia* stem bark extracts, chromosomal bridges occurred at the concentrations of 0.5 g/l and 1 g/l of aqueous extract and 0.125 g/l, 0.5 g/l and 1 g/l of methanol extract. Chromosomal bridges were induced by *Parinari curatellifolia* leaves from 0.125 g/l to 1 g/l with both its aqueous methanol extracts. Treatment with aqueous leaf extract of *Azadirachta indica* resulted in the same type of aberration at 0.5 g/l and 1 g/ while the methanol extract caused bridges at 0.125 g/l, 0.5 g/l and 1 g/l. The c-mitosis was

observed from 0.125 g/l to 1 g/l in *Allium cepa* cells treated with both aqueous and methanolic extracts of *P. curatellifolia* stem bark and leaves as well as *A. indica* leaves. Sticky anaphase was recorded from 0.25 g/l to 1 g/l in *A. cepa* cells treated with *P. curatellifolia* stem bark aqueous extract as well as at 0.25 g/l and 1 g/l of *P. curatellifolia leaf* methanol extracts. *P. curatellifolia* aqueous leaves did not cause sticky chromosomes. However, *A. indica* aqueous leaf extract caused sticky chromosomes at 0.5 g/l and 1 g/l while they were recorded at 0.25 g/l and 1 g/l after treatment with its methanolic extract. Bipolar anaphase was induced with 0.5 g/l and 1 g/l of *P. curatellifolia* stem bark and *A. indica* leaf methanolic extract. Chromosome fragments were observed at 0.5 g/l and 1 g/l after treatment with *P. curatellifolia* stem bark and *A. indica* methanolic extracts. Fragments occurred at 0.125 g/l to 1 g/l of *P. curatellifolia* leaf aqueous and methanol extracts, *P. curatellifolia* stem bark aqueous extract, at 0.5 g/l and 1 g/l of *A. indica* leaf methanolic extract and from 0.25 g/l to 1 g/l of *A. indica* leaf aqueous extract. The mutagenic assay was performed on *Salmonella typhimurium* TA 98 and TA 100 strains. The Ames test revealed a mutagenic activity in *Azadirachta indica* leaf from 0.0625 mg/ml for the aqueous extract with *Salmonella typhimurium* TA98 and TA100 without metabolic activation. The *A. indica* methanol leaf extract was mutagenic from 0.0625 mg/ml to 1 mg/ml with TA 98 and from 0.125 mg/ml to 1 mg/ml with TA 100 strain. *Parinari curatellifolia* leaf aqueous extract was mutagenic on *Salmonella typhimurium* TA 100 at 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml but not mutagenic on TA 98 strain. However, the methanol extract of *Parinari curatellifolia* leaves was only mutagenic at 1 g/l for both *Salmonella typhimurium* TA 98 and TA 100. *Parinari curatellifolia* stem bark aqueous extract was mutagenic from 0.125 mg/ml to 1 mg/ml for both TA 98 and TA 100 while the methanolic extract was mutagenic at 0.25 mg/ml, 5 mg/ml and 1 mg/ml. Results of this study show that leaf and stem bark extracts from the two plants have genotoxic and mutagenic potential against *Allium cepa* and *Salmonella typhimurium* cells. They are therefore likely to have the same effects on human cells. Studies on mutagenic and genotoxic effects of these extracts on human cells should be done.

RESUME (Français)

Dans cette étude, deux plantes médicinales, *Parinari curatellifolia* (Planch. ex Benth.) Kuntze et *Azadirachta indica* A. Juss furent collectées à Sokodé, une ville située dans la région centrale du Togo. La composition phytochimique, les pouvoirs génotoxique et mutagéniques des deux plantes furent evaluées à partir des feuilles et écorces de racines. L'analyse phyto-chimique fut realisée sur les extraits pour la mise en évidence des saponines, tannins, glycosides, alkaloides, flavonoides, phytostérols et des terpenoides. Les classes phyto-chimiques testées au cours de cette étude furent révélées présentes dans les échantillons. L'analyse quantitative des extraits a montré que les fractions aqueuse et méthanolique d'*Azadirachta indica* ont enregistré le taux le plus élevé de phénols et de flavonoides avec 25.46 % (g/100g) pour l'extrait méthanolique et 23.87 % pour l'extrait aqueux. Les phenols totaux étaient estimés à 8.58 % et 7.33% respectivement dans les extraits méthanolique et aqueux d'*Azadirachta indica.* Dans *Parinari curatellifolia*, les taux de flavonoides étaient de 23.63 % et 18.16 % respectivement dans les extraits méthanolique et aqueux. L'extrait aqueux provenant des écorces de racine de *P. curatellifolia* a enregistré le taux le plus élevé d'alkaloides avec 1.75 %, ce qui pourrait être la cause de l'activité génotoxique élevée sur *Allium cepa*. Le test d'*Allium cepa* fut effectué sur les racines des graines d'oignon de afin d'évaluer la potentielle génotoxicité des deux plantes. Quatre concentrations furent utilisées: 0.125 g/l, 0.25g/l, 0.5 g/l et 1g/l. L'effet cytotoxique des extraits provenant des deux plantes fut évalué en tenant compte de leur capacité d'inhibition de croissance des racines des graines germées d'*Allium cepa*. Les extraits ont démontré une propriété inhibitoire sur la croissance des racines des graines germées d'*A. cepa*. Cependant, l'extrait aqueux des feuilles de *A. indica* était plus cytotoxiques avec une CE_{50} (Concentration efficace à 50 %) de 0.26 g/l, suivi de la fraction méthanolique des feuilles de *P. curatellifolia* (CE_{50} = 0.46 g/l). Les extraits aqueux des écorces de racines et feuilles de *P. curatellifolia* ont démontré une cytotoxicité modérée avec des CE_{50} de 1.12 g/l chacun. La réduction de l'indice mitotique fut utilisée comme indicateur de génotoxicité des extraits. Après l'exposition des cellules d'*Allium cepa* aux extraits d'écorces de racines de *P. curatellifolia*, des ponts

chromosomiques ont été identifiés à 0.5 g/l et 1g/l des extraits aqueux, puis à 0.125 g/l, 0.5 g/l et 1 g/l des extraits méthanoliques. Par les feuilles de la même plante, le même type d'aberration fut induit par des concentrations allant de 0.125 g/l à 1 g/l pour les extraits aqueux et méthanolique. Le traitement avec l'extrait aqueux des feuilles *d'Azadirachta indica* a causé les ponts chromosomiques à 0.5 g/l et 1 g/l tandis que l'extrait méthanolique a induit des ponts chromosomiques à 0.125 g/l, 0.5 g/l et 1 g/l. La c- mitose fut observée de 0.125g/l à 1 g/l dans les cellules d'*Allium cepa* exposées aux extraits aqueux et méthanoliques des écorces de racines de *P. curatellifolia* ainsi que les feuilles d'*A. indica*. Les chromosomes collants furent observés de 0.25 g/l à 1 g/l dans les cellules d'oignon exposées aux extraits aqueux et méthanoliques des écorces de racine de *P. curatellifolia*. L'extrait aqueux des feuilles de *P. curatellifolia* ne causèrent pas de chromosomes collants. Cependant, l'extrait aqueux des feuilles de *A. indica* ont induit des chromosomes collants à 0.5 g/l et 1 g/l alors que ces derniers furent enregistrés à 0.25 g/l et 1 g/l après traitement avec l'extrait méthanolique. L'anaphase bipolaire fut induite à 0.5 g/l et 1 g/l des extraits méthanoliques des écorces de racine de *P. curatellifolia* et des feuilles d'*A. indica*. Les fragments chromosomiques furent observés après traitement avec 0.5 g/l et 1 g/l de la fraction méthanolique des écorces de racines de *P. curatellifolia* ainsi que celle des feuilles de *A. indica*. Les fragments de chromosomes furent également observés après traitement avec des concentrations allant de 0.125 g/l à 1 g/l des extraits aqueux et méthanoliques des feuilles de *P. curatellifolia*, de la fraction aqueuse des écorces de racine de *P. curatellifolia*, à 0.5 g/l et 1 g/l de la fraction méthanolique des feuilles d'*A. indica* puis de 0.25 g/l à 1g/l de l'extrait aqueux des feuilles d'*A. indica*. Le test de mutation reverse fut réalisé sur les souches TA 98 et TA 100 de *Salmonella typhimurium.* Le test d'Ames a montré une activité mutagénique induites sur *Salmonella typhimurium* TA 98 et TA 100 à partir de 0.0625 mg/ml par la fraction aqueuse des feuilles d'*Azadirachta indica* sans activation métabolique. L'extrait méthanolique des feuilles *d'A. indica* était mutagénique de 0.0625 mg/ml à 1 mg/ml sur la souche TA 98 et mutagénique de 0.125 mg/ml à 1 mg/ml avec la souche TA 100. La fraction aqueuse des feuilles de *P. curatellifolia* fut mutagénique sur *S. typhimurium* TA 100 à 0.25 mg/ml, 0.5

mg/ml et 1 mg/ml mais pas sur la souche TA 98. Cependant la fraction méthanolique des feuilles de *P. curatellifolia* fut mutagénique uniquement à 1 g/l pour *S. typhimurium* TA 98 et TA 100. L'extrait aqueux des écorces de racine de *P. curatellifolia* s'est révélé mutagénique à partir de 0.125 g/l jusqu'à 1 mg/ml pour les souches TA 98 et TA 100 tandis que l'extrait méthanolique fut mutagénique à 0.25 g/ml, 0.5 mg/ml et 1 g/ml. Les résultats obtenus à partir de cette étude ont prouvé que les extraits méthanoliques et aqueux des feuilles et écorces de racines provenant de ces deux plantes médicinales ont un pouvoir génotoxique et mutagénique respectivement sur *Allium cepa* et les souches TA 98 et TA 100 de *Salmonella typhimurium*. Les extraits provenant des deux plantes pourraient alors éventuellement causer les mêmes effets sur les cellules humaines. Des investigations sur la génotoxicité et la propriété mutagénique de ces extraits sur les cellules humaines devraient être menées.

CHAPTER ONE: INTRODUCTION

1.1 General Introduction

Interest on medicinal plants amongst local communities has increased globally because of their value in the treatment of diseases (Arise *et al*., 2009; Zhang *et al*., 2000). Medicinal plants have played a pivotal role in the health care of many cultures, both ancient and modern (Newman, Cragg & Sander, 2003; Butler, 2004; Balunas & Kinghorn, 2005, Gurib-Fakim, 2006; Newman & Cragg, 2007). According to the WHO, a medicinal plant is any plant that contains in one or more of its organs, compounds that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical synthesis. The important contribution of the traditional medicine in care provision has been recognized by the WHO (http://www.who.int/topics/traditional_medicine/en/). Several secondary metabolites have been characterized in medicinal plants and screened for *in vitro* and *in vivo* activity on various pathogens (Madhava *et al.*, 2008). The World Health Organization (1990) states that 80% of Africans use traditional medicine for their primary health care. In Africa, traditional medicine (TM) suffers from lack of documentation which has the importance of giving information regarding the phytochemistry of plant extracts (Ali, 2011), the most efficient extraction methods, appropriate doses to be administered to sick persons, and possible toxicity or adverse effects (Hamid and Hedayatollah, 2013). A medicinal plant used in therapy can in some cases be substituted for a drug (Kazemipoor *et al*., 2012). Therefore, after a medicinal plant is recognized to have a given therapeutic effect, the toxicity has to be assessed to prove the safety for human consumption. Thus, the lack of enough information concerning the toxicity of the medicinal plants is a problem which needs to be addressed efficiently. In Togo, many medicinal plants have been screened for *in vivo* and *in vitro* activity on human parasites such as helminthes and protozoa (Lakshmi *et al*., 2004; Fujimaki *et al*., 2005; Misra *et al*., 2007). Toxicity testing has also been carried out on several medicinal plants. However there is no information concerning the genetic toxicity of the medicinal plant extracts. Medicinal plants used in Togo are usually prescribed by traditional healers (TH)

in order to treat some diseases such as malaria, gastrointestinal disorders, hemorrhoids, infertility and sexual disorders (Tchacondo *et al.*, 2011). Medicinal plants for a long time have been the first choice for the population in the area, and studies conducted in some parts of the Central region of Togo have shown that some people treated with various medicinal plants presented adverse effects (Tchacondo *et al.,* 2011). Secondary metabolites such as curcumin, quercetin and estragole have been shown to bind to target molecules implicated in human disease (Aggarwal & Shishodia, 2006; Goel *et al.*, 2008; Ji and Zhang, 2008). It has also been reported that some plant secondary metabolites can induce genotoxic effects, and the Ames test (Ames *et al.*, 1975) and *Allium cepa* assay (Akinboro *et al.*, 2007) have been recognized as reliable in the assessment of the genotoxicity of compounds. The genotoxicity and mutagenicity have been assessed on several medicinal plant compounds and a significant number of them has been identified as either potentially genotoxic or carcinogenic or both (Suzanne *et al*., 2011). Genotoxicity may result in DNA mutations, hence increasing the risk of developing cancer. This study aimed at providing information to show whether *Parinari curatellifolia* and *Azadirachta indica* are genotoxic or can cause DNA mutations.

1.2 Background Information

Medicinal plants are a rich source of novel drugs that include the ingredients in the traditional system of medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceuticals intermediate bioactive principles and lead compounds in synthetic drugs (Ncube *et al.*, 2008, Praveen *et al.*, 2010, Tariq *et al.*, 2013). They contain a large number of compounds which can be classified as primary and secondary metabolites. The more important ones in medicine are secondary metabolites (Hill *et al.*, 1995). In the body they undergo a series of transformation leading to the bioactivity and the elimination of the compounds. A compound that is transformed after its enzymatic conversion in the liver into the active drug that can bind its receptors is referred to as a prodrug because to become active its structure must be modified so as to generate the active form of the drug. For instance, during hepatic metabolism involving the

cytochrome P450 enzymatic system, a lipophilic drug can be converted into a polar compound, thus its elimination is facilitated. The pharmacokinetics of xenobiotics include: Absorption, distribution, metabolism, and elimination. *Parinari curatellfolia* is a Chrysobalanacae used in traditional medicine to treat malaria, diarrhea, wound, boils, and fever. A study conducted by Audu and Amutipan (2006) on *Parinari curatellifolia* leaves and stem bark, showed evidence of antimicrobial activity on *Staphylococcus aureus*, *Corynebacterium ulcerans*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella typhi*, *Candida albicans* and *Aspergillus niger*. In the same study, a moderate cytotoxicity was observed with brine shrimp (Audu and Amutipan, 2006). Several studies conducted on *Parinari curatellifolia* have revealed evidence of its benefits on human health (Audu and Amitupan, 2006).

Azadirachta indica is a medicinal plant used to treat malaria, diarrhea, cough, asthma and diabetes. Its leaves are used in many communities. The active compounds isolated from the leaves include gedunin reported as the antimalarial compound nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid. The presence of flavonoids in its leaves makes *Azadirachta indica* potentially cytotoxic to bacteria, fungi and protozoa. The leaves may also be cytotoxic to human cells. Previous studies on the genotoxicity of *Azadirachta indica* using the *Allium cepa* assay have shown that the crude aqueous extract induced chromosomal aberrations and a decrease in the mitotic index in mitotic cells from *Allium cepa* meristems (Akaneme and Amaefule, 2012).

1.3 Statement of the Problem

Parinari curatellifolia (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) are two medicinal plants known for their benefits on human health. In Togo, they are largely used in the rural areas for the treatment of diseases like malaria, fever, diarrhea, asthma, high blood pressure, diabetes and bacterial infections. However when they are prescribed by traditional healers, their dose-related toxicity levels are not considered. During the prescription, the herbalists usually focus only on the disease and the appropriate dosage to give. Very little is known about the phytochemical composition of *P. curatellifolia* and *A.*

indica preparations, though the patient may show some allergenic reactions to some molecules present in their extracts (Amoah *et al.*, 2006). Also, the dosage of the medicinal plant concoctions prescribed by traditional healers may not be accurate, thus can become toxic to the patient and cause adverse effects. Adverse effects of *Parinari curatellifolia* and *Azadirachta indica*, the two selected medicinal plant studied may be the induction of genotoxicity on the *Allium cepa* cells as exemplified in an experiment on *Azadirachta indica* leaves (Akaneme and Amaefule, 2012). *In vitro* testing showed that the genotoxicity is dose-dependent.

1.4 Justification of the Study

Traditional healers use ancestral knowledge to prescribe products to patients. There is no documentation showing a standardization of methods of prescription and the dosage of the drugs. There is a lack of knowledge of phytoconstituents of a large number of local medicinal plants and also their importance in the treatment of diseases or adverse effects. Although various studies have been conducted to assess the toxicological properties of *Parinari curatellifolia* and *Azadirachta indica*, there is still lack of information concerning the genotoxic and mutagenic potential of these two medicinal plants. In many communities in developing countries, traditional medicine is the only health care system available or affordable. Therefore, the people using herbal medicines should be assured that the medicinal plants preparations or concoctions they are buying are safe and effective. Awareness should also be raised among the populations by giving them science-based information on dosage, efficacy as well as toxicological effects of *Parinari curatellifolia* and *Azadirachta indica*. For this to be implemented, global harmonization of legislation is required for the guidance toward a responsible prescription and marketing of the two medicinal plants. If sufficient scientific evidence of benefit is available for *Parinari curatellifolia* and *Azadirachta indica*, then such legislation should allow its appropriate use in promoting public health and treatment of diseases.

Therefore, in this study the genotoxic and mutagenic effects of *Azadirachta indica* and *Parinari curatellifolia* were evaluated to provide information on potential genotoxic or mutagenicity activity in the two plants.

1.5 Research Questions

1. Are *Parinari curatellifolia* (Planch. ex Benth.) Kuntze. and *Azadirachta indica* (A. Juss) extracts capable of causing chromosomal alterations?
2. Are *Parinari curatellifolia* (Planch. ex Benth.) Kuntze. and *Azadirachta indica* (A. Juss) extracts genotoxic?
3. Which phytochemicals are present *Parinari curatellifolia* (Planch. ex Benth.) Kuntze. and *Azadirachta indica* (A. Juss) extracts?

1.6 Hypothesis

1.6.1 Null Hypothesis

Parinari curatellifolia (Planch. ex Benth.) Kuntze. and *Azadirachta indica* (A. Juss) extracts do not have genotoxic and/or mutagenic potential.

1.7. General Objective

To determine phytochemicals in extracts from *Parinari curatellifolia* (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) and evaluate their genotoxic and mutagenic potential.

1.7.1 Specific Objectives

1. To assess the genotoxic potential of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) extracts
2. To assess the mutagenic potential of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) extracts

3. To evaluate the phytochemical composition of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze. and *Azadirachta indica* (A. Juss) extracts.

