TOXICOLOGY AND HERB-DRUG INTERACTION OF SELECTED ANTI-HYPERTENSION PLANTS USED BY LAY PERSONS IN NORTHERN KWAZULU-NATAL (SOUTH AFRICA)

By

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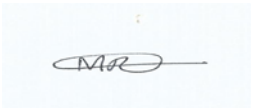
DECLARATION

I, Mmbulaheni Ramulondi declares that this dissertation is my own work. The research documented in this dissertation was conducted in the Department of Botany (University of Zululand), the Department of Pharmacy and Pharmacology (University of Witwatersrand) as well as the Department of Biochemistry and Microbiology (Nelson Mandela Metropolitan University) under the supervision of Prof. H. De Wet and Prof. S. Van Vuuren. It is submitted in fulfilment for the degree of Masters of Botany at University of Zululand. The study has not been previously submitted in any form for any degree or examination at this or any other University.



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ABSTRACT

A recent (2015) ethnobotanical study which was conducted in northern Maputaland showed that the lay people use medicinal plants singularly, in plant combinations, as well as concurrently with conventional drugs to treat hypertension. Because of the lack of studies validating the safety of South African medicinal plants and the limited interactive studies done between medicinal plants and conventional drugs this study was undertaken. The study was designed to validate the safety of 26 medicinal plants used singularly, in 19 plant combinations as well as when combined with conventional hypertension drugs (13 plants). Five medicinal plants (*Citrullus lanatus*, *Cladostemon kirkii*, *Hyphaene coriacea* and *Pyrenacantha kaurabassana* and *Strychnos madagascariensis)* investigated in the current study were evaluated for the first time for any toxicology. Apart from the combination between *Aloe marlothii* and *Hypoxis hemerocallidea*, none of the combinations investigated in the current study have been previously tested for any toxicity.

Aqueous and organic (1:1 dichloromethane-methanol) extracts were prepared. The toxicity was evaluated using three assays [the brine shrimp lethality assay (BSLA), Ames test and MTT assay (human hepatocarcinoma cell line HepG2/C3A)]. The toxicity interaction of the plant combination was evaluated using the sum of fractional inhibitory concentration (ΣFIC). Herb-drug interaction was performed on the aqueous extracts of the 13 medicinal plants which were recorded to be used concurrently with conventional drugs (adcon napamol, aluminum hydroxide, amlodipine, aspirin, enalapril, pharmapress and ridaq). The three assays applied were the CYP3A4 inhibition assay, the beta-glucuronidase inhibition assay as well as the carboxylesterase inhibition assay. These assays were chosen according to the enzymes involved in the metabolism of the hypertension conventional drugs.

The overall toxicity profile of the plant extracts tested in BSLA showed that 17 of the organic extracts were toxic while only two of the aqueous extracts were toxic. The aqueous extracts which were toxic were *Catharanthus roseus* and *Citrus limon* and these two extracts were further tested at varied concentrations to determine dosage. The results showed that as the concentration increases, the mortality percent also

increased. For the extracts tested for mutagenicity, four of the organic extracts were mutagenic while two of the aqueous extracts (*Catharanthus roseus* and *Ozoroa engleri*) were mutagenic toward *Salmonella typhimurium* bacterial strain TA98 and TA100. For the 13 medicinal plants which were tested in MTT, the results showed that *Sarcophyte sanguinea* was hepatotoxic at the concentration of 100 µg/ml. Apart from *Hypoxis hemerocallidea* which had moderate hepatotoxicity, the aqueous extracts of the most frequently used medicinal plants were non-toxic thus considered safe to use as a traditional medicine. However, *in vivo* tests (at preclinical level) are necessary to confirm this assumption.

For the plant combinations, the mortality percentage of the two concentrations tested in BSLA showed a significant correlation between dosage and toxicity i.e. toxicity was dosage dependant. The overall toxicity profile of the two concentrations tested indicated that organic extracts were more toxic than the aqueous extracts. The results of plant combinations when tested in the Ames test also showed that the organic extracts had more mutagenic effect than the aqueous extracts. The aqueous extract combination (*Catharanthus roseus* and *Momordica balsamina*) was the only combination to be toxic in both assays but also the most regularly use combination by the people. In the BSLA, six plant combinations showed antagonistic interactions (aqueous and organic extracts). In the Ames test five combinations from organic extracts had antagonistic interaction with only two aqueous combinations showing antagonistic response.

For the herb-drug interaction studies, all the extracts tested had an inhibitory effect on either of the three enzymes. A high inhibition percentage (97%) was observed on the enzyme CYP3A4, with *Sarcophyte sanguinea* and *Psidium guajava*. The lowest overall inhibitory effect was observed on beta-glucuronidase. The inhibition of the enzymes by the plant extracts was dosage dependent.

The outcome of this study established that low toxicity of the aqueous extracts was observed in most of the assays. Even though all the plant extracts tested for herb-drug interaction showed to inhibit beta-glucuronidase enzyme resulting in improved hypertensive drug efficacy, these medicinal plants cannot be recommended to be used concurrently with hypertensive drugs since they also inhibit the two enzymes

which are responsible for the metabolism of hypertension drugs. It is thus best practice not to take any herbals concurrently with any allopathic medicine.

PUBLICATION AND CONFERENCE PRESENTATIONS

Research publication

De Wet, H., Ngcobo, Z.N., Ramulondi, M., 2016. The use of indigenous medicine for the treatment of hypertension by a rural community in northern Maputaland, South Africa. South African Journal of Botany 103, 78-88. (Appendix A)

Conferences – oral presentations

Ramulondi, M and De Wet, H. Medicinal Plants used by lay people in a rural community in KwaZulu-Natal (South Africa) for the treatment of hypertension. Indigenous Plant Use Forum (IPUF) and Society for Economic Botany (SEB). Clanwilliam, Western Cape, 28 June – 2 July 2015. (Appendix B)

Ramulondi, M., De Wet, H and Van Vuuren, S.F. *In vitro* toxicity analysis of some medicinal plants used in northern KwaZulu-Natal for the treatment of hypertension. Indigenous Plant Use Forum (IPUF). Port St Johns, Eastern Cape, 4 – 6 July 2016. (Appendix C)

E-poster presentation:

Ramulondi, M., Van Vuuren, S.F. and De Wet H. Toxicological analysis of some plant combinations used for the treatment of hypertension by lay people in northern KwaZulu-Natal, South Africa. International Conference on Medicinal and Aromatic Plants. Penang, Malaysia, 1 – 2 December 2016. (Appendix D)

