

**SOMATIC EMBRYOGENESIS AND BIOASSAY STUDIES OF
SCHIZOZYGIA COFFAEOIDES BAIL (MPELEPELE) FOR
POTENTIAL EXPLOITATION OF SECONDARY
METABOLITES.**

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DECLARATION

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

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DEDICATION

To the best parents in the world Mr Bashiloni Gundiri and Mrs Jesty Bashiloni Gundiri thank you so much for loving me and giving me to the best of your abilities. I love you now and always.

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My Sincere appreciation to God almighty for His gracious love and faithfulness throughout this journey. To him be all the glory, honour and adoration forever and ever.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BAP	6-benzylaminopurine
DMSO	Di-Methyl-Sulfoxide
IBA	Indole butyric acid
KIN	Kinetin
MS	Murashige and Skoog (1962) media
NAA	Naphthalene Acetic Acid
NaOCl	Sodium hypochlorite
NaOH	Sodium Hydroxide
PDA	Potato Dextrose Agar
PGR	Plant growth regulators
PTC	Plant tissue culture
TDZ	Thidiazuron
IUCN	International Union of Conservation of Nature.

ABSTRACT

Schizogygia coffeoides (Mpelepele) is an endangered medicinal shrub geographically distributed in Kenya, with substantial antifungal and antibacterial properties. The sustainable utilization of this shrub is hindered by its limited propagation potential through seeds and cuttings. The aim of this study was to develop an appropriate regeneration protocol for *in vitro* regeneration via somatic embryogenesis. Sterilization was optimized using NaOCl at concentrations of 5.2mM, 7.8mM, 10.4mM and 13mM at exposure times of 10, 15, 20 and 25 minutes. The different concentrations of NaOCl were observed to have a significant effect on the survival of explants to sterilization ($P < 0.0001$). Similarly, the time explants were subjected to the sterilization substances was also found to significantly affect their ability to survive the sterilization process. The best callus formation frequency of 73% was observed in MS Media supplemented with 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ, compared to 39% callus in media supplemented with 1.5mg/l BAP+0.6mg/l Kin+0.3mg/l NAA+0.1mg/l TDZ and at 13% observed in media supplemented with 1.0mg/l BAP+0.4mg/l Kin+0.2mg/l NAA+0.05mg/l TDZ. The Callus were maintained on MS media supplemented with 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ for 28 days which led to rapid somatic embryo at all the stages of somatic embryogenesis observed. Roots primordia only formed in shoots cultured in media supplemented with 1.0mg/l BAP+0.5mg/l IBA. Leaf, root, stem and calli were subjected to phytochemicals screening and extracts obtained using ethanol, methanol, acetone, chloroform, hexane di-ethyl and water were subjected to microorganism and observed for their antimicrobial assay by disc diffusion assay method. The zone of inhibition results were compared to Ampicillin, chloramphenicol and Nystatin standard antibiotics at ($p < 0.05$). Leave and root extracts showed a relatively better antimicrobial activity than stem extracts, with callus extracts showing no antimicrobial activity at all. There was a significant difference in antimicrobial activity based on the solvent used for extraction, with methanol, ethanol and water being the best solvents ($P < 0.001$). Followed by diethyl and hexane roots extract. All the antibiotics had significant inhibition against all microorganism. Lower concentrations of Mpelepele leaf, stem and root extracts were all found to be toxic to

brime shrimps compared to higher concentrations ($P < 0.001$). Significant differences were also observed in toxicity based on the solvents used for extraction ($P < 0.005$). The successful development of sterilization protocol and regeneration via somatic embryogenesis will greatly contribute to mass production and conservation of the plant, furthermore through antimicrobial activity and toxicity screening there will be great knowledge available towards the use of mpelepele for pharmaceutical purposes. This research findings will adequately explored areas for maximizing somatic embryogenesis plantlet in mpelepele that will be fully exploited.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The importance of plants and the role they play in the livelihoods of indigenous communities cannot be overlooked. Indeed, plant derived materials remain a very important resource in developing countries as a source of living (Kaeppler et al., 2000). About 60-80% of the world population still relies on plant based materials for traditional medicine to treat common illnesses as well as their use for food and shelter (Sheikh et al., 2013). More than a tenth of the existing plant species in the world are used for development of drugs and other health products. This amounts to over 50,000 plant species being used for medicinal purpose world-wide (Rafieian-kopaei, 2012). Additionally, some plant families not only have more medicinal species than others but are also more endangered species than other families. These plants that suffer genetic erosion and resource destruction are listed as threatened and necessitate the need for sustainable utilization through biotechnological techniques (Chen et al., 2016). However, despite the abundance of plant sources, their diversity is constantly dwindling due to their exploitation for new drug development and validation of traditional medicines. As a result, many plant species are in danger of extinction due to the ever increasing human activities (Atanasov et al., 2015). Most plants are widely located in the forest (Mephora et al., 2012). This challenge can be averted especially that of over exploitation through *in vitro* regeneration for conservation and sustainability of plant (Sucharita, 2010).

The plant family *Apocynaceae* consists of about 250 genera and about 2000 species of tropical trees and shrubs, which are distributed in south-east Asia, India, Africa and Northern Australia (Bruyns, 2002, Meve et al., 2007). *Apocynaceae* has a long historical background in its rich indole-alkaloids properties and nutrient. They have gained popularity in recent years due to the rich metabolites found in them, which exhibit numerous biological activities (Canter et al., 2005). *Schizogygia coffeoides*,

locally known as Mpelepele in Kenya, is an endangered evergreen shrub plant that is rich in alkaloids, glycerides polyphenols and flavonoids, from the leaves, stem, and roots. It belongs to the order *gentianales*, family *Apocynaceae* subfamily *Rauvolfiodeae* and tribe *Tabernaemontanae* (Endress and Bruyns, 2000).

The major constraint in the conventional propagation of mpelepele through the seed is low germination ranging from 10% to 20%. There is therefore need to develop *in vitro* regeneration protocol, which will improve conservation and sustainability of this endangered medicinal plant. Biotechnology involves the use of modern techniques such as tissue culture, cell biology and molecular biology, which offer a great opportunity to develop new germplasm that are well adapted to changing demands (Runyoro et al., 2006). In this regard, tissue culture will ensure conservation and sustainability of *Mpelepele*. *In vitro* techniques offers a powerful tool for mass multiplication of clean and high vigour plant material through somatic embryogenesis or organogenesis (Shargel et al., 2007; Pandey et al., 2010). Bioactive compounds in plants have been used to distinguish the ability of different plants to control diseases caused by pathogenic bacteria fungi (Esimon et al., 2012). Studies on *Apocynaceae* bioactivity compounds have increased the use of herbal medicine as most pathogenic bacteria and fungi are becoming resistant to synthetic drugs. Global attention has been shifted in finding new development of new drugs through the unique elements found in *Apocynaceae* in its molecular diversity and its functionality which is indispensable for novel drug discovery (Sahai et al., 2010).

1.2 Taxonomy of *Schizogygia coffeoides* (Mpelepele)

Schizogygia coffeoides grows to about 1-8 metres high, it has a dark brown bark with pale lenticels, which are red in the inside. Its wood is soft and often yellow in appearance. Its branches are usually yellow to dark brown and lenticellate like in the main branch. The branchlets of this plant often sulcate when dry and are usually glabrous. Its leaves are acuminate and glabrous on both sides, fleshy when young but sub-coriaceous when dry, with a lamina of about 2.4-25 cm long x 1.1-11 cm wide.

The leaves are green with long petioles of about 0.5-9 mm and smooth in appearance. Leaf veins are very conspicuous (Plate 1.1 a). The flowers are usually 7-15 x 12-18 mm in size with narrowly oblong, acute and glabrous bracts on both sides of about 3-5 mm long. The flowers have short and smooth peduncles of up to 3 mm with the individual pedicels measuring about 2-3 mm long. Usually, these flowers have fragrant and free sepals which are sub equal, entire, imbricate, elliptic and acute (Plate 1.1 b). These sepals are smooth, with 5-10 colleters, near the base at the edges of each sepal. The corolla are 6.5- 9.5 mm long, forming cylindrical or areolate hypocrateri form tubes which are 4-5.2 mm long and 1.8-2.5 mm in diameter. The flowers have obliquely and nearly hook shaped lobes with entire margins and glabrous on both sides. Stamens of these flowers are inserted at about 1.5-3 mm below the mouth of the corolla with anthers measuring 2.1-2.7 x 0.8-1 mm. Generally, the pistil measures about 2.5-5 mm with a glabrous or papilated ovary measuring 0.8-1.3 x 1-1.2 x 0.5-1.5 mm and containing 8-15 ovules inside. The style is usually smooth and measures 1-3 x 0-2 mm. The stigma measures about 0.2 mm long. Its fruits are yellow to orange when ripe but green when young (Plate 1.1 c), with ellipsoid carpels, laterally compressed and dehiscent along an adaxial line of dehiscence and contain dark brown seeds (Leeuwenberg, 2003).



Plate 1.1: Mpelepele plant

(A) 8 months old potted seedlings with fully grown leaves (B) Flower (C) Young fruits

Source: Own photo taken from shimba hills Kenya.

1.3 Economic importance of Mpelepele

Schizogygia coffaeoides (*Mpelepele*) is rich in two known alkaloids; schizozygine and insoschizogaline as well as a new indoline alkaloid 6, 7-dehydro-19 β -hydroxyl-schizozygine. These alkaloids are usually found in cells located at the extreme layers of the plant tissue, suggesting that these compounds would be the first line of defence against activity of bacterial and fungal plant pathogens (Kariba et al., 2002). The crude extracts of this shrub have shown substantial antibacterial and antifungal properties which could be attributed to these substances (Magadula and Erasto, 2009).

These secondary metabolites have been reported to confer a broad-spectrum pharmacological effect including antibacterial and antifungal activities. It has been observed that the fruits and flowers of this plant are poisonous, hence only the leaves, roots and stems are used for pharmacological activities (Joseph et al., 2009). For example, in traditional medicine systems in Kenya and Tanzania, barks and leaves of the plant harvested from the wild are used for the treatment of skin related problems, gastro intestinal diseases, sexually transmitted infections, malaria, wounds, eye infections, measles and snake bites (Van et al., 2004). In the Comoro's Islands, the leaves and the fruits are used to coagulate rubber of other plants and decoctions of the stem bark is given to nursing mother while the leaves and bark are popular remedies against fatigue and applied to wound (Handley 1995).

1.4 Geographical distribution of *Schizogygia coffeoides* (Mpelepele)

The shrub is found throughout temperate regions in forests of altitudes 0-1500M above sea level and distributed among East African countries, Angola, Zaire and the Comoro's Island. In Kenya, it is predominately found in the Shimba hills and Kilifi coastal regions, covering an area of 19,250 Hectare (Kariba et al., 2002). This region is rich in a heterogeneous habitat, including forest and shrub land, forming a table plateau boardering the Tana and Taita Taveta rivers. The shrub grows well within a temperature range of 17-24°C, rainfall ranging from 600-1600mm and prefers sandy

or loamy soil. It is mostly harvested from the wild and used as medicine. Its fruits are poisonous and distributed all year round (Vogel, 1991).

1.5 Statement of the problem

Plant resources rich in antimicrobial properties represent a vast tapped and untapped sources of medicine. The biodiversity of these plant sources is rapidly decreasing and some of the potential medicinal plants available are in danger of extinction due to over exploitation and clearance from their natural habitats in search of new lands for settlements and industrialization. Therefore, the multiple of potential usefulness of these plants is at risk of being extinct. *Mpelepele* which is an evergreen shrub and commonly used by the local communities along the Kenyan coast is over-exploited. Its long reproductive cycle and poor seed yield poses a challenge in its improvement through conventional methods. Currently, much remains to be done towards establishing a mass *in vitro* propagation protocol for not only this important shrub but also some members of the *Apocynaceae* family. This, therefore, poses a great challenge considering the high demand of traditional medicines, hence a potential threat of extinction as a result of overexploitation. International union for conservation of nature (IUCN, 2002). Indicate that up to 60% of higher and lower plants species, *Schizogygia coffaeoides* inclusive could be extinct or nearly extinct by the year 2050. Therefore, through proper documentation of active compounds, regeneration, as well as determination of antimicrobial and toxicity activities. It will aid in conservation and sustainable use of mpelepele.

1.6 Justification

The regeneration of *Mpelepele* could be key to its sustainability, thereby ensuring a constant supply to its medicinal materials. With the use of tissue culture techniques to develop *in vitro* regeneration protocol, it could be possible to propagate plants, for the multiple uses of this important shrub. In addition, *in vitro* regeneration could yield a positive effect on plant growth and morphogenesis, resulting in disease free plants for

industrial and pharmacological purposes. *In vitro* propagation as a functional development technique will restore and sustain the plant through mass propagation as it holds a high potential for the production of high quantity and quality plantlets. With the traditional knowledge in the background, the potentially good properties of *Mpelepele* will be conserved through rapid multiplication, the shrub be will extensively available, to meet the increasing demand by chemists and botanists. Therefore, it is essential that efficient *in vitro* regeneration protocol be established for *Schizogygia coffaeoides*, which could act as a guide for not only this plant, but also the beginning point for development of regeneration protocols for other members in the *Apocynaceae* family.

Through bioactivity information, the pharmacological benefits of the secondary metabolites in this plant will be established. Additionally, detailed information concerning the toxicity of extracts from this plant and whether they could be lethal to users will be availed.

1.7 Objectives

1.7.1 General objective

In vitro regeneration and bioassay studies of *Schizogygia coffaeoides* for mass propagation and potential use of secondary metabolites

1.7.2 Specific objectives

1. To evaluate *In vitro* regeneration of *Schizogygia coffaeoides* *Mpelepele* via somatic embryogenesis.
2. To determine phytochemicals and the antimicrobial activity of *Schizogygia coffaeoides* *Mpelepele*.
3. To assess the level of toxicity in *Schizogygia coffaeoides* *Mpelepele* using Brime shrimp test.

1.8 Null Hypotheses

1. Mpelepele cannot be regenerated through somatic embryogenesis.
2. Mpelepele has no antimicrobial and phytochemicals activity.
3. Mpelepele plant extracts are non-toxic.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biotechnology and its role in plant regeneration

Biotechnology refers to the technique that uses living organisms with the aim of making or modifying a product, to improve plants or animals or to develop microorganisms for a certain use (Pathak & Abido, 2014). This technology involves the use of certain old techniques, as well as current advanced techniques such as cell and tissue culture and transgenetics. In addition to improving the production of important agricultural crops, this technique holds a great potential in conserving biodiversity through its advanced techniques especially *in vitro* culture and molecular biology (Gupta & Thorstenson 1993). Through these techniques, new categories of germ plasms have been developed, as well as cell lines with special attributes (Engelmann, 1991). Through other techniques such as cryopreservation, the risk of losing plant genetic resources which are highly vulnerable in general growing and storage is highly reduced (Pence et al., 1996).

2.2 Application of plant tissue culture

Tissue culture is the process through which small pieces of living tissues (explants) are aseptically isolated from organism and grown indefinitely *in vitro* on a nutrient medium (Handley 1995). The very first successful plant tissue culture was done by Goltlieb Haberlandth in the 20th century from leaf mesophyll and hair cell cultures. Plant tissue culture (PTC) is also a quick and non-seasonal biotechnology technique which is efficient in the *in vitro* propagation of plants under sterile conditions. This technique is efficient in developing plant clones which are disease free, virus free and uniform, from a very small part of the mother plant. It relies on the discovery that all plant cell are totipotent and therefore can differentiate into whole plants. The ultimate objective of PTC is to enhance the plants rate of reproduction, by quickly regenerating them without the use of seeds or in other cases by using seeds with very low abilities to germinate (Ellis, 1991). Different tissue culture techniques provide different

advantages of preserving, multiplying and storing plants genetic resources (Bunn et al., 2007). On the other hand, tissue culture medium is composed of a completed mixture of nutrients and growth regulators that can be altered according to the variety of plant at the different staged of cultures (George et al., 2008). The process of tissue culture is divided namely into; initiation, sub culturing for proliferation, shoots, roots and hardening (Harisha, 2007; Jean 2009).

Plant tissue culture techniques can be applied in the conservation of germplasms of important medicinal plants which could be sources of pharmaceutical substances. Actually, through tissue culture, somatic embryos can be conserved either in the medium or long term for different purposes. This technique can also be used to create variations through developing, selecting and isolating somaclones, in a process called somaclonal variations. This ultimately leads to the production of new germplasms, special category clones, elite cell lines and transformed plants with desired traits. Additionally, conserving and domesticating new germplasms in changing environments can positively contribute to increasing biodiversity (Pathak & Abido, 2014). Plant tissue culture provides the ability to rapidly propagate plant species in mass, as well as enable the long-term germplasm conservation in small spaces in a relatively short time, without damaging the existing populations. Since *in vitro* cultures are handled under sterile conditions , this could enable the exchange of plant germplasms and materials at the international level (Sharma & Sharma, 2013). Through *in vitro* techniques, it is possible to multiply and store genetic resources of recalcitrant seeds, plants which can be propagated vegetatively, threatened and rare plants as well as genetically modified plant materials (Lidder & Sonnino, 2012).