CHAPTER TWO: LITERATURE REVIEW

2.1 Description and importance of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze and *Azadirachta indica* (A. Juss) in herbal medicine

2.1.1 Biology and medical benefits of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze.

2.1.1.1 Taxonomy

Kingdom: Plantae

Class: Angiospermae

Subclass: Dicotyledoneae

Super order: Rosanae

Order: Malpighiales

Family: Chrysobalanaceae

Genus: *Parinari*

Species: *Parinari curatellifolia* (Planch. ex Benth.) Kuntze.

2.1.1.2 Ethnobotanical use of *Parinari curatellifolia* (Planch. ex Benth.) Kuntze.

In folk medicine, Chrysobalanaceae species are used for various purposes (Evanilson *et al.*, 2002). *Parinari curatellifolia* is used to treat microbial infections and malaria (Adjanohoun *et al.*, 1986). In African traditional medicine, *P. curatellifolia* is known to treat dysentery, malaria, epilepsy, toothache and veneral diseases (Uyss *et al.*, 2002; Arnold & Gulluman, 1984). In Southern Uganda *P. curatellifolia* leaves are indicated for stomachaches (Ssegawa and Kasenene, 2007). In West Africa it is known as anti-helminthic and used to treat diarrhea (Diehl *et al.*, 2004).

Plate 2.1: *Parinari curatellifolia* leaves and stem barks

2.1.1.3 Scientific studies and biological activities

Karou *et al.,* (2011) studied the antiplasmodial property of *P. curatellifolia* crude extract. Clarkson *et al.,* (2004) found a significant antiplosmodial activity of *P. curatellifolia* roots with dichloromethane extract with IC_{50} values lying between 5 and 7 µg/ml. On the basis chromatographic and spectroscopic techniques, it is found to contain, betulin, β–sitosterol, stigmast-4-en-3-one, stigmasterol and betulinic acid as bioactive compounds.

Ent-kaurane diterpenes isolated from the root of *P. curattellifolia,* show cytotoxicity *in vitro* against several cancer cell lines (Lee *et al*., 1996). *P. curatellifolia*, Diterpenes from with molecular core ent-kaurene derivative of 15-oxozoapatlin were isolated and presented cytotoxic activity (Lee *et al.,* 1996). In a study to find out better drug formulations against multi-resistant *Mycobacterium tuberculosis*, *Parinari curatellifolia* methanol extracts were used in combination with different antibiotics. The accumulation of ciprofloxacin caused by *P. curatellifolia* extract was greater than that caused by the drug efflux inhibitor reserpine. This plant may serve as a source of lead compounds in the search of new antimycobacterials with new mechanisms of action (Chimponda and Mukanganyama, 2010).

2.1.2 Biology and medical uses of *Azadirachta indica* (A. Juss)

2.1.2.1 Taxonomy

Kingdom: Plantae

Super division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Super order: Rosanae/Rutales?

Order: Rosales

Family: Meliaceae

Genus: *Azadirachta*

Species: *Azadirachta indica* (A. Juss)

2.1.2.2 Ethnobotanical use of *Azadirachta indica* (A. Juss)

The neem is an evergreen, fast growing tree and can reach 20 m in height. It can grow in tropical and sub-tropical conditions and is known for its resistance to drought. It is found in tropical Africa, Asia, America and Australia.

The parts of neem tree are generally used for malaria, fever reduction, coughs, asthma, and ulcer and is also used as anti-helminthic, and anti-diabetic. In Africa neem tree is known to have antimalarial properties and the medicinal uses have been described for leaves, fruits and barks (Koul *et al.*, 1990).

Plate 2.2: *Azadirachta indica* leaves

2.1.2.3 Compounds and scientific studies in *A. indica*

Active constituents of neem leaf extract include isomeldenin, nimbin, nimbinene, 6-desacetyllnimbinene, nimbandiol, immobile, nimocinol, quercetin, and beta-sitosterol (Siddiqui *et al*., 2004; Tewary, 1985). Two additional tetracyclic triterpenoids zafaral [24,25,26,27-tetranorapotirucalla-(apoeupha)-6alpha-methoxy-7alpha-acetoxy-1,14-dien-3,16-dione-21-al] (Prakash *et al*., 1988) and meliacinanhydride [24,25,26,27-tetranorapotirucalla-(apoeupha)-6alpha-hydroxy,11alpha-methoxy-7alpha,12alpha-diacetoxy,1,14,20(22)-trien-3-one] (Raizada *et al*., 2001) have been isolated from the methanolic extract of neem leaves **(**Siddiqui *et al*., 2004**).**

The active constituents in the seeds have not been determined with certainty **(**Mukherjee *et al*., 1999). The neem seed extracts vary in each batch in terms of stability and activity. Two new tetranortriterpenoids, azadirachtin H (Tewari, 1985) and azadirachtin I **(**Mukherjee *et al*., 1999**)**, have been isolated from neem seeds (Govindachari *et al*., 1992).

Methanol extracts of leaves have shown antipyretic properties in male rabbits (Okpanyi *et al*., 1981). It is also known to have anti-inflammatory, spermicidal, anti-arthritis, anti-gastric ulcer, antifungal, antibacterial, diuretic, antitumor, immunomodulatory properties.

Aqueous extract of neem leaves significantly decreases blood sugar level and prevents adrenaline as well as glucose-induced hyperglycemia. Recently, hypoglycemic effect was observed with leaf extract and seed oil, in normal as well as alloxan-induced diabetic rabbits. Neem leaf and bark aqueous extracts produce highly potent antiacid secretory and antiulcer activity. Neem seed and leaf extracts are effective against both chloroquin-resistant and sensitive strain malarial parasites. Gedunin (Prakash *et al*., 1988) was reported as the antiplasmodial compound present in *Azadirachta indica* (Khalid *et al*., 1989). According to Jones *et al*. (1994), Azadirachtin has the ability to block the development of the male gamete of *Plasmodium sp.* A spermicidal fraction isolated from the neem oil, NIM-*76* may inactivate directly a virus via the blockage of viral replication. However, NIM-76 did not inhibit the viral multiplication once the infection was present. The antimicrobial property of NIM-76 is due to its ability to stimulate cellular mediated immunity and lymphocyte proliferation (Sai Ram *et al*., 2000).

Extracts of neem leaf, neem oil seed kernels are effective against certain fungi including *Trichophyton*, *Epidermophyton*, *Microspora Trichosporon*, *Geotricum* and *Candida*. Oil from the leaves, seed and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and streptomycin resistant strains. *In vitro*, it inhibits *Vibrio cholerae Klebsiella pneumoniae*, *M. tuberculosis* and *M. pyogenes*. Antimicrobial effects of neem extract have been demonstrated against *Streptococcus mutans* and *S. faecalis*.

Aqueous leaf extract offers antiviral activity against Vaccinia virus, Chikungemya and measles virus. Neem leaf aqueous extract effectively suppresses oral squamous cell carcinoma induced by 7, 12-dimethylbenz[a] anthracene (DMBA), as revealed by reduced incidence of neoplasm. Neem may exert its chemopreventive effect in the oral mucosa by modulation of glutathione and its metabolizing enzymes. Several compounds have been isolated from different parts of neem tree divided into two major classes: the isoprenoids like diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and C-secomeliacins

such as nimbin, salanin and azadirachtin and non isoprenoids which are proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds. Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *A. indica* demonstrated several biological activities (http://www.indianscience.org/essays/t_es_agraw_neem.shtml). From this crude principle, some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated.

2.2 Genotoxicity and mutagenicity

The genotoxicity refers to the ability of a compound to cause the alteration of the genetic material of an organism. Genotoxic compounds can interfere with the DNA or chromosomes in cells and cause either reversible or irreversible mutations. Mutations can involve the replacement or inversion of nitrogenous bases in the DNA molecules and also the deletion of single base of a portion of bases. Genotoxic agents can cause DNA damage, which is either repaired or otherwise, leads to alterations of the DNA. Chromosome aberrations are the consequence of DNA double strand break which was unrepaired or repaired improperly. Broken chromosome ends without telomeres become "sticky" and may fuse with other broken chromosome ends. The result of these chromosomal rearrangements are acentric fragments, dicentric bridges observed in mitotic cells of the first cell cycle after mutagenic treatment or micronuclei in the interphase cell in the next cell cycle.

A large number of substances have the property to bind to DNA molecules. Studies have shown that a large number of compounds of various natures can interfere with DNA molecules and cause mutations. Heavy metals such as Chromium among environmental pollutants are known as genotoxic due to their ability to cause DNA-adducts which lead to mutations. Screening compounds for genotoxicity is important because it allows the identification genotoxic compounds that are potentially carcinogenic. Not all genotoxic compounds are also carcinogenic. Not all genotoxic compounds are mutagenic. The

genotoxicity of a compound can be revealed in DNA molecules as single or double-stranded damage and at the chromosomal level as alterations in structures and in number. Mutagens can be detected cytologically by cellular inhibition, disruption in metaphase, induction of chromosomal aberrations, numerical and structural, ranging from chromosomal fragmentation to the disorganization of the mitotic spindle, and consequently of all subsequent dependent mitotic phases.

2.2.1 Guidelines on the genotoxicity assessment in medicinal plants

The Economic European Community (1987) and the Japanese Ministry of Health (1989) have established guidelines for the testing of pharmaceuticals for genetic toxicity. The guidance on genetic toxicity testing was provided by the FDA's Centre for Drug Evaluation and Research and Biologics Evaluation and Research (CDER and CBER). The guidance on genetic toxicity testing provided by FDA's Centre for Food Safety and Applied Nutrition (58 FR16536, March 29, 1993) is currently considered applicable to pharmaceuticals. The guidelines recommend a standard test battery for genotoxicity and these include a test for gene mutation in bacteria, an *in vitro* test for cytogenetic evaluation of chromosomal damage in mammalian cells or an *in vitro* mouse lymphoma TK assay and an *in vivo* test for chromosomal damage using rodent hematopoietic cells.

In vitro tests for the evaluation of mutations and chromosome damage are really needed prior to first human exposure. Additional testing should be performed in the case an equivocal or positive finding occurs. The standard battery of tests for genotoxicity should be completed prior to initiation of phase II studies. The guidelines specifically stated a set of bacterial strains to be used for genotoxicity testing. The standard set of strains used in bacterial mutation assays should include strains that will detect point mutations at A-T sites, such as *S. typhimurium* TA102, which detects such mutations within multiple copies of *hisG* genes, or Escherichia coli WP2 uvrA, which detects these mutations in the *trpE* gene, or the same strain possessing the plasmid (pKM101), which carries *mucAB* genes that enhance error prone repair. In conclusion, the recommended bacterial strains

for genotoxicity testing are TA98, TA100, TA1535, TA1537 or TA97 or TA97a2; TA102 or E. coli WP2 uvrA or E. coli WP2 uvrA (pKM101).

A preference was given to *Salmonella typhimurium* TA 102 and/or a repair proficient *E. coli* WP2 pKM101strains for the detection of cross-linking agents.

2.2.3 Medicinal Plant Extracts and induction of Chromosomal Alterations

Verma & Sign (2008) showed that many plants extracts and their active component have been described and utilized as therapeutic agents; they further stated that, some plants also synthesize toxic substances as a primary defense against bacteria, fungi, insects and other predators ((Freeman and Beattie, 2008). Studies have shown that a natural sesquiterpene lactone known as Artemisinin extracted from the plant *Artemisia anua* is widely used for treatment of resistant strains of *Plasmodium* (Effertin *et al.*, 2011). Some of the researchers who have carried out *in vitro* studies using Cancer cell line found that artemisinin and some of its derivatively had cytotoxic effects, alter the cell cycle and induce apoptosis (Houe *et al*., 2008, Hi *et al.*, (2008); Youne *et al*., (2008); Zhang *et al.*, (2010). Efferth *et al*., (2001) and Disbrow *et al*., (2005) further argued that there compound, presented cytotoxic activity in mammal are proliferation while on the other hand researchers like Umithn *et al*., (1997); Singh and Lai, (2001) concluded that toxicity was related to high intracellular concentrations of iron. Other researchers, (Oliaro and Taylor, 2004; Sato et *al*., 2007), have shown that action of antimalarial drugs including artemisinin derivatives, cause damage to membrane structure, which can directly influence normal functioning and initiate a series of biochemical reactions resulting in cell death. Studies on human/mammalian cell genotoxicity for antimalarial drugs are scarce. Aquino *et al*., (2011) reported genotoxic effects of artesunate (an antimalarial drug) in liver cells of mice at low doses and clastogenic effect at high doses in bone marrow cells of same animal. Li *et al*., (2008) and Mota *et al.*, (2011) in their studies reported genotoxic effects of artesunate *in vitro* in Chinese hamster ovary cells in human peripheral lymphocytes, respectively.

Rodeiro *et al*., (2006), supported that before the approval of new pharmaceutical products whether natural or synthetic, both their therapeutic and their toxicological effects must be evaluated, so that only substances free from potential mutagenic effects are introduced. Chandra *et al*., (2006) further prove that mutagenic agents can produce carcinogenic effects. Vasquez (2010) carried out a study using the damage inducing agents such as mitomycin C and cyclophosphamide, tested both techniques concomitantly on different tissues of rats and mice and thus reduced the number of animals used without compromising the efficacy of the test. Using the comet assay, it was possible to quantify and distinguish different DNA damage levels, seeing that the evaluation of scores for each experimental group was highly important. In a study carried out by Vasquez, artesunate did not induce DNA damage in the peripheral blood cells of animals. The results on three tested doses have shown that DNA damage was significantly lower than that observed in the negative control group. On the other hand, Vasquez (2010) in liver cells, he outlined that the 5mg/Kg dose also increased the total number of damaged cells but stated that this increase was not statistically significant.

Vasquez's (2010) findings showed that, among the damaged nucleoids, class I comets were predominant. However in the group treated with doxorubicin class 2 and 3 comets predominated. Thus low doses of artesunate were sufficient to produce low genotoxicity in liver cells. Li *et al.*, (2003) after analyzing CHOs (Chinese Hamster ovary cell line) cells and V79-2 (hamster lung fibroblast cell line) cells with comet assay, he outlined that, *in vitro*, artesunate induced DNA damage and apoptosis. Li *et al.*, (2008) and Vasquez, (2010) concluded that, in mammal cells, artesunate generated damage through direct or indirect oxidative stress, resulting in DNA damage strand breakages. From this review, it is noted that there is reported genotoxicity *in vitro* induced by medicinal plant extracts. However there is a gap in investigation concerning the genotoxicity and/or mutagenicity of medicinal plants.

Doumbo, (2006) has shown that genotoxicity of alkaloid rich extracts derived from two plants used traditionally in Mali had strong inhibition of protein synthesis in mammalian

cells but did not exhibit mutagenic or genotoxic activity. He found that an alkaloid rich extract derived from *Nauclea latifolia* could interact *in vitro* with the DNA of bacterial and mammalian cells, leading to G2-M all cycle arrest and heritable DNA damage. Damage to the DNA molecule, such as adducts and breakage of single or double strands, could become permanent lesions and the micronucleus test in an excellent mean of investigating their type of image in genetic materials (Serpeloni *et al*., 2003). *Xanthium strumanum* L. is a member of Asteracae commonly used in Cuba, mainly as diuretic. Some toxic properties of this plant have also been reported and to date very little is known about its genotoxic property. Therefore there is a need to investigate whether these herbs have genotoxic and/or mutagenic potential. The toxicity outlined above on the plants/herbal extracts shows that potential negative effects on human health were elicited by the consumption of herbal products should be remedies. The present work aimed at assessing the potential genotoxic and mutagenic risk of these herbal products. This is because genotoxicity is one of the most important toxic effects that lead to long term effects such as cancer and reproductive diseases.

Medicinal plants contain secondary metabolites which are useful to fight against pathogens (Phan *et al.*, 2001). However, some secondary metabolites such as alkaloids can cause severe adverse effects (Tchacondo *et al*., 2011). Alkaloids can block ion channels, inhibit enzymes or interfere with neurotransmission, producing hallucinations, loss of coordination, convulsions, vomiting and death (http://www.biologyreference.com/Re-Se/Secondary-Metabolites-in-Plants.html). Some other adverse effects included abdominal pain, sweating and diarrhea (Tchacondo *et al.*, 2011). High intakes of tannins and related anthocyanins are correlated to esophageal cancer (Morton, 1980).

Several medicinal plants used to cure sicknesses are revealed to be genotoxic or carcinogenic. Among 30 botanical species considered as nutraceutical plant products, 18 have been identified to contain genotoxic/carcinogenic compounds. Some reported molecules are trans-anethole, β-asarone, aristolochic acids I and II, curcumin, estragole, heliotrine, integerrimine, isosafrole (Suzan *et al.*, 2011). *Acacia nilotica, Terminalia*

chebula and *Tripahala* have been screened for in vitro genotoxicity and found induce genotoxicity at given concentrations (Arora *et al.*, 2005). Vargas *et al.*, 1991 have screened seven medicinal plant species for mutagenic potential. Ames mutagenicity test was used and in all assays significant mutagenicity was observed with microsomal activation. Three medicinal plants showed genotoxicity, namely *Achyrocline saturoides*, *Baccharis anomala*, and *Luehea divaricata*. *A. saturoides* aqueous extract contains four polyphenols including quercetin and 3-methoxyquercetin flavonols, luteolin flavone and caffeic acid mutation with TA98, TA100 and TA102 strains of *Salmonella typhymurium* with metabolic activation. The observed mutagenicity observed with TA102 strain was due to the oxidative activity of quercetin and its quinones derivatives and caffeic acid or the alkylating action of quercetin metabolites (quinones-methides) in the bacterial DNA (Ames *et al.*, 1983; Ravanel *et al.* 1987; Vargas *et al.*, 1990). Many plants synthesize toxic compounds for their defense against pathogens such as bacteria, viruses and fungi (Freeman and Beattie, 2008) and these toxic substances may cause deleterious effects in humans. Several plant phytochemicals have been reported as potentially genotoxic and/or carcinogenic, and these include trans-anethole, β-asarone, aristolochic acids I and II, curcumin, estragole, heliotrine, integerrimine, isosafrole and some many other molecules (Suzanne *et al.*, 2011).

Nauclea latifolia, Nauclea diderrichii and *Nauclea pobeguinii* are medicinal plants used in West Africa treat diarrhea, fevers and malaria. Liu *et al*., 1990 studied the genotoxic potential of *N. latifolia, N. diderrichii* and *N. pobeguinii* and found that the hydromethanolic extracts of barks exhibited a significant clastogenic/aneugenic effect without the S9 mix. Six main saponins were isolated and identified as quinovic acid glycosides. The saponins individually did not exhibit any direct clastogenic/aneugenic activity but when the six saponins were mixed together in equal concentrations they showed a direct clastogenic/aneugenic activity through the micronucleus and the comet assay using the Chinese Hamster Ovary cells. Liu *et al*., 1990 concluded that *Nauclea* bark saponins present in the hydromethanolic extracts have a synergistic effect resulting in the DNA-damage and the genotoxic potential was attributed to the fact that *Nauclea* bark

saponins inhibits the glutathione-S-transferase, therefore decreasing the cells defense against oxidative stress.

