EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND MUTAGENIC PROPERTIES OF *Coccinia rehmanni* AND *Jatropha zeyheri* PLANT EXTRACTS

by

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A thesis submitted in fulfilment of the requirements for the award of Master of Science degree (MSc) in Microbiology

to the

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF MATHEMATICAL & NATURAL SCIENCES

UNIVERSITY OF VENDA

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March 2019

DECLARATION

I, NDOU NZUMBULULO (student number: 11627795) hereby declare that this thesis submitted for the award of a Master's degree in Microbiology at the University of Venda is my original work and has not been previously submitted to this or any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a compiled list of references.

Signature…… Date……..

DEDICATIONS

This work is dedicated to my farther Nthudzeni Robert Ndou (*rest in peace*), my mother Ndou Tshinyadzo Patricia and Ndou Tsakani Martha, my siblings Ndou Freedom Ndou, Ndou Thendo Delight (*rest in peace*), Ndou Rocca, Ndou Roccaline, Ndou Ayanda, Ndou Madembe, Ndou Oritonda.

I would like to dedicate this work to my first born Ndou Ankonisaho Vision.

I would also like to dedicate this work to Tshavhungwa Muvhulawa and RabeBelani mukhwevho.

I would also like to dedicate this work to my family for their support, guidance and patience, Vho-Emelina, vho- Tshabanga Jeremiah, Mabuke Alice, Mphumudzeni Eric, Norman, Donald, and Rofhiwa.

I would also like to dedicate this work to my great grandmother Muofhe Phalanndwa (*rest in peace*), for playing a major role in my upbringing.

ACKNOWLEDGEMENTS

All thanks go to the Almighty God for granting me wisdom, knowledge, kindness, and strength.

I would like to extend my sincere gratitude to my supervisor Prof A.N Traore for guidance, belief, and support that she showed me… words can never be enough!!!

I would like to extend my sincere gratitude to my co-supervisors Prof. N. Potgieter and Dr. M. Sigidi for giving me this oppotunity and provide a good working environment… I couldn't ask for more!!!

I would like to thank, Mr. M. Magwalivha, Dr. Kabue, Dr. E. Musie, Prof. A. Samie, Dr. T. Masebe, Prof P.O Bessong for their support and encouragements that they have given me.

I Would like to thank Prof Tshisikhawe, Mr Magwede and Mr. B. Mabate for support and and helping with plants identifications.

I wish to extend my appreciations again to Marlaine Boukandou, Dr.Anokwuru. Chinedu, Dorcas Tlhapi, Mashilo M.S, Amanda Maluleke, Netshilindi E, and Makungo M, for the mentorship and laboratory skills attained.

I would also like to thank my lab mates: Rikhotso M. Karambwe K, Banda T, Mulondo G, Badugela N, Khumela R, Ajmal A.A, Munzhedzi L, Mnisi N.S, Tshikororo K, Nemutanzhela I, Khwathisi A, Marole T, Tshifularo R, Netshilindi E. Makungo M. you bring the best off me and this accomplishment would never be this way if not for your intervention I thank you for that.

This study was supported by the National Research Fund (NRF).

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LIST OF ABBREVIATIONS

Abbreviations	Definition
%	percentage
°C	Degree Celsius
ADP	Adenosine Diphosphate
ANOVA	One-Way Analysis of Variance
BEA	Benzene/Ethanol/Ammonia hydroxide
C. rehmanni	*Coccinia rehmanni*
CEF	Chloroform: ethanol: Formic Acid
Cm	Centimetre
CO_2	Carbon Dioxide
CPT	Camptothecin
DMD	Duchenne muscular dystrophy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMW	Ethyl Acetate/Methanol/water
$FeCl_3$	iron chloride
FISH	fluorescence in situ hybridisation
G	Gram
H_2SO_4	Sulfuric Acid.
HCl	Hydrochloric acids
HIV	Human Immunodeficiency Virus
I.e. /eg	For Example
IC50	Concentration of an Inhibitor
IR	induction ratio
J. zeyheri	*jatropha zeyheri*
Kda	kilo Daltons
LTR	LysoTracker Red
Mg/ml	microgram per milliliter
Ml	millilitre

ml/well	Microliters per well
mM	millimetre
MS	Mass spectrometry
NAD	Nicotinamide Adenine Dinucleotide
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
PARP	Poly-ADP Ribose Polymerase
RSA	Republic of South Africa.
SE	mean ± Standard error
SPSS	Statistical Package for the Social Sciences
St Dev	Standard Deviation
TCM	Traditional Chinese medicine
TLC	Thin Layer Chromatography
Trad-MCN	Tradescantia micronucleus
USA	United States of America
UV-light	ultraviolet lights
µl	microliter

ABSTRACT

Background: The medicinal value of plants lies in some chemical substances that produce a definite physiological action in the human body. The secondary metabolites help the plants to survive hash conditions and could be used by humans as supplements of their health, as foods additives or for medicinal purposes. This bioactive compounds are not always beneficial to human beings, and some of this plants bioactive compounds can be toxic or genotoxic to human cells. This study used several methods to evaluate of phytochemical constituents and mutagenic properties of *Coccinia rehmanni* and *Jatropha zeyheri* plant extracts.

Methodology: Methanol was used for extraction of the bioactive compounds from the two selected plants, filtered with Whatman filter paper and evaporated with rotary evaporator. The extracts were fractionated using open column chromatography. Chemical and TLC methods were used to determine phytochemicals of the study plants extracts and fractions. The plants extracts and fractions were tested against Vero cell lines in order to evaluate cytotoxicity and genotoxicity of the plants. NucRed and LTR Hoechst 33342 dyes were used for cytotoxicity and genotoxicity respectively. For the evaluation of cytotoxicity and genotoxicity Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope and acquired images analyses using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Antimutagenicity of plants extracts was observed using PARP universal colorimetric assay kit. Acquired data was transferred to an EXCEL spreadsheet and data was analyzed.

Results and discussion: *C. rehmanni* (12.03%) yielded more extract than *J. Zeyheri* (8.20%). the two plants had different compound composition and were in different stages of maturity. The study revealed the domination of Terpenoids, Cardiac glycosides, Phenolic and tannis. With an exception of two fraction fractions all the fractions was found to be toxic to an extent were genotoxicity of such fraction could not be concluded. The reason for such extreme toxicity could be due to the influence of the retained alcohol during rotary evaporation.

Conclusion: this study provides and add to existing knowledge on the phytochemicals mutagenicity and anti-mutagenicity of *C. rehmanni* and *J. Zeyheri* medicinal plants. The study serves as scientific proof that extensive use of this plant in traditional medicine for treatment of various ailments may lead to some irreversible damages.

Key words: Antimutagenicity, bioactive, cytotoxicity, Vero cell lines, medicinal plants, mutagenic, phytochemical, secondary metabolites.

Chapter 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The value of medicinal plants lies in the chemical substances that produce a physiological action in the human body (Mir et al., 2013). The phytochemical constituents of plants have two categories namely primary and secondary metabolites. Chlorophyll, proteins sugar and amino acids are considered primary, while terpenoids, tannins, flavonoids, phenolic compounds and alkaloids are secondary metabolites (Wadood et al., 2013). Secondary metabolites are produced as a means of surviving harsh conditions such as drought are the most important of these metabolites (Saxena et al., 2013).

Despite well understood advantages possessed by some of the plants, some constituents of medicinal plants have been shown to be potentially toxic, carcinogenic and teratogenic (Akinboro and Bare, 2007). Studies have reported some of the Chinese herbal medicines (CHMs) have been known to possess toxic compounds (Zhou et al., 2013). These compounds, generally react with cellular macromolecules, including DNA, causing cellular toxicity and/or genotoxicity (Rietjens et al., 2005). Studies of genotoxicity and anti-genotoxicity can help evaluate the safety and effectiveness of herbal health products (Romero-Jiménez et al., 2005).

These constituents play also an important role in healing various human diseases (Mahesh and Satish, 2008). Medicinal plants are responsible of several biological activities including antifungal, antibacterial and anti-inflammation (Hussin et al., 2009. Boonchum et al., 2011; Boukhatem et al., 2014). The plant metabolites can be associated with the different mutagenicity potentials of the plants' extracts as demonstrated by Déciga-Campos et al., 2007 who demonstrated some of the Mexican plants that are used as traditional medicine.

Another good example is the methanolic extract of *Helichrysum* species that possess mutagenic properties whilst extracts of *Bahinia galpinii* are reported to have anti-mutagenic properties as reported by Verschaeve and Van Staden, (2008). A study that was done by Reid et al, (2006) in South Africa demonstrated mutagenicity of

Bauhinia galpinii, *Clerodendrum myricoides*, *Datura stramonium*, *Buddleja saligna*, *Millettia sutherlandii* and *Sutherlandia frutescens* methanolic extracts.

Animal model for evaluating the potential of extracts' carcinogenicity are quite expensive and time consuming, and it is extremely difficult to conduct such tests on all newly developed chemical products (Baske tter et al., 2012). For this reason, when handling a new chemical product whose carcinogenicity or potential to cause genetic damage is unknown, mutagenicity is one of the toxicities that must be evaluated prior to use. The mutagenicity tests is conducted to predict the possible risk that may be imposed to humans and genetic diseases in the future generations. For new chemical substances, conducting such evaluations before destribution is impotent (Mahmoudi et al., 2012), however, most people from developing countries still rely heavily on traditional concoction's that might pose more harm than good to them.

This study focused on two medicinal plants commonly used around the Vhembe District. One of them being *Jatropha zeyheri* Sond, which is a climbing herb of the *Euphorbiaceae* family with a deep-rooted rhizome and slightly-branched leafy stems mainly found in grasslands and in sandy soils (Van Rensburg et al., 2007). Decoction of the plant is made from the tuber and used for treatment of inconsistent periods, period pain and to ensure a healthy and strong foetus before birth (Mongalo, 2013). It may also be used topically in wound treatment (Luseba et al., 2007).

The second plant regarded in this study is *Coccinia rehmanni* Cogn. This is a non-woody, climbing or scrambling herb, producing stems up to 5 meters long from a large rootstock. The plant is sometimes harvested from the wild for its edible roots and fruit, which are eaten locally. Local traditional healers also prepare concoctions from the bulbs/roots for treatment of different ailments (Sigidi et al., 2017). A decoction of *Coccinia rehmanni* is known to be used in the treatment of gonorrhea, skin diseases, inconsistent periods, period pain and to ensure a healthy and strong fetus before birth (*obtained from interview*). *C. rehmanni* has been used from time to time against, gonorrhea and skin diseases. Studies have shown its activity on diabetes, gastrointestinal disturbances and urinary tract infections (Vadivu and Lakshmi, 2008; and Kumar et al., 2009).

Cytotoxicity testing is a popular method used for the screening of pharmaceutical products and synthetic organic compounds. An important initial step when

investigating possible new therapies or developing new compounds for the treatment of an ailment is the determination of their cytotoxic potential and the determination of their harmful effects. Toxicity screening in the early phase of drug development require assays with high throughput and low amounts of the test compound (Westerink et al., 2011). Typically, fluorescence imaging is employed in a high throughput format and has directly impacted biological research into cellular and systems biology, as well as drug discovery. This approach typically minimizes testing costs by reducing the amounts of test compound and reagent used (Hulkower and Herber, 2011).

Genotoxicity is defined as a destructive effect on a cells genetic material affecting its integrity. Damage of DNA may occur in germ cells causing a heritable altered trait or may occur in somatic cells which may result in malignant transformation and cancer. Genotoxicity is one of the main concerns in drug discovery as alterations to the DNA content can lead to cancer and heritable effects (Tilmant et al., 2013) and thus the potential of drugs to induce genotoxicity is important. Multinucleation, micronucleation and DNA damage are hallmarks of genotoxicity.

1.2 STUDY RATIONALE

Vha-Venda people still depend on the natural environment for their health and survival since the dawn of time, because of their belief and faith in traditional healers. The great dependence could be due to of plants availability, accessibility and affordability. There is an increase in the appreciation of the alternative role traditional medicine plays. Traditional medicine has proven its effectiveness in cases of ill-health and treatment of non-communicable and communicable diseases and improves quality of life in all age groups people and in persons suffering from chronic diseases (Heymann et al., 2015)

There is a continuous need to search for new medical concoctions against a large number of ailments, including cancer. This is because the western type of medications often have unpleasant side effects, some of them have lost the efficiency in inhibiting the replication of pathogens. Epidemiological studies indicate that many cancers are dependent on multiple mutational etiology, as well as on inherited mutator phenotype. The search for mutagenesis inhibitors may therefore be useful as a tool to discover anticarcinogenic agents. There are approximately 25 000 plant species in South Africa

that form a potential pool for the screening of new anti-tumor compounds (Cragg et al., 2014). Such screening may include the screening for inhibitors of mutagenesis.

Coccinia rehmanni and *Jatropha zeyheri* are two medicinal plants extensively used in the Vhembe communities (information obtained from interviews). Despite their popularity, scanty information are documented on the biological activities of these two plants. A preliminary study by Sigidi et al, (2016) showed a wide variety of positive effects on health improvements by the extracts of *C. rehmanni* and *J. zeyheri*. The study demonstrated that *C. rehmanni* significantly inhibited HIV-1 retroviral enzyme, and again the 2 plant extracts inhibited p24 HIV protein, thus reducing the packaging of new viral particles. It was also proven that these plants have pro anti-inflammatory effects.

Previous documented studies have shown that a lot of plants, used as food ingredients or in traditional medicine have *in vitro* mutagenic effects (Monteiro et al., 2006; Déciga-Campos et al., 2007; Razak et al., 2007) and are both toxic and carcinogenic. Although the chosen plants in the current study has proven to be useful in disease treatment and health improvement, there is no documented information on the mutagenicity of both plant species. Verschaeve and Van Staden, (2008), suggested that the majority of medicinal plant extracts used in different communities may exert mutagenic and/or antimutagenic effects.

There is a need to isolate pure active compounds for the chosen medicinal plants and investigate their phytochemical constituents, mutagenicity and any biological effects these extracts, or compounds might have. Thus, it is important to screen medicinal plants for their mutagenic potency. Plants exhibiting clear mutagenic properties should be considered as potentially unsafe and certainly require further testing before they can be recommended for use as alternate therapy.

1.3 OBJECTIVES

1.3.1 MAIN OBJECTIVE

To investigate the phytochemical constituents, mutagenic and antimutagenic properties of *Jatropha zeyheri* and *Coccinia rehmannii* plant extracts.

1.3.2 SPECIFIC OBJECTIVES

- ✓ To determine the phytochemical constituents of the two selected plant extracts
- ✓ To extract the bioactive compounds (fractions) from the plants extracts.
- ✓ To evaluate toxicity of *C. rehmanni* and *J. zeyheri* plants extracts and their fractions of the two selected plants.
- ✓ To investigate the mutagenicity/antimutagenicity of plants crude extracts using a colorimetric test and an advanced fluorescence microscopic method.

Chapter 2

LITERATURE REVIEW

2.1 BACKGROUND

Plants have been used as a source of food and herbal drugs by rural populations in South Africa and other African countries (van vuuren, 2008). Rural areas people uses plants for many purposes including, wood, non-timber forest products, and edibles. Traditional healers and indigenous people in Africa and other countries worldwide are aware of the therapeutic properties of traditional plants in their surroundings. In India, traditional medicine have proven its usefulness 2 000 years (Wadood et al., 2013). Medicinal plants can be consumed as crude extracts as it is from the plants or indirectly like modern drugs. It has been stated that traditional healers in India use about 2 500 plant species for treatment of various diseases such as wounds, diabetes, fungal infection and 100 species of which are considered as natural medicines (Kakkar and Bais, 2014).

The use of traditional medicine by Chinese people dates back to ancient times. Although animal and mineral substances had been in use, the primary source of therapy remains botanical. Five hundred of the 12 000 prepared concoctions made by traditional healers are for routine use as reported in literature (Mothibe et al., 2017). In clinical practice, traditional diagnosis must be followed by prescription of a concoction or individual therapies from plant origins.

Many herbal remedies found their way to the Japanese system of traditional healing from China. Herbs indigenous to Japan were categorized in the first pharmacopoeia of Japanese traditional medicine centuries ago (Saito, 2000). The Japanese traditional medicine has been used in Japanese society for several decades and is subdivided into folk medicine and Chinese medicine (referred to as Kampo medicine).

The indigenous knowledge patterning to the preparation and use of medicinal plants for medicinal purposes have been passed on from generation to generation mostly by plays, person to person poetry and some even went to an extent of dreaming about them, predominantly in African countries (Bidwell et al., 2011). However, the information is at risk of being forgotten as the transmission of information between the generations is not always guaranteed and in accuracy.

It is very important to document information about each medicinal plant from any country globally for their conservation and sustainable use (Borokini and Lawal, 2014). Information of the chemical constituents of medicinal plants is required for screening of new therapeutic agents or drugs. The indigenous knowledge of chemical constituents of medicinal plants helps to disclose the importance of traditional remedies (Viji et al., 2013).

2.2 USE OF MEDICINAL PLANTS IN SOUTH AFRICA

South Africa is rich of indigenous knowledge that has been passed on to generations. South Africa is a significant focal point of botanical and cultural diversity of medicinal plants. The use of medicinal plants by the locals is very significant in their culture. South Africa has a rich diversity of more than 24 000 indigenous plant species, which represents approximately 10% of all higher plants on Earth (Ali-Shtayeh et al., 2008). The people of South Africa have a long tradition of medicinal plant use with an estimated 70% of the population harvesting one if not more from the approximated 3000 species of plants used as traditional medicines (van Wyk and Gericke, 2000; Scott et al., 2004).

The country's vast variety of indigenous floral species together with their diverse medicinal uses indicates a great potential for uncovering of new bioactive chemicals (van Wyk and Gericke, 2000; Louw et al., 2002; van Wyk and Wink, 2004). There estimated more than 4 000 traditional plants that are used for medicinal purposes by different people from different cultures in South Africa (Van Wyk, 2011). Indigenous traditional healers were the first people to collected and administer medicinal plants to their patients for various diseases. Nowadays a lot of people has access to medicinal plants of their choice from the herbal shops in both rural and urban areas, sold as crude, unprocessed drugs in herbal markets in various parts of South Africa. There are more than 100 promising traditional medicine in South Africa that have been studied for their potential in commercialization as medicinal products (Singh, 2015).

2.3 OVERVIEW OF THE STUDY PLANTS

2.3.1 *JATROPHA ZEYHERI*

Jatropha zeyheri also known as Thundamali in Tshivenda is a climbing herb with a deep-rooted rhizome and slightly-branched leafy stems as shown in **Figure 2.1.** It is mainly found in grasslands and in sandy soils. The genus comprises approximately 172 species, all having healing properties (Mong walo et al., 2013). Various ailments (Bhagat and Kulkarni, 2012).

A decoction (prepared by boiling) of the tuber is known to be used in the treatment of inconsistent periods, period pain and to ensure a healthy and strong fetus before birth and It may also be used topically in wound treatment (Luseba et al., 2007). This plant is known for secondary metabolites including Steroids, Alkaloids, Phenol Tannins and Saponins (Gawai et al., 2013; Tshikalange et al., 2016; Lithakong et al., 2018). Naturally dried *J. zeyheri* leaves are used by the Bapedi tribe to make black tea (Lithakong et al., 2018).

Figure 2.1: A picture showing **(a)** digging of Jatropha zeyheri plant, **(b)** the leaves as identified before collection, **(c)** a tuber or rhizome of *J. zeyheri*.

2.3.2 COCCINIA REHMANNI

Coccinia rehmannii also known as Galange in Tshivenda is a non-woody, climbing or scrambling herb, producing stems up to 5 metres long from a large rootstock (**Figure 2.2**). The slender stems scramble over the ground or clamber into surrounding vegetation, attaching themselves by means of tendrils.

The plant is sometimes harvested from the wild for its edible roots and fruit, which are eaten locally. Local traditional healers also prepare concoctions from the bulbs/roots for treatment of different ailments (Sigidi et al., 2017).

Figure 2.2: Pictures showing a *Coccinia rehmanni* bulb **(A)** and flowers **(B)** as identified by the traditional healer.

2.4 EXTRACTION AND FRACTIONATION

2.4.1 EXTRACTION OF BIOACTIVE COMPOUNDS FROM THE PLANTS

There are well known procedures that are used to extract bioactive compounds from plant materials such as maceration, infusion, digestion, decoction, hot continuous extraction (soxhlet) and ultrasound extraction (sonication) (Tiwari et al., 2011). In the extraction process, solvents extract compounds of the same polarities from plant materials. The achievement of extracting bioactive compounds from plants is extremely based on the polarity of solvents used and that of the compounds within the plant (Azmir et al., 2013). An excellent solvents that can be employed for the extraction of bioactive compounds should characteristically be easy to evaporate and have low toxicity. There are various solvents mostly used in the extraction phytochemical compounds including methanol, acetone, ethanol, petroleum ether, diethyl ether and ethyl acetate (Galanakis et al., 2013). Plant materials are ground to a fine powder, before the extraction process may take place. This is done to increase the surface area to volume ratio which enhances rate of extraction. The ratio of solvent and plant material in extraction process is 10:1 v/w (Tiwari et al., 2011). The section below will discuss the methodology used in the present study in more details.

2.4.2 SEPARATION AND PURIFICATION OF COMPOUNDS

Extraction of medicinal plants is the process separation of active portions of medicinal plants from inactive components. It involves the use of chemical solvents of varying polarities ranging from non-polar to polar. This includes class of preparations known as decoctions, infusions, fluid extracts, tinctures, and pilular (semisolid) extracts. The liquid solution obtained from extraction procedure are mixture of liquids of various compounds. There are several extraction procedures used for extraction of compounds from plants such as serial exhaustive extraction, sonification and Soxhlet extraction. Different extraction procedure and solvents are used. Extraction is the first step of separation and purification of compounds from medicinal plants materials (Sasidharan et al., 2011).

There are many extraction techniques used to extracts and separate compounds of interest from a mixture of crude extract and these include the mostly used thin layer

chromatography, column chromatography (**Figure 2.3**) and thin layer chromatography plates that are pre-prepared.

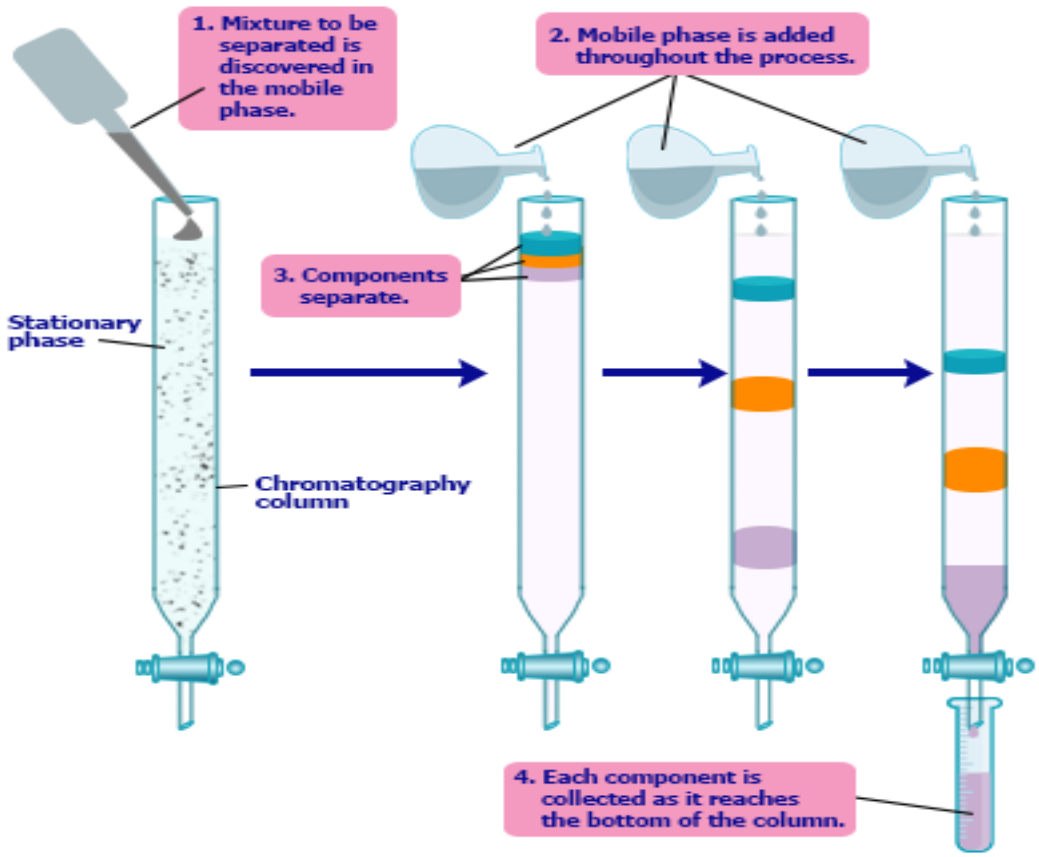


Figure 2. 3: Column chromatography procedure (Sobolev and Dorner, 2002)

The second step of separation and purification of compounds involves the use of column chromatography and preparative thin layer chromatography, to separate components from crude extracts of plants based on the polarities using two or more solvents of varying polarities into fractions for further purification. The last step involves the use of thin layer chromatography (TLC) plates to confirm the purity of separated compounds (Visht and Chaturvedi, 2012). Pure compounds are now analysed for structure elucidation and characterization using nuclear magnetic resonance (NMR) and Mass spectrometry (MS).

2.5 PHYTOCHEMICAL EVALUATION

Phytochemicals are naturally occurring in medicinal plants, leaves, vegetables and roots that have defence mechanism and provide protection against various diseases. Phytochemicals are defined as primary and secondary compounds (Wadood et al., 2013). Chlorophyll, proteins and common sugars are categorized as primary constituents, such compounds generally have no well-known functions in the process of photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation (Mazid et al., 2011), while secondary compounds contain terpenoids, alkaloids and phenolic compounds (Krishnaiah, et al., 2007). Phytochemical compounds are produced in response to abiotic and biotic stress for protection and survival of plants in harsh conditions such as low water content, high temperature, ultraviolet degradation and exposure to herbivores (Sasidharan et al., 2011).

Plants produce a large and diverse range of organic compounds that appear to have no direct functions in growth and development (Karuppusamy, 2009). Such compounds generally have no well-known functions in the process of photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation (Mazid et al., 2011). There are different types of plant derived secondary metabolites such as carotenoids, phytosterols, saponins, alkaloids, tannins, steroids, and flavonoids (Karuppusamy, 2009).

Both phenolic compounds and flavonoids are also extensively dispersed in plants and are described to have multiple biological activities including antioxidant, free radical scavenging abilities, anti-inflammatory, antibacterial and anticarcinogenic activities (Gupta et al., 2014). Secondary metabolites also play an important role in humans when used to boost the immune system, kill pathogenic microorganisms and to scavenge free radical molecules. Diets rich in medicinal plants comprise a variety of secondary metabolites that help to protect the body against various illnesses (Karuppusamy, 2009). Some of the phytochemical compounds are known to have antioxidant and anticancer properties such as triterpens, steroids and tannins (Sawadogo et al., 2012), while other like flavonoids and cardiac glycosides are known to be toxic or cause adverse effects if high dose is ingested (Skibola and Smith, 2000).

2.5.1 FLAVONOID

Flavonoid constitute one of the largest class of naturally occurring plant products mostly phenols either in the free-state or as their respective glycosides (**Figure 2.4**). The main constituent of flavonoid drugs are 2-phenyl-g-benzopyrones (Harborne, 2013). The family includes monomeric flavanols, flavanones, anthocyanidins, flavones, isoflavonoid and flavonols. It is very probable that several herbal remedies, whose constituents are yet unknown, will be shown to contain active flavonoids, quercetin and rutin which are in plant trigonella foenum seed, they are known for its anti-inflammatory properties. The flavonones hesperidin and neohesperidin were shown to protect against the toxicity of hydrogen peroxide, as well as protecting against DNA damage (Clifford and Brown, 2005).