2.3 Somatic embryogenesis in plant tissue culture

This is the developmental pathway by which somatic cells ontogeny structures that resemble zygotic embryo that is bipolar and without vascular connection to the parental tissue through an orderly series of characteristics embryological stages without fusion of gametes (Jimenez, 2001). It occurs via two ways, directly and indirectly. In indirect embryogenesis, embryos form from explant tissues, creating

identical clones as well as revert in organized tissue into callus prior to embryo formation. In direct embryogenesis, embryos form from organized tissue without an intervening callus phase (Hamilton et al., 2004; Sudaha 2006). In the same way, it is an *in vitro* method of plant regeneration by which somatic cells or tissues develop and differentiate without undergoing the process of sexual fertilization. It can be initiated directly from the explants or indirectly by the establishment of mass of unorganized cells. Irrespective of the mode of production it has been argued that the anatomical and physiological features of somatic embryo are highly comparable to zygotic embryos. The morphological and development stage of somatic embryos are similar to that of zygotic embryos, with distinct stages of globular, heart and cotyledon and globular elongated scutellar and coleoptillar stages for monocotyledons (Gupta & Thorstenson 1993; Hamadan et al., 2008).

Plant regeneration through somatic embryogenesis is one of the main prerequisites for the potential application of clonal propagation, genetic transformation and *in vitro* conservation of germplasm of woody plant (Handley 1995). The success of micro-propagation largely relies on the selection of a suitable plant part which is to be used as the starting for the experiment (Prabhat et al., 2009). Somatic embryogenesis is not only a process of regenerating the plants for mass propagation but also a valuable tool for genetic manipulation. The process can also be used to develop plants that are resistant to various kinds of stress and to introduce genes by genetic transformation (Feher et al., 2008).

Somatic embryogenesis has been traditionally divided into two main stages; induction and expression. In the former, somatic cells acquire embryo genetic characteristics by means of a complete re-organization of cellular state, including physiology metabolism and gene expression (Feher et al., 2008). It is usually after a change in one or more cellulite conditions that the induced tissue or cell can reach the expression stage. At the expression stage, cells display their embryogenic competence and differentiate into somatic embryos (Jimenez, 2002).

Somatic embryogenesis is being increasingly exploited for clonal multiplication and *In vitro* regeneration of venerable indigenous germplasm that are threatened with

extinction (Pence, 1996). Therefore by somatic embryogenesis, culture plants hold a great promise for controlled production of myriad of useful metabolites and by extension merits of the whole plants system with those of microbial and animal cell cultures for the production of valuable therapeutic secondary metabolites (James, 2008).

In recent years somatic embryogenesis has become an integral part of advances in plant biotechnology as it allows room for close monitoring and precise, accurate manipulation of plant development and growth. It therefore offers space for rigorous control of physical environment and nutrient status parameters which are difficult to regulate with traditional experimental system (Kluytrmans, 1997). Furthermore, through somatic embryogenesis, the production of transgenic plants which represent an economical alternative to plant made vaccine or antibodies has been enabled. This has ended up reducing screening cost for virus and bacteria toxins, thereby increasing the number of farmers who have incorporated transgenic plants into their production system (James, 2008). Therefore the advantage of somatic embryogenesis over other methods is its unlimited potential in producing clones which can be used as medicinal raw material for secondary metabolites synthesis (Leeuwenberg, 2003).

Additionally reports by other scientist have described success in *in vitro* regeneration through somatic embryogenesis of others Apocynaceae these includes: *Cartharanthus roseus*, *Tylophora indicca*, *inchoma ledgeriana*, *Digitalis spp*, *Rehmannia glutinosa*, *Ruuvolia Serpentina* and *Canarierusis spp*, from leaf disc (Aslam et al., 2010). Somatic embryogenesis together with high germination potential offers a promising tool for rapid and mass clonal product ion of woody plants including angiosperm, gymnosperm and almost all plant families (Reddy et al., 2003). This increases the production of pathogen free materials of various plant species. Since the latter half of the 20th century, there has been an exponential population growth rate, leading to the increased land demand for agriculture and settlement (Shargel et al., 2007; Kumar et al., 2007; Suchariat et al., 2010).

Almost all plant parts such as leaves, stem, bark, root and inflorescences have been used for *In vitro* regeneration (Kumar et al., 2011). Therefore somatic embryogenesis has

become a well-established technique for culturing and studying the physiological behaviour of isolated plant organs, tissue cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions (Tatiya et al., 2011).

2.4 Effects of explants and culture conditions in somatic embryogenesis.

Explant are of various kinds such as shoots, tips leaf disc and node culture (Kacar, 2008). The choice of explant depends on the purpose of culture to be initiated and plant species to be used. (Kim et al., 2002). Before culturing ,in-depth details is vital for the selection and maintenance of the mother plant as the main source by maintaining it in a clean condition that allows an active growth with reduce exposure to diseases. Various explants have been used to initiate somatic embryogenesis, even though a variety of explants can be utilized. The correct developmental stage of the explant is also crucial for the initiation of embryogenic callus (Etienne et al., 2010). As further complexity, different explants tissue from the same mother plant produce embryogenic callus at different frequencies (Zhang et al., 2001). In addition juvenile or young explant produce more somatic embryos than older explant (Zhang et al., 2001). Therefore, the different endogenous phytochrome levels of various explants tissue might be a factor influencing the requirements of exogenous growth regulators. The recalcitrance of some species especially *Mpelepele* (*Schizogygia coffaeoides*) can be overcome by manipulating other media component, conditions and certain factors such as plant growth regulator concentration in the media, light, temperature and pH are major factors in development of regenerated plantlets (Jimenez, 2001).

2.5 Somatic embryogenesis and Organogenesis in plant tissue culture.

The differentiation of a single somatic cell or tissue to generate a relatively large number of plantlets is referred to as somatic embryogenesis. Somatic embryogenesis may be direct or indirect it involves differentiation of organized tissue into callus prior to embryo production whereas direct somatic embryogenesis involves production of embryo form organized tissue without an intervening callus phase (Murugesan et al.,

2008). On the other hand, organogenesis involves the direct development of plant organs (shoots and roots) directly from an explant. One of the major difference between organogenesis and somatic embryogenesis is organogenesis bypass the need for a callus phase whereas direct embryogenesis proceed from cells which are already embryogenically tissue (Arya et al., 2000). These pre-embryogenic cells only appears to favourable conditions such as application of exogenous growth regulators to allow the release into cell division and expression of embryogenesis, such cells tends to be much responsive than those involved in indirect organogenesis (Wernicke et al.,2002).

These two techniques are commonly employed in the regeneration of several endangered plants for conservation purposes (Sadeq et al., 2014b). Culturing explants in suitable media enables the regeneration of whole plants either directly or indirectly through somatic embryogenesis. In direct embryogenesis, plants develop directly from explants without the intermediate step of callus formation and dedifferentiation of the callus to an organised growing plant as involved in direct somatic embryogenesis. The induction of somatic embryos from explants in culture and germinating them to whole plantlets is a technical process which follows several steps and in which different plant growth regulators play a vital role (Sadeq et al., 2014a). Several studies have shown comparison and relationship of morphohogentic ability with varying plant growth regulators (Auxin-cytokinins) combination on the effect of induction and growth of explant in somatic embryogenesis and organogenesis (Janarthanam et al., 2008).

This techniques is greatly applied in the rapid multiplication of medicinal plants which are considered endangered. In somatic embryogenesis the physiological state of explant, composition of the culture medium and the condition of cultivation are vital keys in the process of somatic embryogenesis.

Through somatic embryogenesis it leads to cell suspension cultures development as it offer great advantages which provide basic for morphological, biochemical and molecular studies of the nature of somatic embryo from single-cell to whole plant (Lantcheva et al., 2001). Where as in organogenesis shoots would appear after 4-8weeks on the cultured explants and the regenerated roots can be maintained by serial sub-culturing at 4-8 week intervals indefinitely and when required, the plants can be

regenerated and transferred to the field for conservation purposes (Babu & Subhasree 2009). Somatic embryogenesis however carries the risk of losing materials due to human error or contamination, and in some cases the onset of genetic instability (Pathak & Abido, 2014). Embryo maturation in cell suspension through somatic embryogenesis develop faster and can be used as a source for direct gene transfer via particle bombardment, transit gene expression, microscopy observation and as bioreactors to produce enzymes, medicine and clonal propagation to recover genetically transferred plant (Neve et al., 1999).

The anatomical and physiological features of organogenesis and somatic embryogenesis is highly comparable but similar in terms of their overall size and internal cellular organization (Karuppusamy et al., 2009). In organogenesis mutation does not occur thereby loss of regenerated capacity is less where as in somatic embryogenesis mutation occur leading to various abnormalities such as fused cotyledons and multiple apex formation (Espinosa et al., 2003). In most *Apocynaceae* successful record has been achieved through somatic embryogenesis than organogenesis. This is because most *Apocynaceae* are medicinal plant and large percentage of them use in cell suspension cultures via somatic embryogenesis as it permits large number of somatic embryos for extracts about 60,000 to 1.35million which can be regenerated per litre of medium with long term storage by induction of dormancy (Jualang et al., 2002).

2.6 Challenges of In vitro methods in plant regeneration

Despite its potential, *in vitro* techniques in plant genetic regeneration experiences a lot of challenges. The goal of many propagation and sustainability strategies is to maintain genetic diversity. This however may fail to be achieved by *in vitro* propagation since a new line can be initiated. *In vitro*, genetic clones of these lines are developed, thereby multiplying them. Actually, *in vitro* regeneration increases the genetic diversity of a plant species depending on the culture method used (Pence, 2010).

2.6.1 Technical challenges

Irrespective of the establishment of plant tissue culture methods, different plant species respond differently to successive stages of *in vitro* culture. For example, different plant tissues respond differently to surface sterilisation substances, with some plant species being easy to sterilise while others requiring modifications of existing sterilisation protocols. These challenges which make the *in vitro* propagation process time and resource consuming. These include microbial contamination has been reported as constant problem which can compromise development of all in vitro technique ,the rate of occurrence of contamination were found mostly to be higher in bacterial ranging from 36-49% in explant (Leifert et al., 1989, Ndakidemi et al., 2007). This contamination is higher in the preparatory room and the incubating bench. Tissue culture equipment especially culture bottles vessel are always close sterile, however in Africa due to high cost alternative bottles are reused often for a long period of time and caps are replaced with poly papers thereby making microbial spores gaining asses and travel from one vessel to another contaminating the cultures (Kane, 2003).

The major constraint of contamination tends to cause loss of time. Adverse effects on cultures and erroneous experimental results. Lack of proper training of technician in Africa laboratories in handling and disposal of any equipment for example wearing dirty lab coats are dust magnets placing a dust –laden sleeve into a laminar flow germinates and contaminates cultures. Dry flaky skin is another challenge for expertise as the condition can be aggravated by frequent hand washing in the laboratory, also adequate evaluation of workers to be gloved, gowned and masked whenever on the bench with constant supervisor should be employed (Jony et al., 2013). The used of cheap equipment as replacement to sophisticated standard machine cause challenge in *in vitro* regeneration for instant Bunsen burner should be discouraged as they disrupt the filtering of air and the resulting turbulence can increase the probability of contamination (Baberini et al., 2011). Although it is possible to maintain individual lines in culture, given the limited resources of many laboratories, the larger the number of individual genetic lines, the lesser material for each line can be maintained. This thereby increases the chances of losing a line through contamination or any other unfortunate circumstance like failure to grow.

Another technical challenge to maintaining *in vitro* cultures is the occurrence of somaclonal variations in some species in culture over time, which could involve mutational changes, thereby changing the fidelity of the conserved plant (Kaeppeler et al., 2000, Bairu et al., 2006). Long-time effect of Microbial contamination leads to contamination of explant , failure of explant to grow in culture , necrosis and leaf drops in shoots, inability of shoots to form roots , inability of somatic embryos to germinate and drying of regenerated plantlets at acclimatization.

2.6.2 Economic challenges

In vitro methods are very labour intensive, which eventually translates to higher costs when compared to in situ and ex situ conservation techniques. The cost of producing a plant by tissue culture for conservation purposes has been estimated to be 4-5 times more expensive than it is by use of cuttings. Additionally it has been estimated that the cost of banking a tissue culture line is up to 10 times more expensive than it would cost to bank an accession of seeds (Koo et al., 2003; Reed et al., 2004). The process of cost determination for preservation of an endangered species is different from that of determining the cost of commercial propagation. When conserving endangered species, new protocols must be developed for each species, sometimes dealing with very little plant materials for experimentation, denying the involved persons the economies of scale which come with commercial propagation (Pinto et al., 2002). In commercial enterprises, it's not economically feasible to work with difficult species, the exact opposite with many conservation projects. To overcome cost challenges, either costs can be reduced or funding increased or even resources utilised efficiently. A combination of cost reduction and efficient resource utilization seems to be the best approach.

2.7 Antimicrobial activity of Apocynaceae

Antimicrobial activity of plant can be determine by observing the growth response of various microorganism to the plant extract placed in contact with them (Tiwari et al.,

1998). Traditional medicinal plants have almost maximum ability to synthesize aromatic substance most of which are phenols or their oxygen-substituted derivate (Ramar et al., 2004). *Apocynaceae* are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids and glycosides, which have been found *in vitro* to have antimicrobial properties (Dahanukar et al., 2002). Therefore, antimicrobial properties of medicinal plants are increasingly reported from different parts of the world. The antimicrobial susceptibility test (AST) is widely used to evaluate the plant extracts for antimicrobial activity (Kacar, 2008).

The basic methods of antimicrobial susceptibility test include diffusion, dilution and bioautographic methods. In diffusion technique, plant extract is brought into contact with an organism which is then incubated and the diameter of zone of inhibition measured. Extract that is active against the particular strain of bacteria based on the diameter of the zone of inhibition is directly proportional to the degree of sensitivity and concentration of extract. The dilution method involves the mixture of samples with suitable medium, inoculated with test organism and then reviewed by direct visual observation (Das et al., 2010). Agar disk diffusion assay was developed in 1940 as a modification of that described by Bauer Kirby's Sherri's and Truck commonly known as Kirby-Bauer Test. This technique is a simple method of determining the susceptibility of microorganism to an antimicrobial agent (Pavia et al., 2010).

Plants produce biologically active secondary metabolites often with high complex chemical structures. Such compounds are believed to be mainly concerned with the survival of the plant by defending itself from disease causing organism (Mazid et al., 2011). Compounds such as saponins, flavonoids, phenolics and glycoside have been extracted from plants (Prema et al., 2013). Alkaloids and steroids are the two major classes of plant derived compound used in medicine today (Maynard, 1988). Tannins are also among the most abundant active principles in plants, particularly in trees and shrubs where they are found in the bark (Epstein et al., 1980). Flavonoids on the other hand are another group of compounds with several medicinal properties like anti-allergic, anti-bacterial anti-inflammatory and anti-oxidant (Epstein et al., 1980). Recent studies in Kenya have revealed various compounds of great medicinal value. Other secondary metabolites isolated from plants include coumarins, steroid, Crinitol,

papaverine, Annoniane and mannitol which have inhibitory effects on *B. subtilis*, *C. albican* *C. utilis* (Reed & Rodriguies 2004). The basic driving force for the renewed interest in plant antimicrobial properties which has been synthesized chemically for drug formation, has led to the rapid increase in plant exploitation and overharvesting which is at high risk of being lost irretrievably. In addition the activity of these extract could be influenced by the nature of plant material, parts climatic conditions solvent used for extraction because of the different plants constituents depending on those factors (Bharitkar et al., 2014).

2.8 Plant secondary metabolites

Plants have the ability to synthesize aromatic substances which are referred to as secondary metabolites. These are compound with varied and sophisticated chemical structures, produced not only by plants, but also certain strains of microbial species (Weiss, 1978). The production of secondary metabolites in plants starts when growth is limited by the exhaustion of one key nutrient source of carbon, nitrogen or phosphate. The production of useful metabolites such as alkaloids, essential oils, flavonoids, pigmented proteins, phenols, permethrins, roenioids, sterols and steroids from plant tissue culture has created new avenues for their commercialization (Sarin, 2005). In the recent past, about 12,000 plant derived metabolites have been isolated and characterised (Elumalai & Eswariah, 2012). There are more than 105 secondary structures reported and synthesized to date, with approximately 20% of the carbon fixed by plants through photosynthesis dedicated to their biosynthesis. Various plant extracts have been screened to establish whether they are indeed highly efficient against many pathogenic bacteria. These include alkaloids, flavonoids, Phenylpropanoids, quinines, terpenoids, steroids and tannins. The extracted product have been found to contains a mixture of plant metabolites which can be isolated from each other use of different fractionation techniques. Studies in members of the family *Apocynaceae* have established that secondary metabolites in most plants in this family are localised in root segments in *Cartharanthus roseus*, *Rauwolfia micrantha* and *Tylophora indicca* (Suffredini et al., 2002).

2.9 Importance of cell suspension cultures

Cell suspension cultures are used in modern times for large scale culturing of plant cells from which secondary metabolites could be extracted. It involves developing and transferring the relatively friable portion of the callus into liquid medium and maintaining it under suitable conditions of aeration, agitation light and temperature alongside other physical parameter (Visweswari et al., 2013) .The prospect of using cell suspension culturing techniques is for obtaining secondary metabolites such as active compounds for pharmaceuticals as well as obtaining cosmetic hormones and protein antigens from the harvested cultured cells (Wink, 2015). Cell cultures not only yield high standard phytochemical levels in large volumes but also eliminate the presence of interfering compounds that occur in the field. The advantages of this is that it can ultimately provide a continuous reliable source of natural products as they are an *in vitro* system that can be used for recombinant protein and suitable for large scale application in biotechnology industries (Wang et al.,2006).