A study conducted by Okem *et al.* (2012) on twelve South African medicinal plant species resulted in the conclusion that the extracts were not genotoxic with the Ames test using the *Salmonella typhimurium* TA 98, TA 100 and 1537 without metabolic activation. A genotoxic activity could have been observed if a metabolic activator such as S9 mix was used. Pyrrolizidine alkaloids (PA)-containing plants are among the most important source of poisoning to livestock and human beings through medicinal plants (Chen *et al.*, 2010). Several studies have been conducted to determine the mode of action of genotoxicity or tumorigenesis related to the pyrrolizidine alkaloids-containing plants. Chen *et al*., (2010) found that the PAs with metabolic activation cause DNA adducts, DNA cross-linking, DNA breaks, sister chromatid exchange, micronuclei, chromosomal aberrations, gene mutations and chromosome mutations *in vivo and in vitro*. PAs were found to induce mutations in the cII gene of rat liver and in the p53 and K-ras genes of mouse liver tumors. It has been suggested that all PAs produce a set of (+/-)-6,7-dihydro-7-hydroxymethyl-5H-pyrrolizidine- derived DNA adducts and similar types of gene mutations. Transversion mutations identified were G:C→T:A tranversion and tandem base substitution. *Coccoloba mollis*, a medicinal plant used in traditional medicine to cure various ailments such as insomnia, impaired vision, anemia, memory loss, stress and sexual impotence has been assessed for its effect on the DNA. The plant found to be genotoxic using the comet assay and this latter showed that the leaves and roots induced DNA damage (Tsuboy *et al*., 2010). Four tests were used: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay, comet assay, micronucleus test with cytokinesis block, and an *in situ* test for detection of apoptotic cells with acridine orange staining.

Some *in vitro* studies using cancer cell lines suggested that artemisinin and some of its derivatives have cytotoxic effects, alter the cell cycle, and induce apoptosis (Hou *et al.*, 2008; Li *et al.*, 2008; Young *et al.*, 2009; Zhang *et al*., 2010). These compounds may present cytotoxic activity in mammal cell proliferation (Efferth *et al.*, 2001; Disbrow *et*

al., 2005), and it has been suggested that the toxicity is related to high intracellular concentrations of iron (Smith *et al*., 1997; Singh and Lai, 2001).

Other studies have indicated that the mechanism of action of antimalarial drugs, including artemisinin and its derivatives, causes damage to membrane structures, which can directly influence normal cell functioning and initiate a series of biochemical reactions resulting in cell death (Olliaro and Taylor, 2004; Sato *et al.*, 2007). In addition, it has been shown that endoperoxides of artemisinin trigger the production of reactive oxygen species (ROS), which have also been implicated, in the mechanism of cytotoxicity (Disbrow *et al*., 2005). Embryotoxicity has been observed in rats and rabbits (Clark *et al*., 2004; Longo *et al*., 2006), and neurotoxicity has been observed both *in vitro* and *in vivo* (Schmuck *et al*., 2002).

Reports on mammalian cell genotoxicity are scarce for artesunate. Aquino *et al.*, (2011) reported genotoxic effects of artesunate in liver cells of mice at low doses and clastogenic effects at high doses in bone marrow cells of the same animals. Li *et al.*, (2008) and Mota *et al.*, (2011) reported genotoxic and cytotoxic effects of artesunate in vitro in Chinese hamster ovary cells and in human peripheral lymphocytes, respectively. Programmed cell death or apoptosis is obligatory for harmonious cell life in animals through the removal of infected, injured, or cancerous cells, and caspases are usually involved in this apoptotic process (Denault and Salvesen, 2002). Considering that Reactive Oxygen Species play important roles in the pathogenesis of many diseases, and that superoxide dismutases (SODs) are the major antioxidant defense systems against ROS (Fukai and Ushio-Fukai, 2011), it is important to evaluate expression of these genes when cells are exposed to new chemicals in order to better evaluate the cytotoxicity of these compounds.

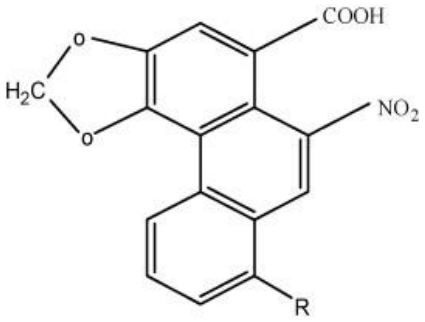
Because artemisinin and artesunate are widely used drugs, with more than 100 million doses administered annually (WHO, 2010), it is essential to develop studies examining their genotoxic potential and to determine the molecular mechanisms of their cytotoxicity in order to evaluate their safe and effective use for malaria and their potential use in cancer treatment. By exposing human hepatocellular liver carcinoma (HepG2) cells to artemisinin and articulate in vitro, this study evaluated both the cytotoxic and genotoxic effects of

these compounds on mammalian DNA, as well as expressions of the caspase 3 (CASP3) and SOD1 genes to obtain more information about its genotoxic mechanism.

2.3 Some plant compounds associated with genotoxic or mutagenic effects

2.3.1 Aristolochic acid

Aristolochic acid (AA) is an herbal drug present in *Aristolochia spp*. And also some plants of the genus *Diploclisia.* These include *Aristolochia clematitis, Aristolochia fangchi, Aristolochia manshuriensis.* AA is a mixture of two related nitrophenanthrene carboxylic acids, aristolochic acid I (AAI) and aristolochic acid II (AAII) (Pailer *et al*., 1955). The chemical formula is $C_{17}H_{11}NO_7$ with a molecular weight of 341.275 g/mol.



Aristolochic Acid I: R = OMe
Aristolochic Acid II: R = H

Figure 2.1: Chemical structures of Aristolochic acid I (AAI) and II (AAII)

AA-DNA adducts have been used as a biomarker of exposure in the investigation of the mutagenic and carcinogenic potential of AA since it has been established that AA has the ability to bind covalently to DNA (Volker *et al*., 2002). DNA binding studies have shown that both AAI and AAII bind to the adenines of the codon 61 in the *H-ras* mouse gene

and preferentially to purines in the human *p53* gene (Volker *et al*., 2002).

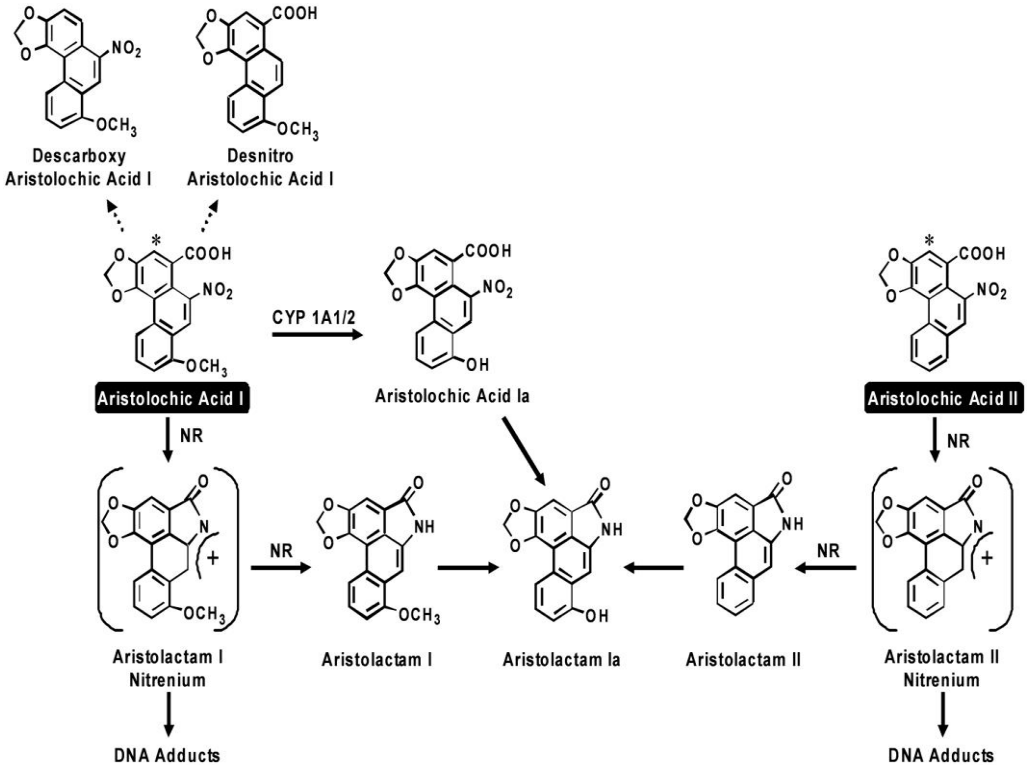


Figure 2.2: Metabolism of aristolochic acids I and II (from Kathleen *et al*., 2011)

A mixture of aristolochic acids I and II indicated to be genotoxic and carcinogenic on rats, mice and rabbits. It is also responsible for urothelial cancer in patients suffering from aristolochic acid nephropathy.

2.3.2 Integerrimine

Integerrimine also called intergerrimine or squalidine is a compound with the formula $C_{18}H_{25}NO_5$. It is a monoterpene belonging to the group of pyrrolizidine alkaloids. It has been isolated from the plant *Crotalaria brevifolia* (Sharma and Ghatak, 1971).

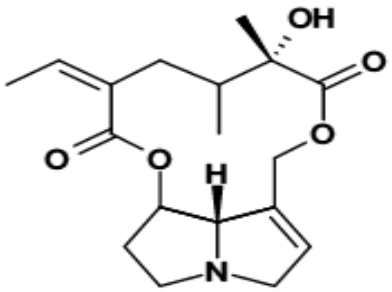


Figure 2.3: Chemical structure of integerrimine

Though the possible carcinogenicity of integerrimine is not established, there is the evidence, proved by several studies, of genotoxic potential induced by the compound (Chen *et al*., 2010).

2.3.3 Estragole

Estragole occurs in a various number of plants including *Foeniculum vulgare* Mill., *Illicium verum* Hook.f. and *Ocimum basilicum* L. Its formula is $C_{10}H_{12}O$ and it has a molar weight of 148.2 g/mol. Estragole is an alkylbenzene, a natural organic compound which chemical structure consists of a benzene ring substituted with a methoxy group and a propenyl group (Figure 2.7).

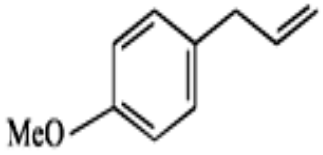


Figure 2.4: Chemical structure of estragole

It is indicated to be genotoxic and carcinogenic in rodents according to the European Food Safety Authority (EFSA).

2.3.4 Trans-anethole

Trans-anethole is a naturally occurring phenylpropene derivative that is estrogenic at lower concentrations. At higher concentrations, it is cytotoxic to cancer cell lines. The metabolism of trans-anethole to 4-hydroxy-1-propenylbenzene confers to the compounds its cytotoxicity effect.

In human volunteers, the major metabolite of trans-anethole was 4-methoxyhippuric acid (Sangster *et al*., 1987). It can be metabolized by Arthrobacter strain (TA13) (Eyal *et al*., 2002). The chemical formula of trans-anethole is $C_{10}H_{12}O$ and it has a molecular weight of 148.205 g/mol.

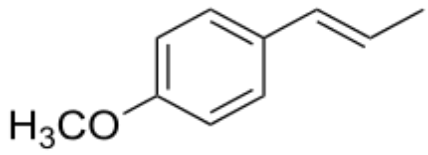


Figure 2.5: Chemical structure of trans-anethole

Trans-anethole is found to be carcinogenic but with no evidence of genotoxicity (Newberne *et al*., 1999).

2.3.5 Lasiocarpine

Lasiocarpine, also called 7-Angelyleuropine or 7-Angelyl-9-lasiocarpylheliotridine is a pyrrolizidine alkaloid. Its molecular formula is $C_{21}H_{33}NO_7$ and 411.495 g/mol its molecular weight.

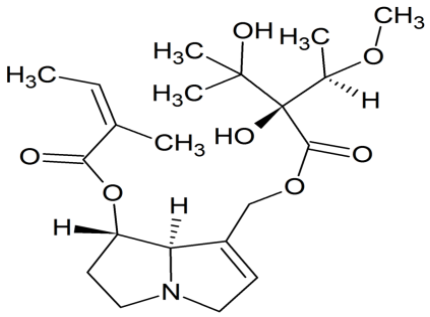


Figure 2.6: Chemical structure of lasiocarpine
(https://commons.wikimedia.org/wiki/File:Lasiocarpine.png)

Angiosarcomas were observed in the liver of both female and male rats after a treatment during 2 years. In addition, several in vitro and in vivo tests have indicated the genotoxic potential of lasiocarpine (Chen *et al*., 2010; Fu *et al*., 2004).

2.3.6 Methyleugenol

Methyleugenol, also called is an alkenylbenzene occurring in a large variety of plant species including *Illicium anisatum* L., *Laurus nobilis* L. and *Zingiber officinale* Roscoe. In the European Food Safety Authority (EFSA) compendium, it has been indicated that methyleugenol along with its 1-hydroxy-metabolite is mutagenic in many sytems and also responsible for DNA adducts and hepatic tumor formation in mice (EFSA)

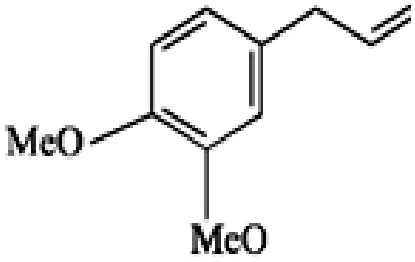


Figure 2.7: Chemical structure of methyleugenol (From Ernane *et al*., 1997)

2.3.7 Heliotrine

Heliotrine is a pyrrolizidine alkaloid occurring in *Helotropium spp*. It has the molecular formula $C_{16}H_{27}NO_5$ and its molecular weight is 313.394 g/mol.

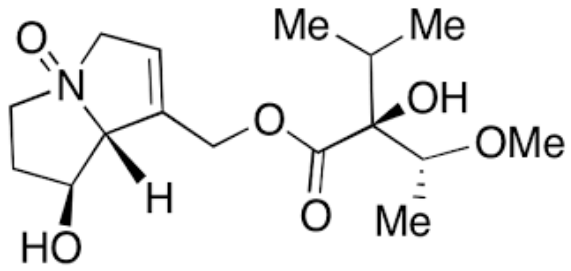


Figure 2.8: Chemical structure of heliotrine

The ability of heliotrine to cause cancer has been investigated in male rats. Heliotrine increased pancreatic islets-cell tumors, transitory cell papillomas of the urinary bladder and also intestinal and testicular tumors were found. Several in vitro and in vivo studies including the DNA-adduct formation and chromosomal aberrations have indicated that heliotrine is genotoxic (Chen *et al*., 2010; Fu *et al*., 2004).

2.3.8 Myristicin

This compound is found in a large variety of plants including *Myristica fragrans* Houtt. and *Sassafras albidum* (Nutt.) Nees. Myristicin is an alkenylbenzene with the formula $C_{11}H_{12}O_3$. Its molecular weight is. It is nomenclature is 1,3-Benzodioxole, 4-methoxy-6-(2-propen-1-yl)- (ACD/Index name) or 6-Allyl-4-methoxy-1,3-benzodioxole (ACD/IUPAC name).

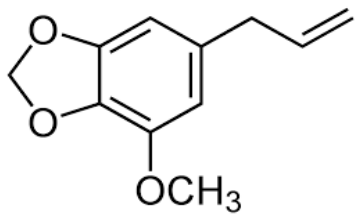


Figure 2.9: Structure of myristicin

The evidence of the genotoxicity of myristicin has been established. It has been indicated that the compound exhibited mutagenic activities and the formation of DNA adducts. 1-hydroxy-myristicin, a metabolite of myristicin is also considered carcinogenic by the European Food Safety Authority compendium.

2.3.9 Quercetin

Quercetin ($C_{15}H_{10}O_7$) is a polyphenolic flavonoid with chemopreventive activity occurring naturally in a wide range of medicinal plant species. It is present in *Cupressus sempervirens* L. and *Camellia sinensis* (L.) Kuntze.

Its molecular weight is 302.236 g/mol. It has an antioxidant property like many other phenolic heterocyclic compounds.

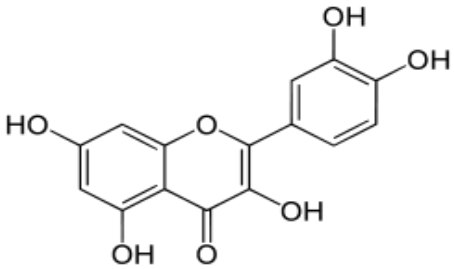


Figure 2.10: Chemical structure of quercetin

Though its mechanism of action is not fully known, quercetin has an antiproliferative property due to the modulation of either EGFR or estrogen-receptor mediated signal transduction pathways. Quercetin produced a decrease in the expression of mutant p53

protein and p21-ras oncogene, induction of cell cycle arrest at the G1 phase and the inhibition of heat shock protein synthesis.

Quercetin also produces anti-inflammatory and anti-allergy effects mediated through the inhibition of the lipoxygenase and cyclooxygenase pathways, thereby preventing the production of pro-inflammatory mediators.

In a study carried out by the NTP (National Toxicological Program), quercetin produced no evidence of carcinogenic activity in female rats and some evidence in male rats. The exhibited carcinogenic activity in the male rats was shown as increased incidences of benign neoplasms. Only in one male rat, exposed to the highest tested dose, an adenocarcinoma was formed. NTP in vitro assays indicated genotoxic activities for quercetin.

2.4.10 Reserpine

Reserpine is an indole alkaloid used to treat high blood pressure and sometimes schizophrenia. It is derived from *Rauwolfia vomitoria* root bark. Its IUPAC name is (3β,16β,17α,18β,20α)-11,17-Dimethoxy-18-[(3,4,5trimethoxybenzoyl)oxy]yohimban-16-carboxylic acid methyl ester. Its molecular formula is C_{33} H_{40} N_2 O_9 and its molecular weight is 608.68 g/mol.

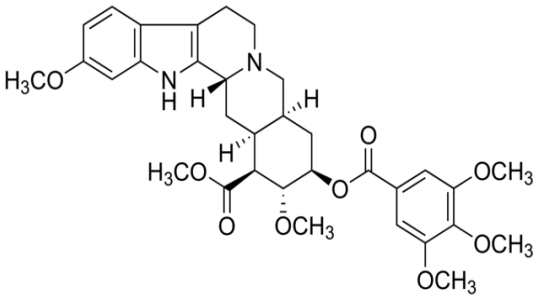


Figure 2.11: Chemical structure of reserpine

2.3.11 Riddeliine

Riddelliine, classified as a pyrrolizidine alkaloid, is a compound isolated first from *Senecio riddellii*. It is also found in the plants of the genus Senecio and also *Jacobaea vulgaris*. Riddelliine is a pyrrolizidine alkaloid of the macrocyclic diester class and exist in the plants as the free-base alkaloid and as an N-oxide. The structure of riddelliine is $C_{18}H_{23}NO_6$ (349.4 g/mol) and that of riddelliine N-Oxide is $C_{18}H_{23}NO_7$.