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ABBREVIATIONS

°C - Degrees Celsius

% - Percentage

µl – Microliter

ΣFIC - The sum of the fractional inhibitory concentrations

4NQO - 4-Nitroquinoline 1-oxide

50X VB salts - Vogel-Bonner medium salts

Ace inhibitors - Angiotensin-converting-enzyme inhibitors

ALP - Alkaline phosphate

ALT - Alanine aminotransferase

BNPP- Bis [p-nitrophenly] phosphate

BSLA - Brine shrimp lethality assay

CCL4 - Carbon tetrachloride

CFUs - Colony forming units

CPY - Cytochrome P450

CFU/ml - Colony forming units per millilitre

D:M - Dichloromethane: methanol

DNA - Deoxyribonucleic acid

DMSO - Dimethylsulfoxide

D2O - Deuterium oxide

EMEM - Eagle’s minimal essential medium

G - Grams

G/kg - Grams per kilograms

IPNI - International plant Name index

LC_{50} - Median lethal concentration

PD - Pharmacodynamics interaction

PK - Pharmacokinetic interaction

p-NPG- p - Nitrophenyl-β-D-glucuronide

PSB - Phosphate buffer

Mg/ml - Milligrams per millilitre

Mg/kg - Milligrams per kilograms

MTT - (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NSAIDs - Nonsteroidal anti-inflammatory drugs

NM - Nanometer

RNA - Ribonucleic acid

SGOT - Serum glutamic oxaloacetic transaminase

SGPT - Serum glutamic pyruvic transaminase

TSA - Tryptone Soya agar

V - Volume

V/V - Volume per volume

W/W - Weight per weight

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Chapter 1

Introduction

1.1 Background of the study

The study area, northern Maputaland (Figure 1.1) is situated in the north east part of KwaZulu-Natal, adjacent to Swaziland and Mozambique. People in this area are very poor (Statistics SA, 2011) and they mostly depend on medicinal plants in and around their homestead to treat various ailments. Even though health care is available free of charge (13 clinics and two hospitals in this region), people still prefer to use medicinal plants due to cultural beliefs and ease of availability. Previous studies done (De Wet et al., 2010, 2012, 2013, 2014, and 2016; York et al., 2011) in northern Maputaland on the use of medicinal plants to treat various ailment have confirmed that medicinal plants still form an important part in the primary health care within this region.

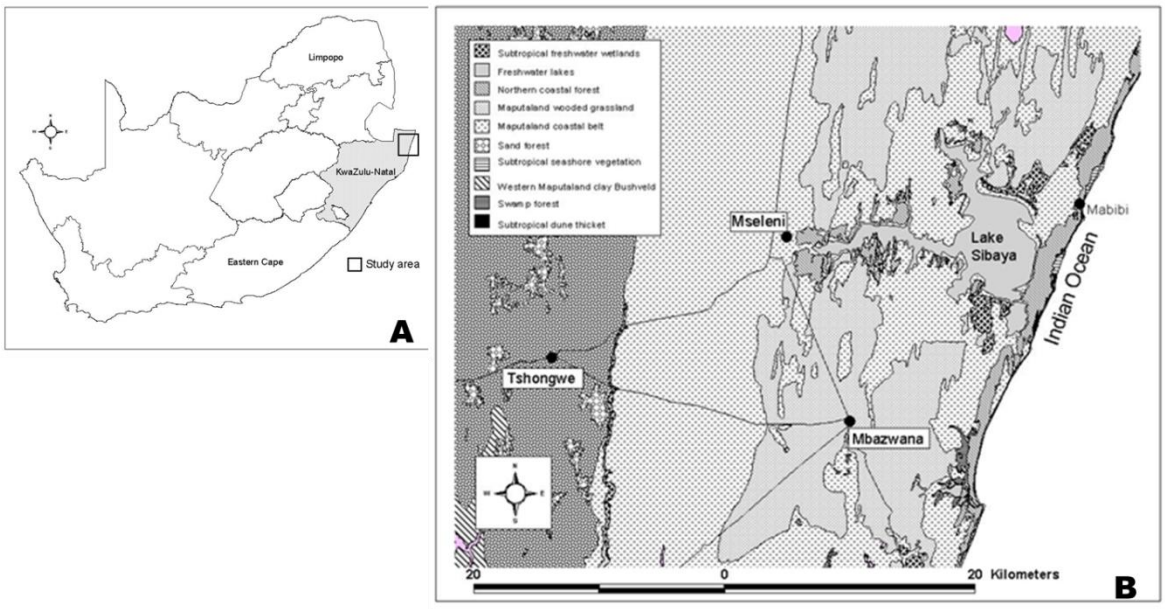

Figure 1.1 (A) Study area in northern Maputaland located in KwaZulu-Natal. (B) A more detail view of the study area and its different vegetation types.

According to WHO (2013), hypertension affects population in low and middle income countries where health care systems are weak. The statistics from 2008 has shown that 33% of South African population have hypertension (WHO, 2013). A survey done by De Wet et al. (2016) on anti-hypertension plants also revealed that 50% of

the respondents who were interviewed in rural communities of northern Maputaland have hypertension. With such a large number of people who have hypertension, and using medicinal plants for the management of hypertension, it is important to evaluate if the medicinal plants being used are safe. In the above mentioned survey where lay people were interviewed about the medicinal plants they used for the treatment of hypertension, 28 medicinal plants were documented. Eighteen of the medicinal plants (*Aloe marlothii* A.Berger*, Albertisia delagoensis (*N.E.Br)*, Carpobrotus dimidiatus* L.*, Citrullus lanatus* (Thunb.) Matsum. & Nakai*, Cladostemon kirkii* (Oliv.) Pax & Gilg*, Hyphaene coriacea* Gaertn.*, Lippia javanica* (burm.F.) Spreng.*, Ozoroa engleri* R.Ferm & A.Ferm*, Pyrenacantha kaurabassana* Baill*, Ptaeroxylon obliquum* (Thunb.) Radlk*, Ricinus communis* L. var. *communis, Sarcophyte sanguinea* Sparm. sups. *Sanguinea, Sarcostemma viminale* (L.) B.r. Saps*, Senecio serratuloides* DC.*, Strychnos madagascariensis* Poir*, Tetradenia riparia* (Hochst.) Codd, *Trichilia emetica* Vahl and *Vangueria infausta* Burch. subsp. *infausta*) (plants without author citation are cited in Table 2.1) were documented for the first time to be used for the treatment of hypertension (De Wet et al., 2016). The plant species which were most commonly used were *Momordica balsamina* followed by *A. marlothii, S. madagascariensis*, *S. serratuloides* and *Hypoxis hemerocallidea* Fisch & Ave-Lall (Figure 1.2).

Sixteen of the 28 medicinal plants recorded (*Albertisia delagoensis, Aloe marlothii, Cannabis sativa, Catharanthus roseus, Cladostemon kirkii, Citrus limon, Carpobrotus dimidiatus, Hyphaene coriacea, Hypoxis hemerocallidea, Lippia javanica, Momordica balsamina, Musa acuminata, Psidium guajava*, *Sarcophyte sanguinea, Senecio serratuloides* and *Trichilia emetica*) were involved in 19 different plant combinations for the treatment of hypertension. Two to four plant species were recorded as being used in various combinations (De Wet et al., 2016). Combinations were used as it is believed that synergism is apparent between different plants. However, there is no scientific documentation that these plants are working together (synergy) or even if they are working against each other (antagonism). None of the combinations recorded have been previously documented as a traditional use for the treatment of hypertension.