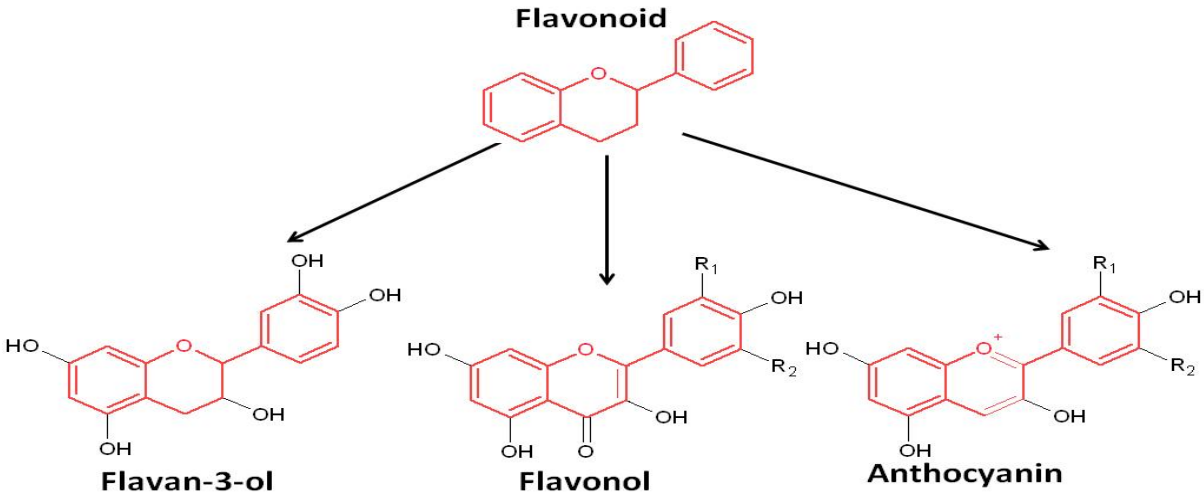


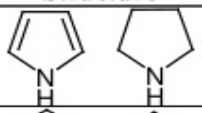
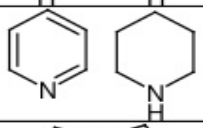
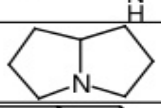
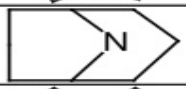
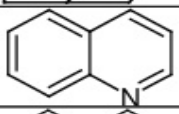
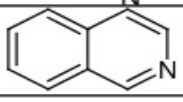
Figure 2.4: Generic structure of flavonoids (Guerrero et al., 2012)

Most flavonoids have anti-tumor properties and are powerful antioxidants (Ru et al., 2012). Onion extract contain quercetin which induce the cellular antioxidant system. Flavonoid structures are different based on their scale and form of hydroxylation, prenylation, alkalinisation and glycosylation reactions that change the general structure of flavonoids (Khoddami et al., 2013).

2.5.2 ALKALOIDS

Alkaloids are low-molecular-weight nitrogen-containing compounds (**Table 2.1**) and, due to the presence of a heterocyclic ring containing a nitrogen atom, they are typically alkaline. Alkaloids are known by their numerous pharmacological effects. These chemicals also demonstrated a broad spectrum of pharmacological properties including sedative, anxiolytic, hypnotic, anticonvulsant, antitumor, antiviral, antiparasitic as well as antimicrobial activities on vertebrates (Cao et al., 2007). These metabolites can be divided into different classes according to their precursor (e.g., indole alkaloids are alkaloids derived from tryptophan), encompassing more than 20 different classes (e.g., pyrrolidine alkaloids, tropane alkaloids, piperidine alkaloids, pyridinealkaloids, quinolizidine alkaloids, and indole alkaloids, among others) (Yang and Stöckigt, 2010). Alkaloids' presences are diverse in the plant kingdom and are much more abundant in plants with relatively complex characteristics (Lu et al., 2011).

Table 2.1: Classification of alkaloids (Roberts, 2013)

Type	Structure	Examples
Pyrrole and pyrrolidine		e.g. Hygrine, coca species
Pyiridine and piperidine		e.g. Arecoline, anabasine, lobeline, conine, trigonelline
Pyrrolizdine		e.g. Echimidine, senecionine, seneciphylline
Tropane		e.g. Atropine, hyoscine, hyoscyamine, cocaine, pseudopelletirine
Quinoline		e.g. Quinine, quinidine, cinchonine, cupreine, camptothecine
Isoquinoline		e.g.Morphine, codeine, emetine, cephaline, narcotine, narceine, d-tubocurarine

Furthermore, several alkaloids have shown some biological activities, such as the alleviation of asthma symptoms as well as pain-relieving and anticancer effects (Lu et al., 2011). Alkaloids are amongst the most remarkable active constituents in natural remedies, and some of these compounds have already been developed into commercial drugs, such as camptothecin (CPT) a topoisomerase I inhibitor (Huang et al., 2007).

2.5.3 TANNINS

They are diverse group of high molecular weight phenolic compounds joined together with capacity to form reversible and irreversible complexes of proteins, polysaccharides and nucleic acids. These compounds are normally found in traditional medicinal plants, fruits such as grapes and blueberries, tea and chocolate (Saxena et al., 2013). Tannins are secondary metabolites of plants with defensive properties. They are plant phenolic polymers with molecular mass range between 600 to 3000 kda. These compounds are commonly toxic to animals and as such, act as feeding repellents to a great diversity of animals. They cause a sharp, astringent sensation in the mouth of many mammalian herbivores dues to their ability to bind salivary proteins.

Many herbivores such as cattle, deer and apes do not eat plants with high tannins content (Kudumela, 2017). These tannins are plant secondary metabolites responsible for different biological activities and have ability to decrease risk of chronic diseases. In India, medicinal plants containing tannins have been used traditionally for treatment of diarrhea, as diuretics and anti-inflammatory remedy (Saxena et al., 2013). These compounds have been used in both pharmaceutical and food industries. In the food industry, tannins are used to clarify wine, beer and fruits juices. They are used as antioxidants in fruit juice, beer, and the wine industry. These compounds also play significant role as caustic for cationic dyes in dyestuff industry and production of inks (Mahgoub, 2017; Saxena et al., 2013).

2.5.4 STEROIDS

They are the most important secondary metabolites of plants which play an essential role in plant cell membranes and plasma membranes as regulatory channels of plant cells. These compounds allow movement of small molecules from outside to the inside of cell by decreasing motion of fatty acid chains. They also play a critical role in the protection of plants from herbivores. For example, milkweeds produce several bitter tasting glucosides (sterols) that protect them against herbivory by most insects and cattle. Triterpene, limnoid are some of the bitter steroids produced by citrus fruits to fight against herbivores (Namdeo, 2007; Mazid et al., 2011).

2.5.5 SAPONINS

They are secondary metabolites that are found in natural sources including plants. Presence of saponins in plants can be confirmed by stable foam formation in aqueous solutions. There are three groups of saponins namely, glycosylated steroids, triterpenoids and steroid alkaloids. Most of these compounds are known to have antimicrobial activity, inhibit molds and protect plants from insect's attack. They also play an important role in traditional medicine through the exhibition of immunostimulant, hypocholesterolaemic, anticarcinogenic, antifungal, antiviral and antioxidant properties (Saxena et al., 2013).

2.5.6 TERPENOIDS

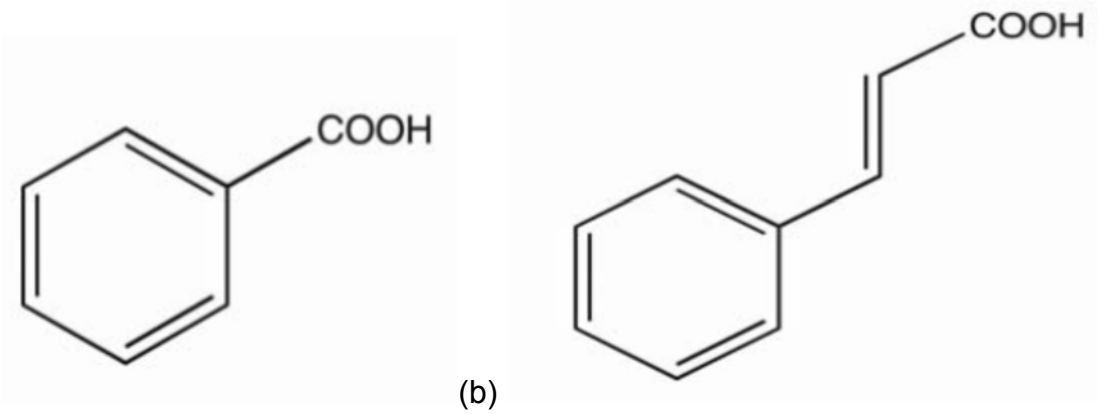
They are groups of secondary metabolites that are responsible for the medicinal activity of some plants. They are derivatives of five carbon isoprene units. Almost all terpenoids molecules have similar structures and differ only in their functional groups and carbon skeletons. These compounds have been used in both food and pharmaceutical industries. In the food industry, terpenoids have been used as flavors and fragrance. Hydrocarbon isoprenes are the building blocks of terpenes.

They are classified according to the number of isoprene units, i.e. hemiterpenoids (one isoprene unit), monoterpenoids (two isoprene units), sesquiterpenes (three isoprene units), diterpenes (four isoprene units), triterpenes (six isoprene units) and tetra-terpenoids (eight isoprene units). This group of compounds is responsible for various biological activities exhibited by plants such as anticarcinogenic, antimalarial, antiulcer and antimicrobial activities (Saxena et al., 2013).

2.5.7 PHENOLIC COMPOUNDS

They are produced by plants as protective mechanism against ecological and physiological attack such as pathogens, insects attack, ultraviolet radiation and wound. These compounds (**Figure 2.5**) have an aromatic ring structure with one or more conjugated hydroxyl groups. They also possess antioxidant properties that play an important role in the prevention of various diseases such as heart diseases, inflammation, diabetes and cancer (Dai and Mumper, 2010). Animals and humans

consume these compounds from plant products such as fruits and vegetables. Legumes are also rich in phenolic compounds. They occur as either simple or polyphenols, due to the number of phenol units that are available in the molecule. Phenols, coumarins, lignins, tannins and flavonoids are the simple plant phenolic compounds (Khoddami et al., 2013).



(a) (b)

Figure 2.5: General structures of phenolic acids (**A**) hydroxybenzoic acid and (**B**) hydroxy-cinnamichydroxycinnamic acid (Khoddami et al., 2013).

There are two major classes of phenolic compounds namely phenolic acids and cell wall phenolics. Phenolic acids are compounds that are found in plants. Phenolic acid exists in the form of esters, glycosides and amide groups. The difference in phenolic acids is on the number and position of hydroxyls groups located around the aromatic ring of the molecules. These acids have two major structures which are hydroxybenzoic (**Figure 2.5a**) and hydroxycinnamic acids (**Figure 2.5b**). Phenolic acids protect cell wall during plant growth against harsh conditions and infections. Cell wall phenolics are associated with other types of cell components. The two major groups of cell wall phenolic are lignins and hydroxycinnamic acids (Khoddami et al., 2013).

2.6 MUTAGENICITY AND GENOTOXICITY

2.6.1 DEFINITION OF TERMINOLOGIES

Mutation are changes in the genetic material brought about spontaneously either by chemical or by physical means whereby successive generations differ in a permanent and heritable way from their predecessors. Current scientific knowledge overwhelmingly supports the concept that many chemicals possess mutagenic properties that present a potential genetic hazard to future generations, as well as a potential cancer risk to the present one. Chemicals that exert adverse effects through interaction with the genetic material, deoxyribonucleic acid (DNA), are said to be genotoxic.

Naturally occurring mutagens have usually been discovered because of outbreaks of diseases in agricultural livestock, or because of epidemiological cancer studies of the liver in man (Sherif et al., 2009). Subsequent work has then shown that the toxic agents responsible often have mutagenic properties. Examples are the pyrrolizidine alkaloids, cycasin, a range of mycotoxins produced by various plants, and at least two unidentified toxic agents in bracken (Hemeryck and Vanhaecke, 2016). Commonly the toxic agent itself does not show high biological activity, but after ingestion it is converted by metabolic processes into the active mutagen or carcinogen (Moon et al., 2006). Some of these toxic substances have been responsible for considerable losses of agricultural livestock and therefore are of economic significance (Boobis et al., 1994).

2.6.2 TYPES OF MUTATION

2.6.2.1 Missense mutation

This type of mutation is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene. Missense mutations can render the resulting protein nonfunctional (Minde et al., 2011), and such mutations are responsible for human diseases such as Epidermolysis bullosa, sickle-cell disease, and SOD1 mediated ALS (Boillée et al., 2006).

2.6.2.2 Nonsense mutation

A nonsense mutation is also a change in one DNA base pair. Instead of substituting one amino acid for another, however, the altered DNA sequence prematurely signals the cell to stop building a protein (Turnpenny and Ellard, 2016). A point-nonsense mutation differs from a missense mutation, which is a point mutation where a single nucleotide is changed to cause substitution of a different amino acid. Some genetic disorders, such as thalassemia and DMD, result from point-nonsense mutations. This type of mutation results in a shortened protein that may function improperly or not at all (Jorde et al., 2015).

2.6.2.3 Insertion mutation

An insertion changes the number of DNA bases in a gene by adding a piece of DNA. As a result, the protein made by the gene may not function properly (Kumar et al., 2014). This can often happen in microsatellite regions due to the DNA polymerase slipping. Insertions can be anywhere in size from one base pair incorrectly inserted into a DNA sequence to a section of one chromosome inserted into another. Insertions can be particularly hazardous if they occur in an exon, the amino acid coding region of a gene (Vogelstein et al., 2013).

2.6.2.4 Deletion mutation

A deletion changes the number of DNA bases by removing a piece of DNA. Small deletions may remove one or a few base pairs within a gene, while larger deletions can remove an entire gene or several neighbouring genes (Thomason et al., 2014). The deleted DNA may alter the function of the resulting protein(s). Deletions are responsible for an array of genetic disorders, including some cases of male infertility, two thirds of cases of Duchenne muscular dystrophy and two thirds of cases of cystic fibrosis (Banavali, 2013).

2.7 METHODOLOGIES USED FOR DETECTING MUTAGENICITY

2.7.1 THE AMES TEST

The Ames test is a well-known bacterial mutagenicity test (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). In this test reverse $His^- \rightarrow His^+$ mutations are visualized by plating *Salmonella typhimurium* in a histidine poor growth medium (**Figure 2.6**). In this medium only His+ mutants can form visible colonies. Different bacterial strains are available to identify different types of mutations. The strains TA98 and TA100 are mostly used in most of the cases as they detect the great majority of mutagens (Elgorashi et al., 2003). Strain TA98 gives an indication of frame-shift mutations, while a positive response from strain TA100 indicates base-pair substitution.

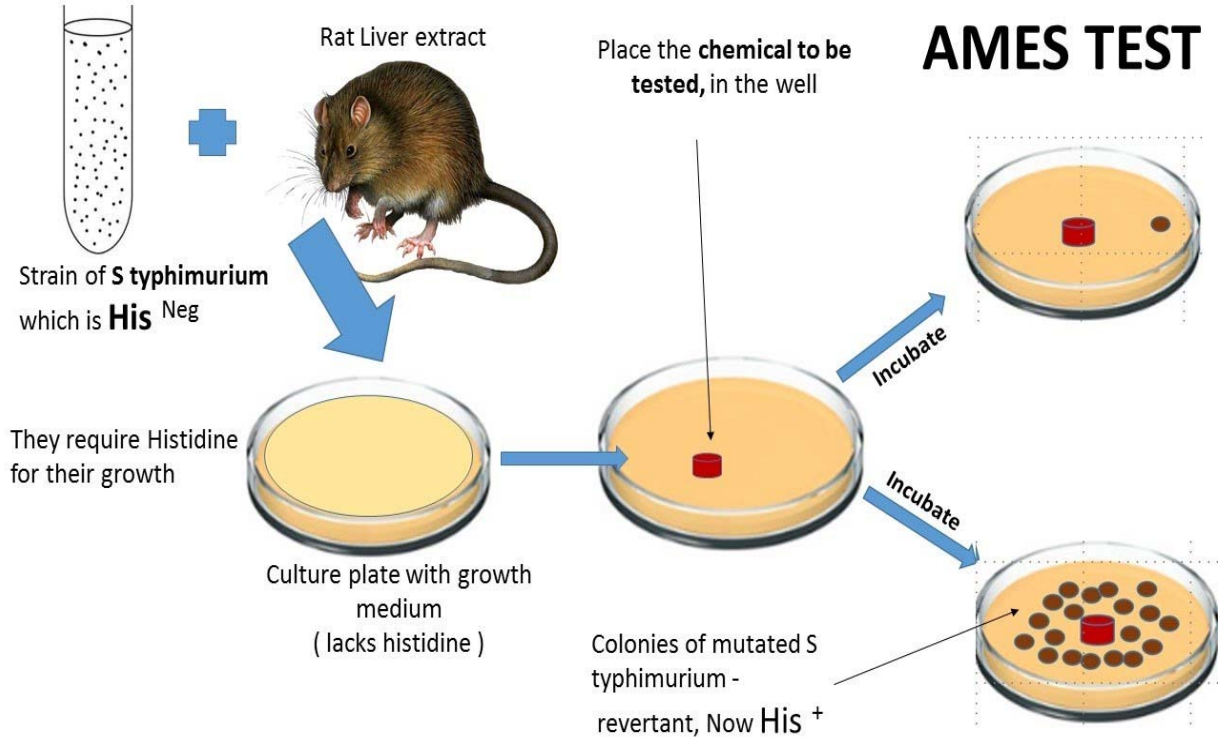


Figure 2.6: The Ames Assay procedure (Kirkland et al., 2011)

For a substance to be considered genotoxic in the Ames test, the number of revertant (revived) colonies on the plates containing the test compounds must be more than twice the number of colonies produced on the solvent control plates (Elgorashi et al., 2003). In addition, a dose–response should be evident for the various concentrations of the mutagen tested. Toxicity can be checked by investigating the background layer of bacteria (Verschaeve et al., 2004; Reid et al., 2006).

2.7.2 THE ALKALINE COMET ASSAY

The so-called “comet assay” or “single cell gel electrophoresis (SCGE) assay” is now considered a very important alternative for the cytogenetic tests (**Figure 2.7**). It is the alkaline comet assay that is mostly used with or without several further modifications (Singh et al., 1988).

Individual cells are briefly are embedded and lysed to unwound DNA on microscope slides in agarose. The DNA is then subjected to gel electrophoresis procedure and this result in a “comet like” figure which is due to the migration of (negatively charged) DNA fragments or DNA loops towards the positive pole.

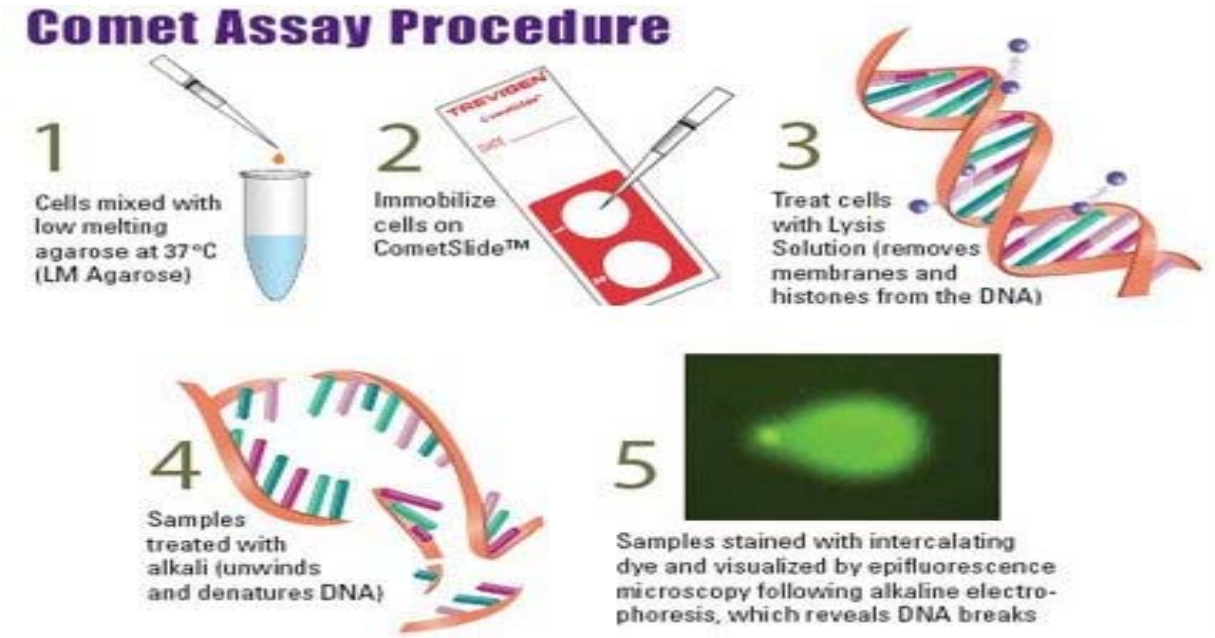


Figure 2.7: The comet assay procedure (Kumaravel et al., 2009)

2.7.3 THE MICRONUCLEUS TEST

The micronucleus test on tetrads of Tradescantia (Trad-MCN) is currently the most widely used bioassay on plants for detecting genotoxins in the environment (**Figure 2.8**). According to Misik et al (2011), approximately 160 chemicals had been tested and 100 articles on complex environmental mixtures have been published. A micronucleus is formed during cell division, a chromosome or a c hromosome fragment becomes separated from the spindle and therefore is not incorporated into one of the daughter nuclei (Fenech, 2000). It then appears under the microscope as a small nucleus (micronucleus) in the bi-nucleated telophase cell or in one of the daughter cells at interphase.

To ensure that cells had undergone mitosis following exposure to the test compound, they can be blocked in telophase by addition of cytochalasin B (which blocks cytokinesis). The micronucleus test can thus detect both clastogenic (chromosome breaking) and aneugenic (e.g., spindle disturbances, genome mutations) events which can be distinguished using fluorescence in situ hybridisation (FISH) techniques (Fenech, 2000).

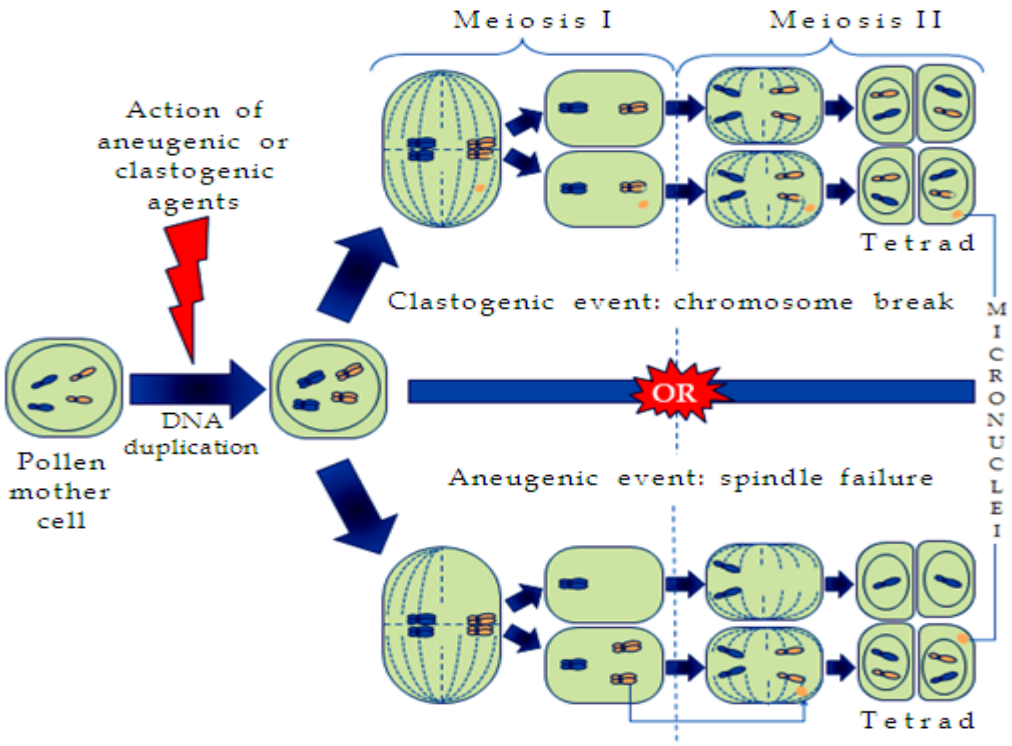


Figure 2.8: Diagram of micronucleus formation (Fenech et al., 2016)

2.7.4 THE UMU-C AND VITOTOX® TESTS

The Umu-C test method detects genotoxicity by using SOS function of *Salmonella typhiurium*. The test is based on the capability of genotoxic agents to induce the umu-C-gene in response to genotoxic lesions in the DNA (Guan et al., 2017). As for the SOS Chromotest, the umuC-gene is fused with the lacZ gene for galactosidase. LacZ is the structural gene for galactosidase (Juers et al., 2012). The tester strain has a deletion for the normal lac region, galactosidase activity is strictly dependent on umu-C expression. The SOS induction will thus result in a measurable galactosidase activity. Galactosidase activity is measured with a simple colorimetric assay. Here, genotoxicity is quantified as an induction ratio (IR) which is the ratio of absorption at 420 ± 20 nm of the sample and of the solvent control, corrected for growth rate. The sample is considered genotoxic when the IR is equal to or higher than 1.5.

2.7.5 PARP UNIVERSAL COLORIMETRIC ASSAY KIT

The PARP Universal Colorimetric Assay Kit is ideal for the *in vitro* screening of candidate PARP-1 inhibitors and determination of IC50 values (Romero-Castro et al., 2011). This ELISA based assay detects biotinylated poly (ADP-ribose) deposited by PARP-1 onto immobilized histones in a 96-well format (Ayyagari and Brard, 2014) as represented in **Figure 2.9**. The addition of Strep-HRP (biotin-binding protein) and a colorimetric HRP substrate yields relative absorbance that correlates with PARP-1 activity (Maciag et al., 2014).

PARP universal colorimetric assay is a process where in poly ADP-ribosylation of proteins nuclear in a post-translational event, that occurs as a results of DNA damage. Poly (ADP-ribose) polymerase (PARP) catalyzes the NAD-dependent addition of poly (ADP-ribose) to itself and adjacent nuclear proteins such as histones. PARP contributes to the sequence of events that occurs during DNA base excision repair (Lord and Ashworth, 2012). Whereas PARP-mediated induction of necrosis can occur by extensive depletion of the intracellular NAD pool (Nikoletopoulou et al., 2013). The cleavage of PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis (Das et al., 2016)).

Experimental models have shown that PARP inhibition prevents tissue damage in animal models of myocardial and neuronal ischemia, diabetes, septic shock, and

vascular stroke (Eltzschig and Eckle, 2011). Moreover, PARP inhibition promotes chemo-sensitization and radio-sensitization of tumors (Abaza et al., 2015). The Trevigen's HT Universal 96-well PARP Assay measures the incorporation of biotinylated poly (ADP-ribose) onto histone proteins. In a 96-well strip well format. This assay is ideal for the screening of PARP inhibitors and determining IC50 values. In this case, we used the technique to screen for PARP inhibitors.

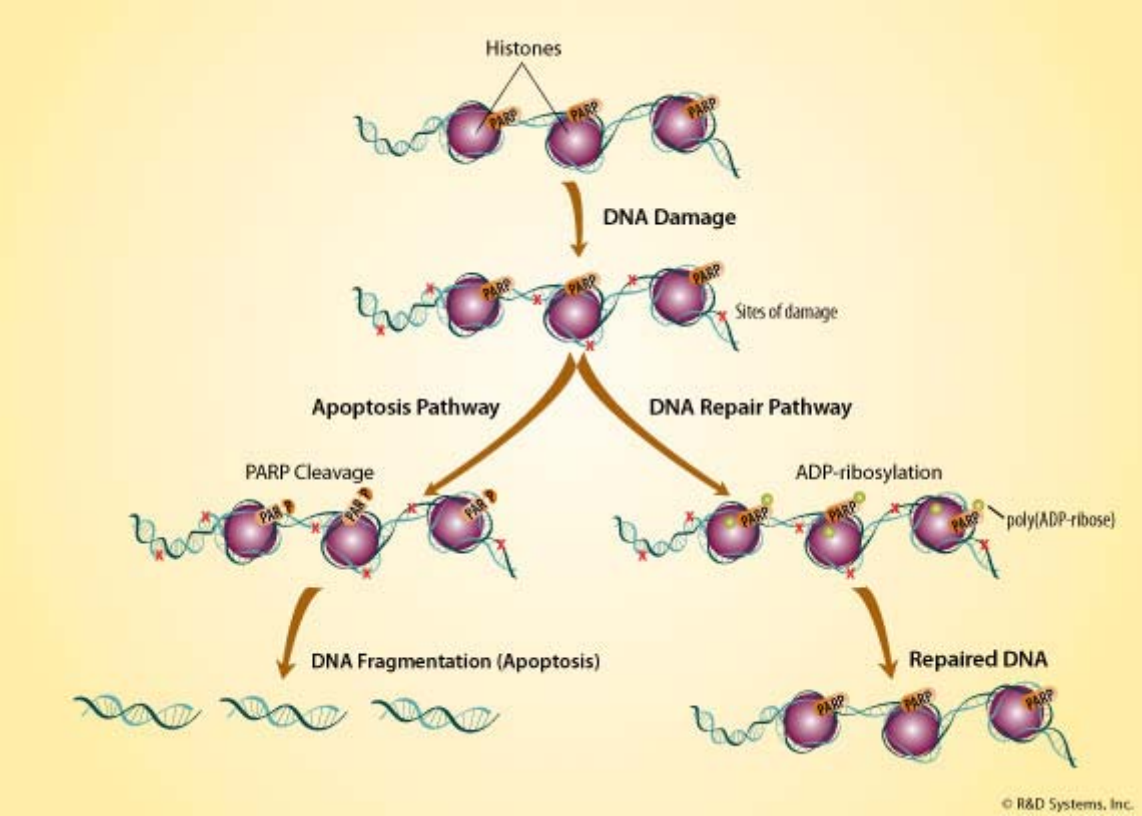


Figure 2.9: Schematic Representation of the Function of PARP Following DNA Damage. Following DNA damage, PARP catalyzes the ADP-ribosylation of itself and adjacent nuclear proteins. This modification is involved in signaling DNA damage and aiding in DNA repair in normal, viable cells (right). During apoptosis, PARP is cleaved by caspases and can no longer be activated by DNA damage, preventing apoptotic cells from repairing their DNA (left) (Fishel et al., 2013).