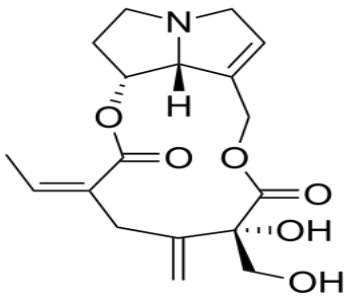


Figure 2.12: Chemical structure of riddelliine
(https://en.wikipedia.org/wiki/File:Riddelliine.svg)

Riddelliine is absorbed primarily via ingestion, distributed in the liver and eliminated in the feces and urine. It is metabolized in the liver to two metabolites, *R*-and *S*-dihydropyrrolizine (DPH) or dehydroretronecine (or dehydroheliotridine) by the cytochromes P450 isozymes CYP3A and CYP2B6. Both R and S-DHPs have been proved to cause tumors in male and female rodents (National Toxicological Program, 2008). The two metabolites have the ability to bind to the DNA, and this may confer them a genotoxic activity (Suzanne *et al*., 2011).

2.3.12 Safrole

Safrole also called shikimol is isolated from sassafras oil or camphor oil. It is a phenylpropene. Its formula is $C_{10}H_{10}O_2$ and it has a molecular weight of 162.19 g/mol. The IUPAC name of safrole is 5-(2-propenyl)-1,3-benzodioxole (also called 5-(2-propenyl)-1,3-benzodioxole or 5-Allyl-1,3-benzodioxole).

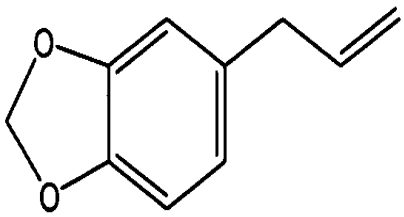


Figure 2.13: Chemical structure of safrole

After oral administration of safrole to guinea pigs and rats, 3'-N, N-dimethylamino-1'-(3, 4-methylenedioxyphenyl)-1'-propanone was identified as major urinary metabolite in guinea pigs and as minor metabolite in rats.

Metabolites resulting from incubation of safrole with adrenal homogenates were 1'-hydroxysafrole, 4-allyl-1,2-dihydroxybenzene, 3'-hydroxysafrole, 2',3'-epoxysafrole and 2',3'-dihydro-2',3'-dihydroxysafrole (Doumas *et al*., 1977).

Safrole was evaluated for its effects on DNA. Dose levels representing 1X, 0.5X, and 0.25X the concentration producing a 50% reduction in plating efficiency were tested. The positive control of 1 µg/mL was 7,12-dimethylbenzanthracene. Concentrations from 12.5 to 100 µg/mL did not influence the frequency of structural chromosome aberrations or polyploidy. At 100 µg/mL, the test substance marginally reduced the plating efficiency (PE) of RL4 cells (derived from a 10-day old male Wistar rat) by 50%. In the sister chromatid exchange (SCE) assay, the mitotic index (MI) was significantly reduced from 7.6% in the controls to 1% in the 100 µg/mL cultures. At the highest dose, there was evidence of cytotoxicity, growth inhibition and cell cycle delay (http://tools.niehs.nih.gov/cebs3/ntpviews/index.cfm?action=testarticle.toxicity&cas_number=94-59-7).

2.4.13 Senkirkine

Senkirkine is a pyrrolizidine alkaloid found in *Jacobaea vulgaris*. It is formula is $C_{19}H_{27}NO_6$ and its molecular weight is 365.426 g/mol. The IUPAC name of senkirkine is

(1R,4Z,6R,7R,11Z)-4-ethylidene-7-hydroxy-6,7,14-trimethyl-2,9-dioxa-14-azabicyclo[9.5.1]heptadec-11-ene-3,8,17-trione.

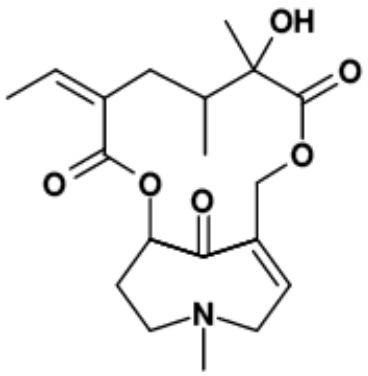


Figure 2.14: Chemical structure of senkirkine

Though the carcinogenic activity is not fully established, several genotoxicity studies on senkirkine revealed positive results (Chen *et al*., 2010; Fu *et al*., 2004; Committee On Carcinogenicity, 2008; International Agency for Research on Cancer, 1983).

CHAPTER THREE: MATERIAL AND METHODS

3.1 Sampling site for the medicinal plants (collection and authentication of plant samples)

1 kg of specimen per medicinal plant species (*Parinari curatellifolia* leaves and stem bark and *Azadirachta indica* leaves) was collected from a forest reserve in the town of Sokode located at 8° 59’ 00’’ latitude and 1° 08’ 00’’ longitude. A visit to the field was done with the assistance of a traditional healer for the harvesting. All collected plants specimen were shade dried and stored in their whole form until used in experiments. A non-governmental organization named CERMETRA (Centre for Study and Research in Traditional Applied Medicine) which promotes research on medicinal plants and their utilization, provided the information regarding the available plant species present in the area which traditional healers use in the preparation of medicinal concoctions. Voucher specimens for the medicinal plants were sent to the botany department of University of Nairobi for further authentication. The reference numbers were KMK2016/002 and KMK2016/008 for *P. curatellifolia* and *A. indica* respectively.

Figure 3.1: Map of Africa showing Togo, the central region and the town Sokode (from http://www.worldatlas.com/webimage/countrys/africa/tg.htm).

Table 3.1: Selected antimalarial plant samples by their harvested parts

Sample code	Scientific name	Parts collected
PCL	*Parinari curatellifolia*	Leaves
PCSB		Stem barks
AIL	*Azadirachta indica*	Leaves

Each harvested plant material was given a code. The stem barks and leaves of the collected plants parts were washed and dried and stored under shade in a greenhouse at the Botany department of Jomo Kenyatta University of Agriculture and Technology.

3.2. Transport of plant samples

The harvested medicinal plants were sealed in transparent plastic bags and transported by flight from Lomé to Nairobi in Kenya. The plants were declared at the airport and their import approved by KEPHIS (Kenya Plant Health Inspectorate Service).

3.3 Preparation of the medicinal plant extracts

3.3.1 Pretreatment of the plants

Both the stem barks and leaves of *Parinari curatellifolia* and leaves of *Azadirachta indica* plants were dried under shade at the greenhouse of the botany department of Jomo Kenyatta University of Agriculture and Technology (JKUAT) and pounded using an electrical mortar.

Plate 3.1: Drying of *Parinari curatelifollia* stem barks and leaves

3.3.2 Preparation of methanol extracts

Methanolic extracts were prepared at laboratory temperature by soaking 250 g of each powdered plant stem barks and leaves in 100 ml methanol for 24 hours. The extracts were then concentrated at 65 °C using a rotary evaporator to evaporate the methanol.

3.3.3 Preparation of aqueous extracts

The water extracts were prepared by soaking 250 grams of the dried and pounded plant material in 1 liter of distilled water at + 4 °C for 3 days and were filtrated using Whatman N° 1 paper. The filtrates were kept at – 20^{o} C before freeze-drying.

3.4 Phytochemical analysis

3.4.1 Phytochemical screening

The phytochemical tests were performed following the method described by Harborne (1984), Edeoga *et al*. (2005) and Yadav and Agarwala (2011), Sasidharan *et al*. (2011), Savithramma *et al*. (2011) and Wadood *et al*. (2013). These tests are usually based on visual observation of color or precipitate formation after addition of specific reagents. One gram of the medicinal plant samples was re-dissolved in 10 mL of distilled water and methanol and subjected to qualitative phytochemical screening for saponins, tannins, alkaloids, flavonoids, phytosterols, glycosides and terpenoids.

3.4.1.1 Detection of saponins

Saponins were tested by diluting 2 mL of the extract in a test tube. The suspension was shaken for 5 minutes and allowed to stand for 10 minutes. A 2cm layer of foam was taken as the indicator for the presence of saponins (Savithramma *et al*., 2011; Wadood *et al*., 2013).

3.4.1.2 Detection of tannins

0.5mL of 5% ferric chloride solution was added to 0.5ml of the sample solution. A dark-green color indicated the presence of tannins (Yadav and Agarwala, 2011; Sasidharan *et al*., 2011).

3.4.1.3 Detection of flavonoids

To 1 ml of extract, 3 drops of ammonia solution was added followed by 0.5ml of concentrated HCl. The formation of a pale brown coloration indicated the presence of flavonoids (Yadav and Agarwala, 2011).

3.4.1.4 Detection of glycosides

Keller-Killiani test was used. Glycosides were tested by adding 1mL of 3.5% ferric chloride in acetic acid to 1mL of the sample solution followed by careful drop-wise addition of 1.5 mL concentrated sulfuric acid by the sides of the test tube to form a separate layer at the bottom. A brown ring at the interface due to the presence of de-oxy sugar characteristic of cardenolides and a pale green color in the upper layer due to the steroidal nucleus was taken as the indicator for the presence of cardiac glycosides (Sasidharan *et al*., 2011).

3.4.1.5 Detection of alkaloids

The Mayer's test was carried out to determine the presence of alkaloids.

In this test, 1mL of Mayer's reagent (potassium mercuric iodine) was added to 1mL of the test solution and observed for a white precipitate, which is a positive indicator for the presence of alkaloids (Harborne, 1998).

3.4.1.6 Detection of phytosterols

1 ml of crude extracts were mixed with 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A red coloration indicated the presence of sterols (Savithramma *et al*., 2011; Tiwari *et al*., 2011).

3.4.1.7 Detection of terpenoids

3 ml of chloroform and 2 ml of concentrated sulfuric acid (H_2SO_4) were added to 2 ml of the extracts. A reddish brown color interface indicated the presence of terpenoids (Sasidharan *et al*., 2011; Edeoga *et al*., 2005).

3.4.2 Quantitative analysis

3.4.2.1 Quantitation of total phenolic content (TPC)

TPC of the leaf extract was determined spectrophotometrically following the Folin–Ciocalteu method described previously with a minor modification (Iqbal *et al*., 2005). Briefly, 20 µl of sample or standard (2.5–50 mg/L gallic acid) plus 150 µl of diluted Folin–Ciocalteu reagent (1:4 reagent: water) was placed in each well of a 96-well plate, and incubated at room temperature for 3 min. Following an addition of 300 µl of sodium carbonate (2:3, saturated sodium carbonate: water) and a further incubation for 2 h at room temperature, the absorbance was read at 765 nm using a spectrophotometer (UV-1601 Shimadzu Corporation, Kyoto, Japan). The phenolic compound content was determined as gallic acid equivalents using the linear equation based on the calibration curve: $C = (c \times V)/m$, where, C = total content of phenolic compounds (mg/g plant extract in GAE), c = concentration of gallic acid obtained from calibration curve (mg/ml), V = the volume of the sample solution (ml), m = weight of the sample (g). All tests were conducted in triplicate.

3.4.2.2 Quantitation of flavonoids

Aluminum chloride colorimetric method was used for determination of flavonoids (Jagadish *et al*., 2009; Marinova *et al.*, 2005). In a 10 mL volumetric flask, 4 mL of distilled water and 1 mL of plant extract were added. After 3 minutes, 0.3 mL of 5 % sodium nitrite solution was added. After 3 minutes, 0.3 mL of 10 % aluminum chloride was added. After 5 minutes, 2 mL of 1 M sodium hydroxide was added and the volume made up to 10 mL with water. Absorbance was measured at 415 nm using UV-Vis spectrophotometer (Shimadzu model UV – 1601 PC, Kyoto, Japan). The amount of total flavonoids was calculated from calibration curve of standard prepared from quercetin.

3.4.2.3 Quantitation of alkaloids

Quantitative estimations of alkaloids were carried out following method of Sairam and Khanna, 1971. To one gram of powder, 0.75 ml 25 % ammonium hydroxide, 1ml 95 % ethyl alcohol and 2 ml ethyl ether were added. The material was allowed to macerate for 12 hours and dried. The dried material was extracted with chloroform for 24 hours in a soxhlet apparatus and the extract obtained was evaporated to dryness and the residue was mixed with 2.5 ml 0.1 Methanol (90 %) H Cl. The extract thus obtained was centrifuged for 10 min at 3000 g to recover the supernatant and discard pellet. The solution was evaporated and the total alkaloids were weighed after drying at 100 ^{0}C.

3.4.3 *Allium cepa* assay (determination of genotoxic potential)

Purple creol onion seed variety was obtained from the Kenya Highlands company and spread on a filter paper moistened in a petri dish for 96 hours to allow seeds to germinate. The test compounds were dissolved in distilled water and diluted to obtain different concentrations (1 g/l, 0.5 g/l, 0.25 g/l and 0.125 g/l) of test solutions. The negative control group was treated with distilled water. The positive control group was treated with glyphosate in different dilutions (1 g/l, 0.5 g/l, 0.25 g/l and 0.125 g/l). The germinated seeds were then transferred to the different concentrations of the medicinal plant extracts for a period of 48 hours. The root growth inhibition test was performed by measuring the

root lengths of treated seeds using a meter ruler together with the positive control group treated with different concentrations of glyphosate and the negative control group for the seeds which were grown in distilled water. The EC50 was determined as the concentration at which the root growth is inhibited by 50 % compared to the negative control. The germinated treated seeds were fixed in Carnoy fixative (Ethanol: acetic acid 3:1) for 24 hours, washed in distilled water and hydrolyzed using HCl 1N at 60 °C for 8 minutes (Asita and Mokhobo, 2013). The seeds were washed in distilled water and about 3 mm of their root tips removed and placed on a microscope slide. 1ml of safranin was added to the root tips and the preparation covered with a coverslip. Three slides were prepared per concentration. The microscopic observation was done at X 1000 magnification with immersion oil using a light microscope coupled with digital camera. 1000 cells were scored per slide and the number is cells in mitosis were counted. The mitotic index was then determined according to Fiskesjo *et al*., (1985) as:

$$M.I = \frac{\text{Prophase} + \text{Metaphase} + \text{Anaphase} + \text{Telophase}}{1000}$$

The cells with chromosomal aberration (bipolar anaphase, chromosomal fragments, c-mitosis, sticky anaphase chromosomal bridges) were also counted and photographs of the types of chromosomal aberrations were made.

3.4.4 Ames mutagenicity test (determination of mutagenic potential)

Salmonella typhimurium TA 98 and TA 100 strains were purchased from Xenometrix and stored at - 80 °C. 50 ml of growth medium was added to the TA 98 and TA 100 vials and the bacterial pellets disrupted using a sterile pipette tip. From each vial, 25 ml of suspension was transferred each to three 50 ml tubes labeled respectively ‘TA 98’, ‘TA 100’ and ‘negative control’. 10 µl of ampicillin and 10 ml of growth medium were added to the tubes. The tubes were incubated overnight at 37°C in a shaker incubator for 16 hours. After incubation, the optical densities of the colonies were determined at a wavelength of 260 nm using a spectrophotometer. An optical density of < 2 for bacterial cultures is an indication of contamination. Hence, such resulted in stopping the

experiment and preparing another culture. For the negative control, the optical density should be less than 0.05. After recording the optical density values, the serial dilution of the test compound *Azadirachta indica* leaves aqueous extract was performed on a 96-well plate. Concentrations of 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µg/µl were prepared. The 1 µg/µl solution was prepared by dissolving 0.05 g of the leaves powder in 50 ml of distilled water. The different concentrations of the extracts were then transferred to a 24-well plate. The negative control was made of distilled water (solvent) and the positive control of Nitroquinoline-N-Oxide 50 µg/ml and nitrofluorene 50µg/ml. A 10 % of the TA98 culture was prepared by adding 0.7 ml of the exposure medium (Xenometrix) to 6.3 ml of overnight culture. For TA 100, 0.35 ml of the exposure medium was added to 0.65 ml of the overnight culture, making a concentration of 20 %. The negative control, the six concentrations and the positive control were added to the wells in triplicates. 2.6-2.8 ml of an indicator medium (Xenometrix) was added to the wells. The experiment was carried out in 96-well plates and for each concentration used per extract as well as the positive (Nitroquinoline-N-Oxide and nitrofluorene) and negative control (distilled water), the tests were done in triplicate. After 2 days, the plates were scored and the number of revertant colonies per extract and strain recorded. The least deviation in color from blue to yellow indicated a positive result.

3.4.5 Statistical analysis

The unpaired t-test was performed to analyze the significance in the root growth inhibition and also the mitotic indices in the treated *Allium cepa* seedlings meristematic cells. The chromosomal aberrations were analyzed using the one-way ANOVA (analysis of variance). For the Ames mutagenicity assay, the unpaired t-test was used. The p-values less than 0.05 indicated statistically significant results in comparison to the negative control.

CHAPTER FOUR: RESULTS

4.1 Phytochemical screening

The phytochemical screening of aqueous and methanolic extracts *Parinari curatellifolia* stem barks and leaves as well as *Azadirachta indica* leaves was based on visual observation of coloured complex formation. The screened phytochemical classes were present in the samples as indicated in Tables 4.1-4.3. Table 4.1 shows the presence of saponins, tannins, alkaloids, steroids, glycosides phytosterols and terpenoids in the aqueous and methanolic extracts of the medicinal plant samples.

Table 4.1: Phytochemical screening of *P. curatellifolia* stem barks

Phytochemical classes	Test	Water extracts	Methanol extracts
Tannins	Ferric chloride test	+	+
Alkaloids	Mayer's test	+	+
Saponins	Froth test	+	+
Terpenoids	Salkowski test	+	+
Glycosides	Keller-Killiani test	+	+
Flavonoids	Ammonia test	+	+
Sterols	Salkowski test	+	+

Table 4.2: Phytochemical screening of *P. curatellifolia* leaves

Phytochemical classes	Test	Water extracts	Methanol extracts
Tannins	Ferric chloride test	+	+
Alkaloids	Mayer's test	+	+
Saponins	Froth test	+	+
Terpenoids	Salkowski test	+	+
Glycosides	Keller-Killiani test	+	+
Flavonoids	Ammonia test	+	+
Sterols	Salkowski test	+	+

Table 4.3: Phytochemical screening of *A. indica* leaves

Phytochemical classes	Test	Water extracts	Methanol extracts
Tannins	Ferric chloride test	+	+
Alkaloids	Mayer's test	+	+
Saponins	Froth test	+	+
Terpenoids	Salkowski test	+	+
Glycosides	Keller-Kiliani	+	+
Flavonoids	Ammonia test	+	+
Sterols	Salkowski test	+	+

4.2 Quantitative analysis

Total phenols, flavonoids and alkaloids were quantified in *Parinari curatellifolia* leaves and stem bark aqueous and methanol extracts and in *Azadirachta indica* aqueous and methanol extracts.