The plant combinations which were frequently used are combinations between *Aloe marlothii* and *Hypoxis hemerocallidea*, *Aloe marlothii* and *Momordica balsamina*,

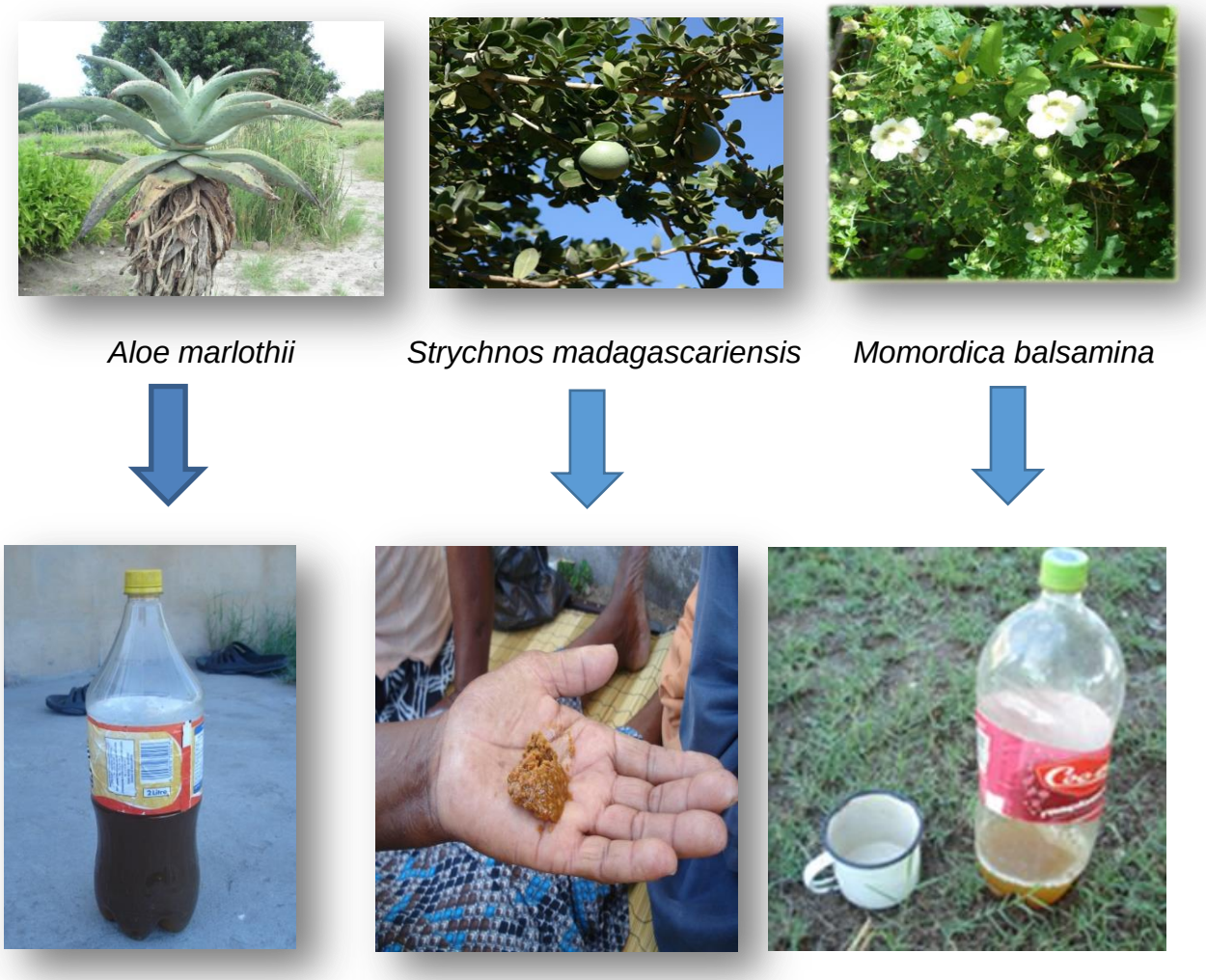

Figure 1.2 Three of the most used medicinal plants with its prepared medicine.

Hypoxis hemerocallidea and *Senecio serratuloides* as well as *Catharanthus roseus* and *Momordica balsamina*.

During the survey it was also found that 50% of the respondents have hypertension and 30% of these people claimed to be using medicinal plants and conventional drugs concurrently to treat hypertension. None of the respondents had revealed to the health care professionals that they take medicinal plants concurrently with conventional hypertension drugs. Neither was it common knowledge that they are also using medicinal plants for hypertension treatment. Therefore it is important to understand and examine the pharmacological interaction of plants when used together with conventional drugs. The lack of adequate knowledge of interactions

between medicinal plants and conventional medicines, as well as the lack of reporting of medicinal plants usage to healthcare professionals can pose a serious risk to patient safety (Butterweck and Derendorf, 2012; Vieira and Huang, 2012). Most of the recent plant interaction studies focus on the synergy that occurs between medicinal plants and conventional drugs. However, studies based on antagonistic interactions between medicinal plants and conventional drug combinations are highly relevant and very little has been documented on South African medicinal plants. Although drug-drug interactions are taken into account up to present, the importance of herb-drug interactions is slowly being recognised as a serious problem especially in elderly people with multiple comorbidities (Sultan et al., 2015). Even though the true occurrence of herb-drug interactions is not known, minimizing the risk of interactions should be a goal in drug and herbal medicine therapy because interaction can result in significant morbidity and mortality (Triplitt, 2006). One of the examples of the interactive studies between medicinal plants and conventional drugs focusing on the antagonistic interaction was done by Hubsch et al. (2014a, b). Six South African medicinal plants (*Agothosma betulina* (Berg.) pillans*, Aloe ferox* Mill., *Artemisia afra* Jacq. ex Willd., *Lippia javanica*, *Pelargonium sidoides* DC. and *Sutherlandia frutescens* (L.) (R.Br.) were antimicrobially tested with seven conventional antimicrobials (ciprofloxacin, erythromycin, gentamicin, penicilin G, tetracycline, amphotericin B and nystatin). The findings showed some notable synergistic interactions as well as the antagonistic interactions.

In the current study, 26 medicinal plants (Table 2.1) and 19 plant combinations (Table 2.2), from the study undertaken by De Wet et al. (2016) were considered for toxicity and herb-drug interaction investigation. The documented medicinal plants were analysed according to the way in which people from northern Maputaland prepares the medicinal plants. Toxicity analysis was undertaken for each plant (used singularly) and those used in plant combinations using the brine shrimp lethality assay (BSLA) and Ames test. A selection of plants were further analysed for hepatotoxicity as well as herb-drug interaction with hypertension conventional drugs. The results would serve to validate the safety of medicinal plants used for the treatment of hypertension in northern Maputaland singularly as well as used in combination or taken with conventional drugs.