2.8 SUMMARY OF LITERATURE REVIEW

Plants have been used as a source of food and herbal drugs by rural populations in South Africa and other African countries. Traditional healers together with native people in Africa and some countries in the rest of the world, are aware of the medicinal

properties of plants in their surroundings. The indigenous knowledge patterning to the preparation and use of medicinal plants for medicinal purposes have been passed on from generation to generation mostly by plays, person to person poetry and some even went to an extent of dreaming about them, predominantly in African countries.

The use of medicinal plants by the locals is very significant in their culture. South Africa has a vast diverse of about 24 000 indigenous plant species, which represents approximately 10% of all higher plants on Earth. *Jatropha zeyheri* also known as Thundamali in Tshivenda is a climbing herb with a deep-rooted rhizome and slightly-branched leafy stems. *Coccinia rehmannii* also known as Galange in Tshivenda is a non-woody, climbing or scrambling herb, producing stems up to 5 metres long from a large rootstock.

The naturally occurring metabolites known as phytochemicals are naturally occurring in medicinal plants, and have defence mechanism and provide protection against various diseases. Phytochemicals are defined as primary and secondary compounds. Different types secondary metabolites such as carotenoids, phytosterols, saponins, alkaloids, tannins, steroids, and flavonoids are found in plants.

Extraction of medicinal plants is a process that involves the separation constituents of medicinal plants from the crude extracts. This is achieved by use varying polarities of solvents ranging from non-polar to polar. Mutation are changes in the genetic material brought about spontaneously either by chemical or by physical means whereby successive generations differ in a permanent and heritable way from their predecessors. There are several methods that are used to screen for mutagens such as the Ames test, the alkaline comet assay, the micronucleus test, the umu-c and vitotox® tests, and PARP universal colorimetric assay kit.

Chapter 3

MATERIALS & METHODS

3.1 ETHICAL CLEARANCE

Ethical approval was obtained from the Health, Safety and Research Ethics Committee of the University of Venda (SMNS/14/MBY/30/1210). Signed informed consent forms were obtained for agreement from all volunteers before collection of

blood specimens. Confidentiality for volunteers was maintained throughout by assigning numerical codes.

3.2 CHEMICALS AND REAGENTS

All chemicals and reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise stated. Potassium ferricyanide was purchased from Rochelle chemicals (Gauteng, RSA), trichloroacetic acid, di-sodium hydrogen phosphate anhydrous, sodium dihydrogen phosphate and ethanol were obtained from Merck chemical (Kenilworth, NJ; USA), ferric chloride hexahydrate and ascorbic acid were bought from Associated Chemical Enterprise (Gauteng, RSA).

3.3 SELECTION AND COLLECTIONS OF MEDICINAL PLANTS

Medicinal plants were selected based on their extensive use for the treatment of different ailments such as gastro-intestinal and urinary diseases and a literature search was conducted to corroborate the scientific findings with traditional findings. Two medicinal plants *Coccinia rehmannii* bulb and *Jatropha zeyheri* rhizoids were chosen and collected from within the geographic locations of the Vhembe District Municipality of Limpopo Province. These plants were identified by their vernacular names and confirmed using taxonomic keys by an ethnobotanical specialist at the University of Venda (Limpopo Province; South Africa).

The plants were collected from March 2017 to June 2018. Upon arrival to the laboratory the plants parts (*Coccinia rehmannii* rhizoids and *Jatropha zeyheri* bulb) were washed with clean water to remove the sand residues on them and chopped to smaller pieces before drying in an oven at 40 °C for 7 to 10 days. The dried parts were then milled to fine powder using an industrial grinder (Buchi Labortechnik AG, Flawil, Switzerland) then stored in clean packets in a cool dark place till further use.

3.4 EXTRACTION PROCEDURE

Extraction of the plant material was done using methanol (99.5 %) as described by Wei et al (2011) with slight modifications. For each plant sample, 1000 g of the powdered materials were macerated in 10,000 ml of methanol for 24 hours and filtered through Whatman No1 filter paper (**Figure 3.1**).

Figure 3.1: Filtration of extract with Whatman No1 filter paper.

The filtrates contained the crude plants extract of ethyl acetate, the solvent was evaporated at 40ºC using a rotary evaporator (Buchi, Flawil, Switzerland) under reduced pressure (**Figure 3.2**). Concentrated crude extracts were obtained and farther dried in an oven at 40 ºC for 24 hours to remove all the solvents residues, weighed and the percentage yield of each was calculated as follows:

$$\text{Extract yield } \% = W_1 / W_2 \times 100$$

W_1= Net weight of powder in grams after extraction and W_2= Total weight of wood powder in grams taken for extraction (Abubakar et al., 2016). All the crude extracts obtained were separately stored in vessels at room temperature until required.

Figure 3.2: Evaporation of filtrate samples in a rotary evaporator at temperature 40 ºC.

3.5 FRACTIONATION OF PLANTS EXTRACTS

The column chromatography was eluted with varying concentrations of Hexane, Ethyle Acetate and methanol (**Figure 3.3**) as described by Jaime et al, (2013) with slight modifications. A total of 39 and 37 fractions was collected from *J. zeyheri* and *C. rehmanni* fractionation respectively. The collected fractions were run on TLC with different solvents systems. Solvent system that retained well separated bands were used to match the compounds on the fractions. Fractions that had the same band after staining were combined in to one fraction.

Figure 3.3: Fractionation of extracts on an open column chromatography.

3.6 GENERAL PRELIMINARY SCREENING FOR PHYTOCHEMICAL COMPOUNDS

To group the collected fractions into final fractions, the extracted chemical components (10 mg/ml) were analyzed by separating chemical constituents using aluminium-backed Thin Layer Chromatography plates (TLC) (Fluka, silica gel F254, Merck) as described by Adamu et al, (2014). Ten microletter of the extracts was loaded on a TLC plate and developed in saturated chambers using mobile phases of different polarities, i.e. Benzene/Ethanol/Ammonia hydroxide (BEA) (non-polar/Basic) (36:4:4), [Ethyl Acetate/Methanol/water (EMW); and Chloroform : ethanol: Formic Acid (CEF)(32:4:4)

(polar/neutral) (81:11:8)] and Chloroform: Glacial Acetic acid: Methanol: Water (64:32:12: 8) (polar) (Kotze et al., 2002), Chloroform: ethanol: Formic Acid (32:4:4).

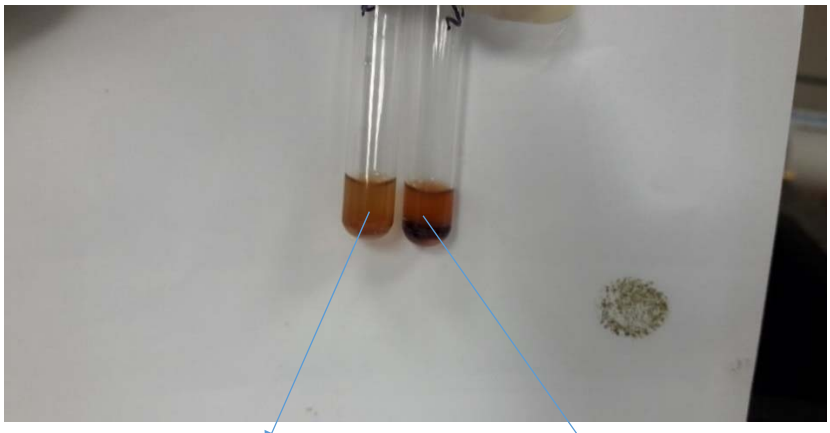
Separated compounds on TLC plates were examined under ultraviolet light (254 and 365 nm) then sprayed with vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid] and then heat 110 °C for optimum color development.

3.7 PHYTOCHEMICALS ANALYSES

Phytochemical analyses were done using two main methods namely (A) biochemical tests that looked at identifying bioactive constituents using different solvents and (B) by Thin layer chromatography that looked at separating bioactive constituents on a plate using their migratory ability.

3.7.1 BIOCHEMICAL TESTS

This section describes the various biochemical testing carried out to determine the presence or not of 2nd metabolites as shown in **Figure 3.4**.



Test tube before addition of reagents Indication of presence of cardiac glycosides

Figure 3.4: Test tubes showing the presence of cardiac glycosides in plant extract.

3.7.1.1 Test for phenols and Tannins

Into a reaction tube 1 ml of crude extract was mixed with few drops of 2 % ferric chloride ($FeCl_3$) (Merck, Kenilworth, NJ; USA). Any black, brown, green or dark blue

colorations was taken as a confirmation of both secondary metabolites (Khanum et al., 2015).

3.7.1.2 Test for flavonoids

In a reaction tube, few drops of a 4 mg/ml sodium hydroxide (NaOH) (Sigma Aldrich St. Louis, MI; USA,) solution were added to 1 ml of crude extract, followed by few drops of 32 % hydrochloric acid (HCl) (Merck, Kenilworth, NJ; USA) solution. A yellowish coloration or a colorless mixture indicated the presence of flavonoids (Resende et al., 2012).

3.7.1.3 Test for Saponins

Into 1 ml of crude extracts was added 5 ml of distilled water into the test tubes. The test tubes were vigorously shaken for 5 minutes. The formation of a persistent foam was considered as an indication of the presence of saponins (Astuti et al., 2011).

3.7.1.4 Test for Cardiac glycosides (celler- kilani test)

Into the reaction tube extracts (1 ml) were mixed with 0.5 ml of glacial acetic acids (Merck, Kenilworth, NJ, USA) (96%) followed by few drops of 2% $FeCl_3$ (Sigma-Alrich, st Louis, MI; USA) and 1 ml of concentrated sulphuric acid (H_2SO_4) (Merck). A brown ring at the interphase or a brown coloration was an indication of the presence of the compounds (Yadav et al., 2011).

3.7.1.5 Test for Steroids

One milliliter of crude extracts was mixed with few drops of concentrated acetic acid (Sigma Aldrich, Saint Louis, MI; USA) and few drops of concentrated sulphuric acid (H_2SO_4) (Merck). A violet green coloration indicated the presence of steroids (Akinmoladun et al., 2010).

3.7.1.6 Test for Terpenoids

A volume of 1 ml extracts was dissolved in 0.5ml of chloroform and 1ml of concentrated sulphuric acid (Merck). A brownish, reddish or dark coloration indicated the presence of this chemical (Yadav et al., 2011).

3.7.1.7 Test for Akaloids

Wagner's reagents were prepared by mixing 2 g of iodine and 6 g of potassium iodide into 100 ml of distilled water. Few drops of the prepared Wagner's reagent were added to 1 ml crude extracts. The observed turbidity or precipitation was considered as an evidence for the presence of alkaloids.

3.7.2 PHYTOCHEMICAL ANALYSIS USING TLC

This section describes the TLC method for the screening of 2nd metabolites as shown in **Figure 3.5**.

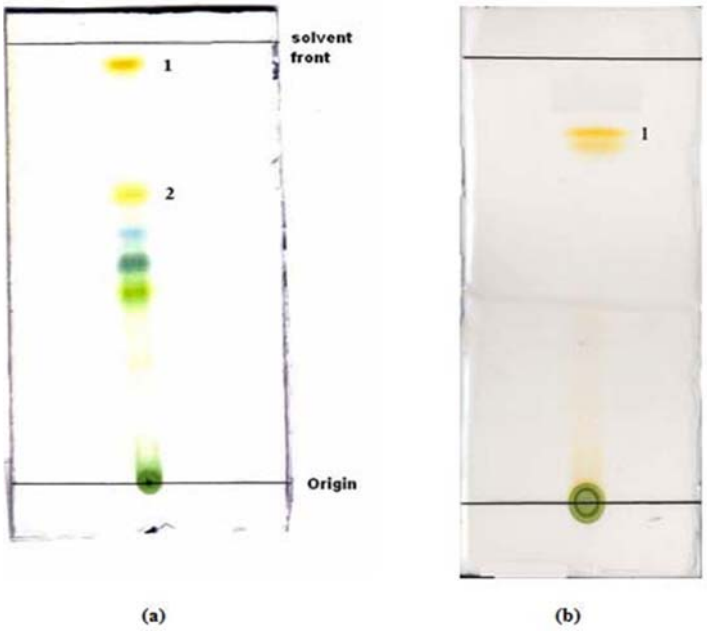


Figure 3.5: TLC plates showing the presence of cardiac glycosides in plant extract (Kreis and Müller, 2018).

3.7.2.1 TLC Separation

Ready to use silica gel plates (Silica gel 60F-254 TLC aluminium sheet 20x20 cm) was used as described by Biradar and Rachetti, (2013) with slight modifications. The plant extracts and their fractions were dissolved in methanol at a concentration of 1 mg/ml, then about 10 µl of each sample was applied manually with a capillary tube on the plate.

For the screening process (separation of components), three eluents solvent systems of different polarities were used, namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1), CEF (chloroform/ethyl acetate/formic acid (5:4:1) and EMW (ethyl acetate/methanol/water (40:5.4:4).

During the screening process, the samples on the plate were kept in TLC glass chambers (solvent saturated). Then the mobile phase was allowed to migrate through adsorbent phase up to 3/4th of the plate, where then they were removed and left to dry. All plates were visualized directly after drying by spraying the plates with the reagent vanillin-sulphuric acid. Components of the samples were visible as bands of different colors.

3.7.2.2 Visualization

Flavonoids

A 1 % (w/v) solution of Aluminium chloride was prepared in ethanol and prayed in developed TLC plates. The plates were viewed on long wave UV light for observation. Yellow band viewed under long-wave UV light was considered as confirmation of flavonoids.

Alkaloids

Wagner's reagents were prepared by mixing 2 g of iodine and 6 g of potassium iodide into 100 ml of distilled water. The developed plates were then observed for any change on the color of bands.

Phenols

Solution **A** was done by preparing 20 % (w/v) of sodium carbonate in water then solution **B** was prepared by mixing Folin-ciocalteu (Sigma-Aldrich, St Louis, MI; USA)

reagent in a ratio 1:3 ratio with water. Plates were sprayed with **A** solution an allowed to briefly dry, and sprayed with solution **B**. Confirmation was done by observing grey to black bands that develops upon spraying with solution **B**.

Terpenoids

Developed plates were sprayed with Vanillin indicator reagent, then incubated at 100 °C for color development. Confirmation of terpenoids was done by observing for bright blue green band.

Tannins

A mixture of 0.5 % of (w/v) vanillin solution with 4 (w/v) HCl was prepared and sprayed on the developed plates. Conformation was done by observation of red bands after spraying.

3.8 CYTOTOXICITY AND GENOTOXICITY OF THE PLANT EXTRACTS

After testing for phytochemicals, the extracts and the fractions were tested against Vero cell lines in order to evaluate their toxicity and genotoxicity. The experiments were carried out at Prof M van de Venter's Laboratory at the Nelson Mandela University (Port Elizabeth; RSA). The methodologies are as follows.

3.8.1 SAMPLE PREPARATION

Test compounds were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/ml. Samples were sonicated if solubility was a problem. Samples were stored at 4 °C until required.

3.8.2 CELL CULTURE AND MAINTENANCE

The Vero cells used for these experiments, are derived from the kidney of an African green monkey (*Cercopithecus aethiops*) and were cultured in a humidified incubator at 37 ºC in DMEM/low glucose:10% fetal bovine serum with 10 % (v/v) penicillin-

streptomycin in culture dishes (Pringle et al., 2018). The cells were passaged when a confluency of 70 % was reached. Cells were seeded in 96 well plates at 3000 cells/well (100 µl aliquots) for the genotoxicity assay and 4000 cells/well (100 µl aliquots) for the cytotoxicity assay and left overnight prior to addition of test compounds to allow for cell attachment to the plate.

3.8.3 TREATMENT OF VERO CELLS

One hundred microliters aliquots of the diluted plant extracts in fresh medium was used to treat cells. Various concentrations (30, 15, 7.5, 3.75 and 1.875 µg/ml) of each extract were tested in quadruplicate. Melphalan (100 mM stock) was used as a positive control for the cytotoxicity assay. Griseofulvin (50 mM stock) was used as the positive control for the genotoxicity assay. Treated cell lines were incubated at 37 °C in a humidified (5% CO_2) incubator (Thermo Fisher, Waltham, MA USA) for 48 hours.

3.8.4 BIOCHEMICAL ANALYSIS OF TREATED CELLS

3.8.4.1 Testing for Toxicity

Treatment medium was aspirated from all wells and replaced with 100 µL of LTR Hoechst 33342 nuclear dye (LysoTracker Red is used to determine the formation of lysosomes in response to treatment of cells with a compound or extract of interest) (5 µg/mL) which was prepared by adding 20 µL PI in 10 mL PBS (+Ca +Mg) and incubated for 10 minutes at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 50 µg/mL in order to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI (ImageXpress Micro XLS Widefield Microscope) using the DAPI and Texas Red filters. Toxicity was determined by looking at cell viability, expressed as nuclei per site of the cells treated with the extracts compared to the untreated control.

3.8.4.2 Testing for Genotoxicity

Treatment medium was aspirated from all wells and 50 µL aliquots of 4 % formaldehyde were added to each well and incubated overnight at 4 ˚C to fix the cells. NucRed (a dye that is used for genotoxicity to determine the formation of micronuclei) working solution was prepared by adding 2 drops per ml PBS (+Ca +Mg). One hundred

microliters of NucRed was added to each well and the plate was incubated for 15 – 30 minutes at 37 °C. Cells were then imaged immediately using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices, manufacturer, HQ) using Cy5 filter.

3.8.5 DATA QUANTIFICATION

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope and acquired images analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet and data was analysed.

An example of how data was interpreted is shown in **Figures 3.6** to **3.9**. The cells were stained with Hoechst 33342 and LysoTracker Red to establish cytotoxic effects and to determine the effect of sample extracts on the formation of lysosomes, respectively. A montage of images results after acquisition and depending on the settings, 9 sites in each well was analysed using MetaXpress software and required data such as total cell number, total “live” cells, total “dead” cells, percentage positively stained cells etc. was captured. This process was followed independently for each dye/fluorescently labelled antibody for each experiment.

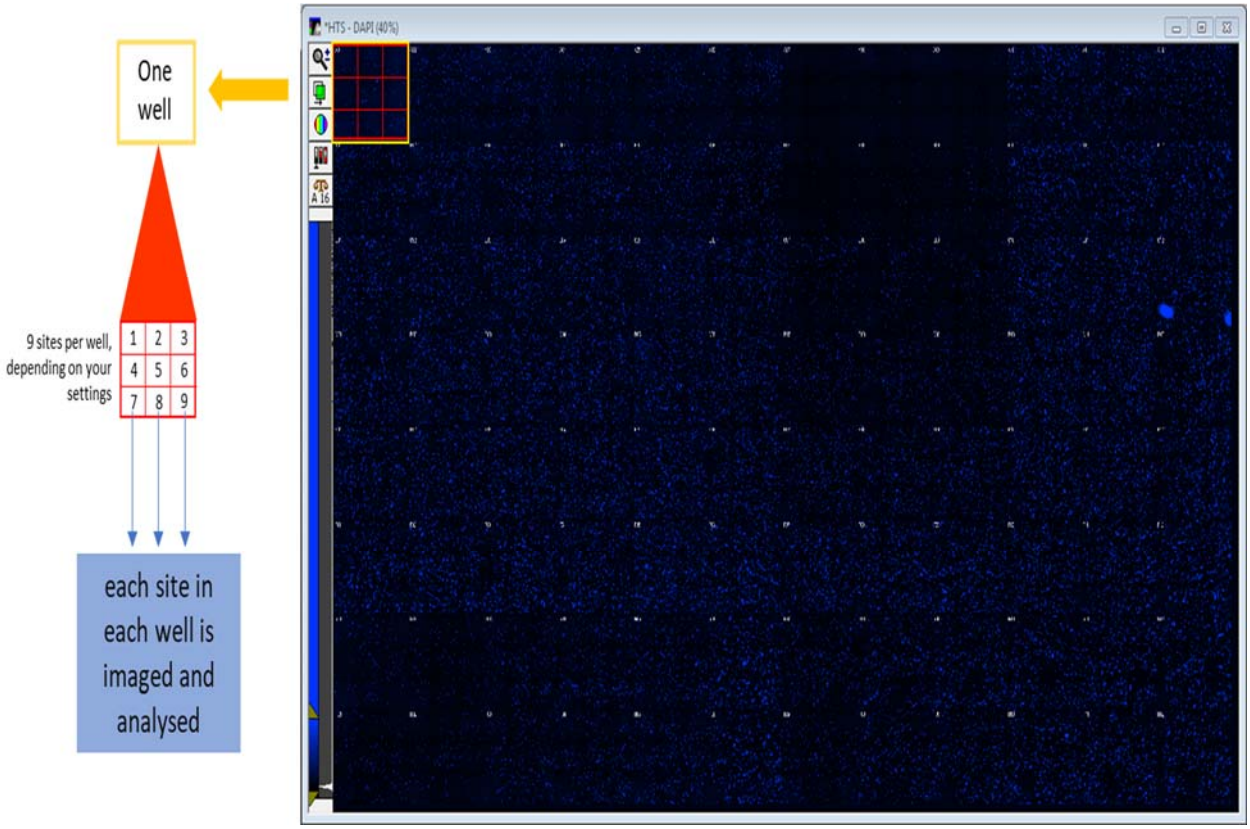


Figure 3.6: Determination of cytotoxic effect of extracts on cells using Hoechst 33342.

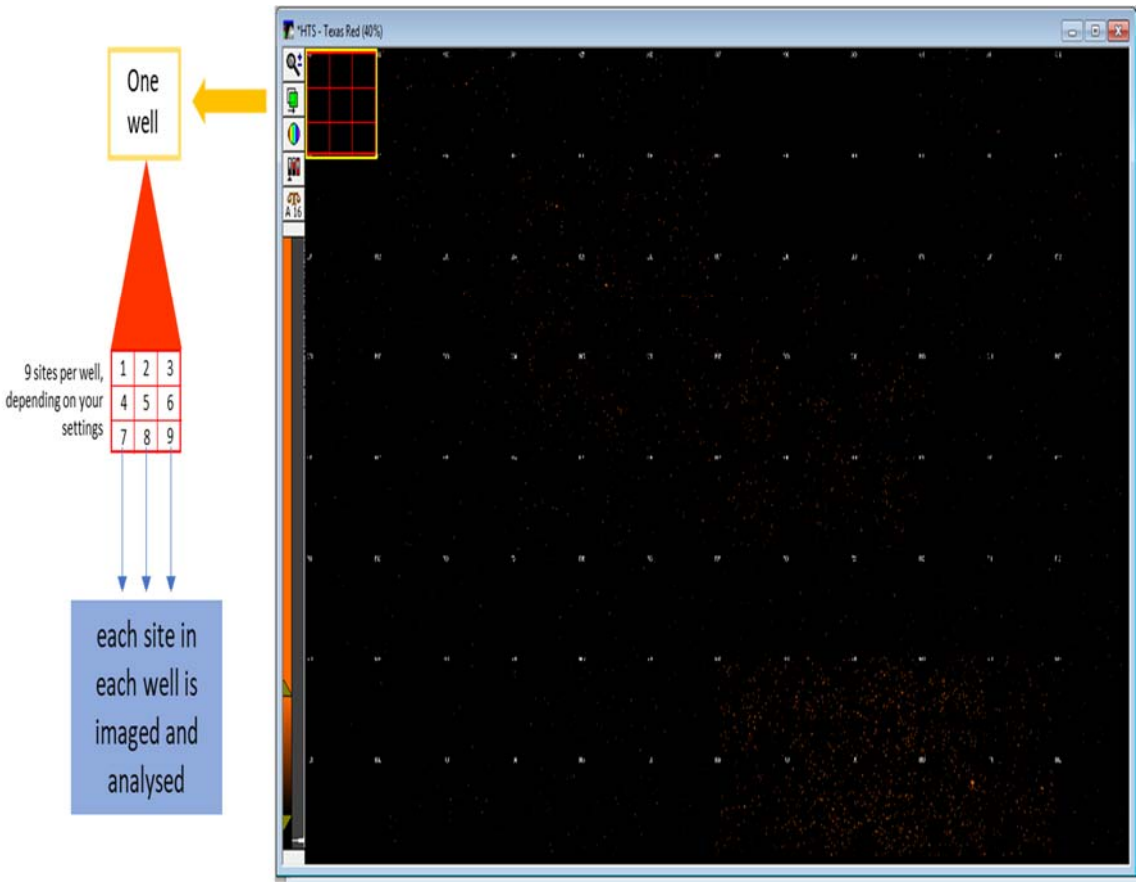


Figure 3.7: Analysis of cells stained with LysoTracker Red for the determination of the formation of lysosomes in response to extract treatment.

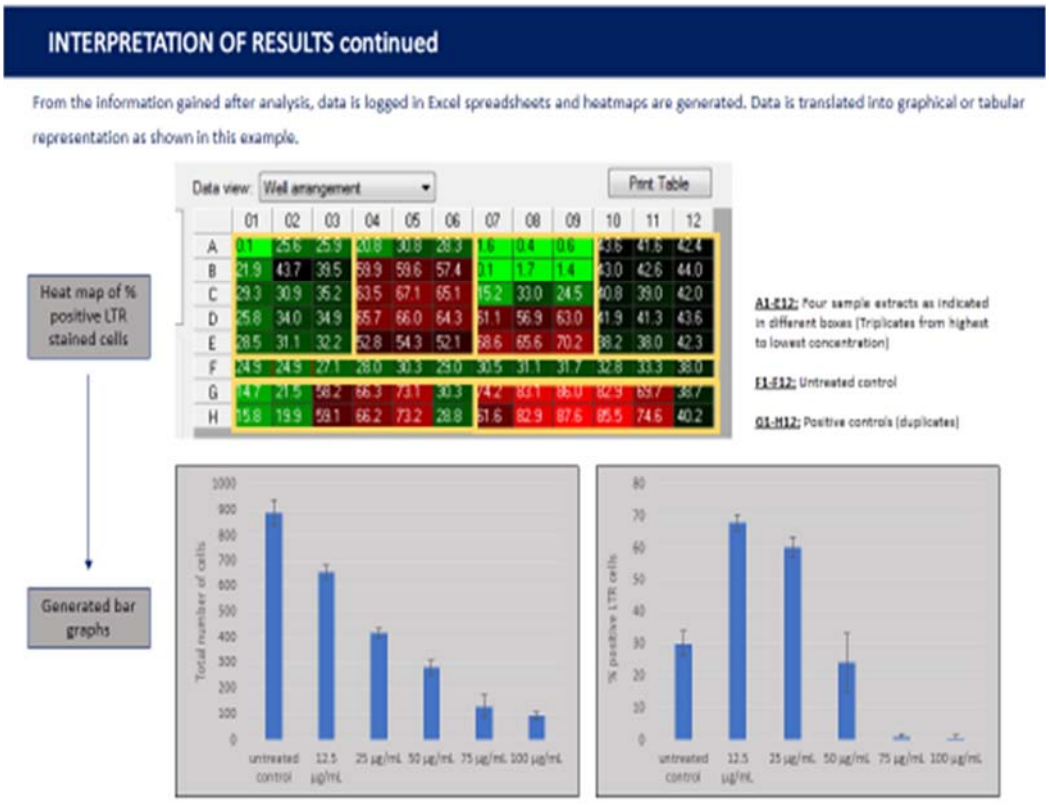


Figure 3.8: Effect of extract (5 concentrations) on total cell number and lysosome formation.