Figure 4.1 shows the correlation between the absorbance and the concentration of flavonoids by quercetin equivalents. The relationship between the concentration of flavonoids (X axis) and the absorbance (Y axis) was determined as y = 0.0015x with a correlation coefficient of R^2 = 0.9846.

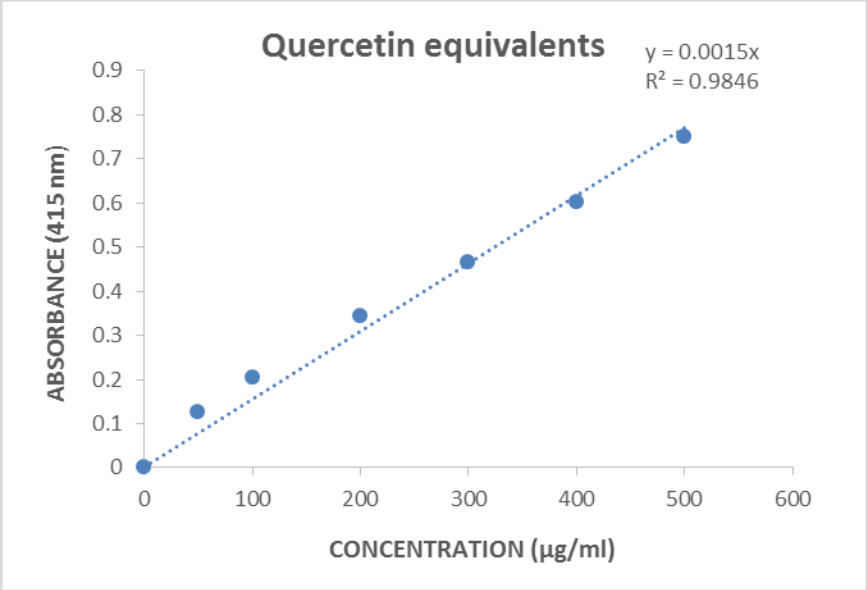


Figure 4.1: Standard curve of the absorbance of flavonoids by concentration of quercetin.

Figure 4.2 shows the relationship linking the concentration of gallic acid equivalents (X axis) to the absorbance (Y axis) was determined as y = 0.0161 x with a correlation coefficient of R^2 = 0.9983.

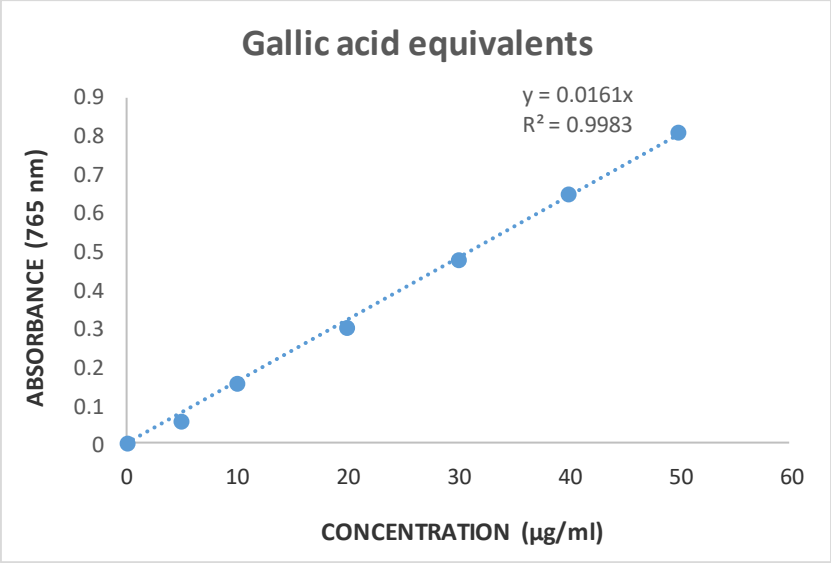


Figure 4.2: Standard curve of the absorbance of total phenols by concentration of gallic acid.

Figures 4.3 to 4.5 respectively show the amount of total phenols, flavonoids and alkaloids in the aqueous and methanolic *P. curatellifolia* stem bark and leaf extract as well as *A. indica* leaf extract. In *P. curatellifolia* stem bark the values of total phenolics were 3.55 ± 0.11 g/100g and 3.86 ± 0.05 g/100g for the aqueous and methanolic extracts respectively. The content of phenolics in *P. curatellifolia* leaves was 3.80 ± 0.12 g/100g for the aqueous extract and 2.59 ± 0.14 g/100g in the methanolic extract. In *A. indica*, phenols were in higher proportion with 7.33 ± 0.17 g/100g and 8.58 ± 0.25 g/100g in the water and methanol extracts respectively. The flavonoids were estimated at 18.16 ± 1.10 g/100g and 23.63 ± 2.64 g/100g respectively in the aqueous and methanol extract of *P. curatellifolia* stem bark, 20.8 ± 0.68 g/100g and 18 ± 1.27 g/100g in *P. curatellifolia* leaf aqueous and methanol extracts respectively. The amount of flavonoids was the highest in *A. indica* with 23.87 ± 1.33 g/100g in the aqueous extract and 25.46 ± 2.10 g/100g in the methanolic extract. The alkaloids content in *P. curatellifolia* stem bark was 1.75 ± 0.25 g/100 g in the aqueous extract and 0.47 ± 0.05 g/100g in the methanol extract. In *P.*

curatellifolia leaves the alkaloids amounted to 1.22 ± 0.02 g/100g and 1.6 ± 0.12 g/100g in the aqueous and methanolic extracts respectively. *A. indica* leaves contained 1.07 ± 0.15 g/100g and 1.6 ± 0.02 g/100g of alkaloids in their aqueous and methanolic extracts respectively.

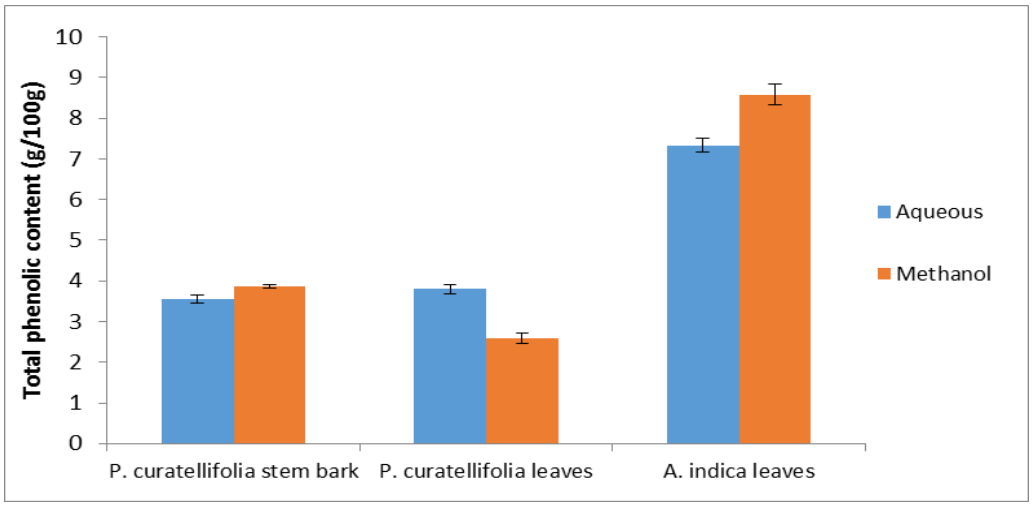


Figure 4.3: Total phenols in the aqueous and methanolic extracts of *Parinari curatellifolia* (stem bark and leaves) and *Azadirachta indica* (leaves)

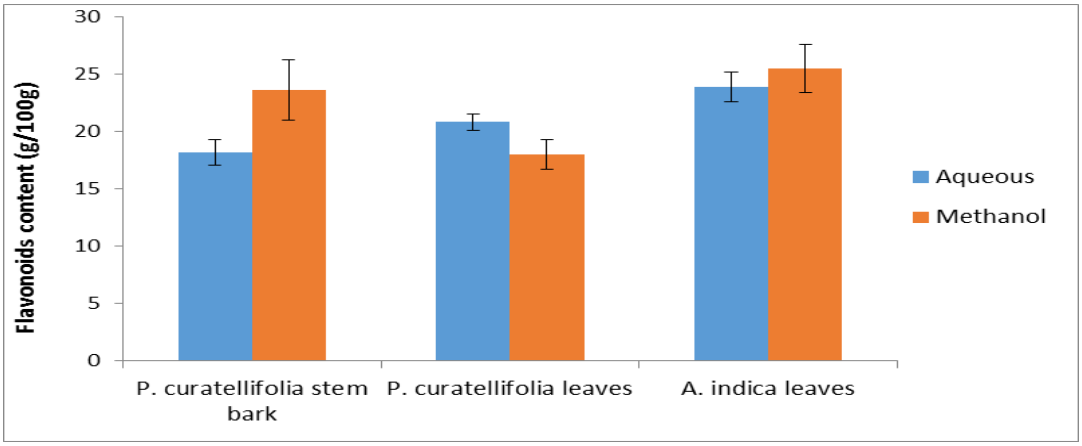


Figure 4.4: Flavonoids content in the aqueous and methanolic extracts of *Parinari curatellifolia* (stem bark and leaves) and *Azadirachta indica* (leaves)

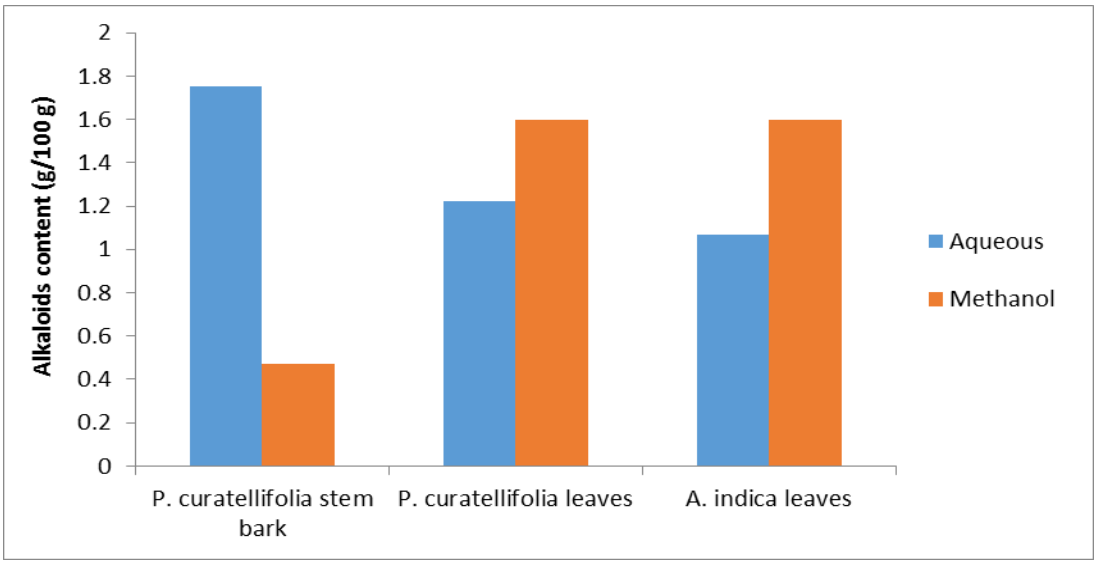


Figure 4.5: Alkaloid content in the aqueous and methanolic extracts of *Parinari curatellifolia* (stem bark and leaves) and *Azadirachta indica* (leaves).

4.3 Genotoxicity assay

The extracts exhibited cytotoxic effects showed by the decrease in the values of the root lengths resulting from treatment of *Allium cepa* seedlings with different concentrations of extracts. The mitotic index, indicators of genotoxicity also decreased in treated *Allium cepa* cells. Chromosomal aberrations (abnormal chromosomal formations occurring during cell division) were observed after exposing the *Allium cepa* seedlings to the aqueous and methanolic extracts of *P. curatellifolia* (Planch. ex Bench.) Kuntze (leaves and stem barks) and *A. indica* A. Juss (leaves).

Tables 4.4 and 4.5 show the cytotoxic effects of the selected medicinal plant extracts on *Allium cepa* seedlings. The cytotoxicity is revealed by the inhibition of root growth. The roots lengths were indicated as mean ± SEM (standard error of mean). The p-values < 0.05 indicated statistically significant values of root lengths in comparison to the negative controls using the unpaired t-test. The EC_{50} was determined for each extract and

estimated to 0.56g/l and 1.12 g/l for *P. curatellifolia* stem bark methanol and water extracts respectively, 0.42 g/l and 1.12 g/l for *P. curatellifolia* leaf methanol and water extracts respectively, and 0.51g/l and 0.26 g/l for *A. indica* leaf methanol and water extracts respectively.

Table 4.4: Cytotoxic effects of the water extracts of the selected medicinal plants on *Allium cepa* cells. R.G: Root growth (% of the control); (*) $P < 0.05$, level of significance of root growth inhibition compared with the control. Values are Mean ± SEM. PCSBW: *Parinari curatellifolia* stem bark water extract; PCLW: *Parinari curatellifolia* leaves water extract; AILW: *Azadirachta indica* stem bark water extract.

	PCSBW			PCLW			AILW		
	Mean root length (cm) ± SE	RG (%) of control	p-value	Mean root length (cm) ± SE	RG% of control	p-value	Mean root length (cm) ± SE	RG% of control	p-value
0	4.44 ± 0.27	100		4.95 ± 0.46	100	-	4.9 ± 0.04	100	-
0.125	3.92 ± 0.2*	88.29	0.02135	4.69 ± 0.25	94.74	0.4519	4.22 ± 0.009*	86	0.0002
0.25	3.28 ± 0.3	73.87	0.05946	4.476 ± 0.3	90.3	0.2195	3.61 ± 0.066*	73.67	$3.905e^{-5}$
0.5	3.1 ± 0.036*	69.81	0.00078	3.354 ± 0.248*	67.75	0.0123	3.07 ± 0.2*	62.65	0.0296
1	2.6 ± 0.2*	58.56	0.00099	2.93 ± 0.65*	59.2	0.01507	2.7 ± 0.27*	55	0.0033
EC50	1.12 g/l	-		1.12 g/l	-		0.26 g/l	-	

Table 4.5: Cytotoxic effects of the methanol extracts of the selected medicinal plants on *Allium cepa* cells. R.G: Root growth (% of the control); (*) P < 0.05, level of significance of root growth inhibition compared with the control. Values are Mean ± SEM. PCSBM*: Parinari curatellifolia* stem bark methanol extract; PCLM: *Parinari curatellifolia* leaves methanol *extract;* AILM*: Azadirachta indica* leaves methanol extracts.

	PCSBM			PCLM			AILM		
	Mean root length (cm) ± SE	RG (%) of control	p-value	Mean root length (cm) ± SE	RG% of control	p-value	Mean root length (cm) ± SE	RG% of control	p-value
0	3.06 ± 0.02	100	-	3.50 ± 0.05	100	-	3.47 ± 0.02	100	-
0.125	1.99 ± 0.07*	65	0.0010	2.17 ± 0.05*	62	0.00143	2.5 ± 0.05*	72.05	0.0002
0.25	1.62 ± 0.1*	53	0.0014	1.85 ± 0.06*	53.43	0.00044	1.8 ± 0.06*	51.87	0.0002
0.5	1.3 ± 0.07*	42	0.0006	1.55 ± 0.005*	44.29	$1.378e^{-10}$	1.3 ± 0.07*	37.46	0.0002
1	1.13 ± 0.005*	37	$3.316e^{-6}$	0.86 ± 0.07*	24.57	$1.378e^{-10}$	1.05 ± 0.12*	30.26	0.0006
EC50	0.56 g/l	-		0.42 g/l	-		0.51 g/l	-	

Figure 4.6 indicates the different linear correlations between the concentrations of each extract and the root lengths of exposed *Allium cepa* seedlings. The EC50 (Effective concentration resulting in the inhibition by 50 % of the root length of the untreated *Allium cepa* seedlings) was determined for each extract using the correlation equation linking y (root length) to x (concentration).

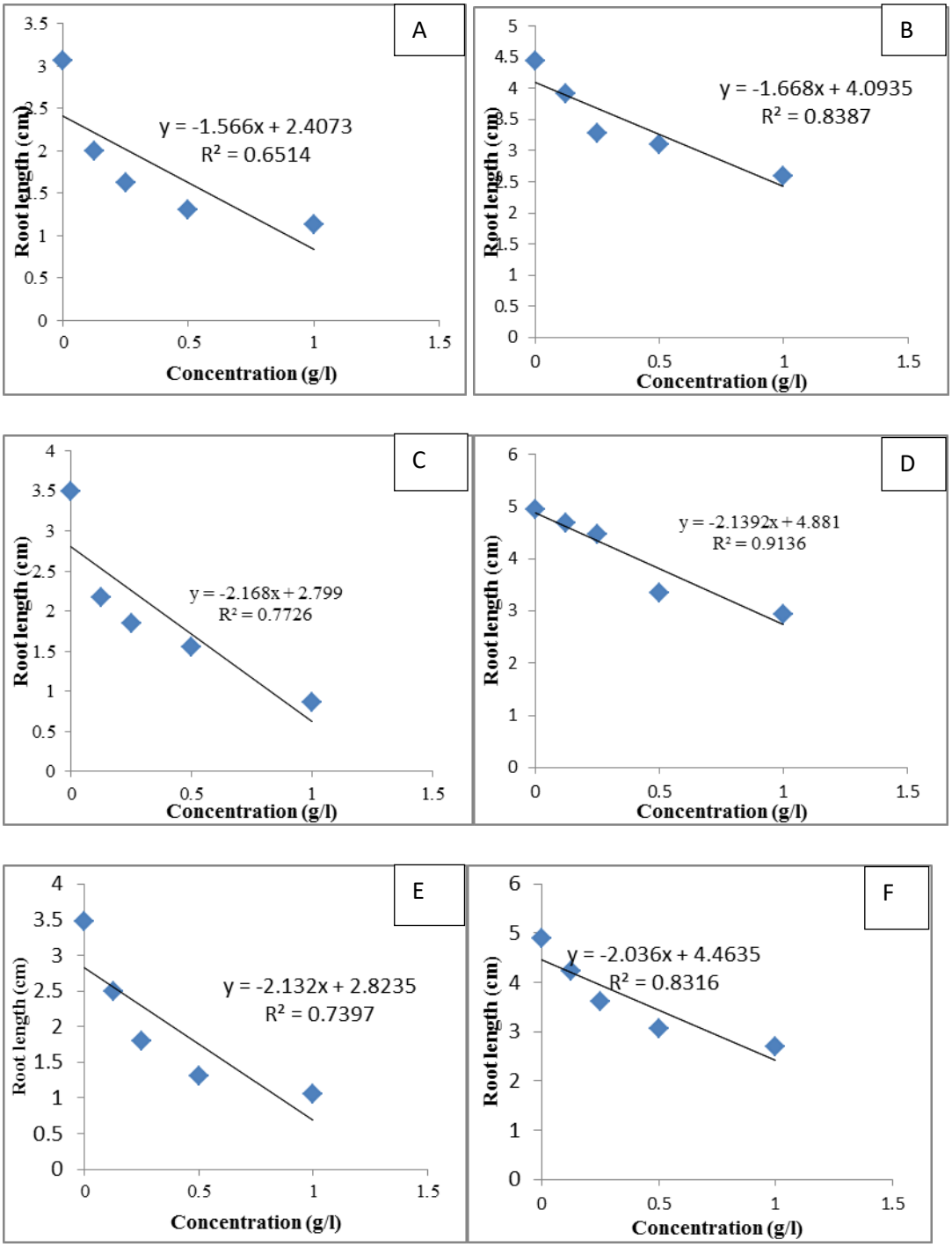


Figure 4.6: Correlations between the concentrations of the selected medicinal plants extracts and the root lengths of *Allium cepa* seedlings exposed to four different concentrations of each of the extracts. A: *Parinari curatellifolia* stem bark methanol

extract; B: *Parinari curatellifolia* stem bark; C: *Parinari curatellifolia* stem bark aqueous extract; D: *Parinari curatellifolia* leaf methanol extract; E: *Azadirachta indica* leaf methanol extract; F: *Azadirachta indica* leaf aqueous extract.