Former studies have validated that traditionally used medicinal plants can be valuable in the management of various diseases such as cancer (Mishra et al., 2013), diabetes (Nammi et al., 2003; Kumar et al., 2010; Tripathi and Chandra, 2010), wound healing (Heggers et al., 1996; Priya et al., 2004; Gupta et al., 2005) and asthma (Saqib et al., 2015) as well as treating infections such as sexual transmitted diseases (Van Vuuren and Naidoo, 2010; Naidoo et al., 2013), skin diseases (Nciki et al., 2015) diarrhoea (Salgado et al., 2006; Acharyva et al., 2009; Saqid et al., 2015; Van Vuuren et al., 2015) and some bacterial and fungal infections (McGaw et al., 2000; Buwa and Van Staden, 2006; Bhalodia and Shukla, 2011; Naz and Bano, 2012; Madikizela et al., 2012; Adamu et al., 2014) as well as viruses (Abad et al., 1997; Mohamed et al., 2010; Dwivedi et al., 2013). However the adverse effect and the possible long term risk of the consumption of these commonly used medicinal plants has not been fully evaluated or documented in literature (Ndhlala et al., 2013). Many studies have acknowledged the need for extensive toxicological research not only for individual plants but also for combinations (Fennel et al., 2004; Adwan et al., 2009). Several studies have acknowledged the importance of herbal remedies and recognised the significance in determining plant safety. These studies were done in countries like South Africa (Ndhlala et al., 2013), Nigeria (Usman et al., 2014), Kenya (Kirira et al., 2006), Cameroon (Assob et al., 2011), Mexico (Déciga-Campos et al., 2007), Brazil (De Sá Ferreira and Ferrão Vargas, 1996), Peru (Bussman et al., 2011), India (Singh and Singh, 2012) and Australia (Kassie et al., 1996). Unfortunately not many of South African medicinal plants have been recorded in literature for toxicity (Popat et al., 2001; Steenkamp and Gouws, 2006; McGaw et al., 2007; McGaw et al., 2010; Ndhlala et al., 2013; Otang et al., 2013). Many of the plants used in South African traditional medicine have been considered safe due to the long history of its use and very little is reported on toxicity (Teschke et al., 2012).

1.2 Toxicity of medicinal plants

Medicinal plants are extensively regarded to be of lesser toxicity compared with conventional drugs, but they are not entirely free from the risk of toxicity or other adverse effects (De Smet, 2004). Their safety is established on their extended term of usage for the management of various conditions according to information accumulated over centuries from one generation to the other (Fennell et al., 2004).

This concept is largely circumstantial and it is important to determine toxicology of plants, especially those that are used frequently over a long period of time (Reid et al., 2006). However, it should be noted that medicinal plants used frequently over a long period of time are not commonly associated with negative effects. Some medicinal plants must be consumed with caution as they may cause adverse effects, especially if consumed in higher doses or if they interact with conventional drugs (Saad et al., 2006). The risk of toxicity accompanying the use of medicinal plants is one of the motives for the hesitancy among health care professionals towards integration of medicinal plants into the health care systems (Ezuruike and Prieto, 2014).

Acute toxicity as a result of medicinal plants in South Africa is not uncommon, but because of inadequate records, estimates of mortality rates vary widely from 8 000 to 20 000 per annum (Thomson, 2000). However, the number is probably higher, as not every death due to toxicity is reported. In rural areas few cases of human poisoning are documented as patients either do not arrive at the hospital in time (distance from homestead) or the plant is not identified (Botha and Penrith, 2008). Toxicity can be defined as undesired harmful effects (including mortality) caused by the collaboration between cells and toxicants. The adverse effects might differ depending on the substance, constituents of the toxicants and the cell membrane. The adverse effects may occur before the binding of the toxicants to the vital organs such as liver and kidneys (Asante-Duah, 2002). Human poisoning from plants usually arises from unintended consumption of toxic plants as medicine (Van Wyk et al., 2002). In most cases, these adverse effects from medicinal plants are only seen when consumed in high doses or prolonged use (chronic toxicity), which would thus not disqualify their continuous usage as medicinal plants as long as there is proper information about their effective dosage concentration (Ezuruike and Prieto, 2014). Most of the plants that are poisonous are as a result of the presence of mixtures of secondary metabolites often belonging to various structural levels that act as a defence mechanism against bacteria, fungi and viruses (Van Wyk and Wink, 2015). These secondary metabolites can have medicinal properties at low concentrations and be dangerous at higher concentrations. Some of these secondary compounds include cardiac glycosides, alkaloids, cyanogenic glycosides, phorbolesters and lectins which are classified as extremely toxic (Ndhlala et al., 2013).

All natural products with a physiological effect on the human body can be potentially toxic/poisonous if ingested in large quantities (Van Wyk and Wink, 2015). The adverse effects of some medicinal plants could be due to irrational uses, such as too high dosages. Thus more attention has to be drawn to the quality of plant material and to standardize the formulations, especially the dosage (Elkhidir et al., 2012). There are five factors which determine if medicinal plants are toxic or not. The first one is the dose (amount of substance ingested at one time). Any substance can be toxic if taken in large quantities. Thus it is the dosage that may be the most important factor to be considered. The second factor is the route of administration. For instance, a substance becomes more toxic when injected into the blood stream than when ingested due to increased bioavailability. The third factor is the preparation methods. Infusion/maceration/ decoction may be harmless whist the tincture may be toxic, since the alcohol can extract both the polar and non-polar compounds. The fourth factor is the frequency of use. Some of the secondary metabolites found in medicinal plants such as pyrrolizidine are not acutely toxic. However, they can cause severe damage over a period of many years. The last factor is the differences between individuals. Some individual's body systems are more sensitive than others. Age and sex may also contribute, since women are more susceptible than men (Matthews et al., 1999; Philomena, 2011; Voncina et al., 2014; Van Wyk and Wink, 2015).

All the medicinal plants documented for treating hypertension (De Wet et al., 2016) were prepared using water and taken orally. On average 250 ml of the decoction was taken three times a day. Thus it is important to investigate if the dose of some medicinal plants could lead to the toxicity. The survey of De Wet et al. (2016) also revealed that some individuals have been using these medicinal plants for up to 22 years. The prolonged use of herbal medicine can be problematic as the users do not take into account the accumulation of some constituents in the body, which could provoke severe side effects and could exaggerate hypertension.

Secondary metabolites from the plants may interfere with various organs, tissues, cells, proteins and enzymes (Van Wyk and Wink, 2015). There are different types of plant toxicities which include acute, sub-acute, nephrotoxicity, hepatotoxicity,

mutagenic, carcinogenic, genotoxicity, teratogenicity and cardiotoxicity. Following is a short explanation of the different types of toxicity and examples of plants causing it.