Cell suspension cultures can easily be grown in shake flasks or fermenters. Currently, more than 2000 plants species have been intensively investigated for the isolation of bioactive compounds predominately from *Euphorbiaceae*, *Asteraceae*, *Labiatae*, *Fabaceae*, *Meliaceae*, *Malvaceae*, *Polygalaceae*, *Apocynaceae* and *Apicaceae* plant families (Yang et al., 2016). Among the metabolites, flavonoids, terpenoids, alkaloids steroids and phenols have showed a pronounced activity against many diseases in humans. The production of useful metabolites from plant tissue culture has created new methodology for their commercialization, among such include alkaloids, antimicrobial, essential oils, flavonoids, pigment proteins, phenols, permethrins, roeniods, sterols and steroids (Sonia et al., 2013). The prospect of using cell suspension culturing techniques is for obtaining secondary metabolites such as active compounds for pharmaceuticals and cosmetic hormones, protein antigens from the harvested cultured cells.

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Cell suspension culture is particularly suitable for physiological, biochemical and molecular studies of the process of somatic embryogenesis and its different stages (Manna &Abulaka 2006). There is a huge variety of plants rich in secondary metabolites which are potential sources of drugs and essential oil of therapeutic importance (Pelka et al., 2000).

Several cells of *Apocynaceae* plant species including, *Cartharanthus roseus*, *Dioscorea delloidea*, *Digitalis lamala* and *Panax notogineseng* have been cultured in various bioreactors for the production of secondary metabolites in controlled environment independently from climate and soil (Rios &Recio 2005, Joseph & Paul 2006). Despite the existence of potent antibiotics and antifungal compounds, resistant microorganism are rapidly appearing. In the case of *Apocynaceae*, this plant family not only possess antimicrobial properties but is also very human friendly (Grigore et al., 2016).

2.10 Brine shrimp tests

This is a simple Bioassay used for the primary screening of crude extract of plant and marine source (Meyer et al., 1982). it is considered a useful tool for preliminary assessment of toxicity, and it has been used for the detecting fungal toxins, plant extract toxicity, heavy metal pesticides of plant materials, from plant extract as it is very simple, inexpensive and also use of low toxins amount to perform the test sufficiently (Palka et al., 2000).

Brine shrimp assay consist of exposing larvae to test sample in saline solution and its lethality is then evaluated at 24 hour. The commercial availability of the inexpensive brine shrimp eggs ease the performance and cost therefore making it very useful bench top method (Barry & Thornberry 1981). A number of studies have indicated the use of brine shrimp assay to screen plant extract (Barry &Thornberry 1981). Bio monitor, complete analysis of the cytotoxicity allows us to understand the location of cytotoxic

substances as it allows the use of smaller quantity of the extract and permit larger number of samples and dilution within shorter time than using the original test vial (Pelka et al., 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection site

Schizogygia coffaeoides Mpelepele is predominately found in shimba hills and Kilifi coastal regions of Kenya. Shimba hills has an area of 19, 250 Hectares above sea level and is located 33 km south of Mombasa. It is rich in heterogeneous habitat including forestland and scrubland forming a plateau. The shimba hills forest is the second largest remaining rainforest in East Africa, covering about 190 square kilometres. It's humid with average temperature of 24°C, and annual rainfall ranges of between 88 mm to 1682 mm yearly.

Kilifi is located in the northwest region of Mombasa and borders Tana River to the north and Taita taveta to the west. It has a square area of 1260 km and is dominated by low range stand and stone hill. Temperatures range from 21-35° C with annual rainfall from 300 mm to 11300 mm.

3.2 Plant material

Mpelepele were obtained from shimba in the coastal region of Kenya. Twelve cuttings were kept in a cool box while roots, stem and leaves were properly collected in sacks and transported to Institute for Biotechnology Research (IBR) in Jomo Kenyatta University of agriculture and technology (JKUAT). The twelve cuttings were transplanted in potting bags containing well mixed forest soil, sand and manure in ratio of 2:1:1 and kept in IBR green house . They were watered six times a week with 500ml water per plant using spraying can and grown from the 4th August 2015 to 12th March 2016. Eight months old plants were used as explant. The research work was carried out at plant tissue culture and molecular laboratories in (IBR) at Jomo Kenyatta University of Agriculture and Technology (JKUAT).

3.3 Media and culture condition.

MS (Murashige and skoog, 1962) (appendix 4) media was used in the experiment. It was supplemented with 3% (w/v) sucrose, 0.28% gelrite and plant growth regulators (PGRs) as required and pH adjusted to 5.8 using 0.1N Hcl and or 0.1NaaOH. The media was then dispensed in 200 ml culture jars (30ml per jar) and autoclaved at 121°C at 15(psi) above atmospheric pressure for 15 mins. The chemicals used were of analytical grade and experiments were carried under sterile conditions in the laminar flow hood. Cultures were grown at 25±2°C under 16hour light and 8hour dark period in growth chambers illuminated by 40Wphilips ® white fluorescent tubes . The intensity of light was between 2500 and 3000µmol⁻² s⁻¹.

3.4 Selection, collection and sterilization of explant

To optimize an efficient *in vitro* propagation protocol for surface sterilization of Mpelepele, the 3rd and 4th fourth pairs of leaves were excised using a new scalpel blade from 8 month old young plants and placed in a clean culture bottle containing tap water and taken to the laboratory for cleaning.

A total of 20 explants were placed under running tap water for 30 minutes. They were then placed in new glass beakers containing 25ml of distilled water with 1ml of detergent and 0.25ml of the antibacterial salvon ® and kept for 10 minutes. This was followed by rinsing the leaves thrice with sterile distilled water .The explants were kept in distilled water containing a fungicide (Ridomil) and 0.1ml of tween 20® for 1 hour. They were thereafter rinsed thrice with autoclaved distilled water and transferred to lamina flow hood. To study the effects of sodium hypochlorite (NaOCl) and time of exposure on these explants, 5 explant were each subjected to (NaOCl) at concentrations of 5.2Mm, 7.8mM, 10.4mM and 13mM as well as exposure time of 10, 15, 20, and 25 minutes and then rinsed thrice using sterile distilled water The explants were timed to 5mm. A total Of 20 trimmed explants were inoculated on full MS media without hormone and kept in the growth chamber. They were monitored for 14 days to check bacterial and fungal contamination as well as those that were scorched.

Explant behaviour in terms of colour change throughout the sterilization period was monitored. Sterilization set up was repeated 3 times with a total of 20 explants in each set up.

Data was collected on the type of contamination observed after sterilization, especially fungal and bacterial contamination as the number of contaminated culture bottles, against the originally cultured bottles and expressed as a percentage. The number of explants surviving the sterilization procedure at the different NaOCl concentrations and exposure times was also determined and expressed as a percentage. A completely randomised experimental design was used.

3.5 Sterilization of Laminar flow hood

Before any culture process, the laminar flow hood was swabbed with 70% ethanol using cotton wool and kept running for about 15minute before working on the hood to sterilize the hood. Precautions were observed by wearing a clean lab coat and all materials (papers, conical flasks, and forceps and scalpel holders) wrapped in aluminium foil and sterilized in autoclave at 121°C at 15(psi) above atmospheric pressure for 15minutes. Forceps and scalpel were placed in steribead[®] sterilizer maintained at 250°C while the laminar flow hood was frequently swabbed with 70% ethanol at suitable intervals while working.

3.6 Callus initiation and induction

A total of 30 clean explants were transferred to culture bottles with callus initiation medium, replicated 3 times (10) in each, three supplemented with: 1.0mg/l BAP+0.4mg/l KIN+0.2mg/l NAA+0.05mg/lTDZ 1.5mg/l BAP+0.6mg/l KIN+0.3mg/l NAA+0.1mg/l TDZ 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ.

Cultures were observed daily and data collected on morphological response: colour, texture and shape of the callus using eye and microscopic observation. Quantitative data involved the callus formation frequency, which was determined as the number of calli forming per treatment as a percentage of the cultured explants.

3.6.1 Callus multiplication

Callus with yellow clumps were sub-cultured to callus multiplication media (2.0mg/l BAP+0.8mg/l KIN + 0.4mg/l NAA+0.5mg/l TDZ) (1.5mg/l BAP + 0.6mg/l KIN + 0.3mg/l NAA + 0.1mg/l TDZ). They were grown on this media to obtain sufficient amount of callus.

3.6.2 Somatic embryo formation and maturation

Callus were sub-cultured to MS medium supplemented with (2.0mg/l BAP+0.8mg/l KIN + 0.4mg/l NAA + 0.5mg/l TDZ). Under a sterile laminar flow hood, cultures were transferred to growth room and incubated at 16 hour and 8 hour photoperiod for a period of one month.

The somatic embryos were observed for stages of embryogenesis from callus to embryoids and finally meristems.

3.6.3 Shoot induction

Mature yellow somatic embryos were excised from somatic embryo maturation medium and transferred to MS based, supplemented with (2.0mg/l BAP + 0.8mg/l KIN+0.4mg/l NAA + 0.5mg/l TDZ) (1.5mg/l BAP + 0.6mg/l KIN +0.3mg/l NAA + 0.1mg/l TDZ) (1.0mg/l BAP + 0.4mg/l KIN + 0.2mg/l NAA + 0.05mg/l TDZ).

Shoot proliferation and number of shoots formed per calli was observed weekly and recorded for each media type.

3.6.4 Rooting of multiple shoots

Multiple shoots were carefully separated with the help of sterile scalpel depending on the number of axillary shoots produced and were rooted in MS media supplemented with 0.5mg/l BAP+0.25mg/l IBA, 1.0mg/l BAP+0.5mg/l IBA and 1.5mg/l

BAP+0.75mg/l IBA and observed weekly. Multiple shoots were observed for formation of root primordia and data collected on the number of roots forming per treatment as well as the time taken for the roots to form.

3.7 Cell suspension cultures

For cell suspension culture, calli from callus multiplication media were transferred to ¼ strength MS (appendix 4) liquid Media supplemented with (0.1mg/l BAP+0.0.5mg/l NAA) (0.75mg/l BAP+0.2mg/l KIN +0.1mg/l TDZ) (0.25mg/l BAP+0.1mg/l KIN +0.2mg/l NAA +0.05mg/l TDZ) and autoclaved distilled water in 200ml sterile bottle and incubate at 100 rpm on rotary shaker at 37°C for 30 days to induce stress and later extract the calli cells.

3.8 Experimental design and data analysis

Completely randomised design (CRD) was used as the study design. Data were replicated thrice and was key in excel spread sheet and analysed using statistical analysis system (SAS) version 9.1. Mean number of contaminated explant by bacterial and fungi as well as those that died as a result of scorching was determined .Two way - ANOVA was used to determine the best suitable concentration and best time of exposure for sterilization as well as the best growth regulators, its interaction and duration of callus induction, number of shoots per calli and the number of roots per shoot. Mean separation was done by Tukey's pairwise comparison at $P < 0.05$.

3.9 Phytochemical screening

Qualitative phytochemical analysis to identify the nature of phytochemical constituents present for major constituents of the plant extract were screened for the presence of biologically active compounds like tannins, flavonoids, alkaloids, saponins and steroid ring. Fresh plant leaves, stem and roots were collected from 4 years old matured plant , dried in the laboratory and then grinded into coarsely

powdered. Extract were prepared by dissolving 1.5g of plant powder and dissolved into 6 extraction solvent (Methanol, Ethanol, Acetone, Chloroform, N-hexane, and Water). About 1.5g of the extract was boiled in 10ml of water in a test tube for 15 minutes. After filtration two drops of 0.1% ferric chloride was added, a brownish – green or blue –black clouration indicated the presence of tannins.

For steroid ring 1.5g of extract (leaves, stem and root powder) was weighed and dissolved in 6ml of chloroform for 5 minutes and filtered, concentrated H₂S04 was added carefully to the filtrate to form a layer, a colour change of reddish brown at the interface showed its presence.

Saponins test was done by addition of 5ml of distilled water to 1.5g of the extract the solution and shaken vigorously a formation of emulsion indicated a presence of saponins.

For Alkaloids 1.5g of the extract was dissolved in 5ml of 1%hydrochloric acid (HCL) on steam bath for 20 minutes after the filtrate was being treated with few drops of Dragendorff reagent its precipitation formation indicated positive results. Flavonoids were tested by using 1.5g of the extract dissolving into 5ml of methanol and heating for 10 minutes , adding a chip of magnesium metal to the mixture then by a few drops of concentrated hydrochloric acid (HCL) Where a red or orange clouration indicates presence of flavonoids.

3.10 Antimicrobial activity

3.10.1 Extract preparation

Plant were separated into Leaf , stem, roots which were dried under room temperature for 3 days then it was crushed into fine powder and sealed separately in paper bags until time of use , callus from cell suspension cultures were crushed and also used for this study.

3.10.2 Procurement of test organism

The following microorganism were used : *Escherichia coli* , *Staphylococcus aureus*, *Pseudomonas aeruginosa* *Bacillus Subtilis* (Bacterial strains) and fungal strains *Candida albicans* and *Aspergillus niger* obtained from microbial type collected at the Institute of Biotechnology for Research (IBR). All the Bacterial strains were cultured on nutrient agar medium (Beef-extract 1.0g, Yeast extract 2.0g , peptone 5.0g sodium chloride 5.0g and Agar 15g) while fungi were cultured on Potato dextrose agar media (potato infusion form 200g, dextrose 20.0g and Agar 15g). They were prepared and sterilized at 151bs and 121°C for 30 minutes then poured into petri dish 30ml each, labelled and maintained.

3.10.3 Standard antibiotics

Standard antibiotics discs namely Chloramphenicol, Ampicillin, Nystatin were used as positive controls.

3.10.4 Preparation of extracts

Powder leaf, stem, roots and callus was weighted at 15g and dissolved in 150ml of the following solvent Acetone, Ethanol, Methanol, Chloroform, N-Hexane, Diethyl and water for 72 hours under room temperature , extract were filtered using Buchner funnel, whatman paper and rotary evaporator, it was further kept in windy oven at 37°C to dried. Each solvent of 10ml was diluted into the extract it was labelled and kept at 4°C.

3.10.5 Antibacterial assay

The disc diffusion assay method was used to determine the growth inhibition of bacterial by plant extracts Diluted bacterial culture (100µl) was spread over nutrient agar plates with a sterile glass rod at 10mg/ml, 20mg/ml, and 50mg/ml of each extract. They were applied to each whatman paper disc (5mm) and allowed to dry before

placed on the plates each was tested in triplicate all plates were well labelled, incubated at 37°C for 24 hours Chloramphenicol and Ampicillin were used as standard control after which the diameter of inhibition was measured.

3.10.6 Antifungal assay

Diluted fungi culture (100µl) were also spread on potatoes dextrose agar (PDA) plates with sterile glass rod 10mg/ml, 20mg/ml, and 50mg/ml of each extract was applied to each Whatman paper disc (5mm) labelled and incubated at 25°C after which the diameter of zone of inhibition was measured by using a standard ruler recorded in millimetres and analysed at 95% confidence level, Nystatin was used as positive control.

All values which are the mean of triplicate observed were expressed as means ± standard deviation. The inhibition zone diameter for all concentration were subjected to Two-way analysis of variance (ANOVA) and $p < 0.05$ was considered significant, comparison of the antibacterial and antifungal activity of plant extract and callus extracts with standard antibiotics were evaluated. Statistical analysis system (SAS) was employed for the analysis.

3.11 Toxicity

Brine shrimps eggs were obtained from Botany department (JKUAT). Artificial seawater was prepared by dissolving 3.8g/l of salt into sterile distilled water to obtain a concentration of 0.5-8.0 mg/ml then brine shrimps eggs was poured into 100 ml beaker containing 80ml of artificial seawater to increase the number (*Artemia saline*). After which the eggs were hatched after 48 hours. They were incubated at room temperature, after 48 hours the larvae (naupli) were collected with a pipette after attracting the naupli to one side of the beaker with the source of light. Stock solution of all extracts were prepared by adding 6ml/L Dimethyl sulfoxide (DMSO). Stock were diluted into different concentrations levels of (200, 400, 600, 800 and 1000ml) by drawing different volumes and then added to vials each containing 10 brine

shrimps (thus the negative control containing brine shrimp artificial sea water . chronic activity was measured after 24 hours according to Meyers 1982. Finally the number of survivor larvae was counted and the various concentration that could kill the shrimps was passed by one way (ANOVA) followed by Tukeys test to ascertain the difference in shrimp mortality between each concentration.

CHAPTER FOUR

RESULTS

4.1 Sterilization of explant

In this study sterilization of *S. coffaeoides* leaf explants was achieved by using a single sterilant; sodium hypochlorite (NaOCl). It was observed that exposing leaf explants to different concentrations of this sterilant and at different times resulted in differences in sterilization levels of the explants based on the level of bacterial contamination as well as that of fungi observed growing in cultures. Irrespective of the exposure time, analysis of variance revealed that varying the levels of NaOCl concentration resulted in significant differences on the levels of contamination (Table 4.1) observed in culture as well as the % number of surviving explants ($P < 0.005$).