Tables 4.6-4.11 show the mitotic indices in treated *Allium cepa* seedlings meristems cells and also the number of abnormal mitotic cells after exposure to the different concentration of aqueous and methanolic extracts of *P. curatellifolia* stem bark and leaves as well as *A. indica* leaves in a total number of 1000 dividing cells. Chromosomal bridges (Plate 4.2 f) during anaphase stage were observed in *Allium cepa* cells treated with *Parinari curatellifolia* stem barks at 0.5 g/l and 1 g/l for the aqueous extract (Table 4.6) and 0.125 g/l, 0.5 g/l and 1 g/l for the methanol extract (Table 4.9). *Parinari curatellifolia* leaves induced chromosomal bridges from 0.125 g/l to 1 g/l with both its aqueous methanol extracts (Table 4.7 and 4.10). The treatment with aqueous leaf extract of *Azadirachta indica* resulted in the occurrence of chromosomal bridges at 0.5 g/l and 1 g/l (Table 4.8) whereas with the methanol extract they were induced 0.125 g/l, 0.5 g/l and 1 g/l (Table 4.11). The c-mitosis was observed from 0.125 g/l to 1 g/l in *Allium cepa* cells treated with both aqueous and methanolic extracts of *P. curatellifolia* stem bark and leaves as well as *A. indica* leaves. Sticky anaphase was recorded from 0.25 g/l to 1 g/l in *A. cepa* cells treated with *P. curatellifolia* stem bark aqueous extract (Table 4.6) as well as at 0.25 g/l and 1 g/l of *P. curatellifolia leaf* methanol extracts (Table 4.9). With *P. curatellifolia leaves*, sticky chromosomes were not found (Tables 4.7 and 4.10). *A. indica* aqueous leaf extract caused sticky chromosomes at 0.5 g/l and 1 g/l (Table 4.8) while sticky chromosomes were scored at 0.25 g/l and 1 g/l after treatment with its methanolic extract (Table 4.11). Bipolar anaphase was induced by the treatment of *A. cepa* cells with 0.5 g/l and 1 g/l of *P. curatellifolia* stem bark (Table 4.9) and *A. indica* leaf methanolic extracts (Table 4.11). The chromosomal fragments were observed at 0.5 and 1 g/l after treatment with *P. curatellifolia* stem bark and *A. indica* methanolic extracts (Tables 4.9 and 4.11). They occurred at 0.125 g/l to 1 g/l of *P. curatellifolia* leaf aqueous and methanol extracts (Table 4.10), *P. curatellifolia* stem bark aqueous extract (Table 4.6), at

0.5 g/l and 1 g/l of *A. indica* leaf methanolic extract (Table 4.11), from 0.25 g/l to 1 g/l of *A. indica* leaf aqueous extract (Table 4.8).

Table 4.6: Effects of *P. curatellifolia* stem bark water extract (PCSBW) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment. PCSBM*: Parinari curatellifolia* stem bark methanol extract; PCLM: *Parinari curatellifolia* leaves methanol *extract;* AILM*: Azadirachta indica* leaves methanol extracts.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	103 ± 6.56	0 ± 0 .00	0.67 ± 1.15	0 ± 0.00	0 ± 0.00	0.67 ± 0.577
PCSBW	0.125	79.67 ± 2.52	0 ± 0 .00	1.66 ± 1.15	0 ± 0.00	0 ± 0.00	2 ± 1
	0.25	74 ± 4.58	0 ± 0.00	2 ± 2	4 ± 2	0 ± 0.00	3.7 ± 1.53
	0.5	64.33 ± 5.86	1.67 ± 2.08	4.33 ± 2.52	3.33 ± 1.53	0 ± 0.00	6 ± 1
	1	44.33 ± 5.86	2.33 ± 0.58	6 ± 2	5 ± 2	0 ± 0.00	6.67 ± 1.53
Glyphosate	0.125	68 ± 4.58	8 ± 3.05	3 ± 0.94	3 ± 1.05	3 ± 2.08	0 ± 0.00
	0.25	47 ± 4	6 ± 2.08	7 ± 2.52	7 ± 2.52	5 ± 2.08	3 ± 2.08
	0.5	29 ± 4.36	5 ± 1.15	12 ± 2	5 ± 3.21	4 ± 1.53	2 ± 1.53
	1	18 ± 6.08	0 ± 0.00	8 ± 2.08	1 ± 0.58	0 ± 0.00	2 ± 1.53

Table 4.7: Effects of *P. curatellifolia* leaf water extracts (PCLW) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	122.3 ± 1 0.8	0.33 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0.67 ± 1.15
PCLW	0.125	96.33 ± 6.03	3.33 ± 1.53	9.3± 2.52	0 ± 0.00	0 ± 0.00	1 ± 1
	0.25	84.0 ± 5.57	5.3 ± 1.53	12.67 ± 4.5	0 ± 0.00	0 ± 0.00	3.3 ± 0.58
	0.5	68.0 ± 3.6	9.0 ± 3	18.67 ± 2.08	0 ± 0.00	0 ± 0.00	4.6 ± 2.08
	1	52.33 ± 4.5	11.67 ± 2.89	25.33 ± 4.5	0 ± 0.00	0 ± 0.00	6.3 ± 3.21
Glyphosate	0.125	68 ± 4.58	8 ± 3.05	3 ± 0.94	3 ± 1.05	3 ± 2.08	0 ± 0.00
	0.25	47 ± 4	6 ± 2.08	7 ± 2.52	7 ± 2.52	5 ± 2.08	3 ± 2.08
	0.5	29 ± 4.36	5 ± 1.15	12 ± 2	5 ± 3.21	4 ± 1.53	2 ± 1.53
	1	18 ± 6.08	0 ± 0.00	8 ± 2.08	1 ± 0.58	0 ± 0.00	2 ± 1.53

Table 4.8: Effects of *A. indica* leaf water extracts (AILW) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	123.33 ± 7.57	0.33 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
AILW	0.125	79.67 ± 2.52	2.67 ± 1.53	4.33 ± 0.58	2.66 ± 2.08	0 ± 0.00	0 ± 0.00
	0.25	89.67 ± 7.5	5 ± 1.73	9 ± 2	3.33 ± 0.58	0 ± 0.00	2 ± 1
	0.5	78.66 ± 5.86	7.66 ± 2.52	16.67± 4.5	6.66 ± 1.53	4.33 ± 0.58	4.33 ± 0.58
	1	58.66 ± 4.16	20.33 ± 7.64	22.33 ± 4.5	10.3 ± 1.53	10 ± 2	10 ± 2
Glyphosate	0.125	68 ± 4.58	8 ± 3.05	3 ± 0.94	3 ± 1.05	3 ± 2.08	0 ± 0.00
	0.25	47 ± 4	6 ± 2.08	7 ± 2.52	7 ± 2.52	5 ± 2.08	3 ± 2.08
	0.5	29 ± 4.36	5 ± 1.15	12 ± 2	5 ± 3.21	4 ± 1.53	2 ± 1.53
	1	18 ± 6.08	0 ± 0.00	8 ± 2.08	1 ± 0.58	0 ± 0.00	2 ± 1.53

Table 4.9: Effects of *P. curatellifolia* stem barks methanol extracts (PCSBM) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	94.33 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
PCSBM	0.125	77.67 ± 2.08	1 ± 1	2 ± 1.73	0 ± 0.00	0 ± 0.00	0 ± 0.00
	0.25	40 ± 5.57	0 ± 0.00	1 ± 1	1 ± 1.73	0 ± 0.00	0 ± 0.00
	0.5	31 ± 2.65	1 ± 1	6 ± 2	0 ± 0.00	5 ± 2	1 ± 1
	1	23 ± 1.73	4 ± 2.65	8 ± 2	1 ± 1	4 ± 1.73	3 ± 1
Glyphosate	0.125	55.33 ± 3.05	8 ± 2.65	3 ± 1.73	3 ± 2.65	3 ± 2.65	0 ± 0.00
	0.25	47 ± 4s	6 ± 2	7 ± 2.83	6 ± 5.86	5.33 ± 1.53	3 ± 2.65
	0.5	29 ± 4.36	5 ± 2.08	12 ± 4.36	5 ± 1.53	4 ± 1.53	2 ± 1.15
	1	20 ± 1	0 ± 0.00	8 ± 1.73	1 ± 0.58	0 ± 0.00	2 ± 153

Table 4.10: Effects of *P. curatellifolia* leaves methanol extracts (PCLM) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	122.3 ± 10.79	0.33 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
PCLM	0.125	96.33 ± 6.03	3.33 ± 1.53	9.3 3± 2.52	0 ± 0.00	0 ± 0.00	1 ± 1
	0.25	84.0 ± 5.57	5.33 ± 1.53	12.67 ± 4.5	0 ± 0.00	0 ± 0.00	3.33 ± 0.58
	0.5	68 ± 3.6	9 ± 3	18.67 ± 2.08	0 ± 0.00	0 ± 0.00	4.67 ± 2.08
	1	52.33 ± 4.5	11.67 ± 2.89	25.33 ± 4.5	0 ± 0.00	0 ± 0.00	6.33± 3.21
Glyphosate	0.125	68 ± 4.58	8 ± 4	4 ± 3.46	3 ± 1	7 ± 3	0 ± 0.00
	0.25	48 ± 6.08	6 ± 3.6	7 ± 2.65	6 ± 1.73	5 ± 1	3 ± 1
	0.5	31 ± 2.65	5 ± 2.65	12 ± 1.73	5 ± 2.65	4 ± 1.73	5 ± 2
	1	20 ± 1	0 ± 0.00	8 ± 3.6	1 ± 1	1 ± 0.00	4 ± 1

Table 4.11: Effects of the methanol extracts of *A. indica* leaves (AILM) on the mitotic division and the chromosomes of *Allium cepa* seedling roots. C.B: chromosomal bridge; C-M: c-mitosis; S.A: sticky anaphase; B.A: bipolar anaphase; F: fragment.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	90 ± 4	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
AILM	0.125	78 ± 2	1 ± 0.00	2 ± 1.52	0 ± 0.00	0 ± 0.00	0 ± 0.00
	0.25	65 ± 2	0 ± 0.00	3 ± 1.73	1 ± 1	0 ± 0.00	0 ± 0.00
	0.5	53 ± 3.6	1 ± 0.58	8 ± 3.6	0 ± 0.00	5 ± 3.6	1 ± 1
	1	38 ± 2	4 ± 1.73	8 ± 2.65	1 ± 0.58	4 ± 0.58	3 ± 2.3
Glyphosate	0.125	68 ± 4.58	8 ± 3.05	3 ± 0.94	3 ± 1.53	3 ± 2.08	0 ± 0.00
	0.25	47 ± 4	6 ± 2.08	7 ± 2.52	6 ± 2.08	5 ± 2.08	3 ± 2.08
	0.5	29 ± 4.36	5 ± 1.15	12 ± 2	5 ± 3.21	4 ± 1.53	2 ± 1.53
	1	18 ± 6.08	0 ± 0.00	8 ± 2.08	1 ± 0.58	0 ± 0.00	2 ± 1.53

Plates 4.1 and 4.2 respectively show representative photographs of normal cells at different stages of mitotic division as well as mitotic cells with chromosomal aberrations. The observation of chromosomal arrangement in the mitotic cells from *Allium cepa* seedlings allowed the identification of their stage of division.

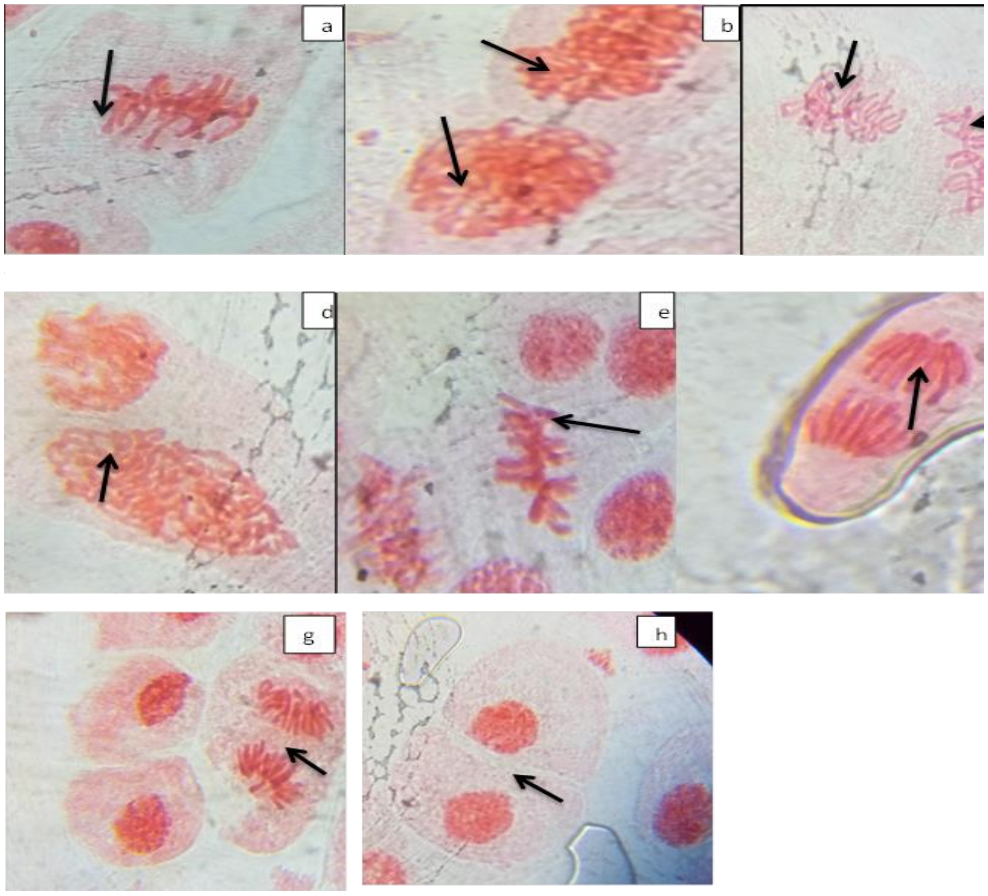


Plate 4.1: Representative photographs of normal *Allium cepa* meristematic cells in the different mitotic stages; a, c, e: Metaphase (M); b, d: Prophase (P); e, g: Anaphase (A); h: Telophase (T). Magnification1000 X.

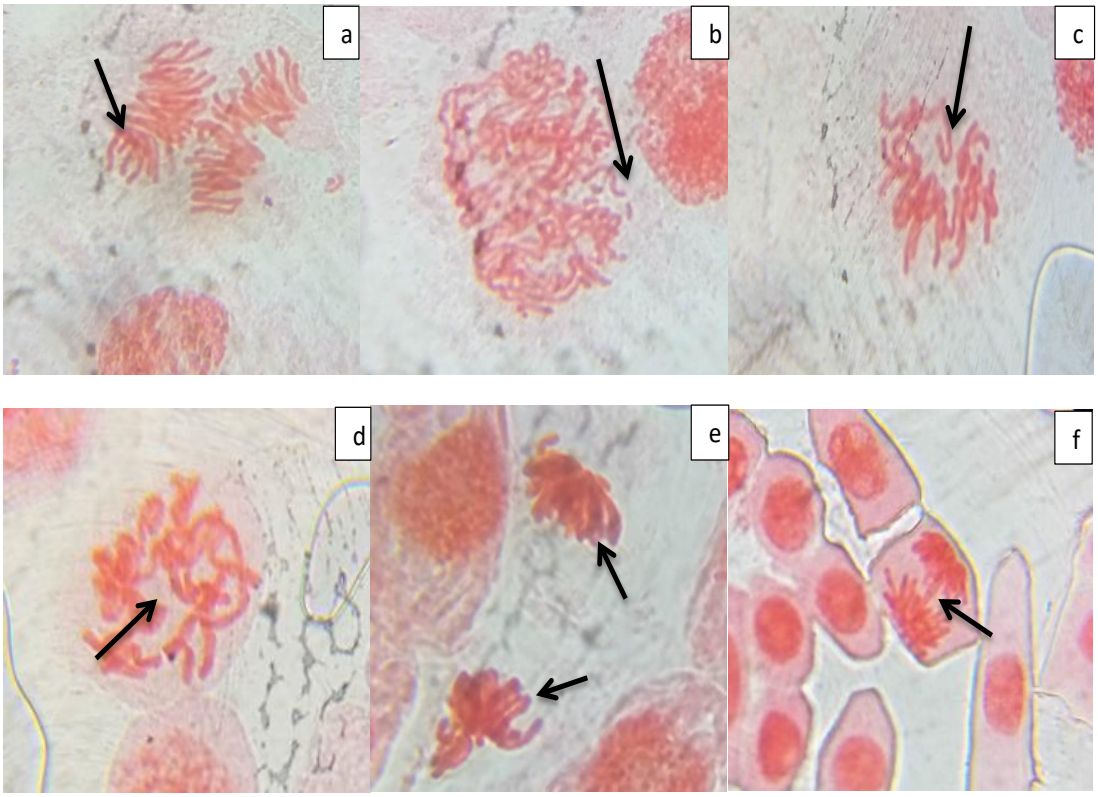


Plate 4.2: Representative photographs of abnormal chromosomes in *Allium cepa* seedlings root cells treated with different concentrations of aqueous and methanol extracts *Parinari curatellifolia* Planch. ex Bench. Kuntze (leaves and stem barks) and *Azidarachta indica* A. Juss (leaves) a: Bipolar anaphase (BA); b: Fragment (F); c: Multipolar anaphase (MA); d: C-mitosis (CM); e: Sticky anaphase (SA); f: Bridge (B). Magnification X1000.