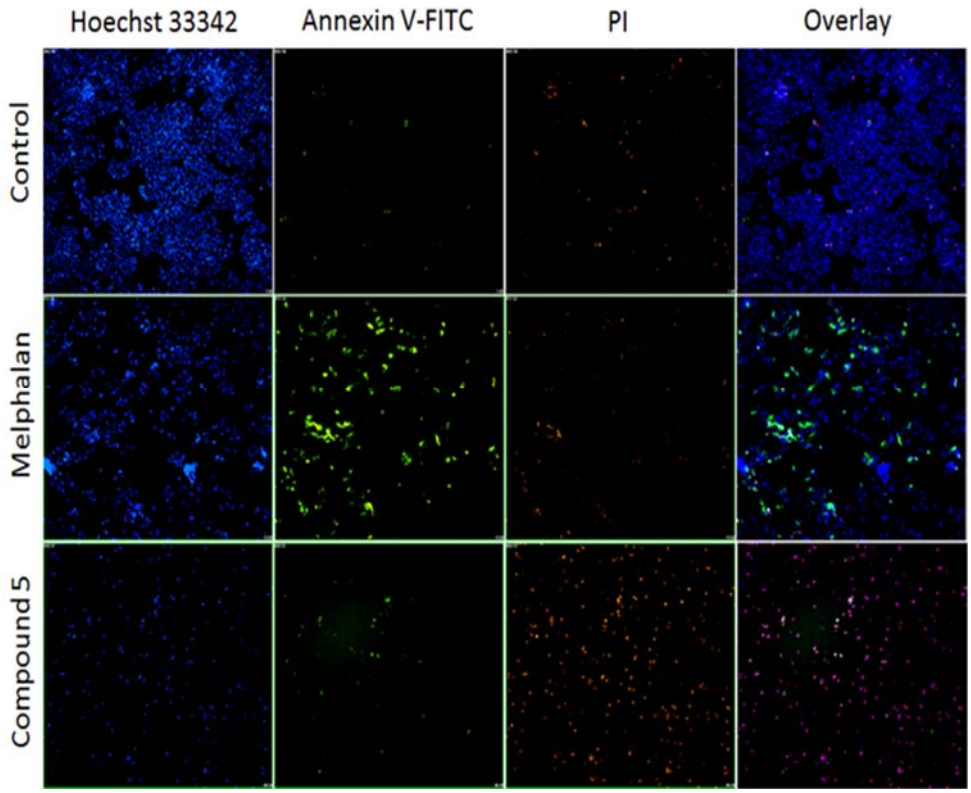


Figure 3.9: C3A hepatocytes were stained with Hoechst 33342 (blue), Annexin V-FITC (green) and propidium iodide (red). In this experiment Compound 5 induced necrotic cell death as opposed to apoptotic cell death as evident with melphalan treatment. This is evident with the high proportion of PI positive cells compared to Annexin V-FITC positive cells as with melphalan. Annexin V has a high affinity for phosphatidylserine on the extracellular surface of the cells, an early indicator of apoptosis.

3.9 MUTAGENICITY OF THE PLANT EXTRACTS USING THE PARP UNIVERSAL COLORIMETRIC ASSAY KIT

This experiment was carried out using the PARP universal colorimetric assay kit (Trevigen, Bio-Techne Ltd, United Kingdom)

3.9.1 REAGENT PREPARATION

Reagents preparations were done following the manufactures instructions as follows: Strep-Diluent (10X) was diluted to a ration of 1:10 with distilled H20 before use. 20X PARP Buffer. The Buffer 20X PARP was diluted to 1X (1:20) with distilled H2O this gave 1X PARP Buffer that was used to rehydrate the histone coated wells, and to dilute the enzyme, PARP Cocktail and the inhibitors to be tested (in this case plants extracts and fractions). Buffer 10X PARP Cocktail was diluted as follows: 10X PARP Cocktail 2.5 µl/well; 10X Activated DNA 2.5 µl/well; and 1X PARP Buffer 20 µl/well

Preparation of PARP Enzyme were as follows: The kit contained 50 µl of PARP-HSA enzyme at a concentration described in the enclosed Product Data Sheet. The enzyme was diluted appropriately with 1X PARP Buffer just before use, Diluted enzyme was used immediately, and any remainder discarded. PARP Inhibitors 3-aminobenzamide (3-AB) that was provided at 200 mM in ethanol as a control inhibitor. 3-AB inhibits the activity of PARP at a wide range of concentrations from 2 µM to 10 mM Serially diluted stock of 3-AB and your PARP inhibitor(s) with 1X PARP Buffer were added to designated wells. Strep-HRP was diluted 500-fold with 1X Strep Diluent just before use. A total of 50 µl/well of diluted Strep-HRP was required for each well in the assay.

TACS-Sapphire (a colorimetric substrate that turns blue in the presence of Horseradish Peroxidase) was pre-warmed to room temperature before use. The addition of an equal volume of 5 % phosphoric acid stops the reaction to generate a yellow color.

3.9.2 PARP INHIBITOR ASSAY PROTOCOL

The strip wells were removed from the wrapper, 50 µl/well of 1X PARP Buffer was added to rehydrate the histones and incubated at room temperature for 30 minutes

before the 1X PARP buffer was tapped out of the well to the paper towel (taking note of not premixing the PARP-HSA enzyme and the PARP Cocktail since PARP will auto-ribosylate in the presence of NAD). A serial dilution of 3-aminobenzamide, plant extracts and fractions (pre-prepared) were added to appropriate wells, followed by the addition of diluted PARP enzyme (0.5 µl/well pre-prepared) to the wells containing inhibitor and incubated for 10 minutes at room temperature.

A negative control (without PARP) was prepared to determine background absorbance. Activity Control for PARP Inhibitor Study (0.5 µl/ well PARP-HAS) without inhibitors was also prepared (these wells provide the 100% activity reference point), 25 µl of 1X PARP Cocktail was distributed into each well using a multichannel pipette to make the final reaction volume of 50 µl. The strip wells were incubated at room temperature for 60 minutes.

3.9.3 DETECTION OF ANTIMUTAGENS

Strip wells were washed 2 times with 1X PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. The strip wells were ensured that all the liquid was removed by following each wash by tapping strip wells onto paper towels. Followed by addition of a total volume of 50 µl/well of diluted Strep-HRP to the strip wells and Incubated at room temperature for 60 minutes. The strip wells were then washed 2 times with 1X PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS ensuring that all the liquid was removed by following each wash by tapping strip wells onto paper towels. A total volume of 50 µl/well of pre-warmed TACS-Sapphire™ colorimetric substrate was finally added and incubate in the dark for 15 minutes at room temperature. The reaction was stopped by the addition of 50 µl of 5% Phosphoric Acid and absorbance were read on a Multi scan MS spectrophotometer (Thermo Labsystems, Waltham, MA; USA) at 450 nm.

Inhibition by the plant extracts was seen as OD values similar to values obtained with the known PARP inhibitor 3-amino-benzamide (provided in the kit).

3.10 STATISTICAL ANALYSIS

Results are expressed as mean ± Standard error (SE). The mean between experimental conditions was compared by One-Way Analysis of Variance (ANOVA) while Pearson correlation was used for correlation analysis. All data analyses were performed using SPSS version 23.0.

Chapter 4

RESULTS AND DISCUSSION

In order for medicinal plants to be rendered important in clinical applications, the prepared extracts must be selectively toxic to the target disease causing pathogen or must, at least, interfere directly with a particular reaction pathway with minimal toxicity to the host cells. Medicinal plants are assumed safe to consume because they are of natural origin and have been used for centuries in treating different ailments (Fennel et al., 2004). A study conducted by Till et al (2003), has, however, revealed that some of the medicinal plants can have long term effects on the human body and one of such effect is DNA damage. The results from this research show high level of selective inhibitory effects from the crude plant extracts. The results also demonstrate toxicity effects on cancerous cell lines. The results obtained are described and discussed below.

4.1 EXTRACTION RESULTS

The results revealed that *C. rehmanni* (12.03 %) yielded more extract than *J. Zeyheri* (8.20 %) these two plants have different compounds composition and were in different stages of maturity. This could be the cause of this observation. To our knowledge, this is the first study to report on the yield percentage of methanolic extracts of *J. zeyheri* and it is the first study to report on the extraction of *C. rehmanni* to date.

The first major step in the analysis of medicinal plants is the extraction of compounds. It is very essential to extract desired chemical components of plant materials for screening and analysis of bioactive compounds using biological activity assays and also for isolation, identification and purification of active compounds (Sasidharan et al., 2011). Water is the common solvent of interest used by traditional healers to extract plant compounds, however water has limitations since it has inability to extract non-polar compounds. In this study only methanolic extracts were use due to Methanol ability to extracts higher mass compares to other solvent as it was once reported by Masoko et al, (2008) who also reported methanol as a better solvent compared to the other solvents.

4.2 PRELIMINARY SCREENING FOR PHYTOCHEMICALS

TLC has been reported to be the most widely used and an impotent technique to separate a mixture of plants extracts (Tekauz et al., 2000). The results showed various secondary metabolites that could be compared and used for grouping of fractions. Phytochemical screening revealed that the plants contained various chemical components in the different extracts of the plants. The notable color changes depicted by individual compounds due to their reaction with the spray reagent used (vanillin/sulphuric acid) could be noticed. Similarities exist between chemical compositions of the components of extracts separated using the same solvent system. **Figure 4.1** shows 34 fractions of *J. Zeyheri* that were then grouped based on the similarity of bioactive compounds. Seven final fractions were obtained. An example, fraction 1-3, 4.7, and 25-32 shows similarities and were as such grouped in to one fraction respectively. Different colours was observed on the TLC chromatograms that confirms the presence of many compounds in *J. zeyheri* fractions. This in in agreement with the finding by Sofowora (1993), who reported that phytochemical analysis might reveal the presence of constituents which are known to exhibit medicinal properties.

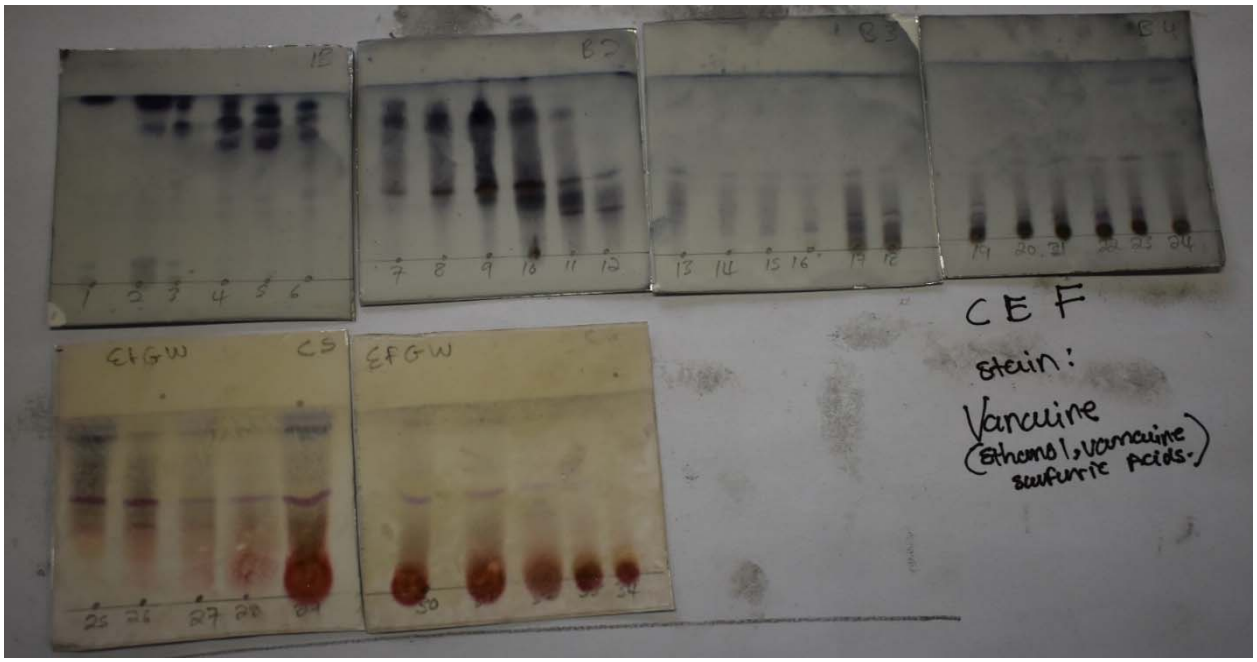


Figure 4.1: TLC- chromatograms shows the profile of crude extracts obtained after extraction. Different colours on the aluminium-backed TLC plates were observed after spraying plates with vanillin-sulphuric acid reagent.

4.2.1 *JATROPHA ZEYHERI* FRACTION RESULTS

The result of the percentage yield of *J. zeyheri* fractions are shown in **Table 4.1** and crude extract percentage is discussed in section *(4.1).* Fraction JZ6 showed higher yield percentage. It could be noticed that first fractions showed low percentage yield compared to the fractions collected last. This could because less polar/nonpolar solvent system was used first and could only elute less polar compounds, this is similar to the observation by Raaman, (2006); Braithwaite and Smith, (2012) and Houghton and Raman, (2012), who reported that when the stationary phase is polar, polar molecules spends more time adsorbed on the stationary phase, while less polar ones will be carried quickly by the non-polar mobile phase. Therefore, it can be concluded that the plant contained more polar compounds than non-polar compounds.

Table 4.1: Separation of *J. zeyheri* with various solvents, mass retained per fractions and the percentage yield.

Fractions	Solvent used		Total mass in grams	Yield percentage
Jz 1	Hexane, Ethyl acetate	90:10	2.69	3.28
		80:20		
Jz 2	Hexane, Ethyl acetate	80:20	4.2	5.06
		70:30		
Jz 3	Hexane, Ethyl acetate	70:30	2.98	3.59
Jz 4	Ethyl acetate Hexane	80:20	1.33	1.60
Jz 5	Ethyl acetate, methanol	90:10	10.13	12.2
		80:20		
		70:30		
Jz 6	Ethyl acetate, methanol	70:30	31.02	37.3
		50:50		
Jz 7	Methanol Ethyl Acetate	70:30	20.51	24.10
		80:20		
		90:10		
	Methanol	100		

4.2.2 *COCCINIA REHMANNI* FRACTION RESULTS

The results revealed that *C. rehmanni* (12.03 %) has the highest yield when compared to that of *J. Zeyheri* as reported in section *(4.1)*. The result of the percentage yield of *C. rehmanni* crude extracts and fractions are shown in **Table 4.2**, with Cr6 showing the highest yield percentage. At this stage, higher percentage of polarity was being increasingly used (Methanol), this solvent is able to elute most of the polar compounds as the polarity increases. The results show that Cr2 and Cr4 had lower yield percentages, these fractions are known to be intermediate and have lower yield percentage when compared to the previous subsequent fractions. This could be because of transaction interval on increasing polarity. As the column elute there is an intermediate transaction of polarity (Watanabe et al., 2009).

Table 4.2: Separation of *C. rehmanni* with various solvents, mass retained per fractions and the percentage yield.

Fractions	Solvent system used		Mass in grams	Yield percentage
Cr 1	Hexane, Ethyl acetate	90:10	0.97	0.81
		80:20		
Cr 2	Hexane, Ethyl acetate	80:20	0.02	0.02
		70:30		
Cr 3	Ethyl acetate Hexane	50:50	0.46	0.38
		60:40		
Cr 4	Ethyl acetate Hexane	70:30	0.21	0.17
		80:20		
		90:10		
	Ethy acetate	100		
Cr 5	Methanol, Ethyl acetate	50:50	1.34	1.11
		60:40		
		70:30		
Cr 6	Methanol, Ethyl acetate	80:10	39.13	10.57
		90:10		
	Methanol	100		

4.3 PHYTOCHEMICAL EVALUATIONS

4.3.1 PHYTOCHEMICAL ANALYSIS BY BIOCHEMICAL TESTS

4.3.1.1 Phytochemical analysis of *J. Zeyheri*

Phytochemical screening of *J. zeyheri* showed the availability of vast bioactive components which includes phenolic, flavonoids, saponins, cardiac glycosides, steroids terpenoids, and alkaloids. Cardiac glycosides, terpenoids, phenolic and tannis were the most prevalent bioactive compounds in the extract and amongst all fractions. This is in support with the study done by Luseba et al, (2007) that reported that *J. zeyheri* has a well-known content of cardiac glycosides and tannins.

Table 4.3: Phytochemical constituents of *J. zeyheri*

Sample or fractions	Phenols and Tannins	Flavonoids	Saponins	Cardiac glycosides	Steroids	Terpenoids	Alkaloids
Jz crude	**++**	**+**	**+++**	**+++**	**++**	**+++**	**+**
Jz1	+	+	-	+++	+	+++	+
Jz2	++	+	-	+++	+	++	+
Jz3	++	++	-	**++**	++	**+++**	+
Jz4	**+++**	**+**	**+**	**+++**	**++**	**+++**	**+**
Jz5	+++	+	+++	++	+	+++	+
Jz6	+++	++	+++	+++	+	+++	+
Jz7	+++	+	+++	+++	+	+++	+

Note: (−), not detectable; (+), low quantities; (++), high quantities; and (+++), very high quantities.

Cardiac glycosides are reported to inhibit the plasma membrane Na^+ pump, and are one of the drug recommended for routine use to treat heart failure, yet their therapeutic window is limited by toxic effects (Liu et al., 2010). This may explain why the fractions that contain a higher content of cardiac glycosides proves to be high in toxicity (Sarkar et al., 2016). Flavonoids and alkaloids were detected in low concentration from both crude extracts and fractions while saponins were not detected from three of the fractions (Jz1, Jz2 and Jz3).

4.3.1.2 Phytochemical analysis of *C. rehmanni*

Phytochemical screening of *C. rehmanni was* done the same as that of *J. zeyheri in* sections *(4.3.1.1)*. The results are presented in **Table 4.4**. This is the first study reporting on phytochemicals found in extracts of *C. rehmanni* plant and also the first project to fractionate this plant extract; However, several phytochemical compounds such as alkaloids, tannins, saponins and phenols have been reported in methanolic extracts of plants (*Coccinia grandis, Coccinia indica* and *Coccinia cordifolia*) that belong to the same family of the study plant (Shaheen et al., 2009; Tamilselvan et al., 2011 and Khatun et al., 2012).

Terpenoids, Cardiac glycosides, Phenolic and tannis were the most detected bioactive compounds respectively from both the crude extract and the fractions. Steroids and alkaloids were the least detected as they were absent in some of the fractions or appear in low concentrations when present.

Table 4.4: Phytochemical composition of *C. rehmanni*

Sample or fractions	Phenols and Tannins	Flavonoids	Saponins	Cardiac glycosides	Steroids	Terpenoids	Alkaloids
Cr crude	**++**	**++**	**++**	**+++**	**-**	**+++**	**-**
Cr1	++	++	-	++	+	+++	+
Cr2	+++	+	++	++	+	+++	_
Cr3	++	++	-	++	+	+++	_
Cr4	++	+	-	**++**	+	**+++**	+
Cr5	**+++**	**++**	**+**	**+++**	**+**	**++**	**++**
Cr6	++	+	++	++	+	++	++

Note: (−), not detectable; (+), low quantities; (++), high quantities; and (+++), very high quantities.

Alkaloids were not detected in the crude extract but detected in the fractions. This maybe because of the process of fractionation where the compounds are basically grouped according to their polarity as such they end up being concentrated in some fraction and not in others (Houghton and Raman, 2012). It can be speculated that these compounds were not detected in the crude extract because of their low content.

4.3.2 PHYTOCHEMICAL ANALYSIS USING TLC

TLC analysis also confirmed the presence of different kinds of phytochemicals *J. zeyheri* and *C. rehmanni* extracts and fractions. **Tables 4.5 and 4.6** report on the R_f values of the plant extracts and fractions after developing in CEF solvent (ration 32:4:4 intermediate poplar/neutral). Some of the samples exhibited more than one spots for active compounds eg. *J. Zeyheri* fraction two (Jz2) shows more spots on alkaloids and tannins while *C. rehmanni* fractions six (Cr6) showed multiple detection of similar compounds in Alkaloids and terpenoids. From the *C. rehmanni* plants it was observed that some samples that's do not appear on the crude extract. This could be because such compounds Terpenoids and Tannins concentrate more to a detectable dose according to the polarity, as such could not be detected in crude but concentrate more on fractions.

Table 4.5: R_f values of *J. Zeyheri* rhizoids extract and its fractions.

Samples	*Flavonoids*	*Alkaloids*	*Phenols*	*Terpenoids*	*Tannins*
Jz crude	**0.50**	**0.051**	**0.40** **0.33** **0.19**	**0.85**	**0.87**
Jz1	1	1	0.91	1	0.81
Jz2	1	-	**0.45** **0.36**	0.83	**0.75** **0.62**
Jz3	0.40	0.45	0.67	0.83	0.78 0.64
Jz4	0.40	0.45	0.42	0.85	0.78
Jz5	0.40	0.51	0.40	0.87 0.33	0.82
Jz6	-	0.32	0.29	0.87	0.82
Jz7	-	-	0.37	-	0.77

From this study it was observed that the R_f values in variation from 0.13 up to 1. This also proves greater separation potential of the CEF solvent system in this methanol extracts. The same degree of separation was made by Masoko and Makgapeetja (2015). Crude extracts of *J zeyheri* shows bands for all the secondary metabolites tested however the R_f values differs with those of the fractions. This could be because the fractions had fewer compounds as such the remaining compounds could move freely on the TLC plates upon development. These findings correlate with the finding by Holmbom et al (1984), although it was observed from different plants and extraction solvent.

Table 4.6: R_f values of *C. rehmanni* extract and its fractions

Samples	*Flavonoids*	*Alkaloids*	*Phenols*	*Terpenoids*	*Tannins*
***Cr* crude**	**0.40**	**0.37**	**0.37**	**-**	**-**
Cr1	-	0.97	0.55	0.39	0.59
Cr2	0.42	**0.97** **0.35**	0.73	**0.32** **0.76**	0.5
Cr3	1 0.84	0.33	0.28 0.73	0.32 0.74	0.48
Cr4	1	0.33	0.38	-	0.52
Cr5	0.42	-	0.38	0.13	0.70
Cr6	0.42	0.36	0.60	-	-

An example of some positive flavonoids and alkaloids bands are shown in **Figure 4.2**.

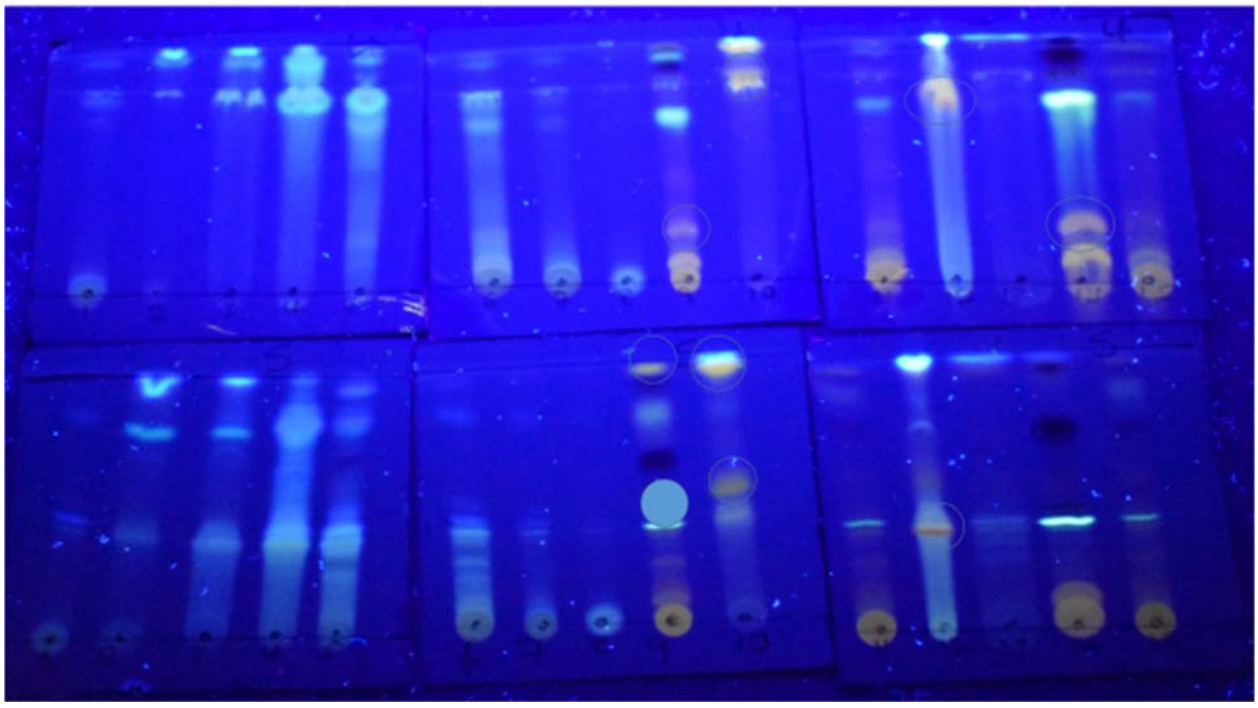


Figure 4.2: TLC plates for positive flavonoids. The plates were developed in CEF (32:4:4) mobile solvent stained with 1 % solution of aluminium chloride prepared in Ethanol. Circled is the fluorescing flavonoids conformational yellow bands viewed under long-wave ultraviolet light.

An example of some positive Alkaloids bands is shown in **Figure 4.3**. Samples separated well and the bands clearly shown. Some samples show orange as a confirmation of Alkaloids presence in the fraction.

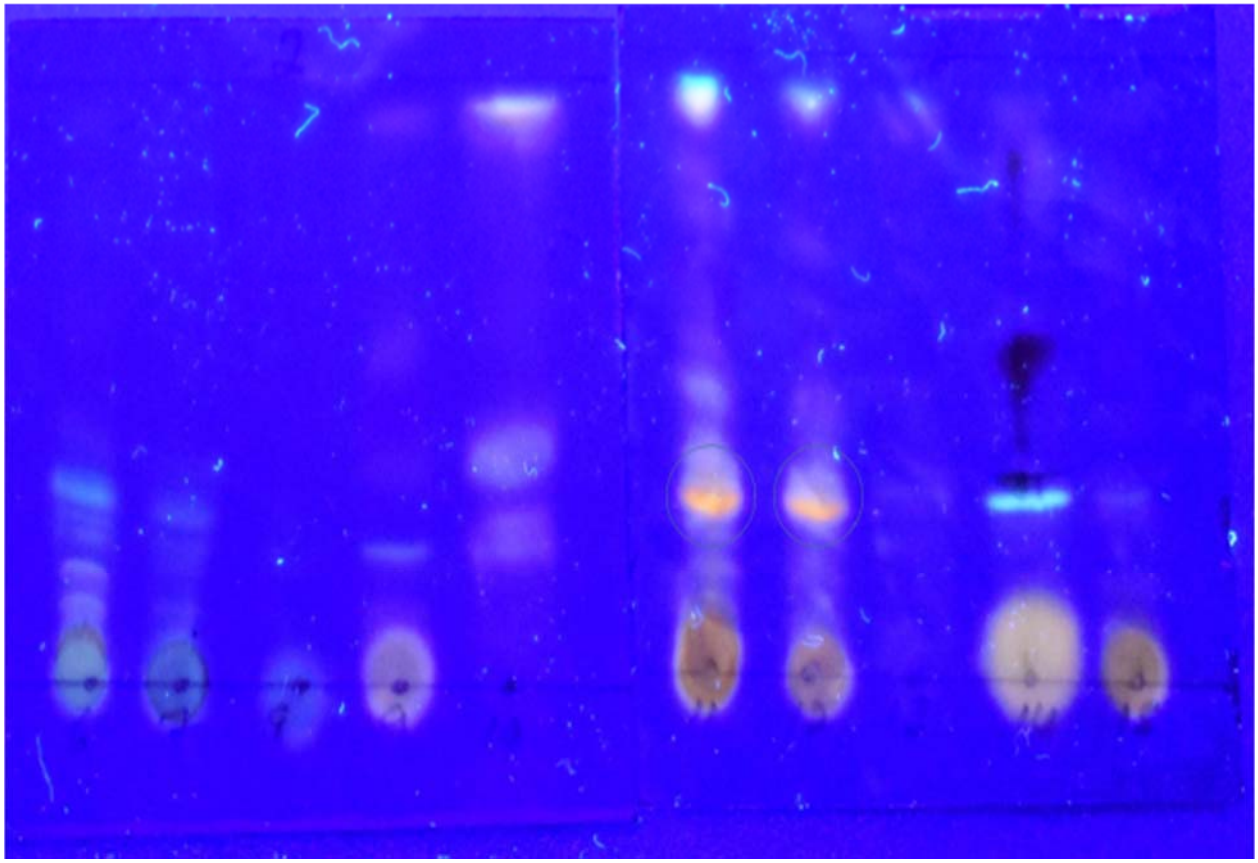


Figure 4.3: TLC plate for positive Alkaloids bands. The plates were developed in CEF (32:4:4) mobile solvent and stained with Wagner's reagents. Circled is the Orange bands conforming the presence of alkaloids viewed under long-wave ultraviolet light.

The presentation of all phytochemical TLC plates is reported in **Figure 4.4**. The reported spots have separated well and show a great deal of varying compounds in the samples being studied. *J. zeyheri* crude extract shows the presence of three types of phenolic compounds. These results were generated based on the change of color when the stains are sprayed on the chromatographs. Some stains needed a UV-light (like stain for Tannins) to be viewed whereas others could be observed directly.