1.2.1 Acute and subacute toxicity

Acute toxicity is defined as the functional damage or biochemical abrasion effect that happens instantly or in a short time after a single or multiple dose of exposure to a substance or chemical (Rhodes et al., 1993; Walum, 1998). Subacute toxicity is the functional or biochemical damage occurring as a result of repeated daily dose of a substance/chemical over a period of several days or weeks (Eaton and Klaassen, 2001). Several South African medicinal plants have been implicated in acute and sub-acute toxicity. One such medicinal plants is *Pterocarpus soyauxii* Taub. Traditionally this plant is used to treat many ailments including hypertension, headaches, diabetes, snake bites, intestinal worms, muscular cramps, boils, skin disease, eczema and itching (Hutchings et al., 1996; Van Wyk and Gericke, 2000; Biennvenu et al., 2002). The symptoms that occur during its use include convulsions, decreased respiratory rate, paralysis of skeletal muscle, respiratory failure and coma (Maphosa et al., 2008). Other South African medicinal plants showing toxicity include *Euphorbia mauritanica* L. var. *mauritanica*, (Botha and Penrith, 2008) *Leonotis leonurus* (L.) R.Br (Maphosa et al., 2008) and *Polygala fruticosa* P. J. Bergius (Mukinda and Eagles, 2010).

1.2.2 Chronic and sub-chronic toxicity

Chronic toxicity is defined as the ability of xenobiotics to cause an adverse effect over an extended period following repeated or continuous exposure, sometimes lasting for the entire life span. Sub-chronic toxicity refers to the adverse effects that occur as a result of a repeated daily dose of xenobiotics for more than one year but less than life time (Adeneye, 2014). Several South African medicinal plants have been screened for chronic and sub-chronic toxicity. One of the medicinal plants which have been reported to have sub-chronic toxicity is *Carica papaya* Linn. The preclinical sub-chronic toxicity of 250, 500 and 1000 mg/kg of the aqueous leaf extract of *C. papaya* has been reported to have sub-chronic toxicity which includes hypoglycaemia and hyperlipidaemia. Leaf extract with ethanol have a sub-chronic toxicity which include hyperuricemia, glomerulosclerosis, tubular clarification,

hepatotoxicity, hypoglycaemia and hyperlipidaemia (Tarkang et al., 2012). A chronic toxicity study done on male Swiss mice were administered 150 mg/kg of *Allium cepa* Linn extract for eight weeks and it resulted in hypothermia, aggression and itchiness on the mice (Alqasoumi et al., 2012). Other South African medicinal plants that have been reported to have chronic and sub-chronic toxicity include *Polygala fruticose* P.J. Bergius and *Tulbaghia violacea* Harv. (Mukinda and Eagles, 2010; Olorunnisola et al., 2012) among others.

1.2.3 Nephrotoxicity

Nephrotoxicity can be described as a renal illness or dysfunction and is often triggered by chemicals, drugs, industrial or environmental toxic agents as well as medicinal plants. Nephrotoxicity results in failure of the kidney to detoxify and excrete xenobiotics (Kim and Moon, 2012). Factors contributing to the nephrotoxicity in medicinal plants use may be associated to the inherent toxicity of plant components, misidentification of medicinal plant or an over-dosage (Zhao et al., 2006; De Smet, 2007; Lai et al., 2009). Several plants have been reported in the literature to cause kidney failure. One of the plants causing acute renal failure is the bean from the tree *Pithecellobium lobatum* Benth. (known as *Djenkol*) which yields a pungent smelling edible fruit. These beans may be eaten raw, fried or roasted. They contain djenkolic acid, a sulphur rich cysteine thioacetal of formaldehyde and is the cause of acute renal failure. Symptoms of poisoning occur soon after or up to 36 hours after ingestion. Symptoms include fever, lower abdominal pains, passage of sandy particles in the urine and hypertension (Singh and Prakash, 2011). Some of the South African medicinal plants that have been implicated in renal dysfunction include *Callilepis laureola* DC. (Wainwright et al., 1977a, b), *Dioscorea quartiniana* Hochst. ex. A. Rich. (Steenkamp and Stewart, 2005) and *Catha edulis* (Vahl.) Endl. (Al-Mamary et al., 2002).

1.2.4 Hepatotoxicity

Hepatotoxicity can be described as damage to the liver or impairment of the liver function as a result of the exposure to xenobiotics such as chlorinated solvents, drugs, alcohol, food additives, peroxidized fatty acids, fungal toxins, a medicinal plant which is used, and radioactive isotopes (Navarro and Senior, 2006; Onaolapo et al., 2013). World-wide implication of medicinal plants in liver diseases and

hepatotoxicity associated to medicinal plants may be greater than expected (Manfo et al., 2014). One of the medicinal plants which is used world widely that has been implicated in hepatotoxicity is *Symphytum officinale* L. (Oberlies et al., 2004). This plant has been traditionally used to treat wounds, abdominal pains, blunt injuries, gout, bone fractures and jaundice (Koll et al., 2004; Stickel and Seitz, 2007; Giannetti et al., 2010). *Callilepsis laureola* DC. (Impila) is a plant indigenous to KwaZulu-Natal and has been used as a traditional remedy to treat snakebites, infertility and tapeworms (Bye and Dutton, 1991). Toxicity of the aqueous extract was observed *in vitro* in human hepatoblastoma Hep G2 cells. After six hours of incubation, 100% toxicity was detected at a concentration of 6.7 mg/ml indicating hepatotoxicity potential. These findings can possibly explain liver necrosis observed in the black population of KwaZulu-Natal in the 1970s (Popat et al., 2001). Another South African medicinal plant which has been reported to cause hepatotoxicity is *Hippobromus pauciflorus* (L.f.) (Pendota et al., 2010). Very few studies have been done in South Africa focusing on the hepatotoxicity of medicinal plants, thus it is essential to continue screening South African medicinal plants for hepatotoxicity potential.

1.2.5 Mutagenicity and carcinogenicity

Mutagens are substances that cause chemical or physical adjustments in the DNA arrangement, following the mistaken duplication of that region of the genome (Cariño-Cortés et al., 2007). The DNA damage as a result of mutagens may be fixed or may result in carcinogenicity as most mutations are in fact silent and do not result in cancer. However, if the damaged cells survive and undergo cell division, mutant cells are formed which results in carcinogenicity (tumour/cancer formation) (Figure 1.2). Carcinogenicity is the process whereby normal cells are transformed into cancer/tumour cells. The progression of carcinogenesis consists of a variation of biological modifications, which may produce the physical and functional adjustment in the genome of the cell. This process includes the initiation, selective functional enhancement (promotion), and progression of the unstable chromosomes (Bajpayee et al., 2005). Some medicinal plants are recognized to comprise of mutagenic, carcinogenic, or co-carcinogenic compounds such as furocoumarins, tannins, cardiac glycosides, anthraquinones, aristolochic acid and flavonoids (De Sá Ferreira and Ferrão Vargas, 1999; Varanda et al., 2002; Rietjens et al., 2005). *Acokanthera*