4.4 Mutagenicity assay

In the Ames mutagenicity assay, the extracts were used to expose *Salmonella typhimurium* strains (TA 98 and TA 100). The results were observed from well plates. The 24-well plates were used for the exposure and the 96-well plates for the scoring. The 98-well plate presented in Plates (Plates 4.3 and 4.4) show 27 positive wells. The change in color from purple to yellow on the 24-well plates showed positive results revealing a metabolic shift

indicated by the synthesis of histidine in treated *Salmonella typhimurium* strains (Plates 4.3 and 4.4).

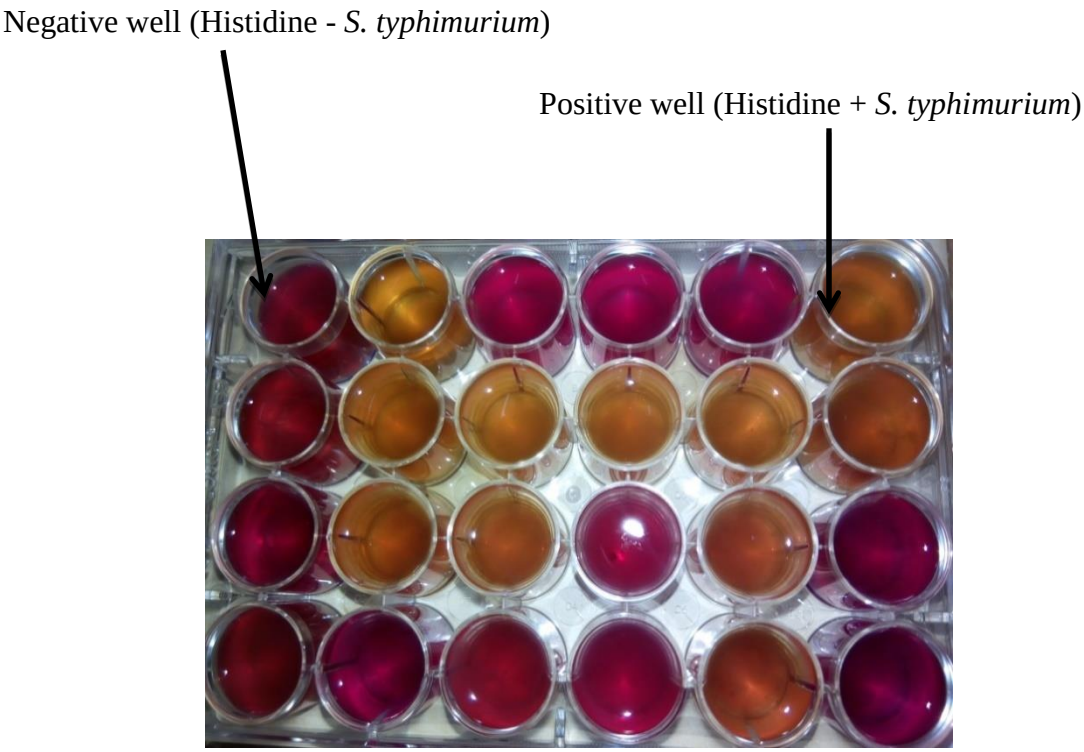


Plate 4.3: 24-well plate showing the positive wells in yellow and the negative wells in purple

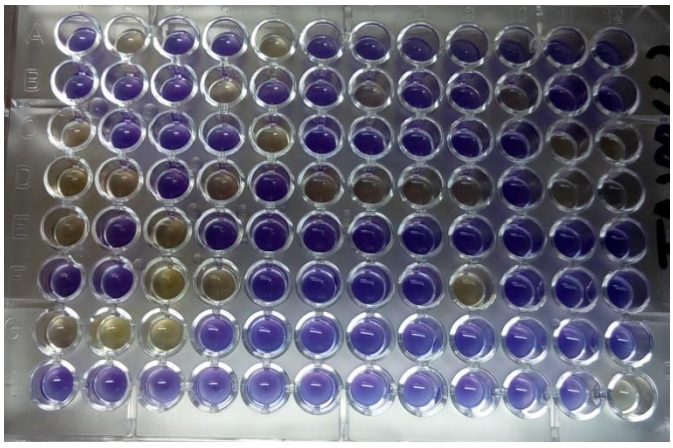


Plate 4.4: 96-well plate showing the positive wells in yellow and the negative wells in blue.

Figures 4.7-4.12 show the number of revertant *Salmonella typhimurium* wells for each concentration of the selected medicinal plant extracts. The stars in the graphs indicate significance at 95 % confidence interval ($p<0.05$) in the number of revertant *Salmonella typhmurium* (with mutagenic activity) in comparison to the negative control using the unpaired t-test. The lines in the graphs show the baseline of mutagenic activity in *S. tyhimurium*.

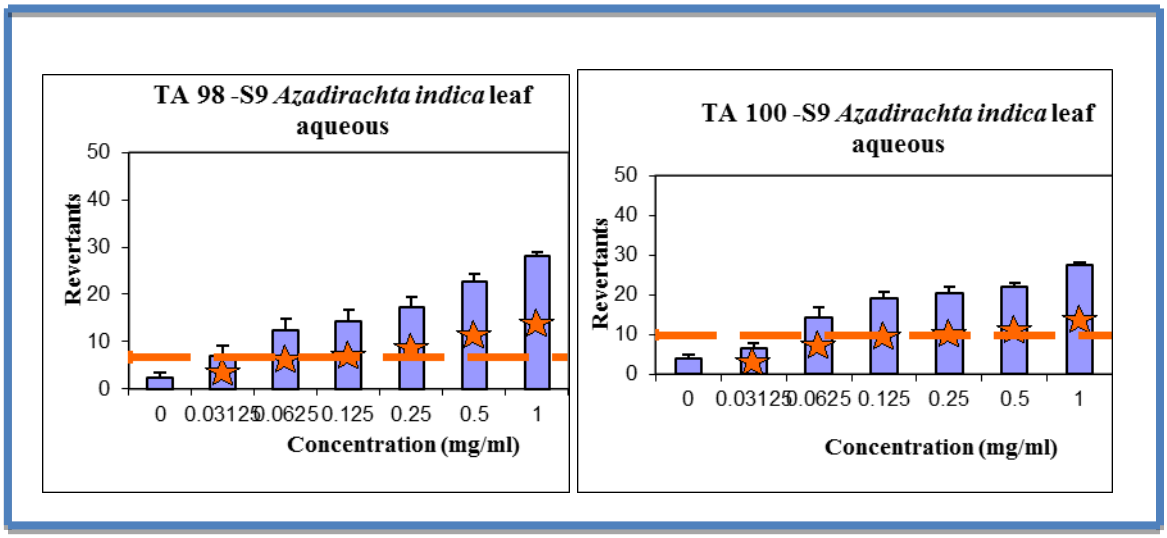


Figure 4.7: Number of revertant *Salmonella typhimurium* TA98 and TA100 by the concentration of *Azadirachta indica* aqueous extracts

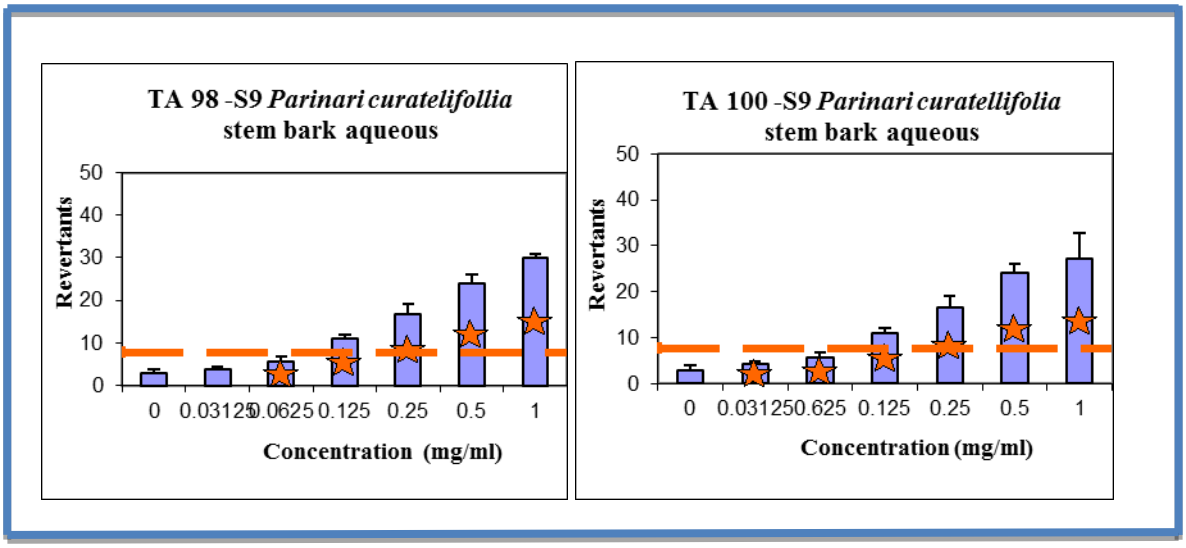


Figure 4.8: Number of revertant *Salmonella typhimurium* TA98 and TA 100 by the concentration of *P. curatellifolia* stem bark aqueous extracts.

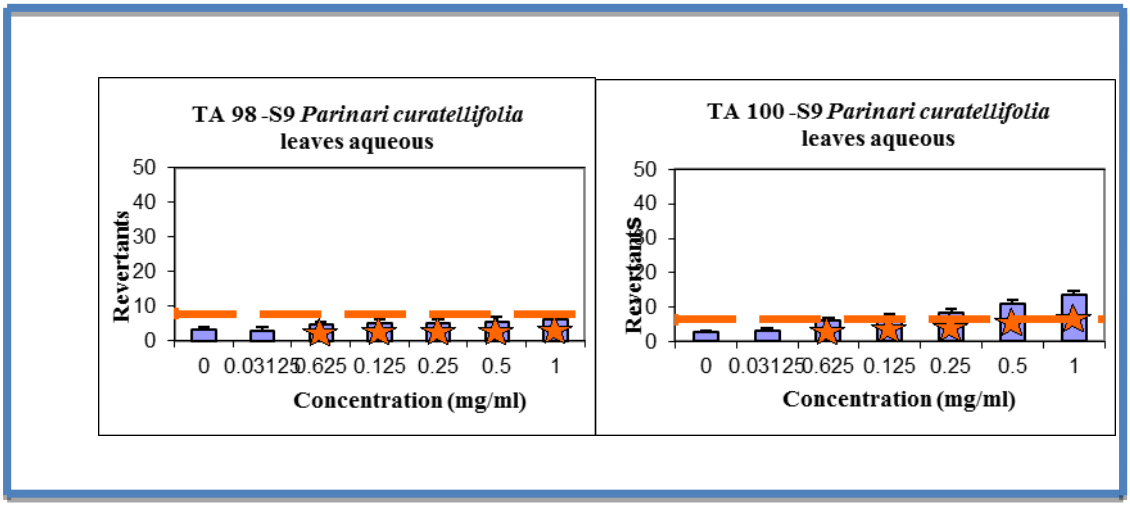


Figure 4.9: Number of revertant *Salmonella typhimurium* TA 98 and TA 100 by the concentration of *P. curatellifolia* leaf aqueous extracts.

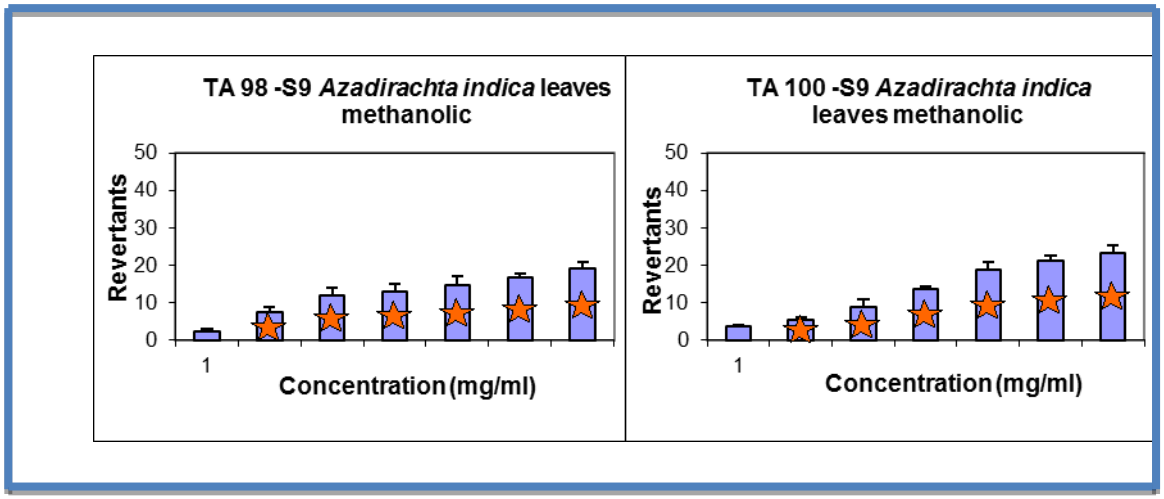


Figure 4.10: Number of revertant *Salmonella typhimurium* TA 98 and TA 100 by the concentration of methanolic *A. indica* leaf extract.

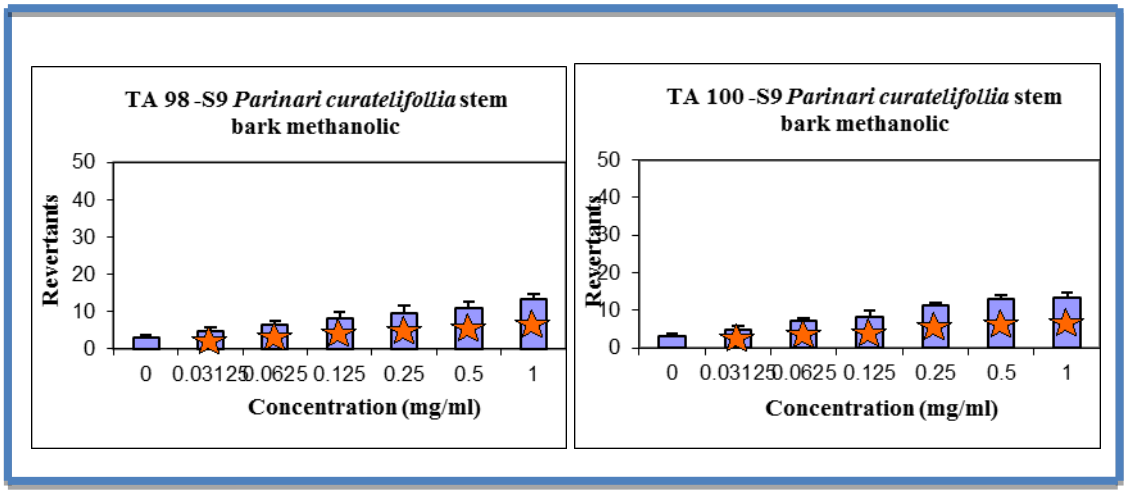


Figure 4.11: Graph showing the number of revertant *Salmonella typhimurium* TA 98 and TA100 by the concentration of methanolic *P. curatellifolia* stem bark extract.

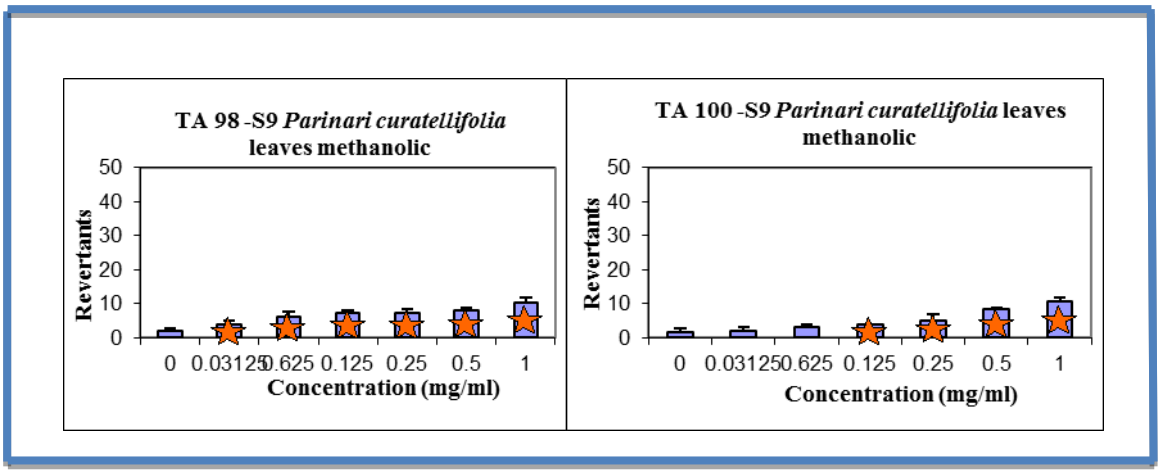


Figure 4.12: Graph showing the number of revertant *Salmonella typhimurium* TA 98 and TA100 by the concentration of methanolic *P. curatellifolia* leaf extract.

Table 4.12 is a recapitulation of the observed mutagenic activity in *Salmonella typhimurium* TA 98 and TA 100 strains after their exposure to six different concentrations of the medicinal plant extracts and the positive control NQO/NF (Nitroquinoline-N-Oxide and 2-nitrofluorene). *($p<0.05$): The statistically significant results at 95 % confidence interval using the unpaired t-test. NS: Non-significant.

Table 4.12: Summary of the number of positive wells after treatment of the *Salmonella typhimurium* TA98 and 100 strains with the selected medicinal plant extracts. NS: non-significant. * Statistically significant ($p<0.005$).