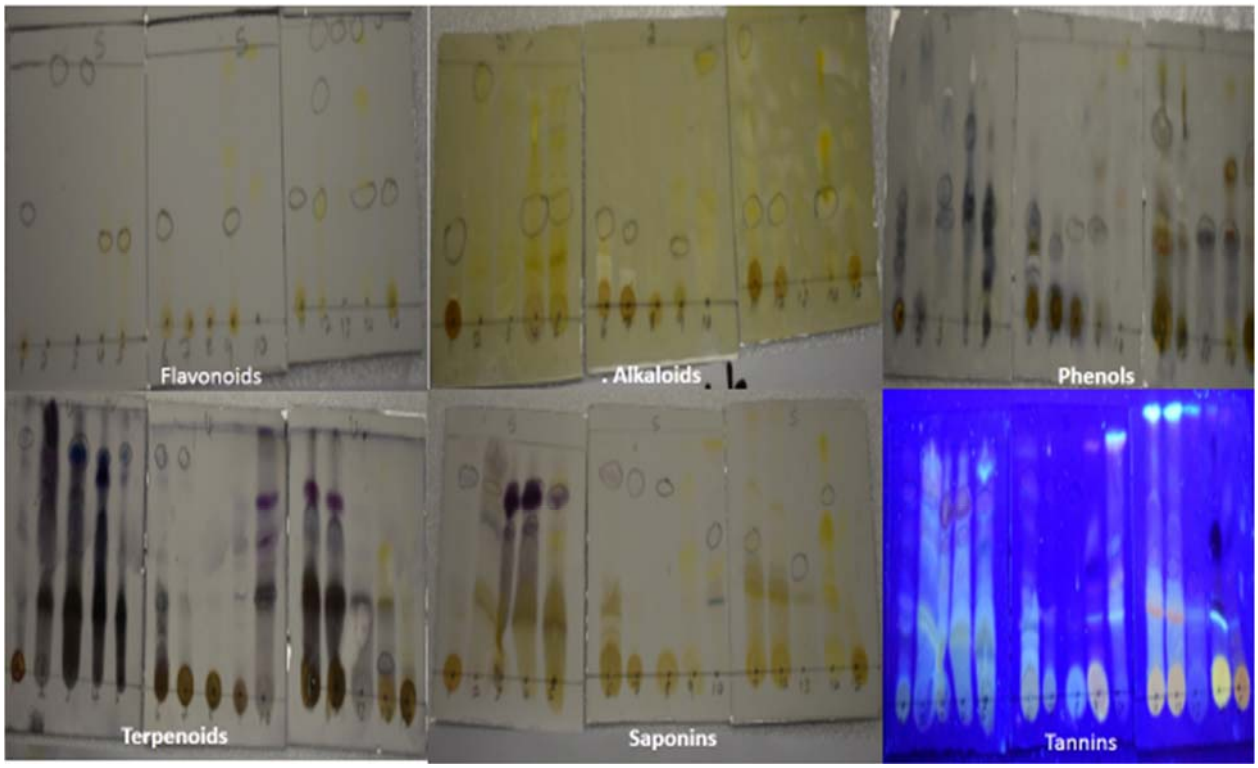


Figure 4.4: Presentation of phytochemical TLC plates developed on CEF (32:4:4) and stained with different stains as an assessment of phytochemicals. Some of the bands could be seen even without UV light, however with Tannins plates were viewed under the UV light long-wave for observation of the red bands.

4.4 CYTOTOXICITY OF PLANT EXTRACTS AND FRACTIONS

Cytotoxicity is defined as the ability of an agent to produce a toxic effect on a cell (Moghimi et al., 2005). Cytotoxicity assays are used to test the ability of cells to proliferate in the presence of a test compound or substance over a specific time period (Rampersad, 2012). Cytotoxicity testing is a popular method used for the screening of pharmaceutical products and synthetic organic compounds (Zanella et al., 2010). An important initial step when investigating possible new therapies or developing new compounds for the treatment of an ailment is the determination of their cytotoxic potential and harmful effects.

4.4.1 CYTOTOXICITY EVALUATION OF *J. ZEYHERI* AND *C. REHMANNI* CRUDE EXTRACTS

To investigate cytotoxicity of *J. zeyheri* and *C. rehmanni* plants, the crude extracts were tested against Vero T68 cell lines (BioAssaix; Nelson Mandela University). Live cells are stained with Hoecsht only while Dead cells are stained with Hoechst and PI. Two concentrations (25 and 100 µg/ml) of the crude extracts were used in this experiment.

All the extracts of *j. Zeyheri* showed an increase in toxicity as the sample concentration increases. *Jatropha zeyheri* crude extracts (**Figure 4.5**) shows a significant toxicity with 46.5% on higher concentration (100 µg/ml) with a statistical significance p <0.0002. *C. rehmanni* Crude extracts, however in contrast to *j. Zeyheri* only shows significance at higher concetration (100 µg/ml) with a p<0.0001. This is the evidence that *C. rehmanni is* less toxic compared to *J. Zeyheri.* As such it could be concluded that the extract had a noticeable cytotoxic effect on Vero cells line in test, although both plants shows a higher magnitude of toxicity.

The reason for such extreme toxicity could be due to the influence of the retained alcohol during rotary evaporation. The toxicity observed in this chapter is supported by the findings of Harun et al (2012) even though a different cell line was used where the potent cytotoxic effects of ethyl acetate extract on MCF-7 cells were demonstrated. Furthermore, another report also revealed that *J. zeyheri* aqua extract exhibited cytotoxic effect on Vero cell lines at a concentration of 90.27 µg/ml by 50% (Olajuyigbe

and Afolayan, 2012; Tshikalange et al., 2016). These previous research findings contradict the results obtained in this study, however, the difference could be due to the different extraction solvents used in each study.

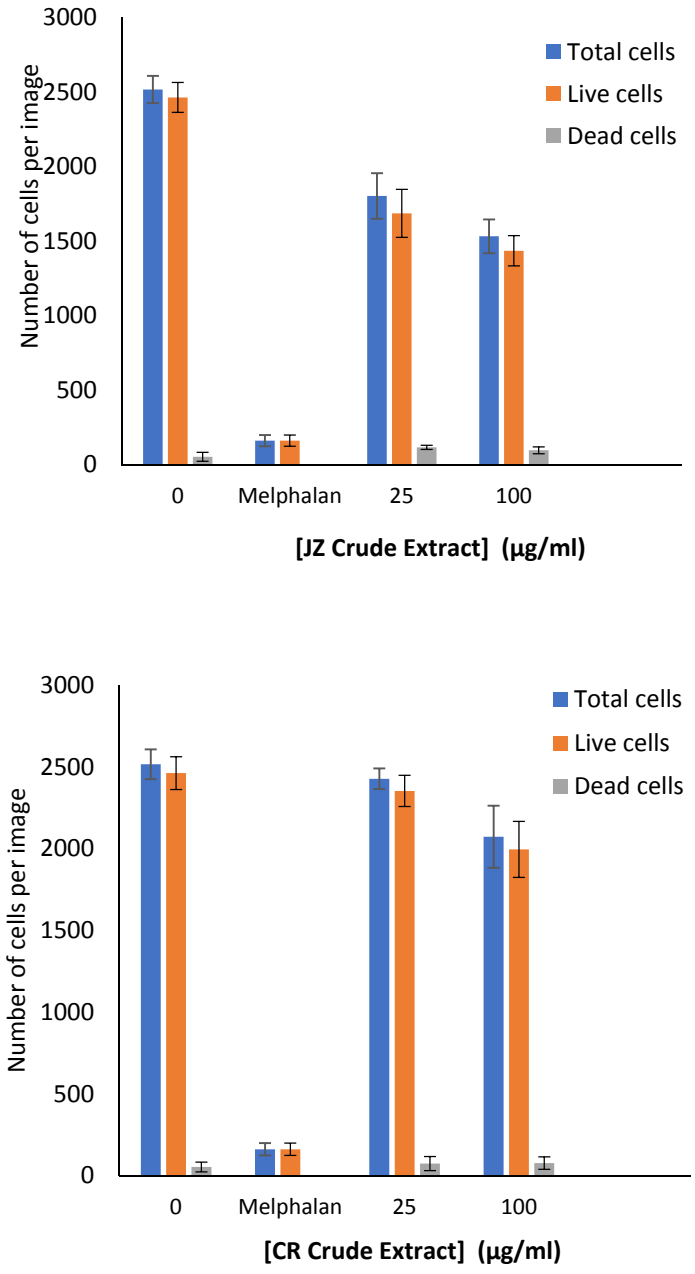


Figure 4.5: Cytotoxicity assessment of methanolic extarcts of *J. Zeyheri and C. Rehmani* on Vero T68 cells. The cells were treated with two concetrations (25 and 100 µg/ml) of plant extracts for 48 hrs. Melphalan was used as a possitive control for toxicity. Results were considered significant when P<0.05.

4.4.2 CYTOTOXICITY EVALUATION OF *J. ZEYHERI* FRACTIONS

This is the first study reporting on the cytotoxicity of *j. zeyheri* in fraction in fact this is by far the only study to fractionate the plants. The results show that all the fractions of *J. zeyheri* showed an increase in toxicity as the sample concentration increases. A gradual toxicity of fraction was observed to increase with the polarity of the fraction as fractions eluted with more polar solvent showed higher toxicity. All fractions of *J. zeyheri* showed an increase in toxicity as concentration increases.

Fractions jz1 and 2 **Figure 4.6** showed a lower toxicity that was displayed when the number of live cells decreased by 10 and 12 % respectively, with statistics that only come significant at 100 µg/ml. This is a minimal inhibition as compared to jz3 and 4 **Figure 4.6** as labelled that inhibited (25 and 31%) respectively, the significance ($p<0.01$) of these extracts is observed even at 25 µg/ml compares. Melphalan inhibited 94 % of the cells, this is a known chemical known to be toxic to cells. This proves that these fractions are toxic to the Vero cells. Jz4 toxicity on total cells was the most toxic and highly significant and had a p value <0.01.

From the observation jz4 was seen to be more toxic than all the fractions and the crude extracts. This magnitude toxicity of this fraction might have risen from the combination of the secondary metabolites. The higher concentration of compounds that are known to be toxic such as Tannins, Cardiac glycosides and terpenoids may explain the toxic nature of this fraction. Studies have demonstrated the toxicity nature of this compounds although it was from different plants and solvent of extraction (Tshikalange et al., 2016). A study reported that adverse effects of flavonoids may outweigh their beneficial ones, and the unborn foetus may be especially at risk, since flavonoids readily cross the placenta. (Skibola et al., 2000).

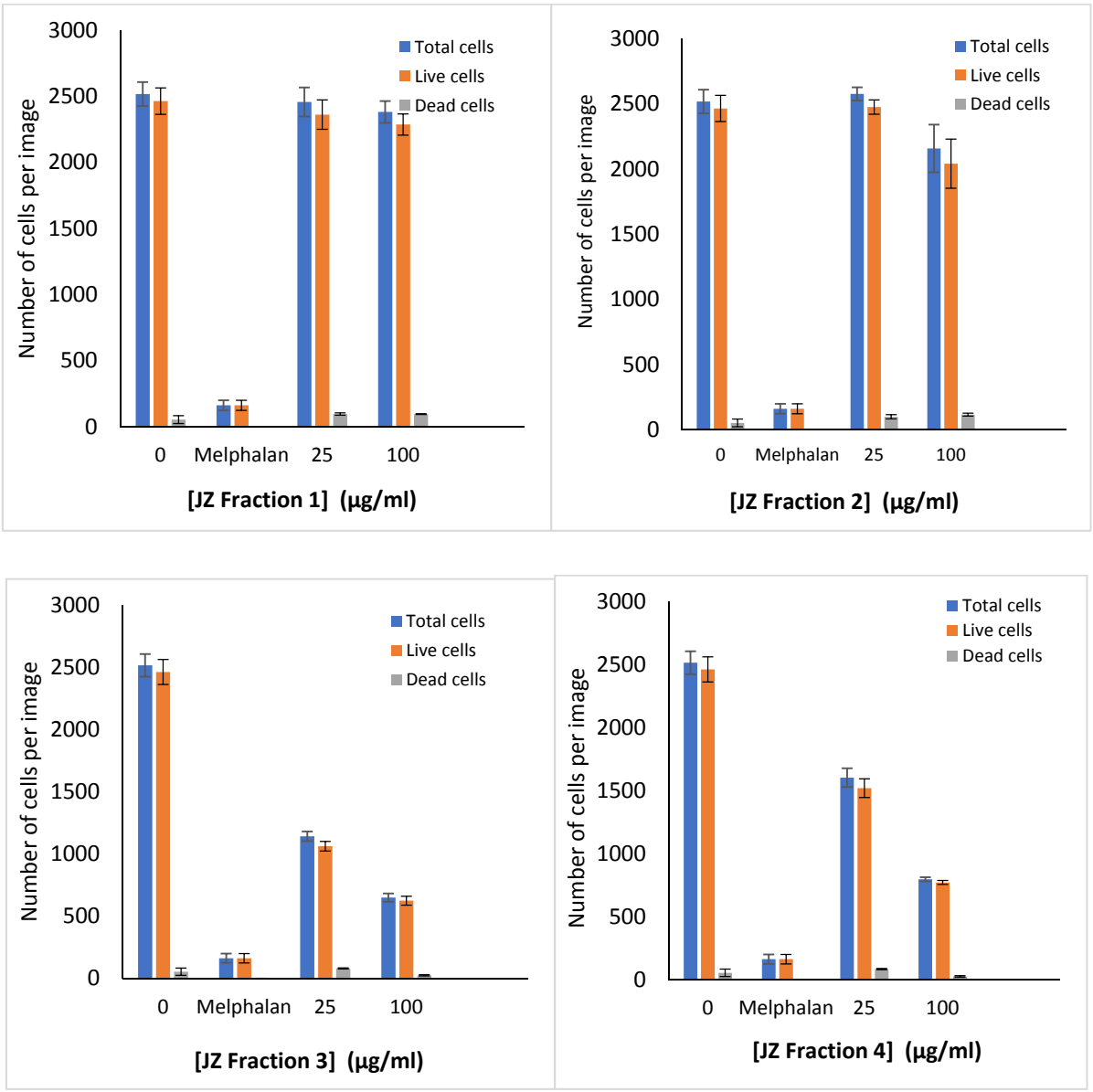


Figure 4.6: Cytotoxicity assessment of *J. Zeyheri* fractions on Vero T68 cells. The cells were treated with two concetrations (25 and 100 µg/ml) of plant extracts for 48 hrs. Melphalan was used as a possitive control for toxicity. Results were considered significant when P<0.05.

4.4.3 CYTOTOXICITY EVALUATION OF *C. REHMANNI* FRACTIONS

Fractions CR1 and 3 (**Figure 4.7**) showed a lower toxicity that was displayed when the number of live cells decreased by 49 and 24 % respectively, with a statistic significance p< 0.01. This is a minimal inhibition as compared to CR4 and 5 (**Figure 4.7**) as labelled that inhibited (66 and 78 %) respectively, with the statistical significance p<0.0001. Melphalan inhibited 94 % of the cells, this is a known chemical known to be toxic to cells. A negative control of untreated cell shows healthy and proliferating cells. This proves these fractions are toxic to the Vero cells. CR4 and 5 toxicity was too high too high and highly significant and had a p value <0.0001.

Similar observation to *J. zeyheri* fractions was made in *C. rehmanni* fractions the increase in toxicity was directly proportional to the increase in concentrations, also the fractions that were eluted with more polar solvent in column chromatography showed higher toxicity. However, *C. rehmanni* showed significance P<0.01 in its toxicity even at lower concentration at fractions cr1 ad 2. This could result from the extracts solvent residues (Methanol). This is the first study to report on toxicity of *C. rehmanni.*

The higher concentration phenols, Cardiac glycosides and terpenoids may explain the toxic nature of this fractions. Studies have demonstrated the toxicity nature of this compounds although it was from different plants and solvent of extraction (Tshikalange et al., 2016, and that higher doses effects of flavonoids may outweigh their beneficial ones, and the unborn foetus may be especially at risk, since flavonoids readily cross the placenta. (Skibola et al., 2000.)

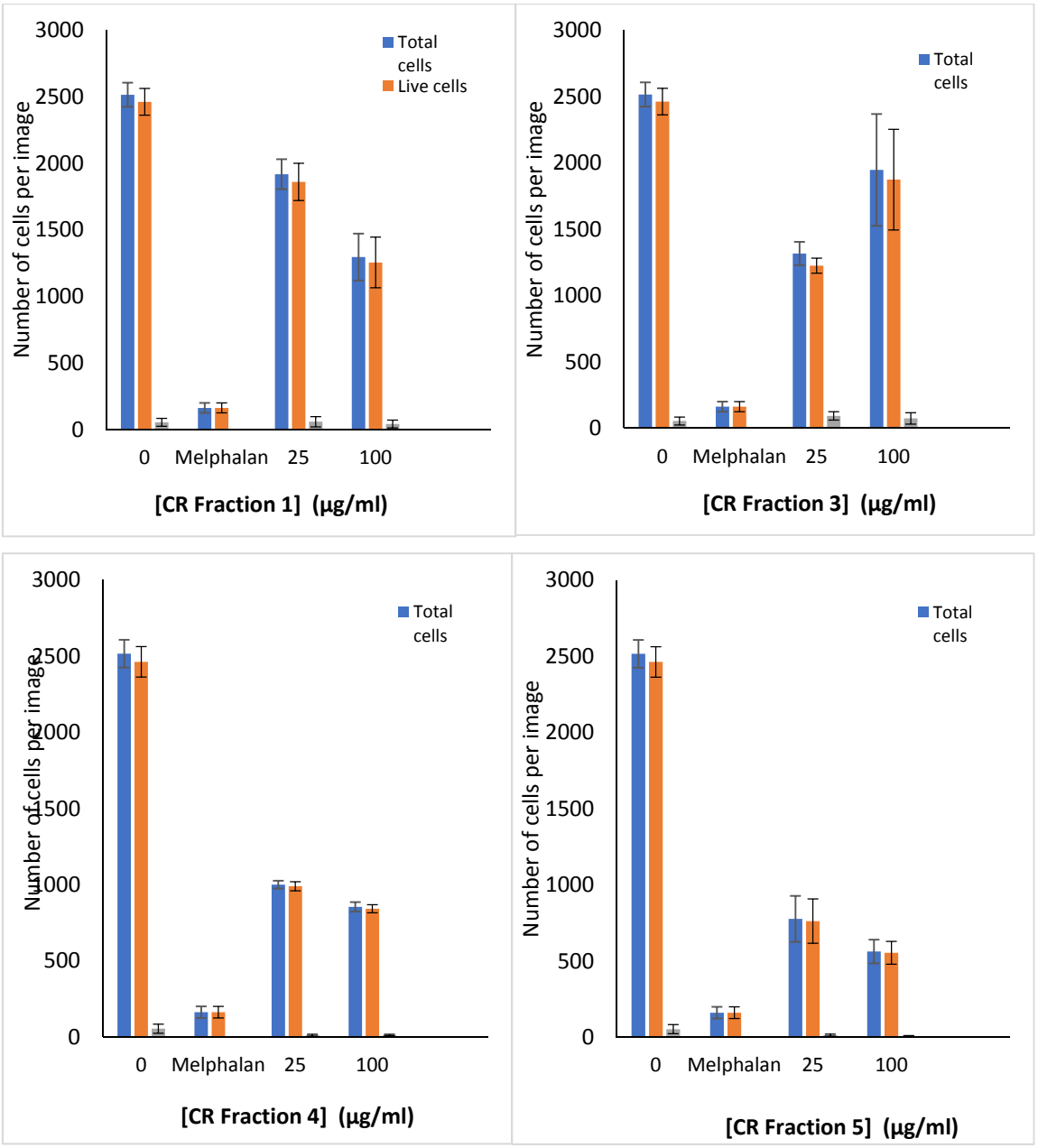


Figure 4.7: Cytotoxicity assessment of *C. Rehmani* fractions on Vero T68 cells. The cells were treated with two concentrations (25 and 100 µg/ml) of plant extracts for 48 hrs. Melphalan was used as a positive control for toxicity. Results were considered significant when $P<0.05$.

4.5 GENOTOXICITY OF PLANT EXTRACTS AND FRACTIONS

4.5.1 GENOTOXICITY ASSESSMENT J. ZEYHERI CRUDE EXTRACTS AND ITS FRACTIONS

The seeded cell counted was 3000 cells/well. *J. Zeyheri* crude extracts showed an increase in toxicity with increase in concentration. Cell density decreased 60% at the highest concentration as shown in **Figure 4.8** as for the genotoxicity, the crude extract also the crude extract also influenced or hinder the mitotic division thereby forming and increase in micro-nucleated cells by 14.7% with an increase in concentration as depicted in **Figure 4.8c.**

There was also an increase in the visibility of micro-nucleated cells with the increase in in concentration the relative cell count percentage decreased by 60% at 200 µg/ml (**Figure 4.8b**)**.** This proves that *J. zeyheri* crude extracts was indeed Genotoxic to Vero cell lines.

The genotoxicity of *J. Zeyheri* fraction 1 was not as severe as that of the crude extract (**Figure 4.9**), the relative cell count remained way above 60% at 200 µg/ml. the highest micro-cells percentage was at nucleated was at 25 µg/ml increasing at 50 and 100 µg/ml and increasing again at 200 µg/ml. however, when compared to the control group there wasn't much distance. Micro-nucleated was also recoded.

Fraction 2 of *J. Zeyheri* showed a significant cytotoxic effect at concentration 100 and 200 µg/ml (**Figure 4.10a)** the cell counts also decreased significantly (p= 0.0235 and 0.000) respectively. The fraction depicts toxicity at concentration 200 and no micronuclei or multinucleated cells formation at non-toxic concentration and therefore the *J. Zeyheri* fraction I concluded not to be genotoxic.

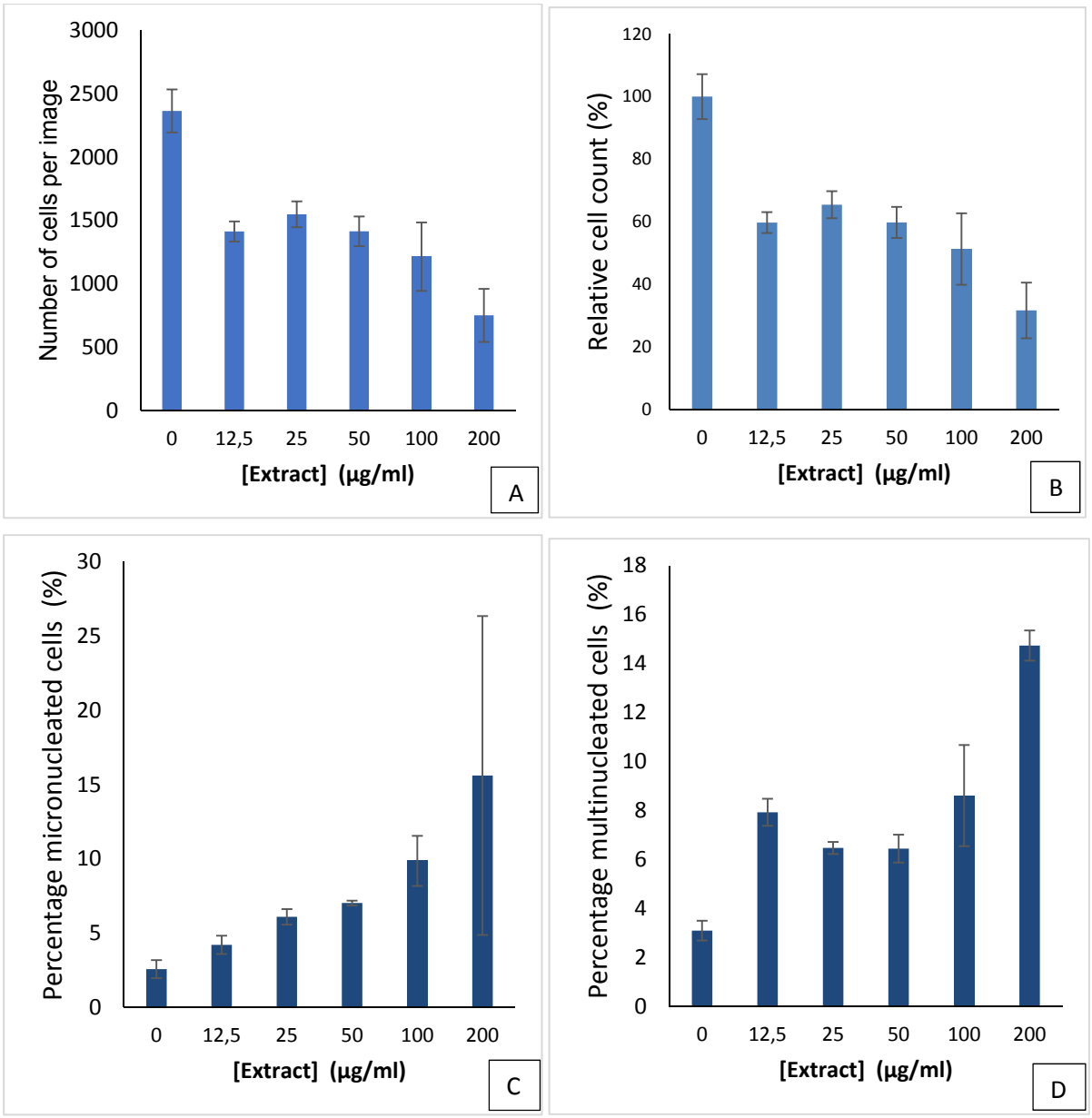


Figure 4.8: Comparative genotoxicity assessment of methanolic crude extracts of *J. zeyheri* plant. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05.

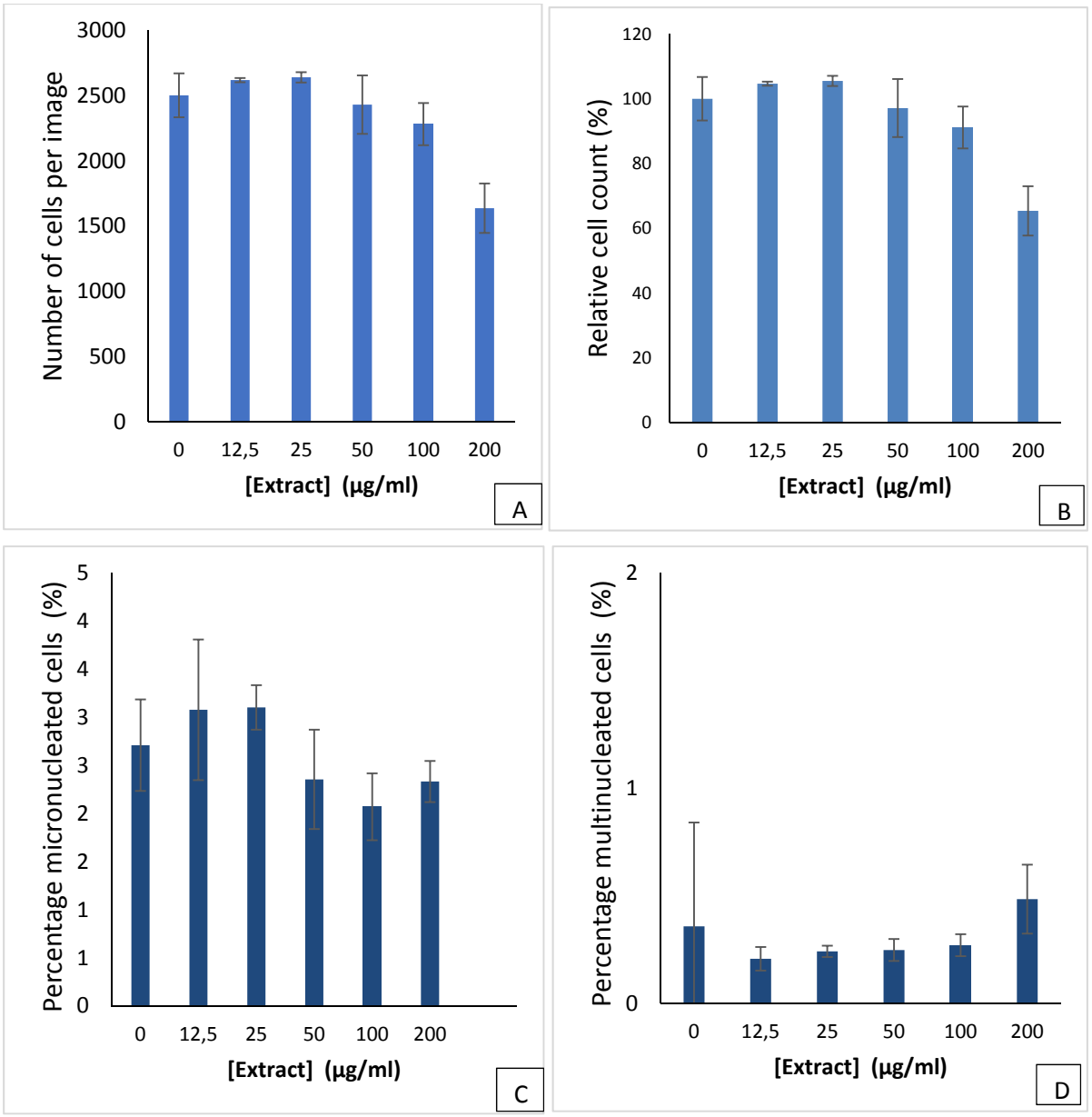


Figure 4.9: Comparative Genotoxicity assessment of methanolic fraction one of *J. zeyheri.* The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05.