At low NaOCl concentrations of 5.2 mM and 7.8 mM, it was observed that the levels of bacterial contamination were high (53.33% and 66.67%) compared to the low contamination levels (33% and 28%) observed at 10.4 mM and 13 mM (Table 4.1).

Although 13.0 mM NaOCl showed lower bacterial and fungal contamination, actually, it was observed that these NaOCl levels (13 mM) lead to most of the explants being scotched hence dead. Therefore, NaOCl levels of 10.4 mM were observed to be ideal for explant sterilization since they produced relatively low levels of bacterial and fungal contamination and the highest levels of un-scotched clean and surviving explants which were regenerable (36%) (Table 4.1).

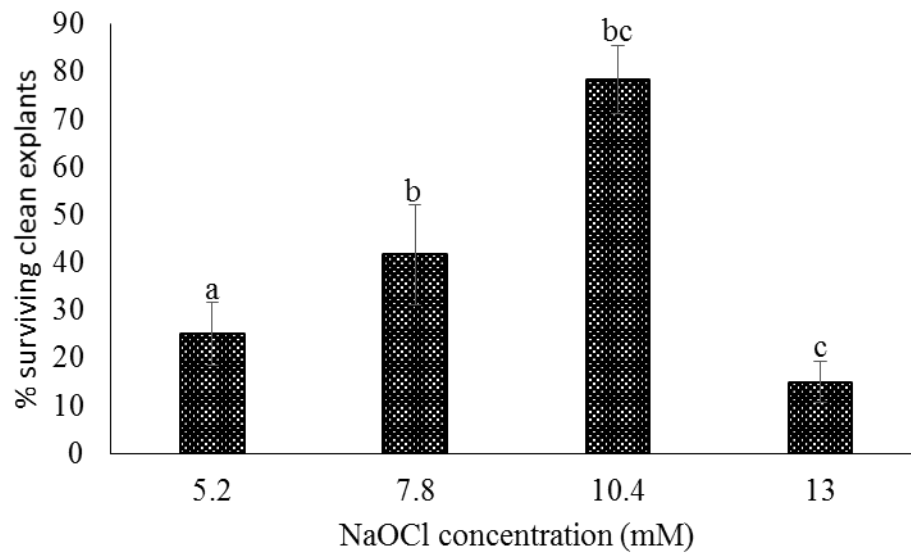


Figure 4.1: The effect of NaOCl on explant survival

Standard errors bars with the same letter are not significantly different by Tukey's pairwise separation ($P \leq 0.05$)

4.2 Effect of time of exposure on explant survival

Analysis of variance also revealed that irrespective of the NaOCl levels, the time of exposure had significant effect on contamination as well as the number of surviving explants that could regenerate (figure 4.2).

It was observed that the ability of the explant to survive and be regenerable in culture was inversely proportional to the exposure time (Figure 4.2).

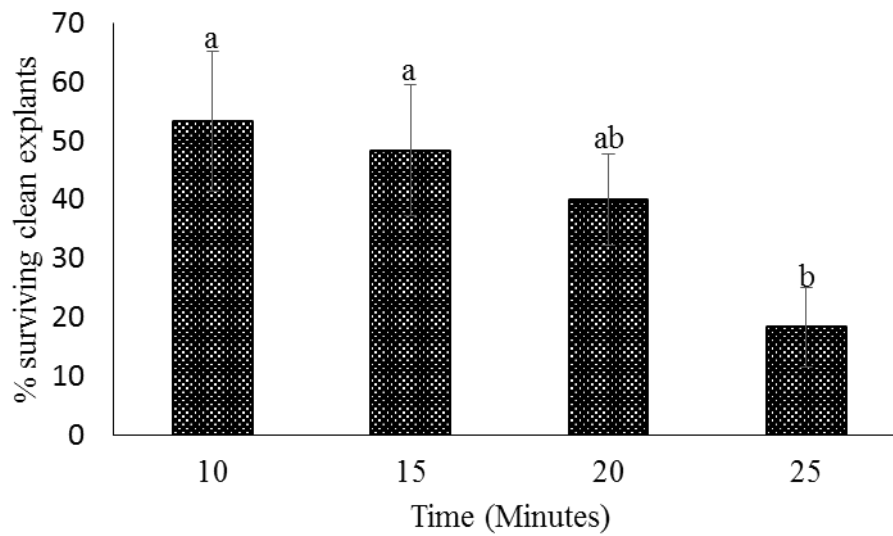


Figure 4.2: The effect of time on explant survival

Standard errors bars with the same letter are not significantly different by Tukey's pairwise separation ($P \leq 0.05$).

4.3 Effect of sodium hypochlorite and Time of Exposure interactions on explant

The interaction of NaOCl concentration and time of exposure on bacterial and fungal contamination as well as explant survival was observed through analysis of variance that the interaction of these two parameters had a significant effect on bacterial and fungal contamination as well as the number of surviving regenerable explants in culture ($P < 0.0001$). In order to reduce fungal contamination, the explants were further exposed to 3% Ridomil® solution that reduced fungal contamination by half the recorded value. As a result, the best interaction level of these two parameters that gave high number of clean and surviving explants was NaOCl at 10.4 mM concentration for 20 minutes followed by exposure to 3% Ridomil® (Table 4.1). It was observed that the ability of the explant to survive and be regenerable in culture was inversely proportional to the exposure time (Table 4.1).

Table 4.1: Effect of NaOCl and time of exposure interaction on explant sterilization.

NaOCl (mM)	Time of exposure (Min.)	Bacterial contamination (%)	Fungal contamination (%)	Surviving explants (%)
5.2 mM	10	53.33±4.33 ^a	66.67±4.67 ^a	48.67±7.64 ^a
5.2 mM	15	53.24±3.04 ^a	64.33±2.34 ^a	46.00±5.00 ^a
5.2 mM	20	47.00±3.55 ^b	61.23±3.20 ^b	43.11±3.33 ^b
5.2 mM	25	40.00±2.45 ^c	58.33±2.67 ^c	40.00±4.17 ^c
7.8 mM	10	41.27±3.67 ^c	60.00±4.26 ^c	42.16±3.67 ^{bc}
7.8 mM	15	38.33±4.64 ^d	58.00±3.35 ^c	41.4±3.13 ^{bc}
7.8 mM	20	35.67±3.37 ^e	56.33±5.23 ^{cd}	39.33±3.04 ^d
7.8 mM	25	32.27±2.17 ^f	54.00±3.55 ^d	37.67±2.47 ^e
10.4 mM	10	33.20±2.28 ^{ef}	55.67±2.55 ^d	38.33±3.67 ^d
10.4 mM	15	31.00±1.14 ^f	52.00±2.07 ^e	36.00±3.55 ^{ef}
10.4 mM	20	29.03±1.03 ^g	50.67±2.27 ^d	36.33±6.67 ^{ef}
10.4 mM	25	26.33±2.13 ^h	49.33±1.93 ^{ef}	34.67±3.33 ^g
13.0 mM	10	28.33±2.33 ^g	48.67±4.67 ^f	30.00±2.55 ^h
13.0 mM	15	24.67±2.17 ^h	42.00±2.05 ^g	28.33±2.47 ^h
13.0 mM	20	20.33±3.07 ⁱ	40.00±3.39 ^g	26.67±1.21 ⁱ
13.0 mM	25	18.50±2.55 ⁱ	32.33±1.33 ^h	20.0±0.00 ^j
L.S.D _(0.05)		2.45	2.71	2.17

Values with the same letters in the same column are not significantly different by Tukeys pair-wise comparison ($P \geq 0.05$).

4.4 Initiation and callus induction

The effect of plant growth regulators BAP, KIN, NAA and TDZ at different combinations to induced calli was assessed. When leaf explants were cultured in three media containing these hormones, it was observed that callus started developing at 9 days of culture from the edge of the leaf in treatment (2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ) and extended into the interior in the responding leaves (Plate 4.1a) while (1.0mg/l BAP+0.4mg/l KIN+0.2mg/l NAA+0.05mg/l TDZ) (plate 4.1b) occurred at 13 days with significant difference observed between the three treatments in time of induction, texture and colour.

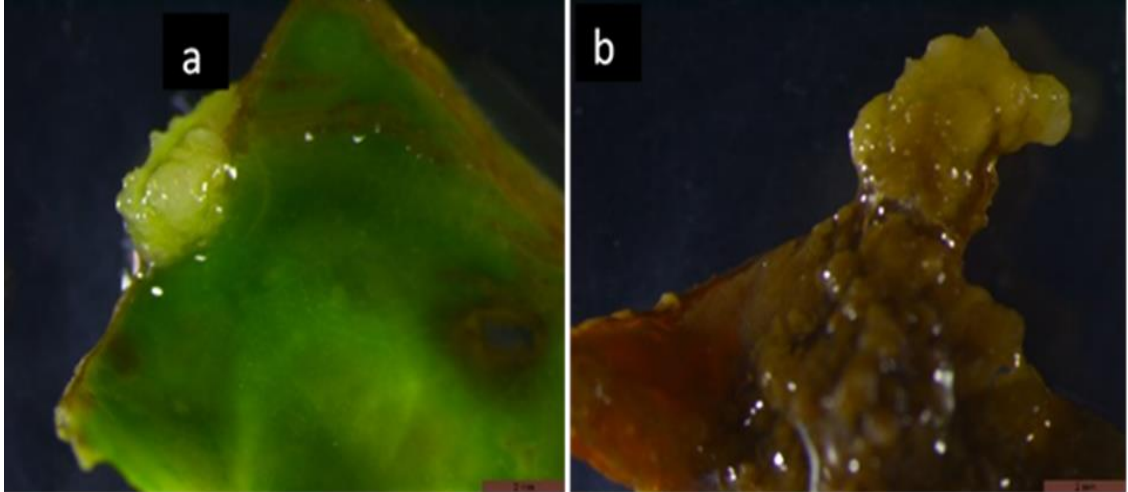


Plate 4.1: Appearance of callus formation on different plant growth regulators

a) Callus appearance in 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ.

b) Callus appearance in 1.0mg/l BAP+0.4mg/l KIN+0.2mg/l NAA+0.05mg/l TDZ.

4.5 Callus multiplication

Cream yellow compact calli were observed on 2.0mg/l BAP+0.8mg/l Kin+0.4mg/l NAA+0.5mg/l TDZ hormone combination which started forming after 9 days of culture (Plate 4.2a). This hormone combination showed the highest callus formation frequency (73%) while at 1.5mg/l BAP+0.6mg/l Kin+0.3mg/l NAA+0.1mg/l TDZ and 1mg/l BAP+0.4mg/l Kin+0.2mg/l NAA+0.05mg/l TDZ, at frequency of (39 and 13%) soft friable white loosely packaged calli were observed (Plate 4.2b). Although these two low hormone combinations formed reasonable embryogenic calli, these soft friable calli could not survive beyond two subcultures and turned brown and died after the second sub-culturing. It was observed that when the white friable calli from the low hormone combinations were transferred to the high hormone combination, they survived multiple subcultures (Plate 4.2).

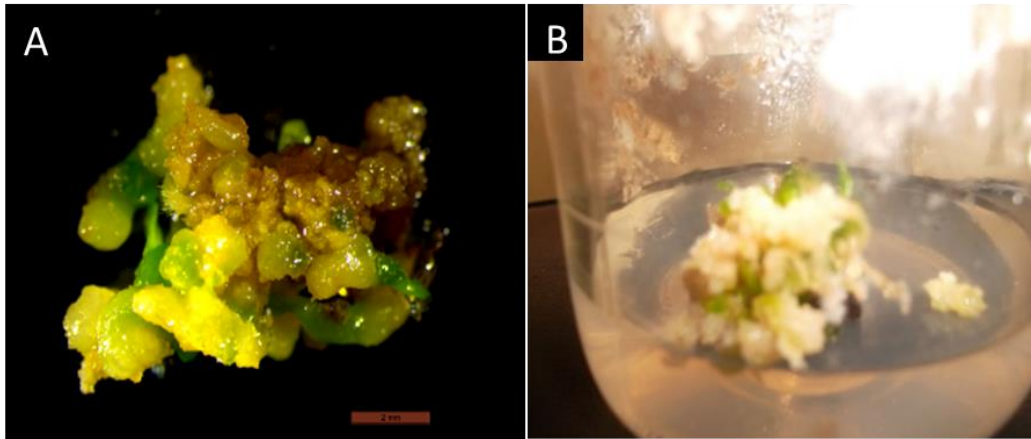


Plate 4.2: Callus growth after 28 days in culture.

(A) Hard compact cream yellow callus forming at high hormone combination

(B) Soft white callus forming at low hormone combination medium.

Data analysis revealed that there was a significant difference in callus formation using the three hormone combinations ($P < 0.001$), with most calli forming at the 2.0mg/l BAP+0.8mg/l Kin+0.4mg/l NAA+0.5mg/l TDZ combination and the least calli forming at 1mg/l BAP+0.4mg/l Kin+0.2mg/l NAA+0.05mg/l TDZ combination embryogenic cultures were highest at mean 68 ± 4 with (68%) frequency and lowest at 13 ± 4.5 with frequency of (13%) (Table 4.2).

Table 4.2: The effect of different growth regulators on callus formation

Treatments (mg/l) BAP+KIN+NAA+ TDZ	Callus induction (%)	Callus texture	Callus colour
1.0+0.4+0.2+0.05	13 ± 4.5^a	Friable	White
1.5+0.6+0.3+0.1	39 ± 8.2^b	compact	Yellow
2.0+0.8+0.4+0.5	68 ± 8.4^c	Hard compact	cream,yellow& green

Values represent the mean \pm SE with the same letter in the same column are not significantly different by Tukeys pair-wise comparison ($P \geq 0.05$).

4.6 Somatic embryo formation

Callus in multiplication medium designated for regeneration of somatic embryo, embryos were produced in masses and contained all the stages of somatic embryogenesis that is globular, heart torpedo and cotyledonary in 30 days (plate 4.3a) and (plate 4.3b) which were light green at the early stage and turned dark green at maturity green nodular structure shoots which were fragile as well as formation of leaves were noticed emerging on the callus (plate 4:3c) and (plate 4.3d).

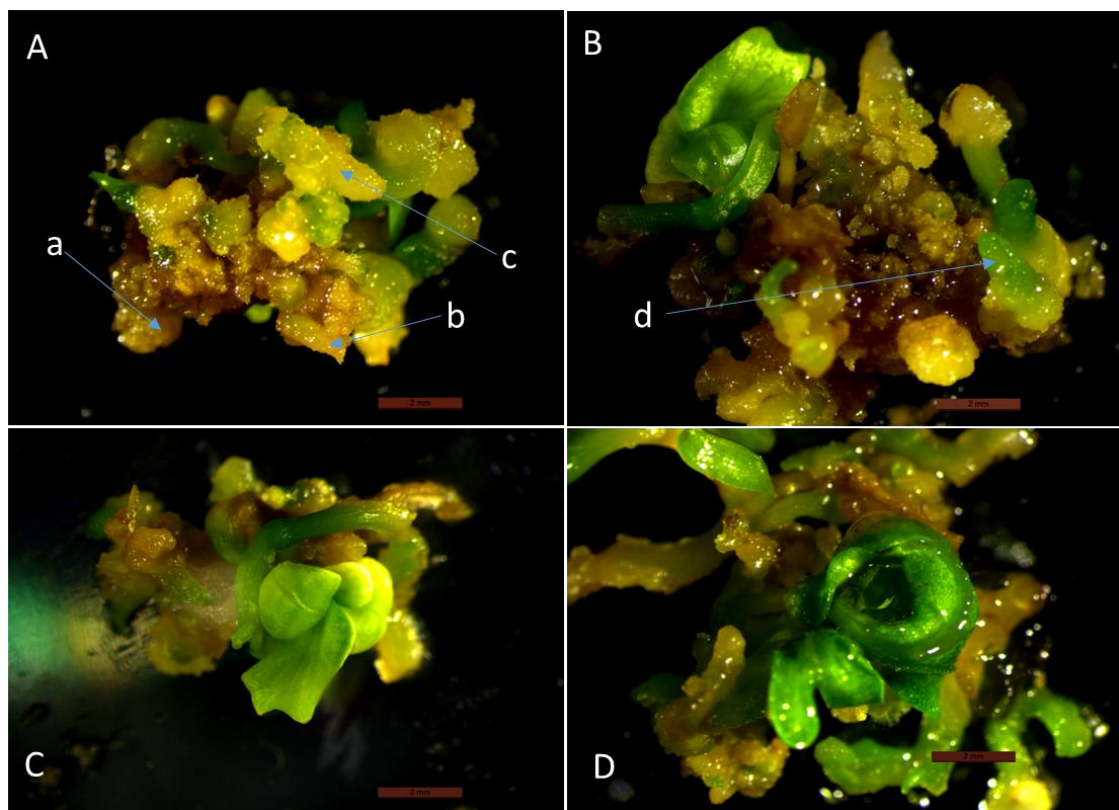


Plate 4.3: Formation of somatic embryos in calli

(A) Somatic embryo development (a) Globular stage (b) Heart stage (c) Torpedo (d) Cotyledonary

(B) Mature somatic embryo (C) Shoots forming on callus (D) Green leaves forming.