oppositifolia (Lam.) Codd from the family Apocynaceae which is found in southern and central Africa has been found to have a mutagenicity effect. This plant contains cardiac glycosides. The plant has been used traditionally for the treatment of headaches, abdominal pains, convulsions and septicaemia. Aremu et al. (2013) demonstrated that the methanol extracts of the root exhibited mutagenic effects in the Ames test. Some South African medicinal plants that have been reported to have mutagenicity effects include *Bauhinia galpinii* N.E.Br, *Catharanthus roseus* (L.) G. Don, *Chaetacme aristata* E.Mey. ex Planch and *Crinum macowanii* Baker (Elgorashi et al., 2003; Verschaeve et al., 2008). Some of the plants which are used in Chinese herbal medicine from the genus *Aristolochia* (containing aristolochic acid compound) have been implicated in carcinogenicity. Species in this genus have been reported to cause upper tract urothelial carcinoma (carcinoma of the bladder) (Hoang et al., 2013; Poon et al., 2013). *Aristolochia villosa* L. was also reported to produce malignant fibrous histiocytoma in rats (Pradhan et al., 1974). To the best of my knowledge, the only South African medicinal plant that has been implicated in carcinogenicity is *Senecio latifolius* D.C (Steenkamp et al., 2001).

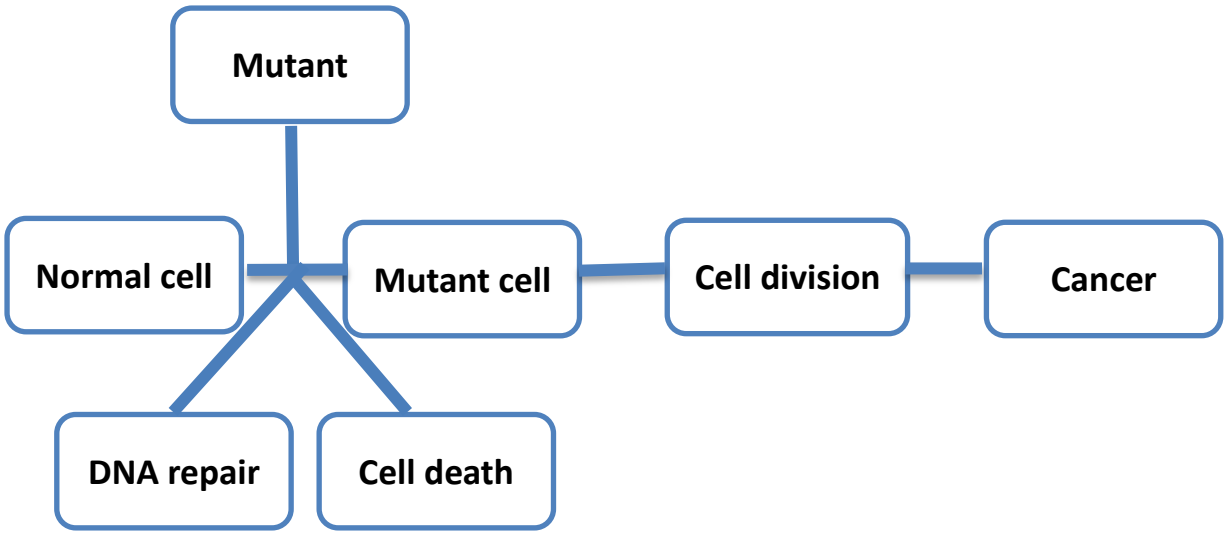

Figure 1.3 The role of mutagens in carcinogenesis.

1.2.6 Genotoxicity and teratogenicity

Genotoxicity can be caused by many substances that result in an extensive variation of potential damage to the genome, stretching from various DNA segments to single- and double-strand damages, DNA–DNA and DNA–protein cross-links or even

chromosomal damage (Ogura et al., 2008; Cavalcanti et al., 2010; Wang et al., 2012). Pyrrolizidine alkaloids are well known secondary metabolites that are genotoxic substances causing DNA damage (Seukep et al., 2014). Teratogenic substances induce abnormalities of functional development of a foetus (Seukep et al., 2014). *Allium sativum* L. (also known as garlic) has been reported to possess genotoxicity (Lim-Sylianco and Shier, 1989). This plant is considered as a vegetable in local communities and has many medicinal uses such as antidiabetic, antimicrobial, fibrinolytic activity as well as hypocholesterolaemic activity (Augusti, 1996). Genotoxicity of South African medicinal plants has been reported with plants such as *Boophane disticha* (L.f.) Herb.*, Catha edulis* (Vahl)Forsk. ex Endl.*, Hypoxis hemerocallidea, Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro, *Kigelia africana* (Lam.) Benth. and *Ricinus communis* (Taylor et al., 2003; Abderrahman and Modallal, 2008*).* Medicinal plants reported from other studies to have teratogenic effect include *Garinia kola* Heckel, *Lupinus formosus* Greene., *Rauwolfia vomitoria* Afzel and *Treculia africana* Decne. ex Trecul (Panter et al., 1990; Lawal, 1997; Akpantah et al., 2005; Eluwa et al., 2013). To the best of my knowledge, the only South African medicinal plant that has been reported to possess teratogenic effect is *Senecio latifolius* DC. (Steenkamp et al., 2001).

1.3 Toxicity of plant combinations

The lay people and healers do not exclusively depend on a single plant but regularly combine several plant species for treatment in the belief that the efficacy may be enhanced (Van Vuuren and Viljoen, 2011). The knowledge of plant combinations is transmitted from one generation to the other in the confidence that combination remedies may improve efficacy, lessen toxicity, reduce adverse effects, increase bioavailability and lower the dosages (Van Vuuren and Viljoen, 2011). Plant combinations have been used for centuries, due to the beneficial effects of a combination (Biavatti, 2009). The applications of plant combinations have gained a wider acceptance for the treatment of various infections (Van Vuuren and Viljoen, 2011). Combination therapy, although frequently used, may also be dangerous when the interaction is antagonistic, since it may result in higher toxicity (Meletiadis et al., 2010). Several studies have been conducted in South Africa on the efficacy of plant combinations to treat various diseases (Kamatou et al., 2006; Suliman et al., 2010; Ncube et al., 2012; Naidoo et al., 2013; Zonyane et al., 2013). However, little has

been done to scientific determine the safety of South African medicinal plants when they are used in combination.