Sample	Concentration	Number of positive wells			
		TA 98		TA 100	
		Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Parinari curatellifolia stem bark	0 mg/ml (Distilled water)	2.33 ± 0.52	3.00 ± 0.89	3.00 ± 0.98	3.00 ± 0.89
	0.03125 mg/ml	3.33 ± 0.58*	5.00 ± 1.00*	4.33 ± 0.58*	5.00 ± 1.00*
	0.0625 mg/ml	4.67 ± 1.53*	7.33 ± 0.58*	5.67 ± 1.15*	7.33 ± 0.58*
	0.125 mg/ml	8.33 ± 0.58*	5.33 ± 1.53*	11.00 ± 1.00*	8.33 ± 1.53*
	0.25 mg/ml	11.00 ± 1.00*	11.33 ± 0.58*	16.67 ± 2.52*	11.33 ± 0.58*
	0.5 mg/ml	15.33 ± 1.53*	13.00 ± 1.00*	24.00 ± 2.00*	13.00± 1.00*
	1 mg/ml	13.67 ± 5.13*	13.33 ± 1.53*	27.33 ± 5.51*	13.33 ± 1.53*
	NQO/NF	37.33 ± 1.53	39.00 ±2.65	37.33 ± 1.53	39.00 ± 2.65
Parinari curatellifolia leaves	0 mg/ml (Distilled water)	1.00 ± 0.89	1.67 ± 1.03	3.00 ± 1.10	1.67± 1.03
	0.03125 mg/ml	2.67± 1.15 (NS)	2.00 ± 1.00 (NS)	14 ± 5.29*	2.00 ± 1.00 (NS)
	0.0625 mg/ml	4.67 ± 0.58*	3.00 ± 1.00 (NS)	16.00 ± 4.00*	3.00 ± 1.00 (NS)
	0.125 mg/ml	3.33 ± 1.53*	4.00 ± 0.00*	21.33 ± 1.53*	4.00 ± 0.00*
	0.25 mg/ml	4.43 ± 1.53*	5.00 ± 2.00*	22.33 ± 1.15*	5.00 ± 2.00*
	0.5 mg/ml	5.33 ± 0.53*	8.33 ± 0.58*	22.00 ± 1.73*	8.33 ± 0.58*
	1 mg/ml	5.00 ± 1.00*	10.67 ± 1.15*	24.33 ± 1.53*	10.67 ± 1.15*
	NQO/NF	38.67 ± 2.52	40.33 ± 0.58	39.67 ± 2.52	40.33 ± 0.58
Azadirachta indica leaves	0 mg/ml (Distilled water)	2.33 ± 1.03	3.67± 0.52*	4.00 ± 0.89	3.67 ± 0.52
	0.03125 mg/ml	7.00 ± 2.00*	5.33 ± 0.58*	6.33 ± 1.53*	5.33 ± 0.58*
	0.0625 mg/ml	12.33 ± 2.52*	8.67 ± 2.08*	14.33 ±2.52*	8.67 ± 2.08*
	0.125 mg/ml	14.33 ± 2.52*	13.67 ± 0.58*	19.00 ± 1.73*	13.67 ± 0.58*
	0.25 mg/ml	17.33 ± 2.08*	18.67 ± 2.08*	20.33 ± 1.53*	18.67 ± 2.08*
	0.5 mg/ml	22.67 ± 1.53*	21.33 ± 1.15*	22.00 ± 1.00*	21.33 ± 1.15*
	1 mg/ml	28.00 ± 1.00*	23.33 ± 2.08*	27.33 ± 0.58*	23.33 ± 2.08*
	NQO/NF	38.33 ± 1.53	38.67 ± 1.53	39.67 ± 1.15	38.67 ± 1.53*

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Phytochemical screening and determination of total phenols, flavonoids and alkaloids

The screened phytochemicals: tannins, saponins, flavonoids, alkaloids, glycosides, phytosterols and terpenoids in this study were showed to be present in all the plant extracts (Tables 4.1-4.3). This confirmed the work of Biu *et al*., (2009) regarding the phytochemical evaluation of *Azadirachta indica* which showed that the aqueous leaves of *Azadirachta indica* contained saponins, terpenoids, alkaloids, tannins, flavonoids, glycosides. In the same study anthraquinones and coumarines were found to be absent in *A. indica* aqueous leaf extract. Peni *et al*., (2010) in a study involving the investigation of phytochemicals found that both water and methanolic extracts of *Parinari curatellifolia* stem bark contain saponins, glycosides, flavonoids, tannins, phenols and alkaloids. Chukwudi *et al*., (2004) found that the crude extracts of *P. curatellifolia* leaves and stem bark contain saponins, sterols, phenols, flavonoids, terpenoids and carbohydrates. A large number of medicinal plants have shown to contain toxic compounds. Some of them are assessed as genotoxic and even carcinogenic. According to Vikram *et al*. (2014) a high level of antioxidants is associated with genotoxic effects. Plants containing flavonoids (flavones, isoflavones, flavonones, anthocyanins and catechins), polyphenols (ellagic acid, gallic acid and tannins) possess a remarkable antioxidant ability (Gupta and Sharma, 2006).

Investigations regarding the genotoxic, mutagenic or carcinogenic activities of plants secondary metabolites carried out on male and female rats by Suzanne *et al*. (2011) showed that plant compounds such as estragole, intergerrimine, eugenol, methyleugenol, quercetin, lasiocarpine and reserpine have either genotoxic or carcinogenic activity or both.

The quantitative analysis of the extracts showed that the aqueous and methanol extracts of *Azadirachta indica* had the highest quantity of total phenols and flavonoids. For all the plant samples used in this study, the total phenols were higher in methanolic extracts than in aqueous extracts. The highest amount of total phenols was observed in the methanolic extracts of *A. indica* leaves.

Alkaloids were the highest in the aqueous *Parinari curatellifolia* stem bark aqueous extract (Figure 4.5). Because alkaloids are known to intercalate within the cell wall and/or DNA strands (Philipson and O'nell, 1987), the high concentration of alkaloids in aqueous *P. curatellifolia* stem bark extract may be a cause to the increased mutagenic activity in both *S. typhimurium* TA 98 and TA 100 strains at 0.25 µg / µl, 0.5µg / µl and 1 µg / µl (Figure 4.8). However, in the present study the high amount of alkaloids was not always linearly correlated to the mutagenic activity.

It has been shown that specific families of alkaloids can cause DNA mutations. Fu *et al*., (2004) have proposed the mechanism of mutagenicity induced by pyrrolizidine alkaloids in *cII* and *p53* genes in rats as well as K*ras* gene in mice. In a study carried out by Vargas *et al*. (1990), the positive mutagenic activity observed with *S. typhimurium* TA 102 strain exposed to the aqueous extracts of *Achyrocline satureoides*, *Luehea divaricata*, and *Baccharis anomala* was related to tannins and flavones with 5,7-hydrolyl substitution patterns (Macgregor and Wilson, 1988; Vargas *et al*., 1989).

5.1.2 Assessment of genotoxic potential of *P. curatellifolia* and *A. indica* extracts

The *Allium cepa* assay used to detect the effects of the medicinal plant extracts on the chromosomes and the root growth of *Allium cepa* cells was introduced by Levan (1938), described by Fiskesjo (1985) and modified by Rank and Nielsen (1993). The different concentrations of extracts from *P. curatellifolia* stem bark and leaves as well as *A. indica* leaves used in this study reduced the mitotic index in the meristematic cells of *Allium cepa* seed roots. The EC_{50} was determined for each of the concentrations per extract and revealed the concentrations at which the root growth of the negative control was reduced

by 50 %. The inhibition of the root growth is an indicator that the extracts contain compounds which interfere with cell division. All the values of root lengths for *P. curatellifolia* stem bark and leaves as well as *A. indica* leaves were statistically significant at 95 % confidence interval (p-values < 0.05) compared to the negative controls treated with distilled water (Tables 4.4 and 4.5). The EC_{50} was lower for the aqueous extracts, showing that they have a higher inhibitory effect on *Allium cepa* cells than the methanol extracts (Tables 4.4 and 4.5). The M.I (mitotic index) is an indication of the frequency of mitotic division and has shown to be reduced while the concentrations increased (Tables 4.6 and 4.11). The dividing cells scored were in prophase, metaphase, anaphase and telophase. The mean value for the mitotic index of the untreated groups decreased when the treatment was done with different concentrations of extracts (Tables 4.6-4.11). At the lowest concentrations used a significant moderate reduction in the mitotic index (M. I) was observed whereas at the highest concentration (1 g/l) the M.I was low. All the extracts showed to reduce significantly the frequency of mitosis in *Allium cepa* roots meristematic cells as the dividing cells were less frequent than those which were untreated, showing that the mitodepressive effects are due to the treatment with the methanol and aqueous extracts of *P. curatellifolia* leaves, stem bark and also *Azadirachta indica* leaves.

The chromosomal abnormalities observed included chromosomal bridges, c-mitosis, multipolar anaphase, sticky chromosomes and chromosome fragments. The chromosomal bridges, multipolar and sticky chromosome were observed in anaphase cells. The negative control groups exhibited no aberrant cell division while aberrations were observed for the treated cells. The most common chromosomal aberration observed in treated cells appeared as c-mitosis (Plate 4.2 d). The c-mitosis occurs when the mitotic spindle fails to form or is destroyed indicating the presence of spindle poisons similar to colchicine (Renjana and Thoppil 2013) in the aqueous and methanol extracts of *P. curatellifolia* stem bark, *P. curatellifolia* leaves and *A. indica*, which leads to the scattering of the chromosomes in a random fashion inside the cell. The chromosome bridges (Plate 4.2 f) and sticky chromosomes (Plate 4.2 e) reveal a risk of lethality to the cells. According to Evandri *et al*., (2000) chromosomal bridges (Plate 4.2 f) and fragments (Plate 4.2 b) are

the result of clastogenic effects of the extracts, characterized by chromosome and chromatids breaks. Most of these aberrations are lethal and can cause either somatic or inherited genetic effects (Swierenga *et al*., 1991).

The reduction of the mitotic index in the treated root tips may be an indication of the inhibition of DNA synthesis (Sudhakar *et al*., 2001) or the arrest of one or more mitotic stages (Kabariti *et al.,* 1980), or the cell cycle might be blocked in the G2 stage, preventing the cells to enter mitosis (Sharma & Sharma, 1990). The extracts have the ability to slow down cell progression through the hindering of DNA replication.

The inhibition of the mitotic activity caused by the extracts reveals that they contain cytotoxic compounds. In a previous study conducted by Soliman (2001), the aqueous leaf extract of *Azadirachta indica* was reported genotoxic on *Allium cepa* cells. In the same study, Soliman proved that the aqueous extract of *Azadirachta indica* leaves reduced the mitotic index and also induced chromosomal aberrations in *Allium cepa* meristematic cells. The chromosomal aberrations observed in the carried out study were c-mitosis, sticky chromosomes, chromosome bridges, micronucleus and multinucleated cells, non-congression metaphase, laggards, polyploidy and disturbed ana-telophase.

Renjana and Thoppil (2013) found that the roots of *Strobilanthes hyneanus* Nees were genotoxic to *Allium cepa* cells. The treatment of *Allium cepa* cells with different concentrations (0.01 %, 0.05 %, 0.1 % and 0.5 %) of the methanolic extracts of *Strobilanthes hyneanus* Nees roots induced a decrease in the mitotic indices in a dose-dependant manner and also an increase in the frequency of chromosomal aberrations. The chromosomal aberrations observed in that same study were nuclear and chromosome lesions, anaphase, bridges, c-mitosis, pulverization, stathmo-anaphases, diagonal orientation and chromosome fragments. In addition, Khan and Awasthy (2003) reported the induction of structural and numerical changes in spermatocytes as well as synaptic disturbances at their first metaphase as results of their treatment with *A. indica* leaf extracts. Rosenkranz and Klopman (1995) proposed azadirachtin as the major active principles in *A. indica*. At least 5 copies of biophores were identified in azadirachtin

which was predicted to be a potent carcinogen. In addition, azadirachtin was also found contain a furan moiety that was thought to undergo epoxidation during biotransformation. Rosenkranz and Klopman (1995) concluded that the electronegativity of Azadirachtin was of the same order of magnitude as that for DNA-reactive molecules. All these, according to the authors, point to the fact that azadirachtin has the features of a potential mutagen that has the capability of inducing damage in genetic material including some clastogenic changes (Akaneme and Amaefule, 2012). However the correlation between the property of the selected medicinal plants to increase the frequency chromosomal aberrations and their ability to induce genetic material loss needs further investigation. The genotoxicity of the plants extracts do not necessarily mean that they are also mutagenic/carcinogenic and vice versa. Some studies have shown that some secondary metabolites may cause genotoxicity with no evidence for carcinogenic activity, and also may exhibit a carcinogenic with no evidence of genotoxicity (Suzanne *et al*., 2011).

5.1.3 Assessment of mutagenic potential of *P. curatellifolia* and *A. indica* extracts

The Ames test assay is a test using prokaryotic systems such as various strains of *E. coli* or *Salmonella typhimurium* (Ames *et al*., 1971). In this study, the Ames mutagenicity assay gave the indication that *P. curatellifolia* (Planch. ex Benth.) Kuntze leaves (methanolic extract), *P. curatellifolia* stem bark (water extracts) as well as *A. indica* A. Juss leaves (aqueous and methanolic), caused mutations in the DNA of *Salmonella typhimurium* TA 98 and TA 100 strains. The genotypic profile of the TA98 strain show that the mutation in the TA98 strain is due to the damage in the DNA is a frameshift-type mutation targeting the GCGCGCGC sequence in the *hisD3052, rfa* cell wall gene and *uvrB* gene of *S. typhimurium*. In the TA 100 strain, the mutation type is a base-pair substitution in the targeted GGG loci present in the *hisG46,* a deletion in *rfa* gene (cell wall gene). For both TA98 and TA100 strains, the *uvrB* DNA repair system is affected, resulting in the elimination of accurate excision-repair mechanism. In addition, the mutation in the *rfa* gene renders the lipopolysaccharide layer that coats the cell surface to be defective, resulting in the loss of pathogenicity and in an increase in permeability to

bulky chemicals. The comet assay on human normal cell lines should confirm whether the medicinal plant extracts can cause single or double stranded breakages in human DNA. Because one single test can not suffice to conclude about the potential genotoxicity of a medicinal plant, a battery of tests should be used. The *Allium cepa* assay and the Ames test have shown their complementarity in the determination of the ability of *Parinari curatellifolia* an *A. indica* to cause chromosomal damage in exposed cells and mutations in the DNA molecules. For the medicinal plant extracts to be carcinogenic, they should induce repeated mutations in the exposed eukaryotic cells.

Therefore this study confirms the crucial importance to assess the genotoxic and mutagenic potential of medicinal products before their use to treat various ailments. Other studies should allow the determination of the genotoxic or mutagenic human doses using mammal or human cells models. In addition, in vitro assays such as the mouse lymphoma TK assay should show the relationship between the observed chromosomal damages and changes in base pair with the carcinogenesis. There was a significant mutagenic activity of *A. indica* leaves and *P. curatellifolia* leaf and stem bark methanolic and aqueous extracts.

5.2 Conclusions

1) Phytochemical screening of the leaf extracts of *Parinari curatellifolia* (Planch ex Bench.) Kuntze, *Azadirachta indica* A. Juss and also of the stem bark extracts of *Parinari curatellifolia* have shown the presence of the tested phytoconstituents.

2) All the extracts (leaves and stem barks of *Parinari curatellifolia* and *Azadirachta indica* leaves) indicated a genotoxic activity.

3) The *Allium cepa* and Ames mutagenicity assays used in this study have shown that various concentrations of aqueous and methanolic leaf and stem bark extracts of *P. curatellifolia* as well as *Azadirachta indica* leaf extracts were genotoxic and also can cause mutation in DNA. However there was no mutagenic activity with *P. curatellifolia* aqueous leaf and methanolic extracts.

4) The current study showed genotoxic and mutagenic potential of the aqueous and methanolic extracts of *Parinari curatellifolia* leaves and stem barks as well as *Azadirachta indica* leaves.

5.3 Recommendations

- The extracts of *P. curatellifolia* stem bark *and Azadiracha indica* are potentially genotoxic and mutagenic, hence can induce the same reported effects on human cells.
- Only *P. curatellifolia* aqueous leaf and methanolic extracts are not mutagenic and can be considered safe at the given concentrations used in this study.
- DNA sequences of the TA 98 and TA 100 in *Salmonella typhimurium* strains should be compared to those of the mutated TA 98 and TA 100 strains. This would allow the confirmation of the mutagenic property of the plant extracts.
- The Ames test should be done with metabolic activator to confirm the presence of eventual pro-mutagenic compounds.
- The *Allium cepa* and Ames test should be supplemented with another test such as the comet assay to confirm the evidence of the genotoxicity of the medicinal plants extracts in human cells.

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APPENDIX (Plant importation permit)

No. 0011722

PERMIT No. KEPHIS/I[illegible]16

REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE & RURAL DEVELOPMENT

KENYA PLANT HEALTH INSPECTORATE SERVICE (KEPHIS)

PLANT IMPORTATION PERMIT

(Plant Protection Act Cap 324)

Date 5 April, 2016

The importer must furnish the supplier with a copy of this import permit before plant material is despatched.

*Permission is hereby granted to KATABALE MASSAMA KOSSI

of JKUAT, P.O.BOX 62000-00200, NAIROBI,KENYA.

to import from KATABALE MASSAMA KOSSI, LOME.DZIDZOLE, HOUSE 445., PHONE NO. 0022890546127, TOGO.

the following Dried plant samples

1 Kgs Plant samples

subject to the following conditions

1) All Dried plant samples to be the produce of and grown in TOGO

2) The consignment to be inspected on arrival and the importing authority reserves the right to treat, destroy or refuse the importation.

3) Plants or plantparts must be entirely free from soil, chaff and/or leaf mould.

4) Each consignment shall be accompanied by an original copy of this import permit and Phytosanitary Certificate (International Model or its equivalent) from country of origin;

Additional Declarations:

i) Nature of research to be declared on request by a KEPHIS inspector.
ii) Packed in sealed containers.
iii) To be used only for scientific research at the approved Institution/Facility i.e JKUAT.
NB:Details to be stated on the phytosanitary Certificate.

Failure to furnish the required certificates may result in prohibition of entry of the plant materials.

5) Packaging The following materials must **not** be used: banana leaves, maize, rice, sorghum, palm, wheat straw soil or leaf mould. If any other plant residue is used as packaging material, the consignment must be accompanied by a certificate stating: all seeds, pathogens and insects have been killed before use of the material either by heating to 180°F / 83°C for ten minutes or by chemical treatment (N.B:- Details to be stated on Phytosanitary Certificate).

This permit is valid for six months from date of issue, but may be cancelled at any time by the Director of Agriculture or by the officer issuing the permit on his behalf

Official Stamp

MINISTRY OF AGRICULTURE PLANT HEALTH INSPECTORATE SERVICE KENYA PLANT INSPECTOR KEPHIS

Janet Odongo

(Signed) 05 APR 2016

for Director of Agriculture

"Import of genetically modified material will require clearance from the National Biosafety Authority in compliance with the Biosafety Act"

The permission hereby granted is additional to any permission or licence required under any other law.
Full name and address of supplier to be stated