4.7 Shoot induction

Somatic embryos were considered mature as soon as the shoot (epicotyl) and elongated with presence of radicle structure which is a precursor to root formation. The effect of different media on shoot induction and multiplication was evaluated by counting the number of emerging shoots after 4 weeks of culture. Shoot were observed within 21 days of transfer to new media. Shoot primordia started at 16-18days developing more cotyledonary nodal explant

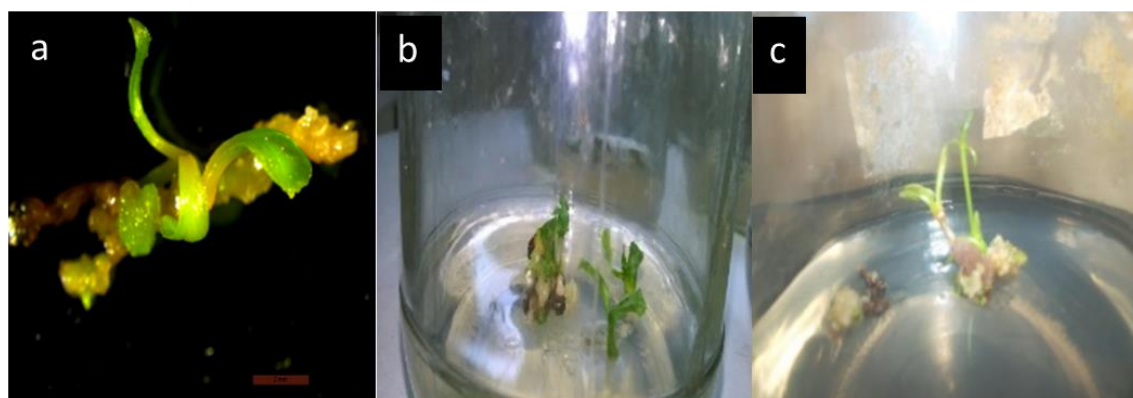


Plate 4.4: Shoot induction

(a) Shoot proliferation (b) Shoot multiplication (c) Shoot elongation

Table 4.3: The effect of plant growth regulators on shooting

Treatment (mg/l)	Shoot induction (%)
1.0BAP+0.4KIN+0.2NAA+0.05TDZ	2.0±0.00 ^a
1.5BAP+0.6KIN+0.3NAA+0.1TDZ	9.0±2.8 ^a
2.0BAP+0.8KIN+0.4NAA+0.5TDZ	29.0±6.6 ^b

Values with the same letter in the same column are not significantly different by Turkey's pair wise comparison ($P>0.05$).

Statistical analysis revealed that there was a significant difference in the number of shoots in the three media types with most shoots forming in 2.0mg/l BAP+0.8mg/l

KIN +0.4mg/l NAA+ 0.5mg/lTDZ with mean of 29.0±6 emerging shoots observed compared to other treatments (Table 4.3).

4.8 Rooting

Medium supplemented with BAP and IBA at different concentration, after 8 weeks of culture under light condition, root primordia were observed in one treatment. This revealed that there was a significant difference in the number of roots emerging from the shoots ($P < 0.003$). A mean of one root per shoot was observed in the treatment supplemented with 1.0 BAP+0.5 IBA extending from the shoots (plate 4.5). The other treatments supplemented with 0.5 BAP+0.25 IBA and 1.5 BAP+0.75 IBA did not form any roots at all. (Table 4.4)

Table 4.4: The effect of BAP and IBA on root formation

BAP+IBA (mg /l)	Roots (No)	Roots (No)
0.5+0.25	0.00 ^a	0
1.0+0.5	1.00 ^b	1
1.5+0.75	0.00 ^a	0

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$).

A mean of six roots per shoot was observed in the treatment supplemented with 1.0 BAP+0.5 IBA extending from the shoots which were fragile (plate 4:5a). The other treatments supplemented with 0.5 BAP+0.25 IBA and 1.5 BAP+0.75 IBA did not form any roots at all.

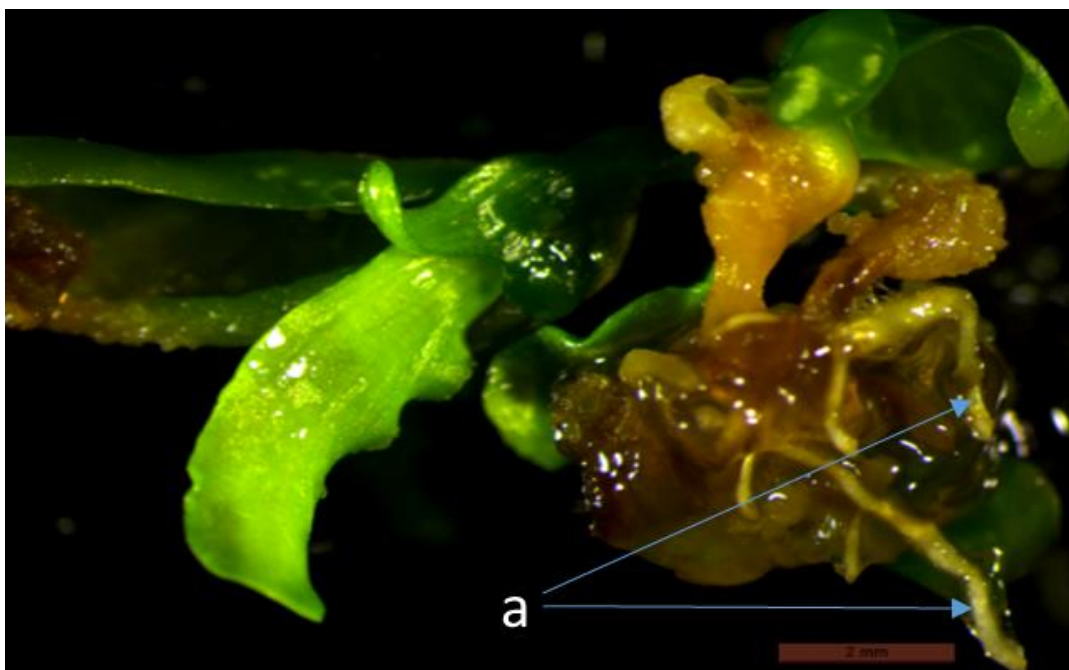


Plate 4.5: (a) Roots forming on media supplemented with 1.0 BAP+0.5 IBA.

4.9 Phytochemical analysis

After screening for the presence of secondary metabolites in the extracts of Mpelepele, it was observed that different extraction solvents have different abilities to extract the metabolites from plants. In the leave extracts, it was observed that saponins were present in all extracts using the different solvents, although chloroform and water extracts showed relatively higher concentrations for this metabolite. Alkaloids, Tannins, and steroids were observed to be highly present in most of the solvent extracts used except for acetone, hexane and di-ethyl ether. Flavonoids were observed to be in trace amounts in the leave extracts irrespective of the solvent used for extraction. Alkaloids were observed to be highly concentrated in ethanol and water extracts (Table 4.5).

Table 4.5: Secondary metabolites present in leave extracts of Mpelepele

TEST	SOLVENT						
	Ethanol	Methanol	Acetone	Chloroform	Hexane	D-ethyl	Water
Saponins	+	+	+	++	+	+	++
Tannins	++	++	+	++	+	+	++
Flavonoids	+	+	+	+	+	+	+
Steroids	++	++	+	++	+	+	++
Alkaloids	++	+	+	+	+	+	++

“++” indicates high presence; “+” indicates moderate presence

From the root extracts, it was also observed that all the screened metabolites were available in all the solvent extracts under study. Saponins and steroids were highly detected only in ethanol and methanol extracts, with all other extracts exhibiting moderate concentrations for these two secondary metabolites. Tannins in root extracts were detected in moderate amounts in all the different solvent extracts under study, except for the water extracts where their concentration was found to be relatively high. The best extraction solvents for flavonoids from the roots were found to be ethanol, methanol and water, since they were found in higher concentrations in extracts of these solvents. Alkaloids in the roots were found to be higher, based on the number of extraction solvents which showed that they were in high concentration. Actually, only acetone, hexane and di-ethyl ether solvent extracts showed moderate concentrations of alkaloids in the roots (Table 4.6)

Table 4.6: Secondary metabolites present in root extracts of Mpelepele

TEST	SOLVENT						
	Ethanol	Methanol	Acetone	Chloroform	Hexane	D-ethyl	Water
Saponins	++	++	+	+	+	+	+
Tannins	++	+	+	+	+	+	++
Flavonoids	++	++	+	+	+	+	++
Steroids	++	++	+	+	+	+	+
Alkaloids	++	++	+	++	+	+	++

“++” indicates high presence; “+” indicates moderate presence

From the stems, it was found out that saponins could not be detected in chloroform, hexane and di-ethyl ether solvent extracts, although they were detected in minimal amounts in ethanol, methanol, acetone and water extracts. Similarly, steroids were not detected in chloroform and di-ethyl ether extracts but were detected in moderate amounts in all other solvent extracts. Tannins and flavonoids were detected in moderate amounts in all the solvent extracts used. Alkaloids were found in moderate concentrations in all extracts except in chloroform extracts where they were detected in high concentrations (Table 4.7). Generally, all the secondary metabolites screened in this study were found to be relatively available substantially in the roots and leaves than in the stems.

Table 4.7: Secondary metabolites present in stem extracts of Mpelepele

TEST	SOLVENT						
	Ethanol	Methanol	Acetone	Chloroform	Hexane	D-ethyl	Water
Saponins	+	+	+	-	-	-	+
Tannins	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Steroids	+	+	+	-	+	-	+
Alkaloids	+	+	+	++	+	+	+

“++” indicates high presence; “+” indicates moderate presence; “-” indicates absence

4.10 Antimicrobial activity of plant extracts based on plant part source

Based on the source of plant extracts (leaves, roots, stems or calli). In this study it was observed that the antimicrobial activity of the different plant extracts differed greatly. Generally, Mpelepele plant extracts from roots and leaves were more active on the microorganisms used than extracts from stems, with extracts from calli not showing any antimicrobial activity at all. From the bacteria test organisms used, there was a Significant difference in the antimicrobial activity of the plant extracts based on source, only in *B subtilis*(0.84mm) and *S aureus* (0.81mm)and not in *E coli* (0.098) and *P aeruginosa* (0.182). In *B subtilis*, Leave extracts showed a significant

antimicrobial activity since they produced larger halos (0.52 mm) than root extracts (0.42 mm) and stem extracts (0.33mm). Similarly, in *S aureus*, larger halos were observed in leaf extracts (0.81 mm), compared to the halos observed in root (0.59 mm) and stem (0.49 mm) extracts (Table 4.8). Compared to bacteria, Mpelepele plant extracts were less active in fungi. Although bigger halos were observed in *C albicans* than in *A niger*, the halos observed in *C albicans* were not significantly different based on their source. In *A niger* however, the small halos observed in leaf and stem extracts (0.03 mm and 0.02 mm) were significantly different to the inactivity observed when this fungi was subjected to the root extracts.

Table 4.8: Effect of plant extract source on antimicrobial activity

M/organism	Leaf	Root	Stem
<i>B subtilis</i>	0.52±0.08 ^a	0.42±0.08 ^b	0.33±0.06 ^c
<i>A niger</i>	0.03±0.01 ^a	0.02±0.01 ^{ab}	0.00±0.00 ^b
<i>C albicans</i>	0.14±0.04 ^a	0.19±0.04 ^a	0.14±0.06 ^a
<i>E coli</i>	0.46±0.07 ^a	0.48±0.41 ^a	0.44±0.10 ^a
<i>P aeruginosa</i>	0.31±0.07 ^b	0.39±0.09 ^a	0.25±0.05 ^c
<i>S aureus</i>	0.81±0.12 ^a	0.59±0.09 ^b	0.49±0.08 ^c

Values with the same row not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$). Zone of inhibition in mm.

4.11 Effect of extraction solvents on antimicrobial activity

Different solvents used in the extraction of plant extracts were observed to have different effects on the antimicrobial activity of the plant extracts. The general observation was that plant extracts obtained using ethanol, methanol and water had higher antimicrobial activity than those obtained from acetone, hexane, chloroform and di-ethyl-ether. After subjecting *B subtilis* to Mpelepele plant extracts, there was a significant difference ($P < 0.001$) on the antimicrobial activity observed based on the solvent used to extract them. Larger zones of inhibition were observed on extracts obtained from methanol (0.84 mm) and ethanol, (0.82 mm) with intermediate inhibition zones observed on extracts obtained from Water (0.45 mm), acetone (0.30 mm), hexane (0.22 mm) and chloroform (0.18 mm). The least inhibition zones under *B subtilis* were observed in extracts obtained from Di-ethyl-ether. Subjecting *E coli* to these plant extracts also revealed that there was a significant difference in the inhibition zones observed, with the largest inhibition zones observed in extracts obtained from water (1.68 mm) and ethanol (1.20 mm) and the least observed in extracts obtained from chloroform (0.14 mm) and di-ethyl-ether (0.12 mm). Analysis of variance also revealed that there were significant differences on the inhibition zones observed in *P aeruginosa* and *S aureus* after subjecting them to Mpelepele extracts obtained from the different solvents. In *P aeruginosa*, the largest inhibition zones were observed in extracts obtained from ethanol (0.93 mm) and methanol (0.53 mm), with intermediates observed in acetone (0.31 mm) and water (0.24 mm) extracts and the smallest inhibition zones observed in chloroform (0.10 mm) and di-ethyl-ether (0.03 mm). In *S aureus*, statistical analysis revealed that the largest inhibition zones were observed in methanol extracts (1.21 mm), with intermediates observed in ethanolic (0.97 mm) and water (0.64 mm) extracts, while the smallest inhibition zones were observed in acetone (0.52 mm), chloroform (0.36 mm) and di-ethyl-ether (0.34 mm) extracts. Antimicrobial activity of the plant extracts on the two fungi used revealed that there were significant differences on the inhibition zones observed with observed in *A niger* and *C albicans*. In *A niger*, the largest inhibition zones were observed in acetone extracts (0.33 mm) with all other extracts showing very small zones of inhibition. In *C albicans*, the largest inhibition zone was observed after subsection to

methanolic extracts (0.41 mm) with ethanolic and water extracts showing intermediate antimicrobial activities of 0.22 mm and 0.19 mm respectively. Acetone, chloroform and di-ethyl-ether extracts showed very small zones of inhibition of 0.10 mm, 0.10 mm, 0.07 mm and 0.03mm respectively (Table 4.9).

Table 4.9: Effect of different extraction solvents on antimicrobial activity (see appendix 5)

Micro-organism	Acetone	chloroform	D.ether	Ethanol	Hexane	Methanol	Water
<i>B subtilis</i>	0.30±0.09	0.18±0.05 ^d	0.13±0.04 ^d	0.82±0.14 ^a	0.22±0.05 ^d	0.84±0.17 ^a	0.45±0.11 ^b
<i>A niger</i>	0.33±0.02 ^a	0.002±0.00 ^b	0.00±0.00 ^b	0.02±0.01 ^b	0.03±0.03 ^b	0.00±0.00 ^b	0.06±0.02 ^b
<i>C albicans</i>	0.10±0.03 ^b	0.10±0.04 ^b	0.03±0.01 ^b	0.22±0.06 ^{ab}	0.07±0.03 ^b	0.41±0.13 ^a	0.19±0.06 ^{ab}
<i>E coli</i>	0.25±0.07 ^{ab}	0.14±0.04 ^b	0.12±0.06 ^b	1.20±0.23 ^{ab}	0.45±0.12 ^{ab}	0.79±0.15 ^{ab}	1.68±0.94 ^a
<i>P.aeruginosa</i>	0.31±0.09 ^{bc}	0.10±0.04 ^c	0.03±0.01 ^c	0.93±0.18 ^a	0.08±0.03 ^c	0.53±0.10 ^b	0.24±0.06 ^{bc}
<i>S aurias</i>	0.52±0.14 ^c	0.36±0.08 ^c	0.34±0.13 ^c	0.97±0.17 ^{ab}	0.38±0.08 ^c	1.21±0.21 ^a	0.64±0.15 ^{bc}

Values with the same letters in row are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$). Zone of inhibition in mm

4.12 Effect of extract concentration on antimicrobial activity of Mpelepele

The effect of different concentrations of the plant extract on the antimicrobial activity of fungi and bacteria was observed to be significant in all the test organisms involved. The general trend observed in most of the microbes under study was the direct relationship between extract concentrations and the microbial sensitivity. Except for *A niger*, all other microbes under study showed that the higher the concentration of the extract, the more it is effective against microorganisms. There were significant differences in the inhibition zones observed in all microorganisms at the different concentrations ($P < 0.001$), with the largest inhibition zones observed when the microorganisms were subjected to the crude extracts. In *A niger*, the crude extracts had the highest effect on the microorganism at 50% dilution (0.005 mm). In *C albicans*, antimicrobial activity of the plant extracts were only observed on subjection to the crude extract and the 50% dilution, with 25% and 15% concentrations of the extract showing no antimicrobial activity at all. In *E coli*, although the extracts did not show any activity at 15% dilution, their activity at crude and 50% dilution were very high. Exposure of this microorganism to the crude extracts of Mpelepele led to the largest zones of inhibition (1.753 mm) at this concentration, with the same being observed at 50% (0.862 mm) dilution. The activity of Mpelepele plant extracts on *S. aureus* was observed to be effective at all concentrations. At 50% dilution, this microorganism was the most sensitive to these extracts, with inhibition zones of 0.812 mm observed suggesting that *S aureus* was the most susceptible microorganism to Mpelepele plant extracts (Table 4.10).