1.4 Pharmacokinetic herb-drug interactions

Herbal products including medicinal plants and dietary supplements have become a popular supplement with conventional medicine globally (Tripathi et al., 2014). For medical purposes, these herbal products are used to relieve pain, encourage healing, improve digestion, cure infections, treat chronic illnesses such as hypertension, diabetes, depression and heart diseases as well as treating non-communicable diseases such as cancer and malaria (Thomford et al., 2015). Because of their medical use in daily life, these medicinal plants are usually used together with conventional medicine (Sachar and Ma, 2013). Drug-drug interactions have been known and studied for years. One of the steps involving the development of a new drug is the authentication of potential drug-drug interaction (Veber et al., 2002; Umehara and Camenisch, 2011). However, medicinal plants are not subjected to the same clinical evaluation (Zhou et al., 2010). Medicinal plants themselves consist of a mixture of active compounds which may interact within the plant as well with the prescribed conventional medicine to either increase (synergize) or lessen (antagonize) the healing effect (Hemalswarya and Doble, 2006). When medicinal plants are repeatedly used in combination with conventional medicines, there is an increased possibility of interaction between medicinal plant components and conventional drugs (Wang et al., 2007). There is evidence in the literature that medicinal plants have pharmacological properties and may lead to antagonistic interactions when used together with conventional medicine (De Smet, 1995; Poppenga, 2002; Singh and Levine, 2004). Since the components of medicinal plants consumed need also to be eliminated from the body by the same mechanism that removes drugs, there is a possibility that medicinal plants may induce/inhibit the drug metabolizing enzyme responsible for the metabolizing of the drugs (Thomford et al., 2015). Despite the popular use of medicinal plants, combinations are poorly controlled, and may contain contaminations, which increases the concern about the possibility of herb-drug interactions (Sachar and Ma, 2013). Very little information has been published on herb-drug interactions. However, the use of herbal medicine concurrently with conventional drugs is progressively growing across the world (Nivitabishekam et al., 2009). Van Vuuren and Viljoen (2011) and Hubsch et al.,

(2014a, b) have highlighted that studies on the potential effects of the usage of medicinal plants with conventional medicines have been neglected and thus require further investigation. Assessment of medicinal plants for potential herb-drug interaction is as important as the assessment of its safety and efficacy (Ezuruike and Prieto, 2014).

Medicinal plants are usually complex mixture of many molecular compounds such as tannins, coumarins, saponins, glycoside, alkaloids and flavonoids, which may have the potential to affect the pharmacokinetic (PK) and pharmacodynamics (PD) of the drug (Skalli and Bencheikh, 2012). A pharmacokinetic herb-drug interaction occurs when the medicinal plant interacts with absorption, breakdown and elimination of the drug. A pharmacodynamic herb-drug interaction occurs when the compounds in the medicinal plant interact with the molecular target of the drug (Sachar and Ma, 2013). Most of the herb-drug interactions arise a through a pharmacokinetic mechanism and involves either induction or inhibition of drug-metabolising enzymes which may affect the fate of conventional drugs in the body (Tripathi et al., 2014). Secondary metabolites from the medicinal plants can act as an inducer or inhibitor of drug metabolizing enzymes and drug transporters such as CYP450 and P-glycoprotein. Induction of drug metabolizing enzymes can lead to an increased degree of drug clearance, resulting in low plasma concentration of the drug, thus decreasing drug efficacy. Inhibition of drug metabolizing enzymes can lead to the increased blood concentration of the drug and increases the possibility of toxicity and adverse drug reactions (Colalto, 2010; Sachar and Ma, 2013). Changed drug protein binding and different drug elimination process may also occur. However, the amount of vulnerability differs from one person to the other (Lin and Lu, 1998; Ioannides, 2002).

Hypericum perforatum L. which is well known as St John's Wort with its main constituents of hypericin, hyperforin and flavonoids, is one of the most frequently used medicinal plant for the treatment of slight to moderate depression in western countries. It is a supplement that people can buy over the counter at any pharmacy or health store. It can have an important effect on the efficacy, safety and outcome of a variety of drug treatments. When St John's is taken concurrently with warfarin, St John's Wort reduces the anticoagulant effect. The possible mechanism is the induction (pharmacokinetic) of drug metabolizing enzyme. Warfarin is metabolised

by cytochrome P450 (CYP) 1A2 in the liver, which is induced by St John's Wort (Izzo et al., 2005). St John's Wort also induces the human CYP enzymes CYP 3A4 and CYP 2C9 (Izzo and Enerst, 2001; Henderson et al., 2002; Kawaguchi et al., 2004) upon chronic exposure, resulting in the decreasing of blood plasma concentration of the drug and thus decrease the drug efficacy (Komoroski et al., 2004).

The possibility of herb-drug interactions needs serious attention. These findings also need to be relayed to the general public to ensure safety. Some studies have been done on the interaction between medicinal plants and conventional drugs (Betoni et al., 2006; Rosato et al., 2007, 2008, 2009; D'Arrigo et al., 2010; Jarrar et al., 2010; Hubsch et al., 2014a, b). *Hypoxis hemerocallidea,* also known as African potato has demonstrated some interactive properties when combined with nevirapine. This plant is commonly used to boost immunity in HIV patients. It has shown to interact with the efflux mechanism of nevirapine through intestinal epithelial cells, which may lead to an increase in the bioavailability of nevirapine, thus results in the rise of drug toxicity (Mills et al., 2005). Other South African medicinal plants that has been reported to have herb-drug interactions include *Momordica charantia* L. with metformin (diabetes drug) (Tripathi et al., 2013) and *Sutherlandia frutescens* (L.) R.Br. with antiretroviral drugs (Fasinu et al., 2013). In spite of the extensive studies (Fasinu et al., 2013; Posadzk et al., 2013; Thomford et al., 2015) that have already been reported across the world and in Africa on the interactions between medicinal plants and conventional drugs, very few studies could be found in the literature that provides information of possible interactions of relevant, southern African medicinal plants with the enzymes involved in the metabolism of conventional hypertension drugs (Mills et al., 2005; Lee et al., 2007; Nair et al., 2007; Fasinu et al., 2013b; Calitz et al., 2015).

1.5 Aims and objectives of the study

Taking into consideration the ethnobotanical study done by De Wet et al. (2016) and the lack of toxicological data validating the safety of medicinal plants used to treat hypertension in northern Maputaland; this study is designed to address these concerns. Although there are a number of directives in assessing toxicity, this study will focus on preliminary cytotoxicity, mutagenicity as well as the hepatotoxicity. The main aim of the study is to evaluate the safety of the plants used to treat

hypertension by using a number of different assays. In order to reach this aim, the following subsequent objectives (Figure 1.3) are to be followed:

- To prepare aqueous and dichloromethane:methanol (1:1) plant extracts of the 26 plant species reported to be used for the treatment of hypertension in northern Maputaland.
- To perform different toxicity studies (brine shrimp assay, Ames test and hepatotoxicity) on all 26 plant extracts (individual species and the 19 different combinations documented).
- To assess the interactions between the plants used in combinations using the sum of the fractional inhibitory concentration (ΣFIC) to determine efficacy.
- To undertake herb-drug interaction assays using the CYP3A4 inhibition assay, carboxylesterase inhibition assay and beta glucuronidase inhibition assay.