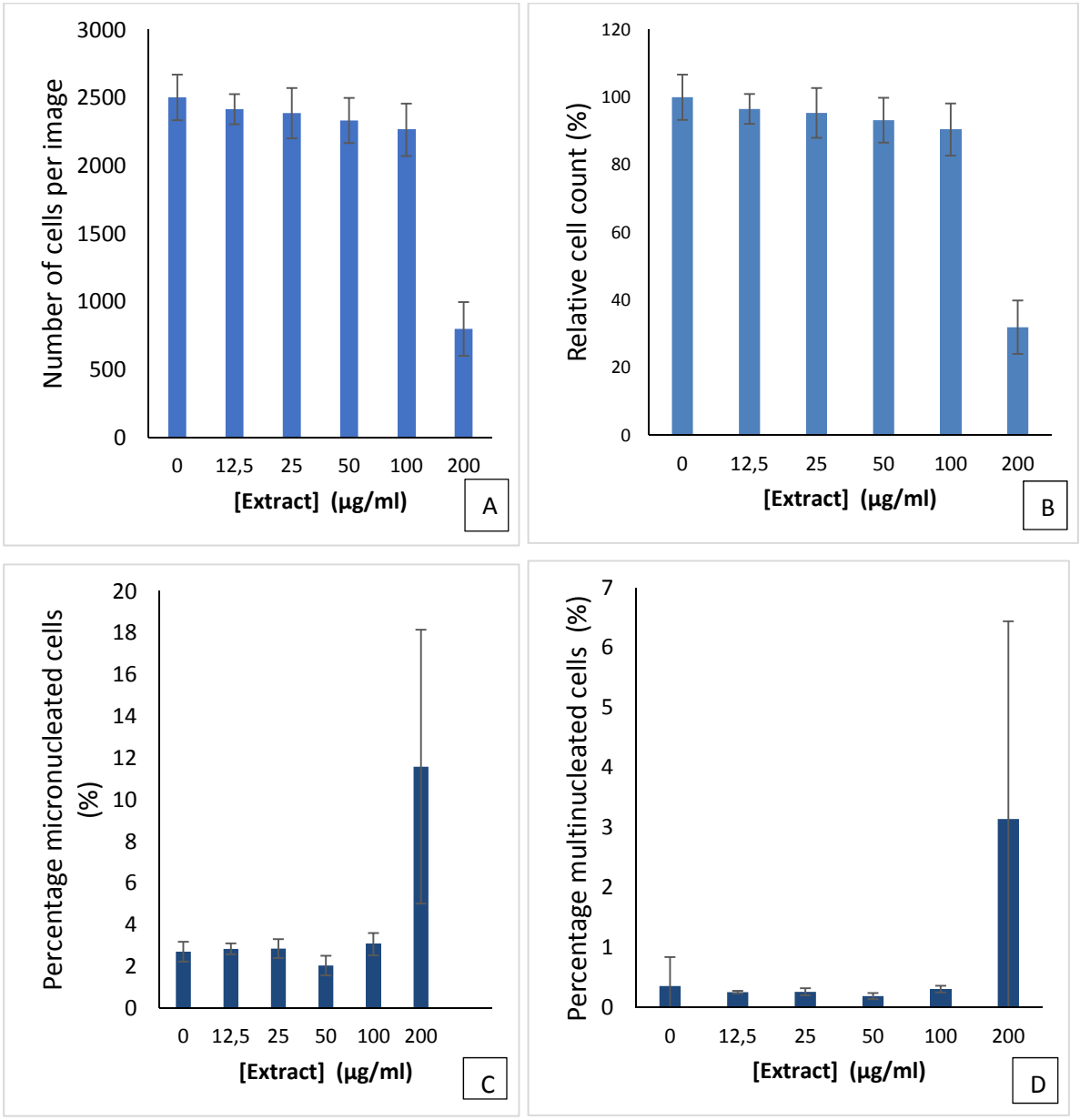


Figure 4.10: Comparative Genotoxicity assessment of methanolic fraction two of *J. zeyheri*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05.

Jatropha zeyheri fraction 3 was found to be genotoxic. The fraction shows a significant increase in toxicity with the increase in concentration the cell count decrease from 2400 up to below 500 cells at concentration 100 µg/ml the formation of micronuclei increased significantly from concentration 12.5 up to 100 µg/ml (from blow 5 % up to 10 %) (**Figure 4.11a, Figure 4.11c** and **Figure 4.11d**). The formation of multi-nucleated increased significantly up to 15 % at concentration 100, and shows a decrease at 200 µg/ml.

Jatropha zeyheri extract fraction 4 shows a significant increase toxicity, the relative cell count decreased from 2400 down to below 500 cells (**Figure 4.12a**). The formation of micronuclei increased significantly from 2 % in control well up to 11 % at concentration 25 µg/ml while, concentration 50 and 100 µg/ml shows higher percentage of micronuclei **Figure 4.12c**. The formation of multi nucleated cells increased significantly from 25 % of the control cells u to 25% at 50 µg/ml concentration. In this fraction it was found that the fraction is very toxic, the relative cell count was < 50 % at lowest concentration 12.5 (**Figure 4.12b**), therefore the genotoxicity of this fraction cannot be concluded. However, it can be said that the fraction was found to be very toxic.

The evidence deduced from the evidence of this study herin proves that *J. Zeyheri plant* is genotoxic to the vero cell lines. Although the genotoxicity of fraction 4 could not be concluded due to the high toxic nature. *J. Zeyheri* fraction 2 was found not to be genotoxic. Numerous studies have reported that some tannin components such as gallic acid or ellagic acid can inhibit the mutagenicity of certain mutagens Wood et al., 1982 this could be the case in this study as it has been found tha this fraction contained high content of Tannins. However, in evaluating the antimutagenic activity of many plant phenolics, inhibitory effect on the growth of test organisms have also been reported (De Flora et al., 1992; Mortelmans and Zeiger, 2000). The present study demostrated that different composition of compounds amoungst fractions of *J. zeyhri* can either be cacinogen or toxic to Vero cell lines.

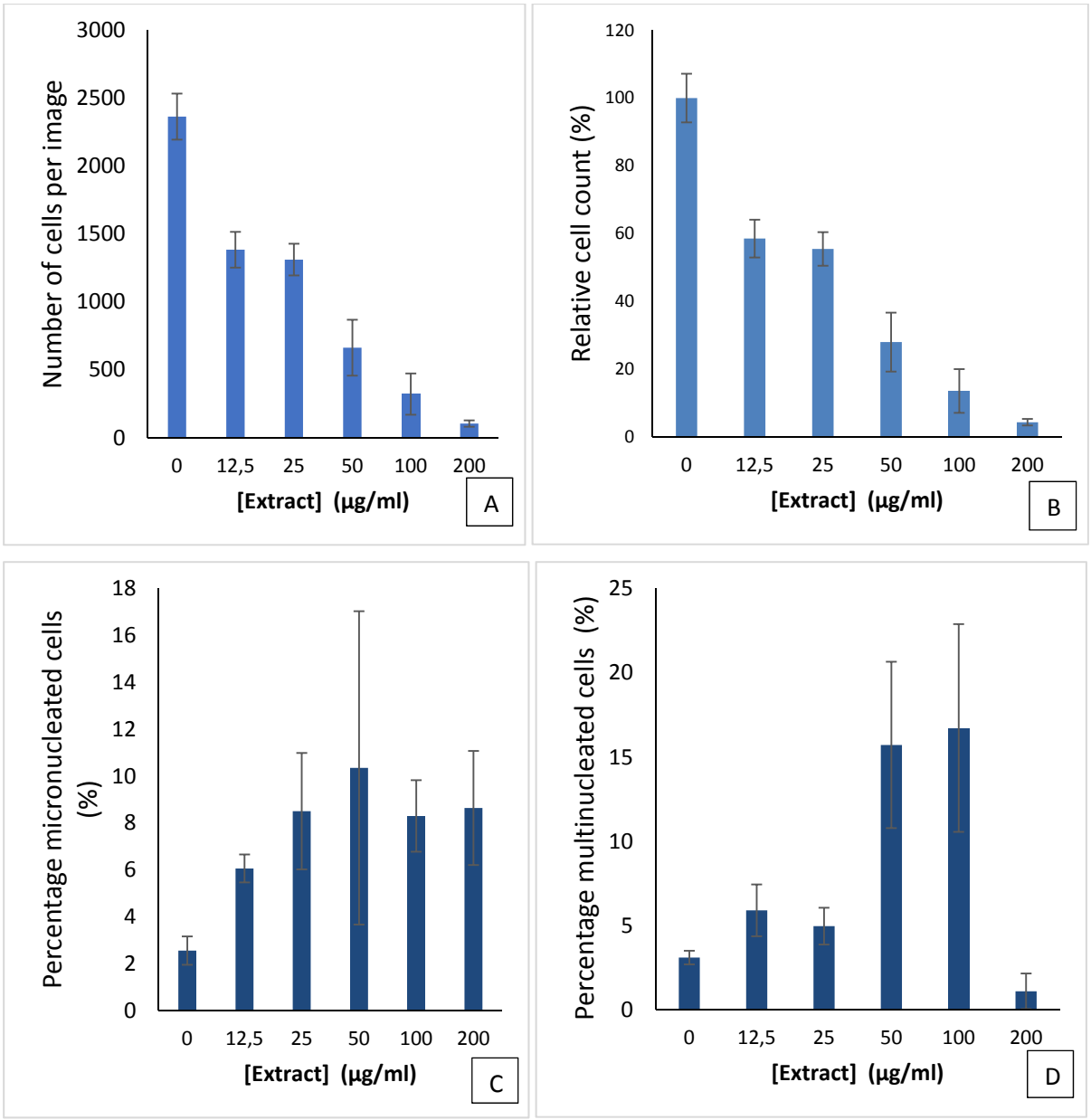


Figure 4.11: Comparative Genotoxicity assessment of methanolic fraction three of *J. zeyheri*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is $P<0.05$.

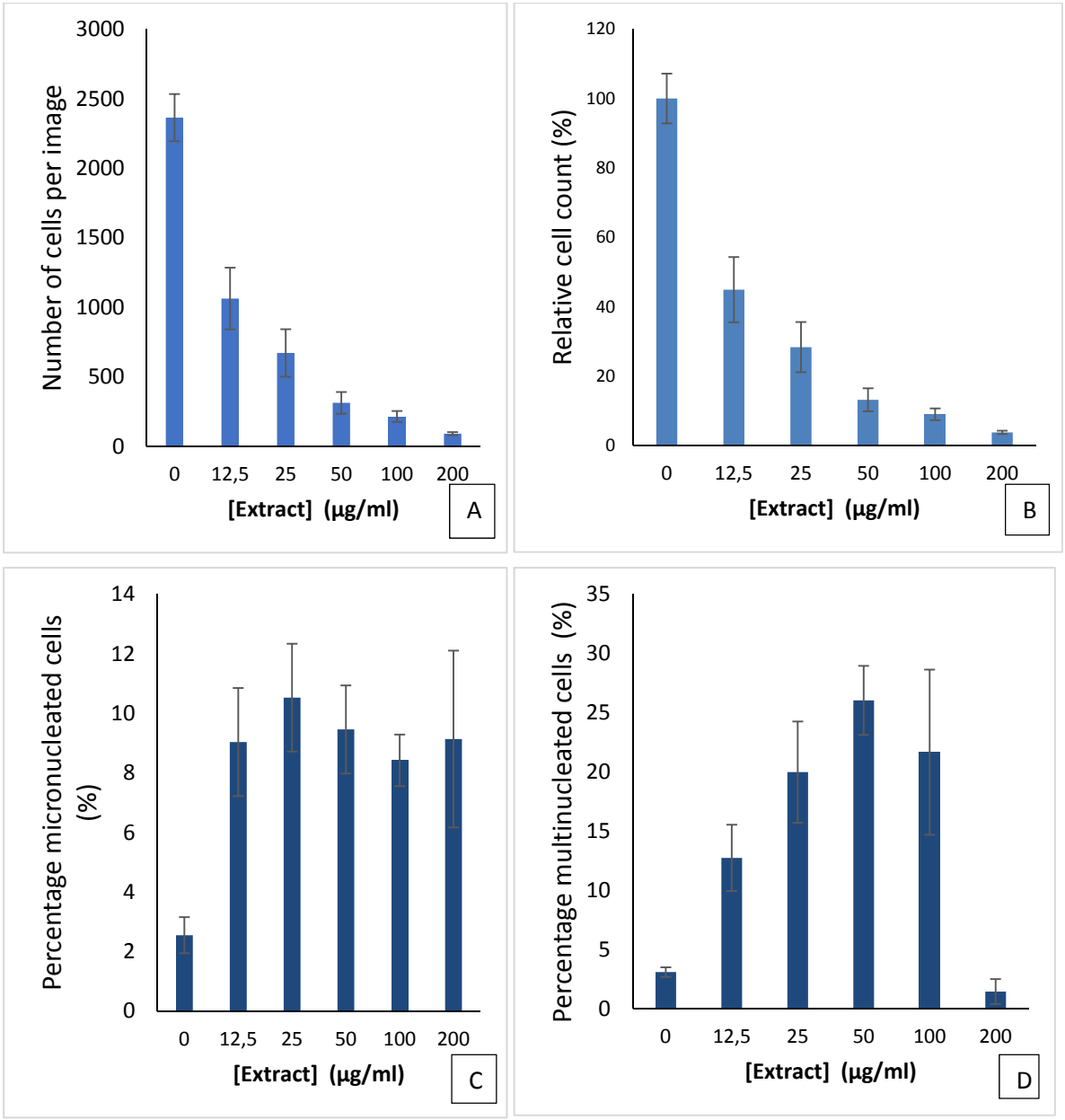


Figure 4.12: Comparative Genotoxicity assessment of methanolic fraction four of *J. zeyheri*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.005

4.5.2 GENOTOXICITY ASSESSMENT *C. REHMANNI* CRUDE EXTRACTS AND ITS FRACTIONS

The toxicity of *C. rehmanni* crude extracts increased with an increase in concetration, however the only significant toxicity was found on 50, 100 and 200 µg/ml. the number of cells remained above half of the of the half of the total cells and cell count eve In higher concetration (**Figure 4.13**). The formation of micronuclei increased significantly from lowest concetration compares to the control cells (**Figure 4.13c**).

While the formation of the multinucleated cells increased from the second concetration and significacy increases as the an increase in concetration. This *C. rehmanni* crude extracts proves to be genotoxic but with lower concetration as compares to that of ***J. zeyheri***.

The toxicity of CR1 increaed as the increase with a strong significance as the concetration increases. Number of cells per well decreased from 2800 on the nagative control to 500 cells/well at concetration 200 µg/ml (**Figure 4.14a**). Formation of the micronuclei shows a significance at concetration 100 and 200 µg/ml although remained below 2 % (**Figure 4.14c**). A significant gradual increase was observed in the formation of multi nucleated cells (**Figure 4.14d**) up to 12 % at concetration 200 µg/ml.

Cytotoxicity in Fraction 3 increased gradually with increase with significance ranging from (0.0004 to 0.000) from the lowest concentration 12.5 to 200 µg/ml respectively (**Figure 4.15**), the micro nuclei also increase compares to the negative control cells by 4.5 % with a significant value. The number of multinucleated cells had significance only on higher concentration (100 and 200 µg/ml), as such this fraction can be said to have a lower genotoxic effect.

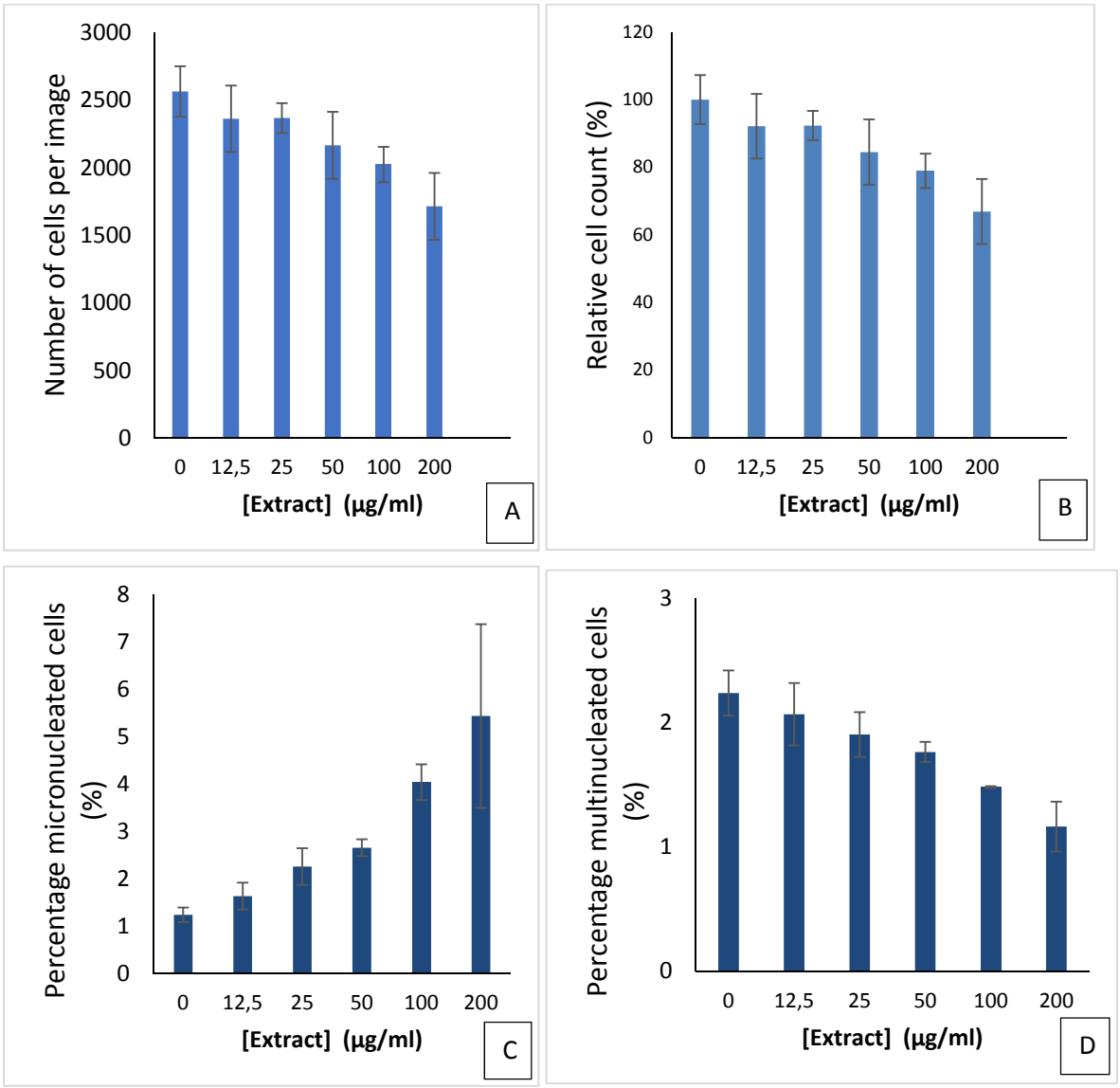


Figure 4.13: Comparative genotoxicity assessment of methanolic crude extracts of *C. rehmanni* plant. The test was done in five concentrations (12.5, 25, 50, 100 and 200) on (A) number of cells per image (B) relative cell counts (c) percentage micro-nucleated **(D)** percentage multi-nucleated. P value indicating significance is P<0.05

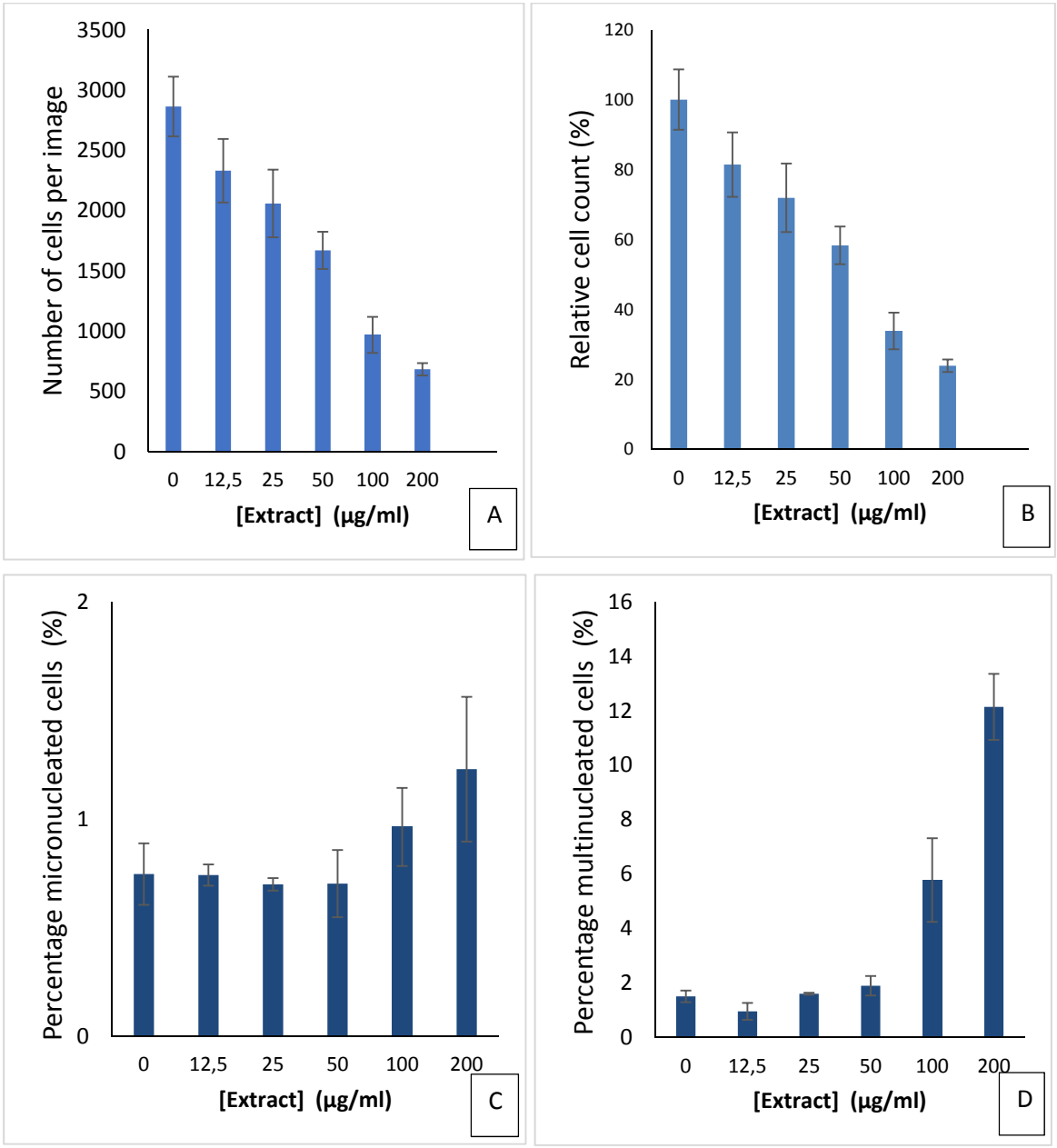


Figure 4.14: Comparative Genotoxicity assessment of methanolic fraction one of *C. rehmanni.* The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05

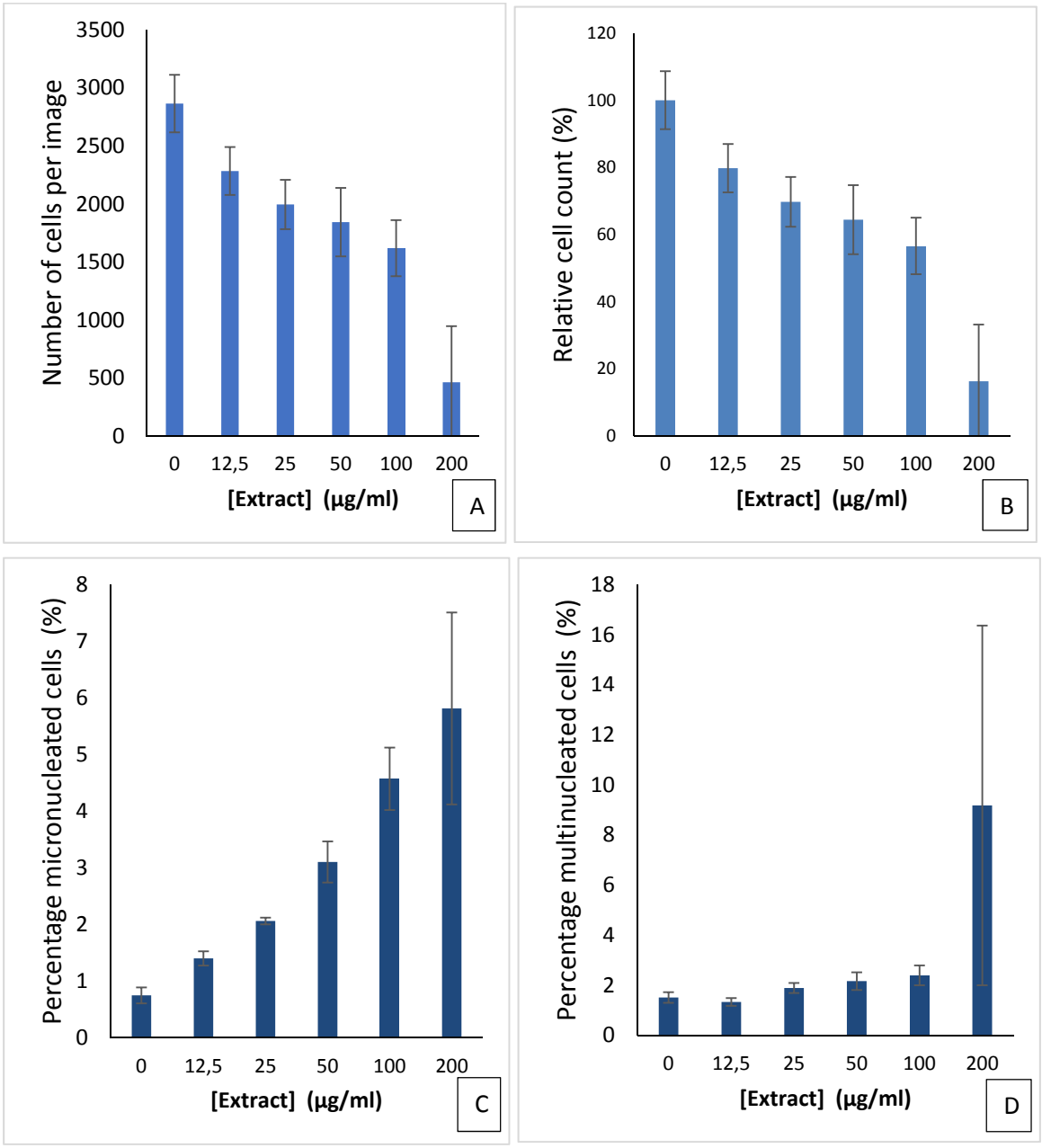


Figure 4.15: Comparative Genotoxicity assessment of methanolic fraction three of *C. rehmanni*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05

Coccinia rehmanni fraction 4 exhibit a huge decrease in number of cells, in relative cell count and number of cells (**Figure 4.16b**) goes below <50% even in the lowest concentration. The fraction shows a great deal of formation of multinucleated cells that range from 4 to 8 %, the percentage of the multi nucleated cells increased by 18 % at the third concentration. However due to the highly decreased number of the cells even in the lowest concentration, the genotoxicity of this fraction cannot be concluded. It can only be said that the fraction toxicity is very high. In a toxic sample like this one, apoptotic nuclei may be mistaken for micronuclei by the software

Coccinia rehmanni fraction 5 exhibit a huge decrease in number of cells, in relative cell count (**Figure 4.17b**) and number of cells (**Figure 4.17a**) goes below <50% even in the lowest concentration. The fraction shows a great deal of formation of multinucleated cells that range from 4 to 8 %, the percentage of the multi nucleated cells increased by 18 % at the third concentration. However due to the highly decreased number of the cells even in the lowest concentration, the genotoxicity of this fraction cannot be concluded. It can only be said that the fraction toxicity is very high. In a toxic sample like this one, apoptotic nuclei may be mistaken for micronuclei by the software