Table 4.10: The effect of extract concentration on antimicrobial activity

M/organism	Extract concentration (mg/L)			
	15(%)	25(%)	50(%)	100(%)
<i>B subtilis</i>	0.009±0.004 ^b	0.024±0.014 ^b	0.560±0.075 ^b	1.089±0.103 ^a
<i>A niger</i>	0.000±0.000 ^c	0.000±0.000 ^c	0.005±0.002 ^b	0.057±0.015 ^a
<i>C albicans</i>	0.000±0.000 ^b	0.000±0.000 ^b	0.078±0.023 ^b	0.560±0.078 ^a
<i>E coli</i>	0.000±0.000 ^b	0.030±0.01 ^b	0.862±0.133 ^{ab}	1.753±0.034 ^a
<i>P aeruginosa</i>	0.000±0.000 ^c	0.011±0.006 ^c	0.356±0.066 ^b	0.898±0.110 ^a
<i>S aurias</i>	0.011±0.0.04 ^c	0.102±0.040 ^c	0.925±0.106 ^b	1.478±0.124 ^a

Values with the same letter in the same row are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$).

4.13 Toxicity effect of Mpelepele

After subjecting brine shrimps to Mpelepele leave, root and stem extracts, it was observed that the toxicity of these substances differed based on the concentrations of the extracts and on the extraction solvents used. Based on the concentrations of the extracts, statistical analysis revealed that there were significant differences in the toxicity of all the leave, root and stem extracts based on the different concentrations. In the leave extracts, there was a significant difference in toxicity ($P < 0.001$) based on the different concentrations, with the crude extracts being more toxic to the brine shrimps, causing death of about 8 brine shrimps. At 200ml dilution level toxicity was less and led to the death of 9 brime shrimps. On further dilution of the leaf extract to 400 ml, the toxicity further reduced and led to the death of 5 shrimps . Further dilution of the leave extract concentration to 1000 ml and 800 ml led to no deaths of the brine shrimps.

On investigating the toxicity of the stem extracts, statistical analysis revealed that there was a significant difference in the toxicity of these extracts at the different concentrations ($P < 0.001$). The crude extract was the most lethal to brine shrimps since it led to death of approximately 8 shrimps, while concentrations of 1000 and 800 were not toxic to brine shrimps at all. At 600ml dilution level extract concentration caused death of only 3 brine shrimps, suggesting that this extract concentration has intermediate toxicity to that of the crude extract and the low concentration extracts.

Statistical analysis on the toxicity of stem extracts also revealed that there was a significant difference in its toxicity based on the different concentrations investigated. The crude root extracts were very toxic and led to the death of almost all the brine shrimps. On reducing the concentration of the extract by 400 ml, its toxicity slightly reduced and led to the death of 7 brine shrimps. The extract concentration of 600 ml led to the death of half of the brine shrimps and therefore this concentration was not very lethal as the higher extract concentrations. The lowest extract concentrations of 400 ml and 200 ml did not lead to the live of any brine shrimps. Generally, Root extracts of mpelepele were more toxic with the stem extracts being the least toxic on brine shrimps (Table 4.11).

Table 4.11: Effect of extract concentrations on toxicity of brine shrimps

Extract concentration	Leave	Root	Stem
200	0.00±0.00 ^d	0.00±0.00 ^d	0.00±0.00 ^d
400	0.00±0.00 ^d	0.00±0.00 ^d	0.20±0.20 ^d
600	2.51±0.39 ^c	3.00±0.52 ^c	5.46±0.43 ^c
800	5.83±0.21 ^b	6.78±0.37 ^b	7.46±0.15 ^b
1000	8.14±0.19 ^a	8.37±0.31 ^a	9.54±0.17 ^a

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$).

4.14 The effects of different solvents on toxicity of brine shrimps

When different solvent were used to extract leave, root and stem extracts from Mpelepele, it was observed that the different extracts had different effects on the toxicity of brine shrimps. On analysis of the toxicity of leave extracts, it was observed that there was a significant difference on toxicity based on the extract used to obtain the leave extracts ($P=<0.001$). Ethanol was the most toxic solvent for extracting the substrate from leaves, since it caused death of about 5 brine shrimps from the 10 incubated shrimps. Methanol caused death of 4 shrimps while di-ethyl-ether, chloroform, hexane, acetone and water were the least toxic, causing mean deaths of about 3 brine shrimps per solvent.

Analysis of variance also revealed that there was a significant difference in toxicity of the root extracts based on the solvent used to extract them ($P=<0.001$). Chloroform was the most toxic solvent used to extract active metabolites from roots. This solvent caused death of about 5 brine shrimps. Ethanol, methanol, and acetone had intermediate toxicity, resulting in deaths of about 4 brine shrimps per solvent. The least toxic solvent used to extract active metabolites from roots were diethyl and water, since they resulted in mean deaths of about two brine shrimps per solvent (Table 4.12).

Table 4.12: Effect of different solvents on toxicity of brine shrimps

Solvent	Leave	Stem	Root
Ethanol	4.72±0.82 ^a	2.40±0.61 ^d	5.20±0.88 ^{ab}
Methanol	3.56±0.65 ^b	4.44±0.77 ^{ab}	5.20±0.88 ^{ab}
Chloroform	3.40±0.80 ^{bc}	4.88±0.89 ^a	5.24±0.84 ^a
Hexane	3.08±0.64 ^{bcd}	4.00±1.00 ^{bc}	3.92±0.75 ^c
Acetone	2.80±0.71 ^{cd}	4.40±0.83 ^{ab}	4.52±0.81 ^{bc}
Diethyl	2.76±0.50 ^d	3.68±0.66 ^c	4.60±0.83 ^{abc}
Water	2.76±0.69 ^d	1.60±0.42 ^e	3.04±0.71 ^d

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$).

The different solvent used to extract active metabolites from Mpelepele stems also revealed a significant difference in their toxicity to brine shrimps. Methanol, chloroform, hexane, and acetone and were the most toxic, all causing deaths of about 5 brine shrimps. Ethanol showed intermediate toxicity since it caused death of only 4 brine shrimps while water was the least toxic solvent, causing death of only 3 brine shrimps.

CHAPTER FIVE

DISCUSSION

5.1 Developing an appropriate sterilization protocol for Mpelepele

The first step in any plant tissue culture process is to get explants that are free from microbial contamination. Toward this aim, many sterilants are used, most of which are commercial bleaches. One popular sterilization agent amongst many used to disinfect explants is Jik® bleach, used at time intervals of between 20-45 minutes. From this study, it was observed that optimum sterilization using this commercial bleach was achieved at a concentration of 10.4 mM for 20 minutes and that higher concentrations of 13 mM and longer exposure times of 25 minutes caused scorching to the leaf. (Table 4:1) This adverse tissue damage observed on higher concentrations of NaOCl could be due to the fact that the sterilant is toxic to plant tissue (Colgeceen et al., 2011).

Other studies have reported efficient sterilization of explants using NaOCl, at 10.4mM at 15 minutes (Pasternak et al., 2002, Maryyama et al., 2007). Reported an efficient sterilization of different explants of Brahmi (*Bacopa monniera*) using NaOCl. Although its use alone could not achieve efficient sterilization of explants, its supplementation with a systemic fungicide and an antibiotic enabled it achieve efficient sterilization of these explants. In agreement with my findings, supplementing NaOCl with the systemic fungicide ridomil® ensured efficient sterilization compared to explant sterilization using NaOCl only (Ndakidemi et al., 2013). Working on *B. huillensis* leaf explants reported efficient sterilization using NaOCl concentrations of 1.5% v/v as the optimum concentration for 10 minutes supplemented with tween 20 and the antifungal cefotaxime. Actually, almost all sterilization protocols reported using NaOCl involve additional use of either an antifungal or antibacterial or in most cases both.

5.2 Effect of cytokinins and auxins on somatic embryogenesis

The age of an explant has a vital role on somatic embryogenesis, as it indicates that young plant are likely to be stimulated higher into developmental pathway of Somatic embryogenesis than older cells of explant (Dasti et al.,2001). Somatic embryos arise either directly on somatic explants or on intermediate callus in which cells somehow acquire competence. In this study, the effect of different auxins and cytokinins was observed at increased levels of BAP. It resulted in the development of somatic embryos forming creamy yellow compact calli, which started forming at the cut edges of the explants. (plate 4.2)This observation is in agreement with that of (Hatanaka et al. 1991). who observed that embryogenic calli start forming at the cut edges of *Coffea Canephora* explants, this phenomenon could be due to the fact that the cut surfaces are probable sites for rapid uptake of mineral and hormone resulting in the high percentage of calli formation (Bhat et al.,2008, Othmani et al.,2009). Reported that BAP proved to be efficient and essential for the induction of callus in *Apocynaceae* at different level of concentration (Wang et al., 2006). Result indicated that at 2.0mg/l BAP+0.4mg/l NAA gave an optimum concentration for callus induction which is agreement with this study that the best concentration was at 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ, however the best time for subculture was observed to be 14 days as frequent subculture cause drying and death of callus this could be in agreement with (Daffall et al., 2011). Which state that *Apocynaceae* callus tend to move from stationary phase to end lag phase after 14 days. According to callus texture in the various different media concentration it demonstrate that the concentration level of cytokinins and auxins have significant effect on callus colour, and texture which is based on the level of BAP as it tends to promote chlorophyll in some reported *Apocynaceae* (Pandely et al., 2010, Ndakidemi et al.,2013). Somatic embryo development and maturation appeared to be synchronized at different stages of embryogenesis, in the same culture level showing all the development stages of globular, heart torpedo and cotyledonary stages at higher level it was observed that highest mean of embryo with high percentage of embryogenic formation which is in agreement with (Joseph et al., 2009).

The time taken to regenerate leaf to somatic embryogenesis development started within 28 days and was evident also in *nerium oleander* Apocynaceae (Pandey 2010). Root induction frequency was observed at 1.0mg/l BAP+0,5mg/l IBA which is agreement with (Wang et al., 2006). Indicated the importance of auxins in higher concentration as it increase the rate of ethylene linked to root induction. (King ling et al., 2009). Justified the study that leaf disc were good starting tissue for root induction this is due to the presence of cell associated with the leaf –veins (vascular tissue which can be readily stimulated by adding auxins which tend to manipulate direct pluripotent cell in leaf disc of *Apocynaceae*).

5.3 Antimicrobial activity

The Antimicrobial activity of *Apocynaceae* family has been tested against many gram positive and gram negative bacteria highlighting the importance of this family as alternative therapeutic agent *Apocynaceae* have been shown to have the ability to synthesize aromatic substances, mostly phenols and their oxygen-substitute derivatives. Most of these substances are secondary metabolites which serve as plant defence mechanisms against herbivores, microorganisms and insects (Tiwari, 1998). It has been shown that the most predominant active phytochemicals in most of the plants which have shown activity to most microorganisms are phenolic compounds, with gram positive bacteria being more susceptible to these substances compared to gram negative bacteria and fungi (Ríos & Recio, 2005). In agreement with findings in the current study, the leave and root extracts of Mpelepele showed higher antimicrobial activity on gram positive bacteria *S. aureus* and *B. subtilis* than the gram negative bacteria *P. aeruginosa*, stem extract was higher in *E.coli* based on the size of the inhibition zones observed. The plant extracts also showed very little antimicrobial activity against the fungi *C. albicans* and *A. niger*.(Table 4:9) In an antimicrobial activity test done on 11 Amazonian *Apocynaceae* plant species by (Suffredini et al., 2002). On the microorganisms *S. aureus*, *P. aeruginosa* and *C. albicans*, it was observed that out of the 38 extracts screened, most of the extracts which showed activity against the bacteria species were either purely leave extracts or a combination of leaf and root extracts (Espinosa et al., 2003). Indeed, only one stem extract showed

activity against *S. aureus*. However, among the extracts which showed activity against the fungus *C. albicans* (Chandra et al., 2010). The results from both of these studies suggest that the localisation of the active metabolites against microorganisms differs in plants. In the family *Apocynaceae*, most active antibacterial substances could be localised in the leaves, barks and stem while the roots could contain most active substances against fungal microbes. According to (Suffredini et al., 2002). Alkaloids and saponins were detected in most of the extracts. In the current study, alkaloids were highly detected in root and leaf extracts of Mpelepele and could probably have led to the observed antimicrobial activity. Although previous findings on the antimicrobial activity of calli of *Holarrhena antidysenterica*, a member of the *Apocynaceae* family showed that the root callus have an almost equal antimicrobial activity as the root and seeds (Mahato et al., 2013). Among all the extractions solvent none showed inhibition activity on leaf callus. Based on some studies done in *Apocynaceae* it was observed that cell suspension were active in root cell suspension cultures due to its high potency of metabolites mostly stored in the inner-cellular vascular strand, and the cell wall around parenchyma which stored most flavonoids, alkaloids and saponins in large quantity. (Elloff et al., 2006). In similarly studies. Indicated that most metabolic compound were found in loosely arranged in root callus therefore depending on the explant (leaf, stem and root) in cell suspension cultures shows different degree and level of metabolites extraction (Hassan et al., 2009). In similar studies it revealed that calli cell of *Graveolens* and *calendula officinals* *In vitro* in cell suspension of leaf and root indicate that the leaf failed to produce the same level of alkaloids to inhibit microbes whereas roots show high level of alkaloids and saponins in antimicrobial activity (Gossell et al., 2006). This is in agreement with study that indicates zero zone of inhibition in leaf cell suspension cultures against microbes.

In this study it was noted that standard antibiotics showed higher inhibition than most of the extracts solvents. In another study on *Psoralea corylifolia* *Apocynaceae* on root callus and root extract via methanol extraction solvent showed higher halo against chloramphenicol, penicillin and streptomycin (Tang et al., 2001). In this study some root extract had halos that were close to antibiotics showing the higher concentration thereby suggesting the potential of root to produce the bioactive phytochemical

responsible for antimicrobial activity therefore this gives Mpelepele great potential to be used as a source of antibiotics for drug development against bacterial and fungal infections.

5.4 Plant secondary metabolites

Most of the secondary metabolites in plant extracts are more often found associated with other molecules like polysaccharides, proteins and inorganic compounds, hence require suitable solvents to extract (Tatiya et al., 2011). Since almost all of the known plant active components against microorganisms are aromatic or saturated organic compounds, they are often obtained through ethanol or methanol extraction (Cowan, 1999). This explains why most methanol and ethanol extracts were very active against the test microorganisms under study. Additionally, certain water soluble compounds such as polysaccharides and polypeptides like fabatin and certain lectins are effective antimicrobial agents commonly found in the aqueous phase hence could only be obtained using less polar solvent like water (Cowan, 1999). In agreement with their findings therefore, phytochemical screening revealed that among the active substances in mpelepele extracts are alkaloids and saponins which could be associated with polysaccharides or polypeptides hence the observed high antimicrobial activity in the water extracts. Secondary metabolites in plants(Table 4:5, 4:6 and 4:7) are biosynthesized as active constituents at different times for different purposes, and have over time showed marked pharmacological activities (Raja and M. Sreenivasulu, 2015). Different members of medicinal importance in the plant family *Apocynaceae* have been screened to determine their active secondary metabolites. From these studies, it has been found that the active metabolites which could be tagged to their hypothesised or confirmed pharmacological activity differs. Certain plants like *Cartharanthus roseus Apocynaceae* have been found to contain dimeric Vinci alkaloids, which are linked to the use of this plant in tumour therapy (Wink, 2015). Another plant in this family which has also been used for medicinal purposes in South America is *Tabernaemontanae angulata*, with the observed pharmacological effect attributed to the presence of alkaloids and triterpenes in the plant (Mussallam et al.,

2011). Similarly, all the extracts which showed antimicrobial activity in *Schizogygia coffaeoides* (Mpelepele) had alkaloids being detected, although other metabolites were also detected. Although other substances have been associated with the observed pharmacological effects in other plants in the Apocynaceae family, the activity of the commonly known secondary metabolites like alkaloids, terpenes and phenols cannot be ruled out. the activity of *Rauvolfia reserpina* for treatment of high blood pressure has been attributed to the presence of reserpine(Chah et al.,2006) while that of *Strophantus gratus* in treatment of heart failure has been attributed to the presence of ouabain (Wink, 2015).

5.5 Toxicity test

From the current study, it was observed that irrespective of the substance used to extract active substances from plants, higher concentrations of plant extracts were more toxic to brime shrimps than lower concentrations,(Table 4:11) hence led to more deaths. Similarly, other studies have also showed similar results, with brine shrimp toxicity being directly proportional to the concentration of the plant extracts. In a study done to determine the toxicity of *Lantana camara*, *Chromolaena odorata*, and *Euphorbia hirta* plant ethanoic extracts on brine shrimps, it was observed that lower concentrations of 200 µg/ml of the plant extracts were all causing 100% mortality of the brine shrimps. Additionally, 4000 µg/ml concentrations of the plant extracts caused at least 50% mortality, with concentrations of 800 and 1000µg/ml of the extracts being less toxic hence considered safe. From the current study, 200 and 400 concentrations were very lethal to brine shrimps, with all the other higher concentrations being relatively safe. Similar results were also observed by (Rajalakshmi et al., 2013). With chloroform, methanol and ethanol extract showing a higher levels of toxicity to brine shrimps compared to water, hexane and acetone extract at lower concentration.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. An efficient surface sterilization protocol for *S. coffeoides* was established with significant difference between concentration of NaOCL and time of exposure was observed, confirming the importance of determining the proper concentration of sterilant, duration of exposure and the required sequence of sterilant

2. An alternative regeneration to conventional breeding was established via somatic embryogenesis. Callus was successfully induced from leaf explant on MS media supplemented with 2.0mg/l BAP+0.8mg/l KIN+0.4mg/l NAA+0.5mg/l TDZ under light condition. Additionally there was a significant interaction and difference between auxins and cytokinins on embryo formation and shoot induction. Rooting was successfully induction on 1.0mg/l BAP+0.5mg/l IBA.

3. The Medicinal activities of plants extracts as well as antimicrobial activity have been found to be active against some selected pathogens, through phytochemical screening it shows clearly the rich source of metabolites for antimicrobial test. The various level of plant extract toxicity to brime shrimps was clearly determined.

6.2 Recommendations

Since it was possible to regenerate Mpelepele via somatic embryogenesis and bioassay studies. The following recommendation are made.

1. The sterilization and regeneration protocol of Mpelepele established in this study using leaf explant can be adopted for tissue culture purpose for other members of the Apocynaceae family.
2. Comparison between Organogenesis and somatic embryogenesis should be tested on Mpelepele, for the production of somatic embryo which can be

exploited to regenerate clonal materials for genetic transformation by *Agrobacterium*.

3. New extraction techniques and methods should be used in order to detect new compounds
4. Through antimicrobial activity direct callus extraction and cell suspension cultures should be used with different extractions, solvent in order to compare the relationship between whole callus and cell suspension cultures on antimicrobial activity of extract.

REFERENCES

- Aslam, J. Mujib, A. Fatima, Z. & Sharma. M.P. (2010) Variation in vinblastine production of different stages of somatic embryogenesis embryo and field grown plantlets of *caratharanthus roseus*(L) . *Journal of plant science* 73:32-42.
- Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M., & Stuppner, H. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, 33, 1582–1614.
- Arya, S. R., Kalia, & Arya, I. (2000). Induction of somatic embryogenesis in *pinus roxburghii*. *Journal of Plant cell* 19:775-780.
- Babu, P.D., & Subhasree, R.S. (2009). Antimicrobial activities of *lawsonia inermis* Academy. *Journal of plant science* 2(4)231-232.
- Bairu, M. W., Fennell, C. W., & Van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (*Musa A* cv. “Zelig”). *Scientia Horticulturae*, 108, 347–351.
- Barry, A.L., & Thronsberry, C. (1981). Susceptibility testing in manual of clinical microbiology. *American society for microbiology Washington DC*, PP:561-574A.
- Bhat, T. R., Ariff, M. Gaur, A.K., & Rao, R.G. (2008). *Rauwolfia serpentina* protocol optimization for In vitro propagation. *African journal of biotechnology* 7(23)4265-4268.
- Bharitkar, Y. P., Bathini, S. Ojha, D. Ghosh, S. Mukherjee, H. Kuotsu, K. Chattopadhyay, D. & Mondal, N. B. (2014). Antibacterial and antiviral evaluation of sulfonoquinovosyl diacylglyceride: A glycolipid isolated from *Azadirachta indica* leaves. *Letters in Applied Microbiology*, 58, 184–189.
- Bunn, E. Turner, S. Panaia, M. & Dixon, K. W. (2007). The contribution of in vitro technology and cryogenic storage to conservation of indigenous plants.

Australian Journal of Botany, 55, 345–355.

- Bruyns, P.V. (2003). Three new succulent species of *Apocynaceae*(*Asepiadoicaceae*) from southern Africa. *Current science* 582,427.
- Canter, H. Thomas, N.& Erist, E. (2005). Bringing medicinal plants in to cultivation opportunities and challenges for Biotechnology. *Trends in Biotechnology* 23:180-185.
- Chandra, S. Bandopadhyay, R. Kumar, V.&Chandra, R. (2010). Acclimatization of tissue cultured plantlets: From laboratory to land. *Biotechnology Letters*, 32, 1199–1205.
- Chah, K. F., Eze, C. A., Emuelosi, C.E.,& Esimone, C. O. (2006) Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. *Journal of Ethnopharmacology* 104:pp164-167.
- Chen, S. L., Yu, H. Luo, H. M., Wu, Q., Li, C. F., & Steinmetz, A. (2016). Conservation and sustainable use of medicinal plants : problems, progress, and prospects. *Chinese Medicine*, 1–10.
- Çölgeçen, H. Koca, U. & Toker, G. (2011). Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb. *Tubitak*, 35, 513–520.
- Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12, 564–582.
- Daffalla, H .H. , Abedellatef, E. Elhadi, E .A.,& Khalaffa, M. M. (2011). Effect of growth regulators on In vitro morphogenic response of *Boscia sengalensis Lam* using mature Zygotic embryo explants. *Biotechnology Research international*.45-49.
- Dahanukar, S.A, Kulkarni, R A.,& Rege, N. N. (2002). Pharmacology of medicinal plants and natural products. *Indian Journal of pharmacology*, 32:S81-S118.
- Das, K,Tiwari, R. K .,S.&.Shirvastava, D. K. (2010). Review Techniques for Evaluation of Medicinal Plant Products as Antimicrobial Agent current method

- and future trends. *Journal of medicinal plant Research* 4(2)pp104-111.
- Dasti, A. A., Fatima, K. & Malik, S. A. (2001). Storage time on seed dormancy and germination ineti mutants of *Arabidospsis t (L) Heynh.* *Journal of Research (science)* 12(1) pp 34-42.
- Ellis, R. H. (1991). The Longevity of Seeds. *Scientia Horticulturae*, 26, 1119–1125.
- Elumalai, A. & Eswariah, M. C. (2012). Herbalism – A review. *International Journal of Phytotherapy*, 2, 96–105.
- Ellof, J.N., & Mcgrow, L .J. (2006). Plant extract used to manage Bacterial ,fungal and parastic infections in south Africa Ahmad, F. Aqil, and Mowais, eds (2006). Modern phytomedicine turning medicinal plant to drugs. *Journal of medicinal plant* 3(2)57-60.
- Endress, M. E., & Bruyns, P. V. (2000). A Revised Classification of the *Apocynceae* s.l. *The Botanical Review*, 66, 1–56.
- Engelmann, F. (1991). *In vitro* conservation of tropical plant germplasm - A review. *Euphytica*, 57, 227–243.
- Etienne,H. Bertrand ,B. Rib, A .S.,. Lashermes, P. Malo, E., Montagnon, C., Alpizar E., Bobadilla, R., Simpson, J., Dechamp, E., Jourdan, I.,& Georget, F. (2010).Somatic embryogenesis of trees conference on Advances in somatic embryogenesis of treesand its application for the future forest and plantations August 19-21 . *Suwon Republic of korea*. 39-41.
- Epstein, J. B., Pearsall, N. N.,& Truelove, E. L. (1980). Quantitative relationships between *Candida albicans* in saliva and the clinical status of human subjects. *Journal of Clinical Microbiology* 12, 475–476.
- Esimone, C.K., Anthony, A. A., Kwaliafon, S. M., Nneka ,N.. I., & Kennedy, F.C. (2012). Antimicrobial activity of *psidium guajava* Linn.stem extracts against methicillin-resistant staphylococcus aureus. *African Journal of Biotechnology* .11(89):pp15556-15559
- Espinosa, A.C., Pijut, P. M.,& Micher, C.H.(2003). Adventitious shoot regeneration

- and rooting of prunus sevtina in vitro. *Journal of Horticulture science* 4:193-201.
- Fehers, A. (2008). The intitation phase of somatic embryogenesis what we kow and what we don't .*Acta Biol szeged* 52:53-56.
- Gossell, Willam, M.Simon, O. R., & West, M. E. (2006). The past and present use of plant for medicine used. *Indian medical journal* 55(4)pp 217-218.
- George, E.F. (2008). Plant propagation by tissue culture the technology 2nd cambridge. *University press cambridge* .89-91.
- Grigore, A. Pirvu, L. Bubueanu, C. Colceru-mihul, S. Ionita, C.& Ionita, L. (2016). Medicinal Plant Crops - Important Source of High Value - Added Products. *Scientific Papers, LIX*, 298–307.
- Gupta, P.D.& Thorsteinson, A.J. (1993). Food plant relationship of the diamondback moth (*Plutella maculipennis curt I Austation and olfaction* in relation to botinical specifically of the larva entomologia. *experimentalis et applicata* :241-250.
- Hassan, A. Rah man, S. Deeba, F. Mahmud, S.H. (2009). Antimicrobial activity of some plant extracts having hepatoprotective effects. *Journal of medicinal plant research* 3 (1)pp20-23.
- Handley, L.W.(1995). Future uses of somatic embryogenesis in woody plantation species. *Current science* 78:35-39.
- Hamdan, R. H., Musa, N. Musa, N. Wei, L. S.,& Sarman, A. (2008). Isolation and Enumeration of Coliform Bacteria and *Salmonella spp* . from Short Necked Clam at East Coast , Malaysia. *International Journal of Food Safety, 10*, 58–64.
- Hamid, H. A., Ramli, A. N. M., & Yusoff, M. M. (2017). Indole Alkaloids from Plants as Potential Leads for Anti-idepressant Drugs: A Mini Review. *Frontiers in Pharmacology, 8*, 96.
- Hamilton, A. C. (2004). Medicinal plants, conservation and livelihoods. *Biodiversity and Conservation, 13*, 1477–1517.

- Hatanaka, T. Arakawa, O. Yasuda, T. Uchida, N. & Yamaguchi, T. (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Reports*, 10, 179–182.
- Harisha, S. (2007). Biotechnology procedures and Experiments Handbook Infinty science press ,LLC.
- Interational union for conservation of Nature (2002) IUCN Red list of threatened species www.Iucnredist.org .
- James, C. (2008).Global status of commercialized Biotech GM ISAAA Brief NO39 - 045. *Der pharmacia* 2:(2)311-314.
- Janarthanam ,B. & Seshadri, S. (2008). Plantlets regeneration from leaf derived callus of *vanilla planifolia*. *In vitro cell development biology plant* 44:84-89.
- Jean ,N.W. (2009).Tissue culture derieved variation in crop improvement. *Euphytica* 118:153.
- Joseph, J., Magadulla, & Paul Erastus,W. (2009). Bioactive natural products derived from the east Africa Flora. *Turkey journal of biology* 31:53-58.
- Jony, M. Hafsha B. C., Abdullah, A.F.,& Sourav,D. (2013). Pharmacological profile of *cathranthus roseus* (*Apocynaceae*). *Asian journal of pharmaceutical Research and development* vol 1:(3)01-06.
- Jimenez, V. (2001). Regulation of in vitro somatic embryognesis with empasis on the role of endogenous hormones. *Revista brasileira de fisologia vegetal* 13:196-223.
- Jimenez, V. M., & Thomas,C. (2002). Particpation of plant hormones in determination and progression of somatic embryognesis. *Plant cell monographs* vol 5: 103-111 *Springer Berlin /Heideberg*.
- Jualang, G.A., Marziahi , M. Radzali, M. & Joari R. (2002) . Establisment of physalis minima hairy roots culture for the production of *physalians*. *plant cell tissue culture journal* 69:271-278.
- Kacar, D. (2008). Screening of some plant speciesfor their total Antioxidant and

- Antimicrobial Activities . *Journal of phytomorphology* 55:259-266.
- Kaeppler, S. M., Kaeppler, H. F., & Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology*, 43, 179–88.
- Kane ,M. (2003). Bacterial and fungal indexing of tissue cultures. *Journal of Allergy and Immunology* 94:393-400.
- Kariba, R. M., Houghton, P. J., & Yenesew, A. (2002). Antimicrobial activities of a new schizozygane indoline alkaloid from *Schizozygia coffaeoides* and the revised structure of isoschizogaline. *Journal of Natural Products*, 65, 566–569.
- Karuppsamy, S. Kiranmai, C. Aruna, V. & Pullaiah, T. (2009). In vitro conservation of *ceropogia intermedia* -An endemic plant of south india. *African journal of Biotechnology* 8:(17)4052-4057.
- King ,Ling, A .P., Kok, K . M., Hussein, &Son ., S.. L. (2009). Effect of plant growth regulators on Adventitious roots induction from different explant of *orthosiphon* American -Eurasian. *Journal of sustainable Agriculture* 3(3)pp493-501.
- Kim, M .H. H., Lee ,M. S., Chung,& .Joo, J. (2002). Factors affecting efficiency of shoot induction in citrus . *M.sc Thesis National University Dagu korea*.
- Kumar, S. S. S., Suri, K. U., Sonie, K.G., & Ramzwat, H. (2007). Establishment of embryonic cultures and somatic embryogenesis in callus culture of *guggul-commiphorowaigh(Arnot)Bhandari* . *Indian Journal of experimental biology* ,41:69-77.
- Kumar, P, Chandaria, S, Musra, J ,J. .Lupin ,D.W., M. S., Arun, Kumar ,T, &Veehl, T, J. (2011). Ethno botanical and pharmacological study of *Altstonia (Apocynaceae)* A review. *Journal of Pharmateucial science and research*.8:(17)23-25.
- Kluytmans, J. Belkum Van, A. &Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: Vemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Reviews*, 10, 505–520.
- Koch, G. (1995). Protected areas in Europe and their importance for conservation. In

- Conservation and Management of Forest Genetic Resources in Europe. *Zvolen: Arbora Publishers*.pp 513-533.
- Koo, B. Pardey, P. G., & Wright, B. D. (2003). The economic costs of conserving genetic resources at the CGIAR centres. *Agricultural Economics*, 29, 287–297.
- Lantcheva, A. Vlahovo, M. Trinh, T. H., & Brown, S. (2001). Assessment of polysomaty, embryogenic potential, embryo formation and regeneration in liquid media for different species of diploid *Annals medicago*. *Journal of plant science* 160:621-627.
- Leeuwenberg, A. M. (2003). Series of revisions of *Apocynaceae* 53. *Melodinus*. *Systematics and Geography of Plants*, 73, 3–62.
- Leifert, C. Waites, W. M., & Nicholas, J. R. (1989). Bacterial contamination of micro propagated plant cultures. *Journal of Applied Bacteriology* 67:353-361.
- Lidder, P. & Sonnino, A. (2012). Biotechnologies for the Management of Genetic Resources for Food and Agriculture. *Advances in Genetics* (Vol. 78).
- Manna, A. Abulaka, M. E. (2006). Two preliminary screening of the various extracts of *physales angulata*. *Journal of phytochemistry* 23:19-21.
- Magadula, J. J., & Erasto, P. (2009). Bioactive natural products derived from the East African flora. *Royal Society of Chemistry*, 26, 1535–1554.
- Mahato, S. Mehta, A. Roy, S. Woon, R. & Jharkhand, R. (2013). Studies on Antibacterial Effects of Bark, Seed and Callus Extracts of *Holarrhena Anti dysenterica*. *Journal of pharmateucial science and research* 8, 717–721.
- Maruyama, E. Hosoi, Y. & Ishikawa, K. (2007). Somatic embryogenesis and plant regeneration in *yakutanegoyou pinus armandil franch var amamiana* (koidz) Hatusima, an endemic and endangered species in Japan. *In vitro cellular dev. Biol-plant* 43:28-34.
- Maynard, C. A. (1988). Interaction between cloning success and forest tree improvement in valentine F.A. (ed) *Forest and crop biotechnology progress and prospects*. *Springer-Verlag New York* pp:335-346.