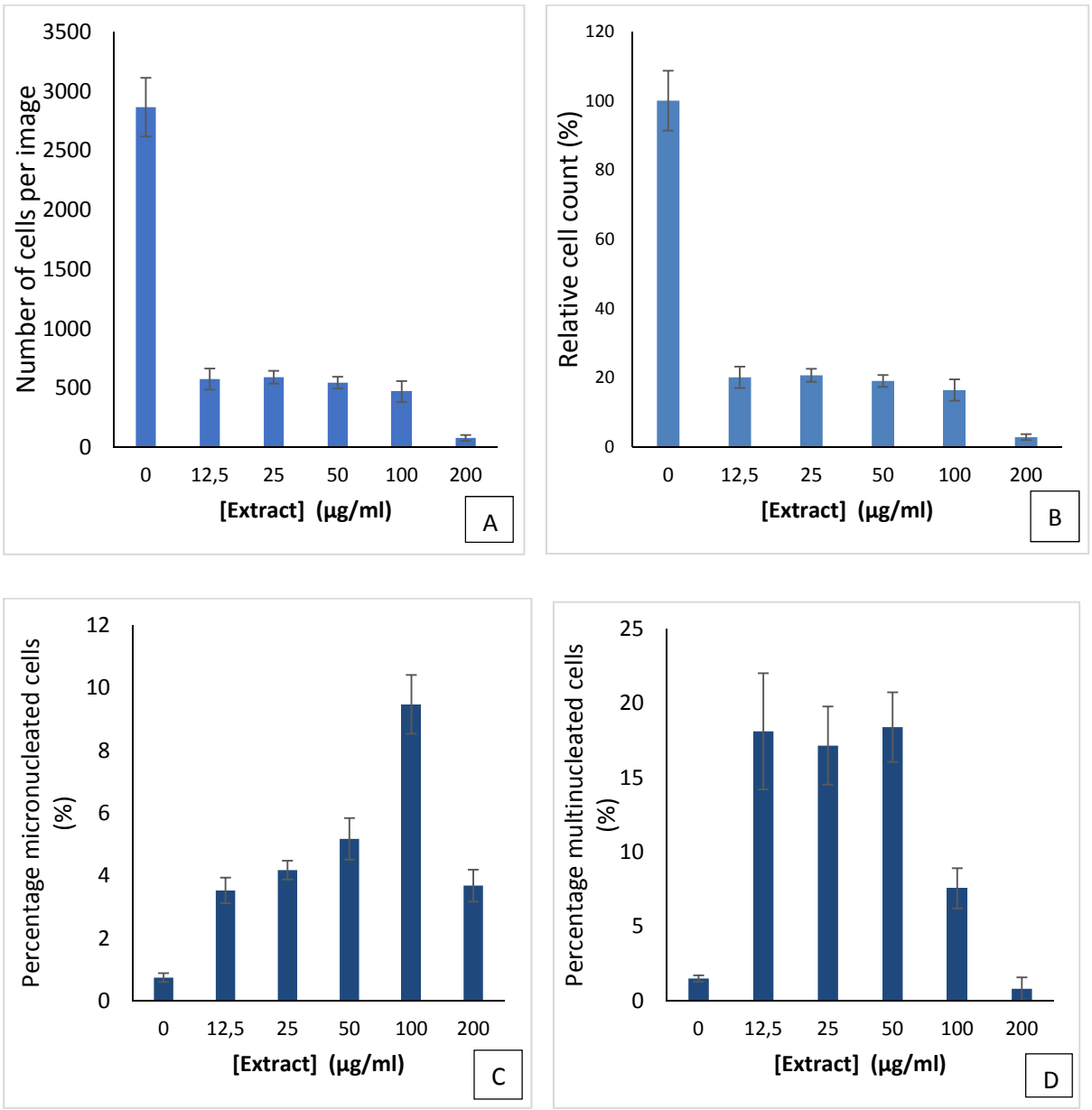


Figure 4.16: Comparative Genotoxicity assessment of methanolic fraction four of *C. rehmanni*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05

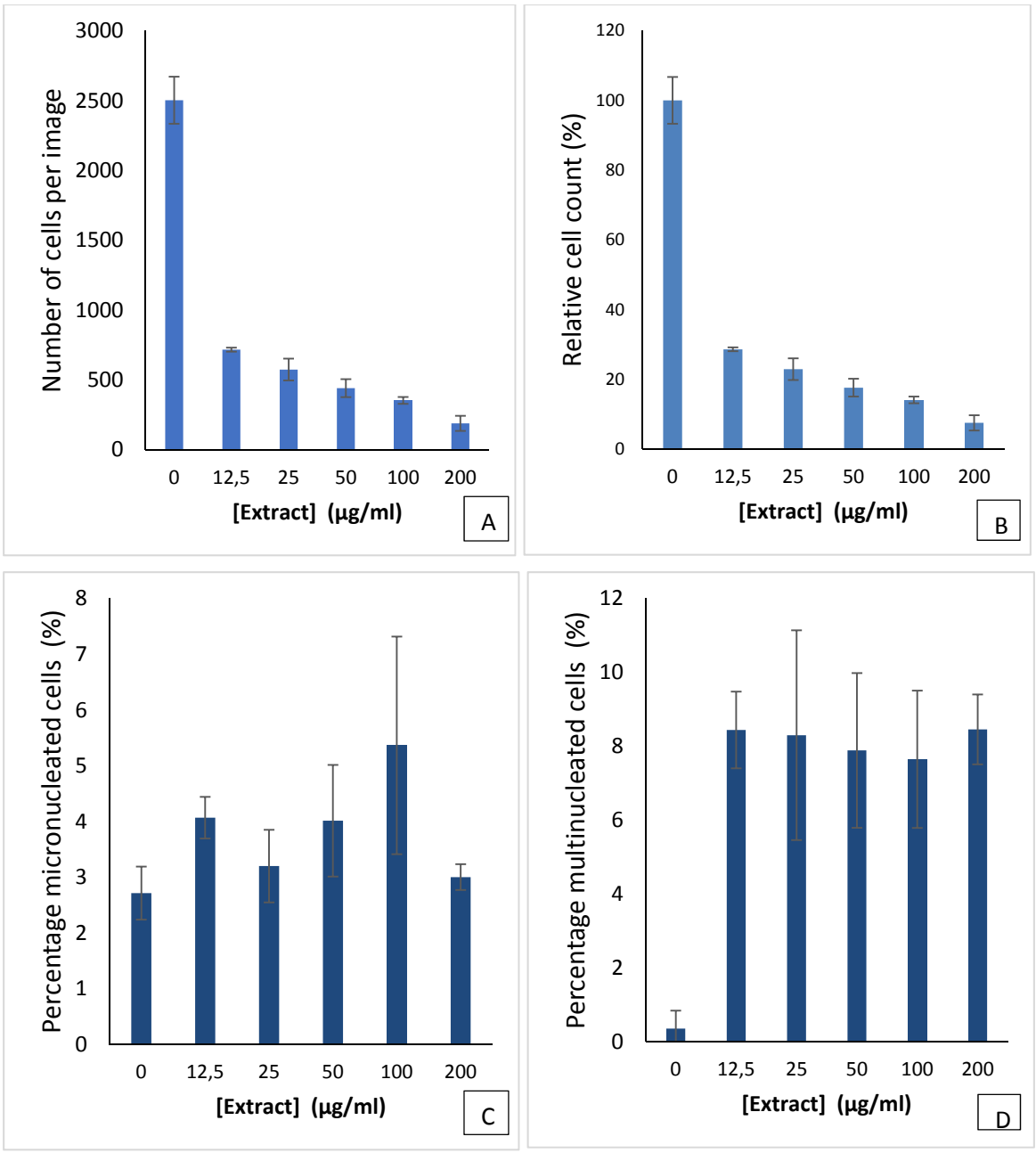


Figure 4.17: Comparative Genotoxicity assessment of methanolic fraction five of *C. rehmanni*. The test was done in five concentrations (12.5, 25, 50, 100 and 200 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is $P<0.05$.

Below, are presentations (**Figures 4.18 – 4.20**) of all negative controls; Griseofulvin, a known genotoxic chemical.

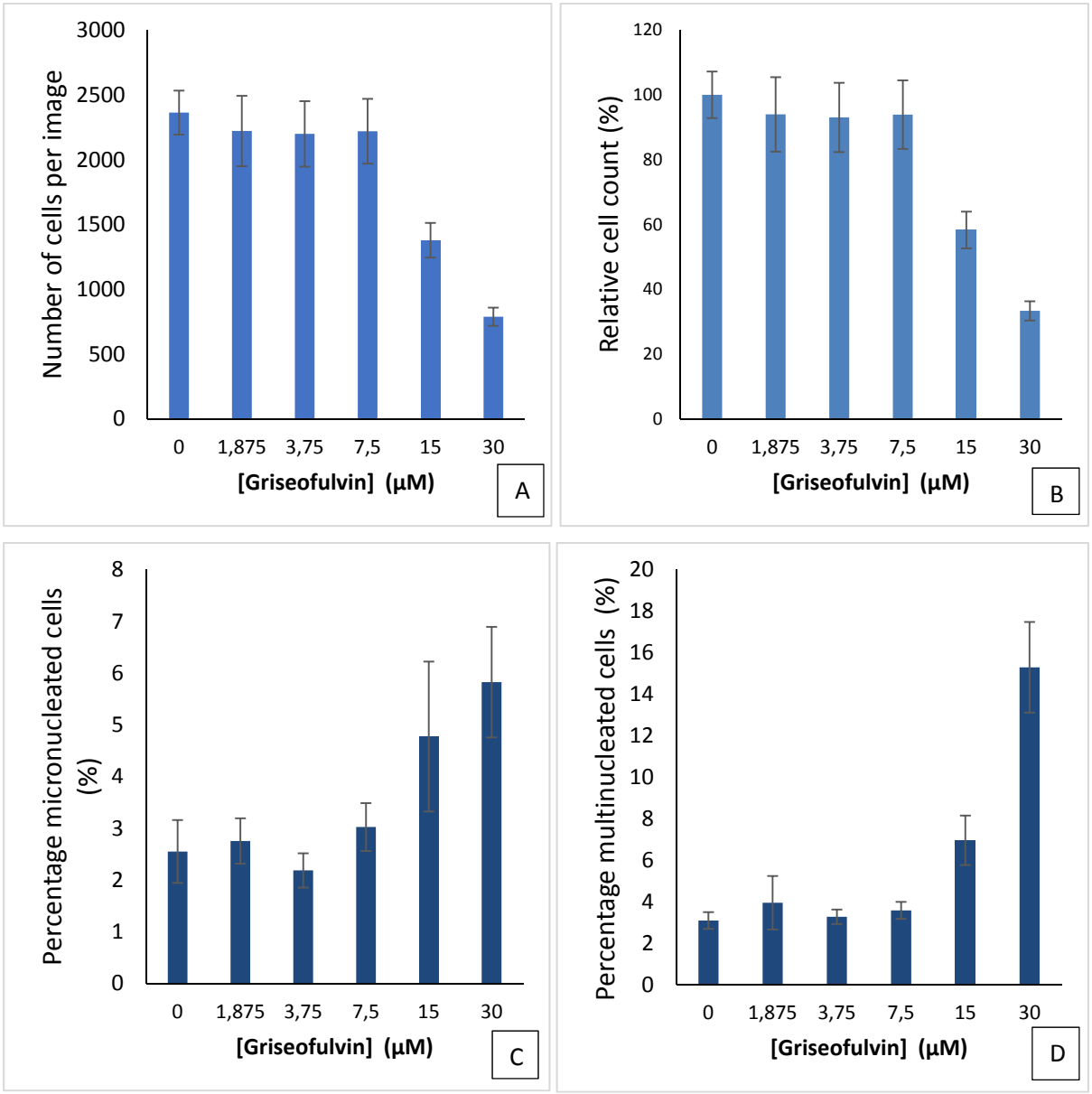


Figure 4.18: Comparative Genotoxicity assessment griseofulvin 1 as a positive control. The test was done in five concentrations (1.875, 3.85, 7.5 15 and 30 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05.

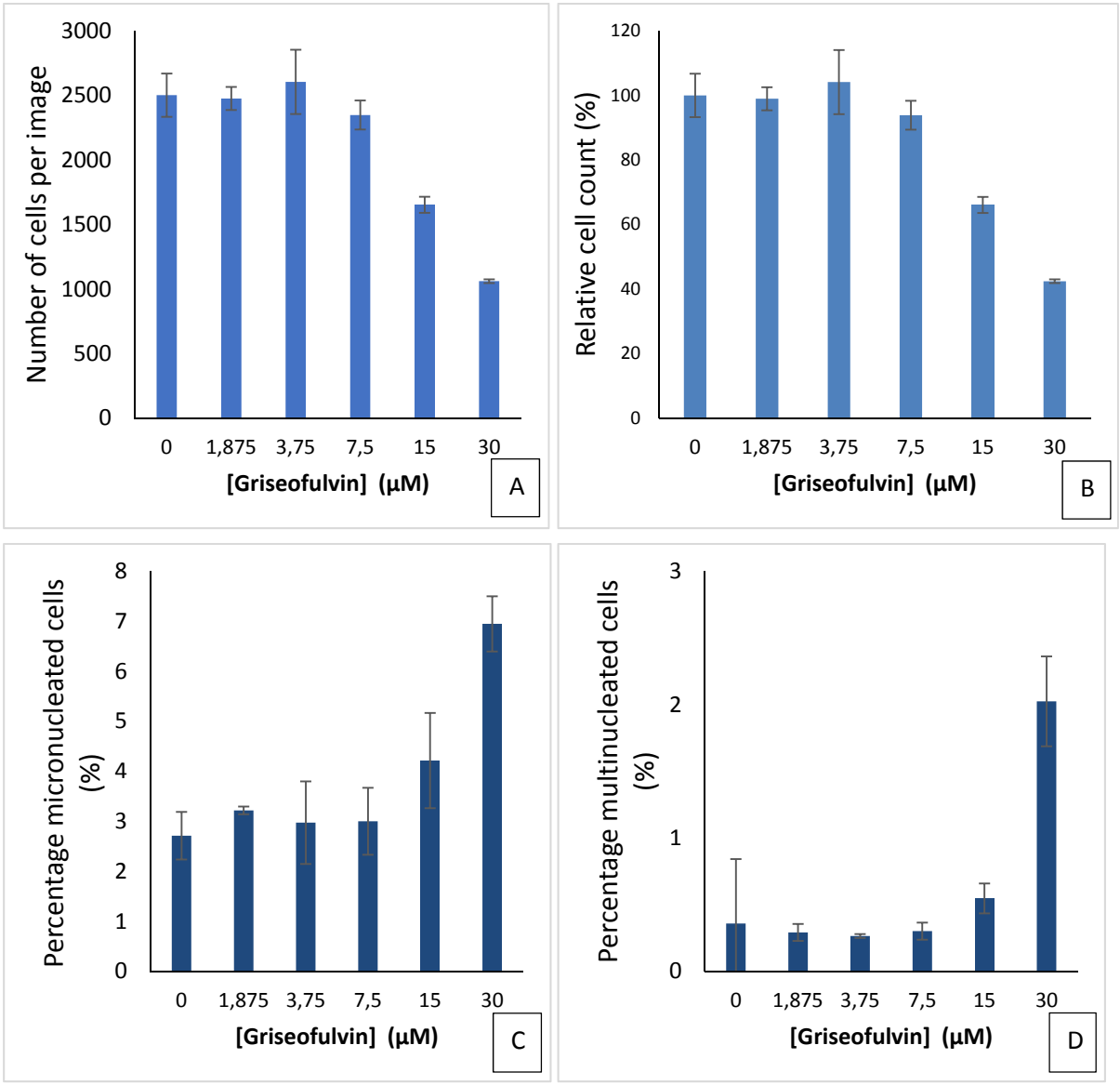


Figure 4.19: Comparative Genotoxicity assessment griseofulvin 2 as a positive control. The test was done in five concentrations (1.875, 3.85, 7.5 15 and 30 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.05.

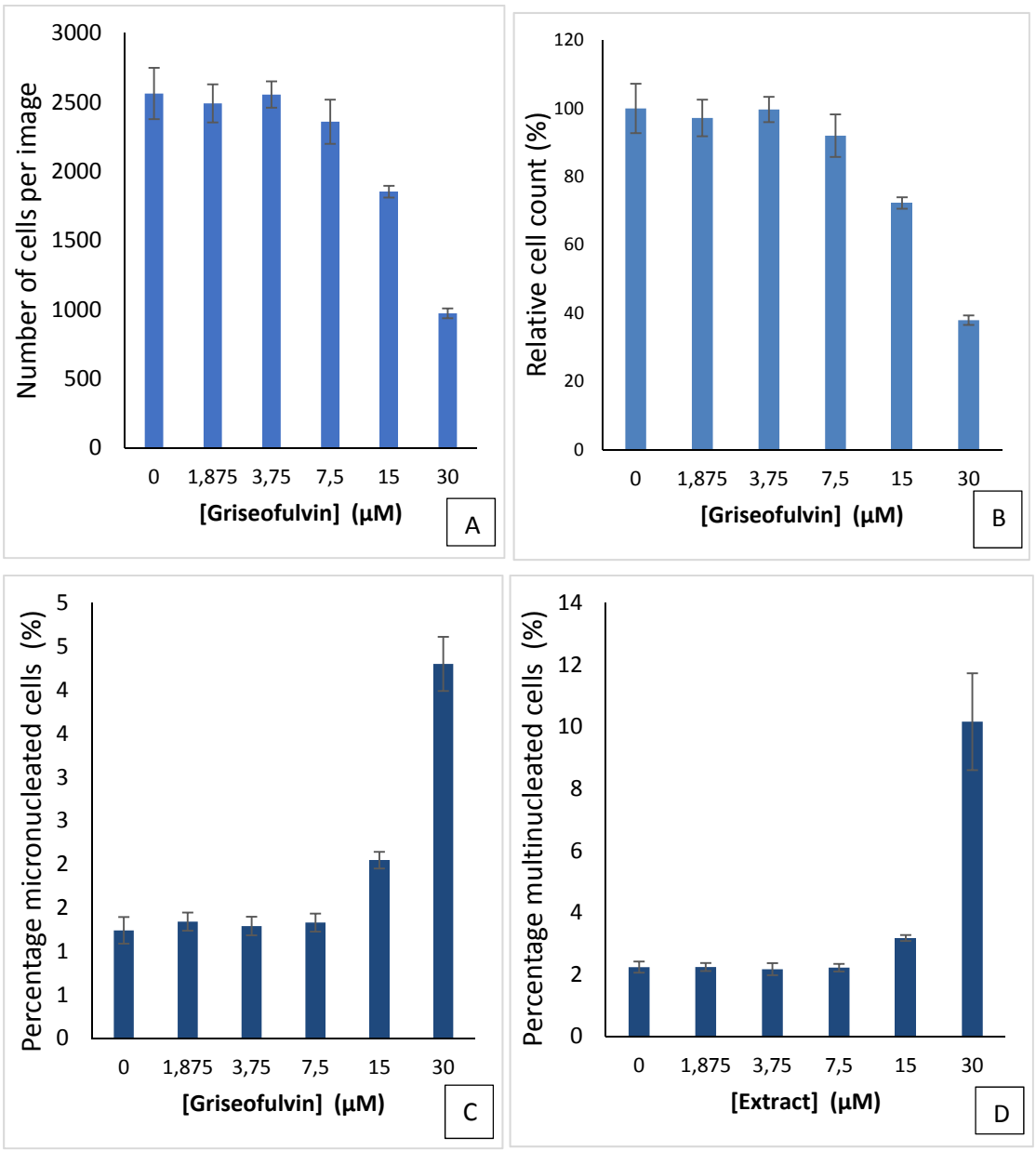


Figure 4.20: Comparative Genotoxicity assessment griseofulvin 3 as a positive control. The test was done in five concentrations (1.875, 3.85, 7.5 15 and 30 µg/ml) on **(A)** number of cells per image **(B)** relative cell counts **(c)** percentage micro-nucleated cells **(D)** percentage multi-nucleated. P value indicating significance is P<0.005

4.6 HARP COLORIMETRIC ASSAY

Crude extracts and fractions from *J. Zeyheri* and *C. Rehmanni* were tested for their ability to inhibit PARP-1 using a colorimetric assay kit at 2 different concentrations (50 µg/ml and 100 µg/ml). **Table 4.7** shows the OD values for a well-known inhibitor, 3-AB. There is a correlation between the concentration of the inhibitor and the OD values. The absorbance values decrease as the concentration of the inhibitor increases. Indeed, the more concentrated the inhibitor is, the stronger the inhibition seen as a decrease in OD values.

Table 4.7: OD values of PARP inhibition activity by 3-aminobenzamide (3-AB)

	3-aminobenzamide	
	Mean	St Dev
100 µg/ml	0.18	0.022
50 µg/ml	0.18	0.003
25 µg/ml	0.18	0.201
12.5 µg/ml	0.37	0.462
6.25 µg/ml	**1.59**	**0.031**
3.13 µg/ml	2.26	0.259

The results of the tested crude extracts and their fractions (**Table 4.8**), indicated that only Fractions 4 of the extracts at both concentrations tested show inhibitory potency close to the know control inhibitor (3-AB) at 6.25 mM In terms of their mean ± SD Absorbance values, fraction 4 of *J. Zeyheri* at 50 µg/ml was 80 % potent inhibitor as 3-AB while the same fraction at 100 µg/ml was 85 % as potent inhibitor as 3-AB.

Fraction 4 of *C. Rehmanni* at 50 µg/ml was 80 % potent inhibitor as 3-AB while the same fraction at 100 µg/ml was only 81 % potent inhibitor as 3-AB. Here the concentration does not play a role in the inhibition of PARP1. The crude extracts of *C Rehmanni* show inhibitory potential to the level of 3-AB at 3.13 µg/ml.

Table 4.8: OD values of PARP inhibition assay by *J. Zeyheri* and *C. Rehmanni*

	J. Zeyheri		*C. Rehmanni*	
	[JZ] at 50 µl/ml	**[JZ] at 100 µl/ml**	**[CR] at 50 µl/ml**	**[CR] at 100 µl/ml**
Fraction 1	2.87	2.88	2.99	3.01
Fraction 2	2.89	2.89	3.09	2.86
Fraction 3	2.95	2.87	2.27	2.67
Fraction 4	**1.99**	**1.87**	**1.99**	**1.96**
Fraction 5	2.67	2.99	2.68	2.91
Fraction 6	3.12	3.20	2.97	3.10
Crude extracts	2.89	3.04	**2.11**	2.29
Control inhibitor (3-AB)	1.59	1.59	1.59	1.59

The findings described in the present study have demonstrated that extracts of *C. Rehmanni* and *J. Zeyheri* to be very toxic to cells seen as a decrease in more than 50 % of viable cells. This toxicity leads to cell death by apoptosis or necrosis. It is reported that apoptosis and necrosis are directly triggered by excess level of Reactive oxide species (Al-Khayal, 2017). Furthermore, high level of ROS also promotes genotoxicity. Poly (ADP ribose) polymerase, or PARP, is required during the repair of damaged DNA (Gohlke et al., 2015). When DNA is exposed to toxic compounds, DNA breaks occur leading to structural damage. PARP recognizes these defects by binding to NAD+, and starts the synthesis of poly ADP ribose chains (Pandya et al., 2010). The results obtained here show the inhibition of PARP-1 activity by Fractions 4 of *C. Rehmanni* and *J. Zeyheri*. Thus, a plausible mechanism for genotoxicity of both plants would be direct inhibition of PARP activity blocking the repair of DNA damage caused by excessive ROS, this in turn would lead to cell death by apoptosis or necrosis.

Chapter 5

CONCLUSION AND RECOMMENDATIONS

This study was aimed to investigate the phytochemical constituents, mutagenic and antimutagenic properties of *Jatropha zeyheri* and *Coccinia rehmanni* plant extracts. This was archived by screening various phytochemicals in *C. rehmanni* and *J. Zeyheri,* Fractionation of these plants extracts, cytotoxicity screening, and determination of mutagenicity and antimutagenicity of the selected plants. The main objective was achieved by the four derived specific objectives.

Secondary objective number one was to determine the phytochemical constituents of the two selected plant extracts. Phytochemical compounds were extracted from *C. rehmanni* and *J. Zeyheri*. Methanol solvent showed to be the better solvent as it resulted in a greater yield of plant extract. Traditional healers commonly uses water which has limitation as it has inability to extract compounds that are non-polar compounds. The findings on this study leads to a recommendation that solvents used to extracts plants must be able to extracts nonpolar compounds; CEF was the best solvent system to separate most of phyto-constituents in most of crude extracts. Further test on other extractant such as Hexane, Ethyl Acetate and chloroform is recommended.

The second objective was to extract the bioactive compounds (fractions) from the plants extracts. Open column chromatography was used to fractionate the plants extracts. The study revealed that both *C. rehmanni* and *J. Zeyheri* plants contained a large number of intermediate polar compounds due to bands observed in the intermediate-polar solvent system.

The study further find out that a lot of phytochemical compounds (alkaloids, tannins, steroids, terpenoids, flavonoids, cardiac glycosides and saponins) present in *C. rehmanni* and *J. Zeyheri* are responsible for therapeutic purposes of this plant in the treatment of different ailments. Further studies in the investigation of other secondary metabolites of these compounds is recommended. This study also provides an insight to existing knowledge on fractionations of plants. The compounds separated very well and had different activities. Therefore, we recommend the use of column chromatography in similar study.

The third objective was to evaluate toxicity of *C. rehmanni* and *J. zeyheri* plants extracts and their fractions of the two selected plants. *Jatropha zeyheri* crude extracts (**Figure 4.5**) shows a significant toxicity with 46.5 % on higher concentration (100 µg/ml) with a statistical significance p <0.0002. *C. rehmanni* Crude extracts, however in contrast to *j. Zeyheri* only shows significance at higher concetration (100 µg/ml) with a p<0.0001. Jz4 was seen to be more toxic than all the fractions and the crude extracts. Similar observation to *J. zeyheri* fractions was made in *C. rehmanni* fractions the increase in toxicity was directly proportional to the increase in concentrations, also the fractions that were eluted with more polar solvent in column chromatography showed higher toxicity

Finally the last aim of the present study was to investigate the mutagenicity/antimutagenicity of plants extracts, colorimetric test and an advanced fluorescence microscopic method proved to be effective on this regard. Toxicity and genotoxicity of these plants were determined on high doses. As such consumption of these plants as food or as an ailment in higher doses is not recommended. The present study serves as a scientific evidence that extensive use of this plant in traditional medicine may lead to some irreversible damages.

REFERENCES

Abaza, M.S.I., Orabi, K.Y., Al-Quattan, E. and Raja'a, J., 2015. Growth inhibitory and chemo-sensitization effects of naringenin, a natural flavanone purified from *Thymus vulgaris*, on human breast and colorectal cancer. *Cancer cell international*, **15**: 46.

Abubakar, N., Shehu, K., Yahaya, M.M., Tafinta, I.Y. and Imonikhe, M.A., 2016. Phytochemical Screening and Thin Layer Chromatographic Studies of *Guierasenegalensis* GF Gmel (Egyptian mimosa). *Annals of Biological Sciences,* **4**: 26-30

Akinboro, A. and Bakare, A.A., 2007. Cytotoxic and genotoxic effects of aqueous extracts of five medicinal plants on *Allium cepa* Linn. *Journal of Ethnopharmacology*, **112**: 470-475.

Akinmoladun, A.C., Obuotor, E.M. and Farombi, E.O., 2010. Evaluation of antioxidant and free radical scavenging capacities of some Nigerian indigenous medicinal plants. *Journal of Medicinal Food*, **13**: 444-451.

Ali-Shtayeh, M.S., Jamous, R.M., Al-Shafie, J.H., Elgharabah, W.A., Kherfan, F.A., Qarariah, K.H., Isra'S, K., Soos, I.M., Musleh, A.A., Isa, B.A. and Herzallah, H.M., 2008. Traditional knowledge of wild edible plants used in Palestine (Northern West Bank): a comparative study. *Journal of Ethnobiology and Ethnomedicine*, **4**: 13.

Al-Khayal, K., Alafeefy, A., Vaali-Mohammed, M.A., Mahmood, A., Zubaidi, A., Al-Obeed, O., Khan, Z., Abdulla, M. and Ahmad, R., 2017. Novel derivative of aminobenzenesulfonamide (3c) induces apoptosis in colorectal cancer cells through ROS generation and inhibits cell migration. *BMC cancer*, **17**: 4.

Astuti, S.M., AM, M.S., Andayani, R. and Risch, A., 2011. Determination of saponin compound from *Anredera cordifolia* (Ten) Steenis plant (binahong) to potential treatment for several diseases. *Journal of agricultural science*, **3**: 224.

Ayyagari, V.N. and Brard, L., 2014. Bithionol inhibits ovarian cancer cell growth in vitro-studies on mechanism (s) of action. *BMC cancer*, **14**: 61.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N. and Omar, A.K.M., 2013.

Techniques for extraction of bioactive compounds from plant materials: a review. *Journal of Food Engineering*, **117**: 426-436.

Banavali, N.K., 2013. Partial base flipping is sufficient for strand slippage near DNA duplex termini. *Journal of the American Chemical Society*, **135**: 8274-8282.

Basketter, D.A., Clewell, H., Kimber, I., Rossi, A., Blaauboer, B.J., Burrier, R., Daneshian, M., Eskes, C., Goldberg, A. and Hasiwa, N., 2012. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing-t4 report. *ALTEX-Alternatives to Animal Experimentation*, **29**: 3-91.

Bhagat, R.B. and Kulkarni, D.K., 2012. Evaluation of larvicidal and antifeedant potential of three *Jatropha* species against *Spodoptera litura* (*Lepidoptera: Noctuidae*) and two predators (*Coleoptera: Coccinellidae*). *Annals of Biological Research*, **3**: 2911-2916.

Bidwell, N.J., Winschiers-Theophilus, H., Koch-Kapuire, G. and Chivuno-Kuria, S., 2011. Situated interactions between audiovisual media and African herbal lore. *Personal and Ubiquitous Computing*, **15**: 609-627.

Biradar, S.R. and Rachetti, B.D., 2013. Extraction of some secondary metabolites & thin layer chromatography from different parts of *Centella asiatica L*.(URB). *American Journal of Life Sciences*, **1**: 243-247.

Boillée, S., Velde, C.V. and Cleveland, D.W., 2006. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron*, ***52***: 39-59.

Boobis, A.R., Lynch, A.M., Murray, S., de la Torre, R., Solans, A., Farré, M., Segura, J., Gooderham, N.J. and Davies, D.S., 1994. CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. *Cancer research*, **54**: 89-94.

Boonchum, W., Peerapornpisal, Y., Kanjanapothi, D., Pekkoh, J., Amornlerdpison, D., Pumas, C.H.A.Y.A.K.O.R.N., Sangpaiboon, P. and Vacharapiyasophon, P., 2011. Antimicrobial and anti-inflammatory properties of various seaweeds from the Gulf of Thailand. *International Journal of agriculture and biology*, **13**: 31.

Borokini, T.I. and Lawal, I.O., 2014. Traditional medicine practices among the Yoruba people of Nigeria: A historical perspective. *Journal of Medicinal Plants Studies*, *2*(6), pp.20-23.

Boukhatem, M.N., Ferhat, M.A., Kameli, A., Saidi, F. and Kebir, H.T., 2014. Lemon grass (Cymbopogon citratus) essential oil as a potent anti-inflammatory and antifungal drugs. *Libyan Journal of Medicine*, **9**: 25431.

Braithwaite, A. and Smith, J.F., 2012. Chromatographic methods. *Springer Science & Business Media*, **2**: 21

Cao, R., Peng, W., Wang, Z. and Xu, A., 2007. β-Carboline alkaloids: biochemical and pharmacological functions. *Current medicinal chemistry*, **14**: 479-500.

Clifford, M. and Brown, J.E., 2005. Dietary flavonoids and health—broadening the perspective. In *Flavonoids* (330-381). CRC Press.

Cragg, G.M., Grothaus, P.G. and Newman, D.J., 2014. New horizons for old drugs and drug leads. *Journal of natural products*, **77**: 703-723.

Dai, J. and Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, ***15*:** 7313-7352.

Das, S., Filippone, S.M., Williams, D.S., Das, A. and Kukreja, R.C., 2016. Beet root juice protects against doxorubicin toxicity in cardiomyocytes while enhancing apoptosis in breast cancer cells. *Molecular and cellular biochemistry*, **421**: 89-101.

De Flora, S., Camoirano, A., D'Agostini, F. and Balansky, R., 1992. Modulation of the mutagenic response in prokaryotes. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **267**: 183-192.

Déciga-Campos, M., Rivero-Cruz, I., Arriaga-Alba, M., Castaneda-Corral, G., Angeles-López, G.E., Navarrete, A. and Mata, R., 2007. Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. *Journal of Ethnopharmacology*, **110**: 334-342.

Elgorashi, E.E., Taylor, J.L., Maes, A., van Staden, J., De Kimpe, N. and Verschaeve, L., 2003. Screening of medicinal plants used in South African traditional medicine for genotoxic effects. *Toxicology letters*, **143**: 195-207.

Eltzschig, H.K. and Eckle, T., 2011. Ischemia and reperfusion from mechanism to translation. *Nature medicine*, **17**: 1391.

Fenech, M., 2000. The in vitro micronucleus technique. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **455**: 81-95.

Fenech, M., Knasmueller, S., Bolognesi, C., Bonassi, S., Holland, N., Migliore, L., Palitti, F., Natarajan, A.T. and Kirsch-Volders, M., 2016. Molecular mechanisms by which in vivo exposure to exogenous chemical genotoxic agents can lead to micronucleus formation in lymphocytes in vivo and ex vivo in humans. *Mutation Research/Reviews in Mutation Research*, **770**: 12-25.

Fennel, T., Bertsch, G.F. and Meiwes-Broer, K.H., 2004. Ionization dynamics of simple metal clusters in intense fields by the Thomas-Fermi-Vlasov method. *The European Physical Journal D-Atomic, Molecular, Optical and Plasma Physics*, ***29*:** 367-378.

Fishel, M.L., Vascotto, C. and Kelley, M.R., 2013. *DNA base excision repair therapeutics: summary of targets with a focus on APE1* (233-287). CRC Press, FL, USA.

Galanakis, C.M., Goulas, V., Tsakona, S., Manganaris, G.A. and Gekas, V., 2013. A knowledge base for the recovery of natural phenols with different solvents. *International Journal of Food Properties*, **16**: 382-396.

Gawai, D., Das, G. and Rout, G.R., 2013. Phytochemical screening and comparative analysis of antimicrobial activity of root and leaf extracts of *Tinospora coridifolia*, *Phyllanthus niruri* and *Abrus precatorious*, important medicinal plants. Journal of Medicinal Plants Research, **7**: 2208-2213.

Gohlke, B.O., Overkamp, T., Richter, A., Richter, A., Daniel, P.T., Gillissen, B. and Preissner, R., 2015. 2D and 3D similarity landscape analysis identifies PARP as a novel off-target for the drug Vatalanib. *BMC bioinformatics*, **16**: 308.

Guan, Y., Wang, X., Wong, M., Sun, G., An, T., Guo, J. and Zhang, G., 2017. Evaluation of genotoxic and mutagenic activity of organic extracts from drinking water sources. *PloS one*, **12**: e0170454.

Guerrero, L., Castillo, J., Quiñones, M., Garcia-Vallvé, S., Arola, L., Pujadas, G. and Muguerza, B., 2012. Inhibition of angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. *PloS one*, **7**: 49493.

Gupta, R.K., Patel, A.K., Shah, N., Chaudhary, A., Jha, U., Yadav, U.C., Gupta, P.K. and Pakuwal, U., 2014. Oxidative stress and antioxidants in disease and cancer. *Asian Pac Cancer Prev*, **15**: 4405-4409.

Harborne, J.B., 2013. The flavonoids: *advances in research since 1980*. Springer. **1**: 65.

Harun, S.W., Ismail, M.A., Ahmad, F., Ismail, M.F., Nor, R.M., Zulkepely, N.R. and Ahmad, H., 2012. A Q-switched erbium-doped fiber laser with a carbon nanotube based saturable absorber. *Chinese Physics Letters*, **29**: 114202.

Hemeryck, L.Y. and Vanhaecke, L., 2016. Diet-related DNA adduct formation in relation to carcinogenesis. *Nutrition reviews*, **74**: 475-489.

Heymann, D.L., Chen, L., Takemi, K., Fidler, D.P., Tappero, J.W., Thomas, M.J., Kenyon, T.A., Frieden, T.R., Yach, D., Nishtar, S. and Kalache, A., 2015. Global health security: the wider lessons from the West African Ebola virus disease epidemic. *The Lancet*, **385**: 1884-1901.

Holmbom, B., Voss, R.H., Mortimer, R.D. and Wong, A., 1984. Fractionation, isolation and characterization of Ames-mutagenic compounds in kraft chlorination effluents. *Environmental science & technology*, **18**: 333-337.

Houghton, P. and Raman, A., 2012. Laboratory handbook for the fractionation of natural extracts. *Springer Science & Business Media*. 56.

Huang, J., Li, Q., Sun, D., Lu, Y., Su, Y., Yang, X., Wang, H., Wang, Y., Shao, W., He, N. and Hong, J., 2007. Biosynthesis of silver and gold nanoparticles by novel sundried Cinnamomum camphora leaf. *Nanotechnology*, **18**: 105104.

Hulkower, K.I. and Herber, R.L., 2011. Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics*, ***3***: 107-124.

Hussin, N.M., Muse, R., Ahmad, S., Ramli, J., Mahmood, M., Sulaiman, M.R., Shukor, M.Y.A., Rahman, M.F.A. and Aziz, K.N.K., 2009. Antifungal activity of extracts and phenolic compounds from *Barringtonia racemosa L.(Lecythidaceae*). *African Journal of Biotechnology*, **8**: 65

Jaime, M.F.V., Redko, F., Muschietti, L.V., Campos, R.H., Martino, V.S. and Cavallaro, L.V., 2013. In vitro antiviral activity of plant extracts from *Asteraceae* medicinal plants. *Virology journal*, **10**: 245.

Jorde, L.B., Carey, J.C. and Bamshad, M.J., 2015. *Medical genetics e-Book*. *Elsevier Health Sciences.* **5***:* 91.

Juers, D.H., Matthews, B.W. and Huber, R.E., 2012. LacZ β-galactosidase: structure and function of an enzyme of historical and molecular biological importance. *Protein Science*, ***21***: 1792-1807.

Kakkar, S. and Bais, S., 2014. A review on protocatechuic acid and its pharmacological potential. *ISRN pharmacology*, **2014**: 1-9

Karuppusamy, S., 2009. A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *Journal of Medicinal Plants Research*, ***3***: 1222-1239.

Khanum, R., Mazhar, F. and Jahangir, M., 2015. Antioxidant evaluations of polar and non-polar fractions of *Cajanus Cajan* seeds. *Journal of Medicinal Plants Research*, **9**: 193-198.

Khatun, S., Pervin, F., Karim, M.R., Ashraduzzaman, M. and Rosma, A., 2012. Phytochemical screening and antimicrobial activity of Coccinia cordifolia L. plant. *Pakistan journal of pharmaceutical sciences*, **25**: 757

Khoddami, A., Wilkes, M. and Roberts, T., 2013. Techniques for analysis of plant phenolic compounds. *Molecules*, **18**: 2328-2375.

Kirkland, D., Reeve, L., Gatehouse, D. and Vanparys, P., 2011. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **721**: pp.27-73.

Kotze, M., Eloff, J.N. and Houghton, P.J., 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (*Combretaceae*). *South African Journal of Botany*, **68**: 62-67.

Kreis, W. and Müller-Uri, F., 2018. Biochemistry of sterols, cardiac glycosides, brassinosteroids, phytoecdysteroids and steroid saponins. *Annual Plant Reviews online*, **1**: 304-363.

Krishnaiah, D., Sarbatly, R. and Bono, A., 2007. Phytochemical antioxidants for health and medicine a move towards nature. Biotechnology and Molecular Biology Reviews, **2**: 97-104.

Kudumela, R.G., 2017. *Isolation, characterisation and cytotoxic effects of antibacterial and antioxidant compounds from selected medicinal plants* (Doctoral dissertation). 76.

Kumar, A., Zarychanski, R., Pinto, R., Cook, D.J., Marshall, J., Lacroix, J., Stelfox, T., Bagshaw, S., Choong, K., Lamontagne, F. and Turgeon, A.F., 2009. Critically ill patients with 2009 influenza A (H1N1) infection in Canada. *Jama*, **302**: 1872-1879.

Kumar, P., Henikoff, S. and Ng, P.C., 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature protocols*, **4**: 1073-1081.

Kumar, V., Abbas, A.K., Fausto, N. and Aster, J.C., 2014. Robbins and Cotran pathologic basis of disease, *professional edition e-book*. *Elsevier health sciences*.

Kumaravel, T.S., Vilhar, B., Faux, S.P. and Jha, A.N., 2009. Comet assay measurements: a perspective. *Cell biology and toxicology*, **25**: 53-64.

Lithakong, T.C., Seepe, H.A., Amoo, S.O. and Ejidike, P., 2018. The effect of fermentation conditions on the phytochemical composition and antioxidant activity of Jatropha zeyheri leaves. *South African Journal of Botany*, **115**: 292.

Liu, T., Brown, D.A. and O'Rourke, B., 2010. Role of mitochondrial dysfunction in cardiac glycoside toxicity. *Journal of molecular and cellular cardiology*, **49**: 728-736.

Lord, C.J. and Ashworth, A., 2012. The DNA damage response and cancer therapy. *Nature*, **481**: 287.

Louw, C.A.M., Regnier, T.J.C. and Korsten, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. *Journal of Ethnopharmacology*, **82**: 147-154.

Lu, Y., Luo, J. and Kong, L., 2011. Steroidal alkaloid saponins and steroidal saponins from Solanum surattense. *Phytochemistry*, ***72*:** 668-673.

Luseba, D., Elgorashi, E.E., Ntloedibe, D.T. and Van Staden, J., 2007. Antibacterial, anti-inflammatory and mutagenic effects of some medicinal plants used

in South Africa for the treatment of wounds and retained placenta in livestock. *South African Journal of Botany*, 73(3), pp.378-383.

Maciag, A.E., Holland, R.J., Kim, Y., Kumari, V., Luthers, C.E., Sehareen, W.S., Biswas, D., Morris, N.L., Ji, X., Anderson, L.M. and Saavedra, J.E., 2014. Nitric oxide (NO) releasing poly ADP-ribose polymerase 1 (PARP-1) inhibitors targeted to glutathione S-transferase P1-overexpressing cancer cells. *Journal of medicinal chemistry*, **57**: 2292-2302.

Mahesh, B. and Satish, S., 2008. Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World journal of agricultural sciences*, **4**: 839-843.

Mahgoub, M.E., 2017. *Phytochemical Study of The Leaves of Adansonia digitata* (Doctoral dissertation, Sudan University of Science and Technology). 115.

Mahmoudi, M. and Serpooshan, V., 2012. Silver-coated engineered magnetic nanoparticles are promising for the success in the fight against antibacterial resistance threat. *ACS nano*, **6**: 2656-2664.

Maron, D.M. and Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, **113**: 173-215.

Masoko, P. and Makgapeetja, D.M., 2015. Antibacterial, antifungal and antioxidant activity of *Olea africana* against pathogenic yeast and nosocomial pathogens. *BMC complementary and alternative medicine*, **15**: 409.

Masoko, P., Mmushi, T.J., Mogashoa, M.M., Mokgotho, M.P., Mampuru, L.J. and Howard, R.L., 2008. In vitro evaluation of the antifungal activity of Sclerocarya birrea extracts against pathogenic yeasts. *African Journal of Biotechnology*, **7**: 20.

Mazid, M., Khan, T.A. and Mohammad, F., 2011. Response of crop plants under sulphur stress tolerance: A holistic approach. *Journal of Stress Physiology & Biochemistry*, **7**: 27

Minde, D.P., Anvarian, Z., Rüdiger, S.G. and Maurice, M.M., 2011. Messing up disorder: how do missense mutations in the tumor suppressor protein APC lead to cancer? *Molecular cancer*, **10**: 1.

Mir, M.A., Sawhney, S.S. and Jassal, M.M.S., 2013. Qualitative and quantitative analysis of phytochemicals of Taraxacum officinale. *Wudpecker Journal of Pharmacy and Pharmacology*, **2**: 01-05.

Mišík, M., Ma, T.H., Nersesyan, A., Monarca, S., Kim, J.K. and Knasmueller, S., 2011. Micronucleus assays with Tradescantia pollen tetrads: an update. *Mutagenesis*, **26**: 215-221.

Moghimi, S.M., Symonds, P., Murray, J.C., Hunter, A.C., Debska, G. and Szewczyk, A., 2005. A two-stage poly (ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Molecular therapy*, **11**: 990-995.

Mongalo, N.I., 2013. *Antibacterial activities of selected medicinal plants used to treat sexually transmitted infections in Blouberg area, Limpopo Province* (Doctoral dissertation, University of Zululand).

Mongalo, N.I., Opoku, A.R. and Zobolo, A.M., 2013. Antibacterial activity of root and leaf extracts of *Jatropha zeyheri* Sond (*Euphorbiaceae*). *African Journal of Biotechnology*, **12**: 26.

Monteiro, F.A., Cardoso, I., Sousa, M.M. and Saraiva, M.J., 2006. In vitro inhibition of transthyretin aggregate-induced cytotoxicity by full and peptide derived forms of the soluble receptor for advanced glycation end products (RAGE). *FEBS letters*, **580**: 3451-3456.

Moon, Y.J., Wang, X. and Morris, M.E., 2006. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicology in vitro*, **20**: 187-210.

Mortelmans, K. and Zeiger, E., 2000. The Ames Salmonella/microsome mutagenicity assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **455**: 29-60.

Mothibe, M.E., Osuch, E. and Kahler-Venter, C.P., 2017. Commercial herbal medicines used as African traditional medicines: Ngoma Herbal Tonic Immune Booster interferes with a rapid urine drug screening test. *South African Medical Journal*, **107**: 734-737.

Namdeo, A.G., 2007. Plant cell elicitation for production of secondary metabolites: a review. *Pharmacogn Review*, ***1***: 69-79.

Nikoletopoulou, V., Markaki, M., Palikaras, K. and Tavernarakis, N., 2013. Crosstalk between apoptosis, necrosis and autophagy. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, ***1833***: 3448-3459.

Olajuyigbe, O.O. and Afolayan, A.J., 2012. In vitro pharmacological activity of the crude acetone extract of *Erythrina caffra*: antibacterial and antifungal assessment. *Journal of Medicinal Plants Research*, **6**: 1713-1720.

Pandya, K.G., Patel, M.R. and Lau-Cam, C.A., 2010. Comparative study of the binding characteristics to and inhibitory potencies towards PARP and in vivo antidiabetogenic potencies of taurine, 3-aminobenzamide and nicotinamide. *Journal of biomedical science*, **17**: 16.

Pringle, N.A., Koekemoer, T.C., Holzer, A., Young, C., Venables, L. and van de Venter, M., 2018. Potential therapeutic benefits of green and fermented Rooibos (Aspalathus linearis) in dermal wound healing. *Planta medica*, ***84***: 645-652.

Raaman, N., 2006. Phytochemical techniques. *New India Publishing*, **101**: 252

Rampersad, S.N., 2012. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors*, **12**: 12347-12360.

Razak, M.F.A., Aidoo, K.E. and Candlish, A.G., 2007. Mutagenic and cytotoxic properties of three herbal plants from Southeast Asia. *Tropical biomedicine*, **24**: 49-59.

Reid, K.A., Maes, J., Maes, A., Van Staden, J., De Kimpe, N., Mulholland, D.A. and Verschaeve, L., 2006. Evaluation of the mutagenic and antimutagenic effects of South African plants. *Journal of Ethnopharmacology*, **106**: 44-50.

Reid, K.E., Olsson, N., Schlosser, J., Peng, F. and Lund, S.T., 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC plant biology*, **6**: 27.

Resende, F.A., Vilegas, W., Dos Santos, L.C. and Varanda, E.A., 2012. Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test. *Molecules*, **17**: 5255-5268.

Rietjens, I.M., Martena, M.J., Boersma, M.G., Spiegelenberg, W. and Alink, G.M., 2005. Molecular mechanisms of toxicity of important food-borne phytotoxins. *Molecular nutrition & food research*, **49**: 131-158.

Roberts, M.F. ed., 2013. Alkaloids*:* biochemistry, ecology, and medicinal applications. *Springer Science & Business Media*. **4**: 36.

Romero-Castro, A., León-Rivera, I., Ávila-Rojas, L.C., Navarrete-Vázquez, G. and Nieto-Rodríguez, A., 2011. Synthesis and preliminary evaluation of selected 2-aryl-5 (6)-nitro-1H-benzimidazole derivatives as potential anticancer agents. *Archives of pharmacal research*, **34**: 181-189.

Romero-Jiménez, M., Campos-Sánchez, J., Analla, M., Muñoz-Serrano, A. and Alonso-Moraga, Á., 2005. Genotoxicity and anti-genotoxicity of some traditional medicinal herbs. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **585**: 147-155.

Ru, Q.M., Wang, L.J., Li, W.M., Wang, J.L. and Ding, Y.T., 2012. In vitro antioxidant properties of flavonoids and polysaccharides extract from tobacco (*Nicotiana tabacum L*.) leaves. *Molecules*, **17**: 11281-11291.

Saito, H., 2000. Regulation of herbal medicines in Japan. *Pharmacological Research*, **41**: 515-519.

Sarkar, P., Patra, M.K. and Kumar, H., 2016. Strategic treatment with immunomodulators to resolve endometritis in cow: A review. *Agricultural Reviews*, **37**: 36.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, **8**: 11.

Sawadogo, W.R., Schumacher, M., Teiten, M.H., Dicato, M. and Diederich, M., 2012. Traditional West African pharmacopeia, plants and derived compounds for cancer therapy. *Biochemical pharmacology*, **84**: 1225-1240.

Saxena, V., Mishra, G., Saxena, A. and Vishwakarma, K.R., 2013. A comparative study on quantitative estimation of tannins in *Terminalia chebula*, *Terminalia belerica*,

Terminalia arjuna and *Saraca indica* using spectrophotometer. *Asian Journal of Pharmaceutical and Clinical Research*, **6**: 148-149.

Scott, G., Springfield, E.P. and Coldrey, N., 2004. A pharmacognostical study of 26 South African plant species used as traditional medicines. *Pharmaceutical Biology*, **42**: 186-213.

Shaheen, S.Z., Bolla, K., Vasu, K. and Charya, M.S., 2009. Antimicrobial activity of the fruit extracts of *Coccinia indica*. *African journal of Biotechnology*, **8**: 24.

Sherif, S.O., Salama, E.E. and Abdel-Wah hab, M.A., 2009. Mycotoxins and child health: The need for health risk assessment. *International Journal of Hygiene and Environmental Health*, **212**: 347-368.

Sigidi, M.T., Anokwuru, C.P., Zininga, T., Tshisikhawe, M.P., Shonhai, A., Ramaite, I.D.I., Traoré, A.N. and Potgieter, N., 2016. Comparative in vitro cytotoxic, anti-inflammatory and anti-microbiological activities of two indigenous Venda medicinal plants. *Translational Medicine Communications*, **1**: 9.

Sigidi, M.T., Traoré, A.N., Boukandou, M.M., Tshisikhawe, M.P., Ntuli, S.S. and Potgieter, N., 2017. Anti-HIV, pro-inflammatory and cytotoxicity properties of selected Venda plants. *India Journal of Traditional Knowledge*, **16**: 545-552.

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental cell research*, **175**: 184-191.

Singh, R., 2015. Medicinal plants: A review. *Journal of Plant Sciences*, **3**: 50-55.

Skibola, C.F. and Smith, M.T., 2000. Potential health impacts of excessive flavonoid intake. *Free radical biology and medicine*, **29**: 375-383.

Sobolev, V.S. and Dorner, J.W., 2002. Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *Journal of AOAC International*, **85**: 642-645.

Sofowora, A., 1993. Screening plants for bioactive agents. *Medicinal plants and traditional medicinal in Africa*, ***2***: 134-156.

Tamilselvan, N., Thirumalai, T., Elumalai, E.K., Balaji, R. and David, E., 2011. Pharmacognosy of *Coccinia grandis*: a review. Asian Pacific Journal of Tropical Biomedicine, **1**: 299-302.

Tekauz, A., McCallum, B. and Gilbert, J., 2000. Fusarium head blight of barley in western Canada. *Canadian Journal of Plant Pathology*, **22**: 9-16.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N. and Court, D.L., 2014. Recombineering: genetic engineering in bacteria using homologous recombination. *Current protocols in molecular biology*, **106**: 1-16.

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E. and Henikoff, J.G., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome research*, **13**: 524-530.

Tilmant, K., Gerets, H.H.J., De Ron, P., Cossu-Leguille, C., Vasseur, P., Dhalluin, S. and Atienzar, F.A., 2013. The automated micronucleus assay for early assessment of genotoxicity in drug discovery. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **751**: 1-11.

Tiwari, P., Kumar, K., Panik, R., Pandey, A., Pandey, A. and Sahu, P.K., 2011. Hepatoprotective potentials of Butea monosperma stem bark extract against carbon tetrachloride induced hepatotoxicity in albino rats. *International Journal of Medicine and Medical Sciences*, **3**: 252-255.

Tshikalange, T.E., Mamba, P. and Adebayo, S.A., 2016. Antimicrobial, antioxidant and cytotoxicity studies of medicinal plants used in the treatment of sexually transmitted diseases. *International Journal of Pharmacognosy and Phytochemical Research,* **8**: 1891-1895

Turnpenny, P.D. and Ellard, S., 2016. *Emery's Elements of Medical Genetics E-Book*. Elsevier Health Sciences. 56.

Vadivu, R. and Lakshmi, K.S., 2008. In vitro and in vivo anti-inflammatory activity of leaves of Symplocos cochinchnensis (Lour) Moore ssp laurina. *Bangladesh Journal of Pharmacology*, **3**: 121-124.

Van Rensburg, W.J., Van Averbeke, W., Slabbert, R., Faber, M., Van Jaarsveld, P., Van Heerden, I., Wenhold, F. and Oelofse, A., 2007. African leafy vegetables in South Africa. *Water SA*, ***33***: 317-326.

Van Vuuren, S.F., 2008. Antimicrobial activity of South African medicinal plants. *Journal of Ethnopharmacology*, **119**: 462-472.

Van Wyk, B. and Wink, M., 2004. Medicinal Plants of the World. *Pretoria: Brazia Publications*.

Van Wyk, B.E. and Gericke, N., 2000. *People's plants: A guide to useful plants of Southern Africa*. Briza Publications. **5**: 152.

Van Wyk, B.E., 2011. The potential of South African plants in the development of new medicinal products. *South African Journal of Botany*, **77**: 812-829.

Verschaeve, L. and Van Staden, J., 2008. Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants. *Journal of Ethnopharmacology*, **119**: 575-587.

Verschaeve, L., Kestens, V., Taylor, J.L.S., Elgorashi, E.E., Maes, A., Van Puyvelde, L., De Kimpe, N. and Van Staden, J., 2004. Investigation of the antimutagenic effects of selected South African medicinal plant extracts. *Toxicology in vitro*, **18**: 29-35.

Viji, G.S., Vasanthe, B. and Suresh, K., 2013. Screening and antibacterial activity analysis of some important medicinal plants. *Indian Journal of Knowledge*, **8**: 26.

Visht, S. and Chaturvedi, S., 2012. Isolation of natural products. *Current Pharmacological Research*, **2**: 584.

Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A. and Kinzler, K.W., 2013. Cancer genome landscapes. *Science*, **339**: 1546-1558.

Wadood, A., Ghufran, M., Jamal, S.B., Naeem, M., Khan, A. and Ghaffar, R., 2013. Phytochemical analysis of medicinal plants occurring in local area of Mardan. *Biochem Anal Biochem*, **2**: 1-4.

Watanabe, Y.H., Nakazaki, Y., Suno, R. and Yoshida, M., 2009. Stability of the two wings of the coiled-coil domain of ClpB chaperone is critical for its disaggregation activity. *Biochemical Journal*, **421**: 71-77.

Wei, L.S., Wee, W., Siong, J.Y.F. and Syamsumir, D.F., 2011. Characterization of anticancer, antimicrobial, antioxidant properties and chemical compositions of *Peperomia pellucida* leaf extract. *Acta Medica Iranica*, **49**: 670-674.

Westerink, W.M., Schirris, T.J., Horbach, G.J. and Schoonen, W.G., 2011. Development and validation of a high-content screening in vitro micronucleus assay in CHO-k1 and HepG2 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **724**: 7-21.

Wood, A.W., Huang, M.T., Chang, R.L., Newmark, H.L., Lehr, R.E., Yagi, H., Sayer, J.M., Jerina, D.M. and Conney, A.H., 1982. Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. *Proceedings of the National Academy of Sciences*, **79**: 5513-5517.

Yadav, R.N.S. and Agarwala, M., 2011. Phytochemical analysis of some medicinal plants. *Journal of phytology*. **1**: 56.

Yang, L. and Stöckigt, J., 2010. Trends for diverse production strategies of plant medicinal alkaloids. *Natural product reports*, **27**: 1469-1479.**Zanella, F., Lorens, J.B. and**

Zanella, F., Lorens, J.B. and Link, W., 2010. High content screening: seeing is believing. *Trends in biotechnology*, **28**: 237-245.

Zhou, J., Ouedraogo, M., Qu, F. and Duez, P., 2013. Potential genotoxicity of traditional Chinese medicinal plants and phytochemicals: an overview. *Phytotherapy Research*, **27**: 1745-1755.0