- Mazid, M. Khan, T. A., & Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and Medicine*, 3, 232–249.
- Mephora, M. Mujib, A., & Siddiquin, Z. H. (2012). Synthetic seed development and conservation. *Journal of plant pathology* 11:37-43.
- Meve, U. Leide-Schumann, Ceropegia, D. (2007). *Apocynaceae*, *Stapelinae*. *Annals of the Missouri Botanical Garden* 94(2)392-406.
- Meyer, B., Ferrigni, N. Putnam, J. Jacobsen, L. Nichols, D. & McLaughlin, J. (1982). Brine Shrimp: A Convenient General Bioassay for Active Plant Constituents. *Planta Medica*, 45, 31–34.
- Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*.
- Murugesan, D. Doo, H. K., & Seung, B. H. (2008). Efficient plant regeneration via somatic embryogenesis and organogenesis from explants of *catharanthus roseus*. *Journal of plant biology* 44:18-25.
- Mussallam, I. Duwayi, M. & Shibil, R. A. (2011). Micropropagation of *capper sinosa* from wild plant functional. *plant science and biotechnology* 5(special)17-21.
- Neve, L. Duque, S. R. L., Almeida, J. S., & Fevereiro, P.S. (1999). Repetitive somatic embryogenesis in *medicago truncatula* spp *Narbonensis* and *M. truncatula gaertn*. *Journal of plant cell* 18:398-405.
- Ndakidemi, C. F., Mneney, E., & Ndakidemi, P. A. (2013). Development of Sanitation Protocol for Leaf Explants of *Huillensis* for *in vitro* Culture. *American Journal of Plant Sciences*, 4, 2425–2430.
- Othmani, A. Bayouhd, C. Drira, N. Marrakchi, M. Trifi, & Masrerit E, T, (2009). Somatic embryogenesis and plant regeneration In *Gymnema sylvestre*. *Plant Cell, Tissue and Organ Culture*, 38, 71–79.
- Paiva, P. M. G., Gomes, F. S., Napoleon, T. H., S Correia, M. T. S., & Coelho, L., B. (2010). Antimicrobial activity of secondary metabolites and Lectins from *plantin* A. Mendez -Vilas, ed 2010 Current Research Technology and Education Topics

- in Applied Microbiology and Microbial Biotechnology Spain. *Formatex Research Center* pp 396-406.
- Pandey, V. P., Cherian, E.& Patani, G. (2010). Effect of of growth regulators and culture condition on Direct Root induction of *Ruvivolfia Sepentina L(Apocynaceae)* Benth by Leaf explants Tropical. *Journal of pharmaceutical Research* 9(1)pp 27-34.
- Parrott,W. A. ,M. A., Badely, R .E., Durham, & H. V., Mattew,G. (2001). Tissue culture and regeneration in legumes in moss JP .(ed) *Biotechnology in crop improvement in Asia* pp:115-148.
- Pasternak,T. P., Prisen, E. Ayaydin, F. Miskolezi, P. Potters , G. Asard, H .V., Anonckelen, A. Dudits, D.& Feher, A. (2002). The role of auxin ,PH and stress in the activation of embryogenic cell divison in leaf protoplast derived cell of *Alfafa*. *Journal of plant physiology* 129,1807-1819.
- Pathak, M. R., & Abido, M. S. (2014). The Role of Biotechnology in the Conservation of Biodiversity. *Journal of Experimental Biology and Agricultural Sciences*, 2, 353–363.
- Pence, V. C. (2010). The possibilities and challenges of in vitro methods for plant conservation. *Kew Bulletin*, 65, 539–547.
- Pence, V. Clark, J. R., & Plair, B. L. (1996). Wild and endangered species. In In vitro collecting techniques for germplasm conservation. *Plant Cell Reports* 98-101.
- Pelka, M. Danzi, C. Distler, W. & Petschlt, A. (2000). A new screening test of dental materials. *Journal of Dentistry* 28:341-345.
- Probhat S, Annad S, Arvirid, K. Shukla, L, Venna P,& Tapan, K. (2009). *Somatic embryogenesis* and *In vitro* regeneration of an endangered plant . *Plant Cell, Tissue and Organ culture* 24:11-15.
- Prema, R. Paul S, & Therbirag S, B. (2013). Indirect organogenesi of the medicinal plant species cryptotolepis *Granchfora wiright (Apocynaceae)* by tissue culture. *Arfrican Journal of Biotechnology* 3:12-15.

- Pinto, G. Santos, C. Neves, L.& Ajaujo, C.(2002). Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* abill. *plant cell reports* 21:208-213.
- Ramar, M .D., Sultana, N. S.,& Anwar, M. N. (2004). In vitro antimicrobial activity of Holarrifine 2401 Isolatd from the stem bark of *Holarrhina anlidysenterica*. *International J ournal of.Agriculture and Biology* 64:689-700.
- Rafieian-kopaei, M. (2012). Medicinal plants and the human needs. *Journal of HerbMed Pharmacology, 1*, 1–2.
- Raja, R. & Sreenivasulu, M. (2015). Medicinal Plants Secondary Metabolites Used in Pharmaceutical Importance-an Overview. *World Journal of Pharmacy and Pharmaceutical Sciences, 4*, 436–447.
- Rajalakshmi, N. Poongodi, T. S., Asikada, & Banuraviganesh , F .(2013). Antimicrobial activity and phytochemical screening of *cathranthus roseus*. *International journal of scientifc research* volume 2: Issue ISSN No 2277-8179 1-2.
- Reed, B. M., Engelmann, F. & Dulloo, M. E. (2004). Technical guidelines for the management of field and *in vitro* germplasm collections. (IPGRI Hand). Rome, Italy. *International Plant Genetic Resources Institute*.
- Reddy, P. S., Rodriguies, R.& Rajasekharan, R. (2003). Shoot organogenesis and mass propagation of *coleus forskohill* from leaf derived callus. *plant cell tissue and organ culture* 66:183-188.
- Ríos, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology, 100*, 80–84.
- Runyoro, D. Matee, M. Olipa, N. Joseph, C.& Mwamboh, M. (2006). Screening of Tanzanian plant for anti-candida activity BMC complement *Altern Med6:11*. *Free article pubmed*.
- Rotach, P. (2005). In Situ Conservation Methods. Conservation and management of forest Genetic Resources in Europe. *Planta* 222:107-117.
- Sahai, A. Shahzad, A & Sharma, S. (2010). Histology of organogenesisand somatic

- embryogenesis in excised root cultures of an endangered species *Tylophora Indica Asclepiadaceae*. *Australian journal of Botany* 58:198:205.
- Sadeq, M. A., Pathak, M. R., Salih, A. A., & Abido, M. (2014a). Effect of plant growth regulators on regeneration of the endangered medicinal plant *Calligonum comosum* L. *Henry in the Kingdom of Bahrain*, 13, 2513–2523.
- Sadeq, M. A., Pathak, M. R., Salih, A. A., & Abido, M. (2014b). Highly Efficient in Regeneration Method of Endangered Medicinal Plant *Heliotropium kotschy Ramram*. *In the Kingdom of Bahrain. Media*, 5, 736–747.
- Sarin, R. (2005). Useful metabolites from plant tissue cultures. *African journal of Biotechnology*, 4, 79–93.
- Sharma, D. K., & Sharma, T. (2013). Biotechnological Approaches for Biodiversity Conservation. *Journal of infectious disease* 4, 183–186.
- Shargel, S. Matrick, A. N., Journey, P. F., & Swanson, N. (2007). Comprehensive Pharmacology review 6th edition. New York. *Lippincott Williams and Wilkins* pp35:176-188.
- Sheikh, N. Kumar, Y. Mishra, A. K., & Pfoze, L. (2013). Phytochemical screening to validate the ethnobotanical importance of root tubers of *Dioscorea species*. *Journal of Medicinal Plant Studies*, 1, 62–69.
- Sonia, C. Garima, Sharm, S. Anil, & Reshi, K. (2013). Study of Antimicrobial properties of *cartharanthus roseus* by agar well diffusion method. *International Research Journal of Pharmaceutical and Applied science (IRJPAS)* 3:(5)65-68.
- Sucharita, R. Paddam, C. (2010). A nontoxic antifungal compound from the leaves of *cartharanthus roseus* characterized as 5-hydroxy flavones by spectroscopic analysis. *Journal of phytochemistry* 3:77-79.
- Sudaha, G. C., & Seenii, S. (2006). Spontaneous Somatic embryogenesis on In vitro root segments cultures of *Rauwolfia micrantha* hook F-A medicinal plant. *In vitro cellular and development biology plant* 42:119-123.
- Suffredini, I. B., Bacchi, E. M., Sakuda, T. M. K., Ohara, M. T., Younes, R. N., &

- Varella, A. D. (2002). Antibacterial activity of *Apocynaceae* extracts and MIC of *Tabernaemontana angulata* stem organic extract. *Revista Brasileira de Ciências Farmacêuticas*, 38, 89–94.
- Tatiya, A. U., Tapadiya, G. G., Kotecha, S. & Surana, S. J. (2011). Effect of solvents on total phenolics, antioxidant and antimicrobial properties of *Bridelia retusa* Spreng. stem bark. *Indian Journal of Natural Products and Resources*, 2, 442–447.
- Tang, W. Guo, Z. & Ouyang, F. (2001). Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. *In vitro cellula Devopmental Biology of plant*, 37, 558–563.
- Tiwari, V. Singh, B. D., & Tiwari, K. N. (1998). Shoot regeneration and somatic embryogenesis from different explants of *Brahmi* [*Bacopa monniera* (L.) Wettst. *Plant Cell Reports*, 17, 538–543.
- Van, Wyke, B. E., Wink, M. (2004). Medicinal plant of the world 1st edition pretoria South Africa. *African journal of Biotechnology* 6: 44–46
- Visweswari, G. Christopher, R. & Rajendra, W. (2013). Phytochemical Screening of Active Secondary Metabolites Present in *Nerium oleander*. *International Journal of Pharmaceutical Sciences and Research*, 4, 2770–2776.
- Vogel, N. (1991). Similarities between various systems of traditional medicine consideration for the future of *ethno pharmacology* *Journal of eltnopharmacology* 33:35 179–90.
- Wang, H. C., Chen, J. T., & Chang, W. C. (2006). Somatic embryogenesis and plant regeneration from leaf, root and stem-derived callus cultures of *Areca catechu*. *Biologia Plantarum*, 50, 279–282.
- Weiss, E. A. (1979). Some indigenous plants used domestically by East African coastal fishermen. *Journal of Economic Botany* 33, 35–51.
- Wernicke, W. & Gorst J. (2002). Coordination of growth and reproduction its options for differentiation. *American society of microbiology washtington Dc* pp :345–362.

- Wink, M. (2015). Modes of Action of Herbal Medicines and Plant Secondary Metabolites. *Journal of plant Medicines*, 2, 251–286.
- Yang, L. Yang, C. Li, C., Zhao, Q. Liu, L. Fang, X., & Chen, X. Y. (2016). Recent advances in biosynthesis of bioactive compounds in traditional Chinese medicinal plants. *Science Bulletin*, 61, 3–17.
- Zhang, C. Zink, D. L., Ushio, M. Burgess, B. Onishi, R. Maurekar, P. Barrett, J. F., & Singh, S. B. (2001). Isolation Structure and antibacterial activity of thiazomycin A potent thiazolyl peptide antibiotics from *amycolalopsis fastidisa* Bloorg. *Journal of Medicinal plant* 16:8818-8823.

APPENDICES

Appendix 1: ANOVA tables for Antimicrobial analysis

ANOVA for antimicrobial effects of Mpelepele extracts on *Aspergillus niger*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	0.127738	0.042579	5.53	0.001
extract	6	0.105000	0.017500	2.27	0.038
p_part	2	0.052460	0.026230	3.40	0.035
Residual	140	1.849524	0.007706		
Total	251	2.134722			

ANOVA for antimicrobial effects of Mpelepele extracts on *Candida albicans*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	13.70741	4.56914	49.41	<.001
extract	6	3.63927	0.60654	6.56	<.001
p part	2	0.19978	0.09989	1.08	0.341
Residual	140	22.19413	0.09248		
Total	251	39.74059			

ANOVA for antimicrobial effects of Mpelepele extracts on *Bacillus subtilis*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	49.9211	16.6404	91.31	<.001
extract	6	19.2299	3.2050	17.59	<.001
p part	2	1.5770	0.7885	4.33	0.014
Residual	140	43.7363	0.1822		
Total	251	114.4644			

ANOVA for antimicrobial effects of Mpelepele extracts on *Escherichia coli*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	130.059	43.353	9.58	<.001
extract	6	75.829	12.638	2.79	0.012
p part	2	22.153	11.076	2.45	0.089
Residual	140	1086.372	4.527		
Total	251	1314.413			

ANOVA for antimicrobial effects of Mpelepele extracts on *Pseudomonas aeruginosa*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	33.5683	11.1894	64.33	<.001
extract	6	21.8915	3.6486	20.98	<.001
p part	2	0.8881	0.4441	2.55	0.080
Residual	140	41.7474	0.1739		
Total	251	98.0954			

ANOVA for antimicrobial effects of Mpelepele extracts on *Staphylococcus aurious*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Conc	3	92.4583	30.8194	90.14	<.001
Extract	6	24.6432	4.1072	12.01	<.001
p part	2	4.2686	2.1343	6.24	0.002
Residual	140	82.0562	0.3419		
Total	251	203.4264			

Appendix 2: ANOVA tables for Toxicity analysis

ANOVA for effect of leaf extracts on toxicity of brine shrimps

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
concentration	4	1828.5486	457.1371	869.55	<.001
Solvent	6	74.3886	12.3981	23.58	<.001
concentration.solvent	24	132.0114	5.5005	10.46	<.001
Residual	140	73.6000	0.5257		
Total	174	2108.5486			

ANOVA for effect of root extracts on toxicity of brine shrimps

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
concentration	4	2068.5143	517.1286	1046.21	<.001
Solvent	6	214.6171	35.7695	72.37	<.001
concentration.solvent	24	308.5257	12.8552	26.01	<.001
Residual	140	69.2000	0.4943		
Total	174	2660.8571			

ANOVA for effect of stem extracts on toxicity of brine shrimps

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
concentration	4	2583.9200	645.9800	900.77	<.001
Solvent	6	99.9771	16.6629	23.24	<.001
concentration.solvent	24	125.2800	5.2200	7.28	<.001
Residual	140	100.4000	0.7171		
Total	174	2909.5771			

Appendix 3: Effects of NaOCl and exposure time on sterilization

ANOVA for effect of NaOCl and exposure time on bacterial contamination

Source of variation	DF	SS	Mean Square	F Value	Pr > F
Time of exposure	3	2233.333	744.44444	1.60	0.2033
NaOCl conc.	3	17900.000	5966.66667	12.85	<.0001

Source	DF	SS	Mean square	F Value	Pr > F
Model	6	20133.33	3355.56	7.23	<.0001
Error	41	19033.33	464.227		
Corrected	47	39166.67			

ANOVA for effect of NaOCl and exposure time on fungal contamination

Source	DF	SS	Mean Square	F Value	Pr > F
Model	6	1133.33	188.88889	0.38	0.8850
Error	41	20166.67	491.86992		
Corrected total	47	21300.00			

Source	DF	SS	Mean Square	F Value	Pr > F
Time of exposure	3	166.667	55.556	0.11	0.9520
NaOCl	3	966.667	322.222	0.66	0.5844

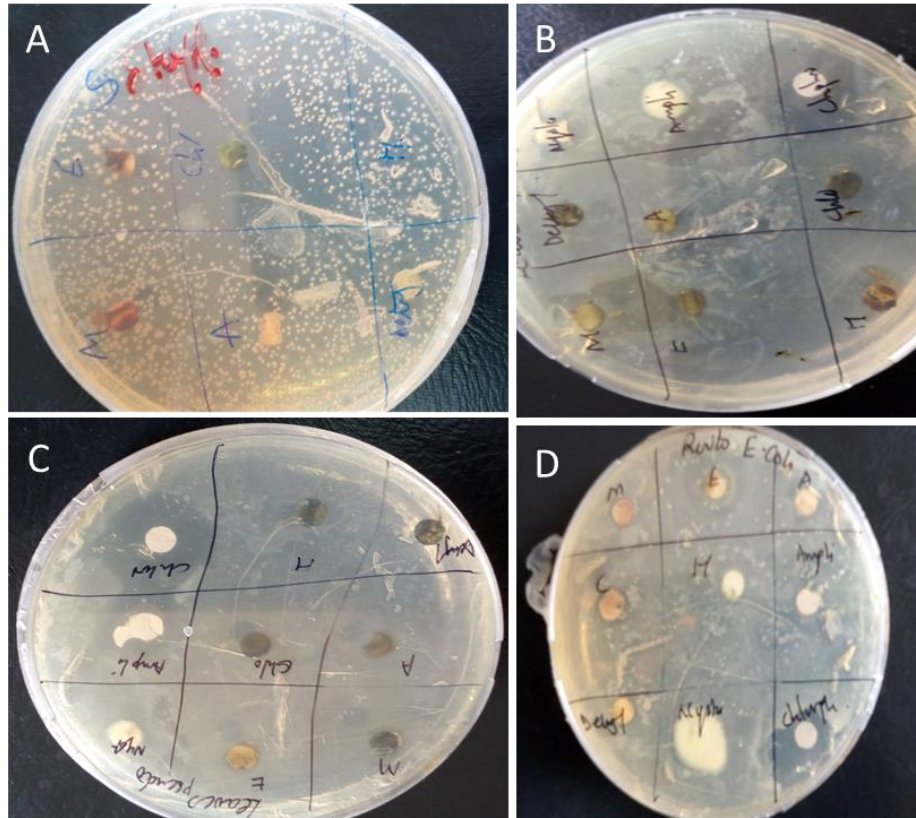
ANOVA for effect of NaOCl and exposure time on clean explants

Source	DF	SS	Mean Square	F Value	Pr > F
Time of exposure	3	8600.000	2866.667	5.56	0.0027
NaOCl	3	27866.667	9288.889	18.02	<.0001

Appendix 4: Murashige and Skoog (MS) basal media composition

MACRONUTRIENTS	MICRONUTRIENTS
NH ₄ NO ₃ (1650 mg/L)	H ₃ BO ₄ (6.2 mg/L)
CaCl ₂ .2H ₂ O (332.02 mg/L)	CoCl ₂ .6H ₂ O(0.025 mg/L)
MgSO ₄ .7H ₂ O (180.54 mg/L)	CuSO ₄ .5H ₂ O (0.025 mg/L)
KNO ₃ (1900 mg/L)	FeNaEDTA (36.7 mg/L)
KH ₂ PO ₄ (170 mg/L)	FeSO ₄ .7H ₂ O (27.8 mg/L)
	MnSO ₄ .H ₂ O (16.9 mg/L)
	KI (0.83 mg/L)
	Na ₂ MoO ₄ .2H ₂ O (0.25mg/L)
	ZnSO ₄ .H ₂ O (8.6 mg/L)

Appendix 5: Zone of inhibition on selected petri-dish cultured



- Zone of inhibition of chloroform stem extract on *S. aureus*
- Zone of inhibition of ethanol root extract on *B. subtilis*
- Zone of inhibition of chloroform leaves extract on *P. aeruginosa*
- Zone of inhibition of ethanol and chloroform roots extracts on *E. coli*