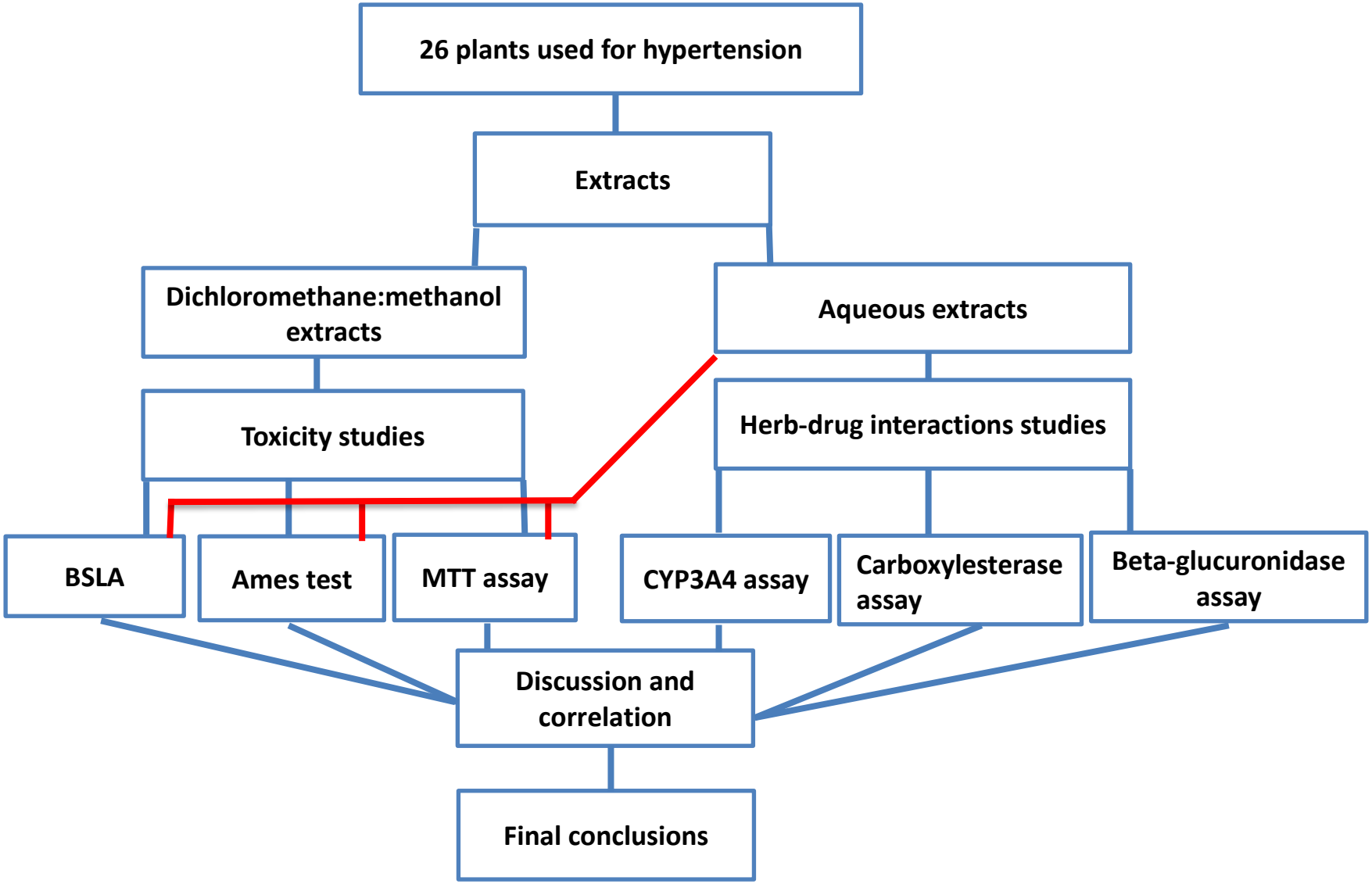

Figure 1.4 A diagrammatic summary of the steps undertaken in assessing the safety of medicinal plants used in northern Maputaland for the treatment of hypertension.

Chapter 2

Toxicity evaluation of plant extracts (individual and combination) using the brine shrimp lethality assay (BSLA)

2.1 Introduction

For the current study, a rapid bioassay known as brine shrimp lethality assay (BSLA) was used to assess preliminary cytotoxicity of selected medicinal plants. *Artemia franciscana* (brine shrimp) are small invertebrates occurring in sea water and other saline ecosystems. *Artemia franciscana* are frequently used as agents in laboratory assays to determine toxicity values (Meyer et al., 1982; McLaughlin, 1991; Cepleanu et al., 1994; Coe et al., 2010). The primary aim of the BSLA is to provide a base line screen that can be backed up by more specific and detailed bioassays (Pisutthanana et al., 2004). This assay is attractive because it offers a quick, simple and a cost-effective way of testing toxicity of plant extracts, and allows a high throughput (Bussmann et al., 2011). This is a straightforward assay as there is only one criteria; the shrimps are either dead or alive (Sudhakesavan et al., 2011). The availability of the eggs, the effortlessness of hatching them into larvae, the rapid growth of the nauplii and the relative ease of maintaining a population under laboratory conditions have made this assay to be the more preferred toxicology screening assay (Tawaha, 2006). *Artemia* spp. have the same purine metabolism as that of mammalian cells. The DNA-dependent RNA polymerases of *Artemia* are also similar to the mammalian type (Solis et al., 1993). Even though the BSLA test does not provide any adequate information regarding the mechanism of action of the plant extracts, it is useful to assess the basic toxicity and indicates the cytotoxic nature of the plants requiring further investigation. The BSLA has been successfully used in predicting biological activities including cytotoxicity, photo toxicity, pesticidal and trypanocidal activities, enzyme inhibition and ion regulation (Mackeen et al., 2000). Several studies have demonstrated good results using the BSLA to determine toxicity (Parra et al., 2001; Pisutthanana et al., 2004; Suad, 2012; Hubsch et al., 2014). The positive control which was used for this assay was potassium dichromate. This is the most commonly used and recommended reference compound for aquatic organisms. The

compound is toxic to aquatic organisms because of the presence of hexavalent chromium (Krejci and Palikova, 2006). This compound is a heavy metal which is commonly found in the environment and it is commercially used in numerous metal finishes, leather tanning and wood preservation (Banu et al., 2008).

Plant combinations were also analysed for toxicity using the BSLA. There is very little done on toxicity of medicinal plants use in combinations. *Aloe marlothii* + *H. hemerocallidea* were the only plant combination relevant to our study which could be found in the literature (Naidoo et al., 2013). Therefore, the aim of this chapter was to evaluate the potential toxicity of the 26 medicinal plants (singularly) and 19 plant combinations which were reportedly used for the treatment of hypertension (De Wet et al., 2016) using the BSLA.

2.2 Methods

2.2.1 Plant material

The collection of plant material occurred during February and March 2014. Various plant parts were collected including leaves, roots, corm, flowers, fruits and the whole plants, depending on the traditional use. Voucher specimens were prepared and deposited in the herbarium of the Botany Department at the University of Zululand. Botanical identification was made by Prof H De Wet, Dr THC Mostert and further confirmation of authenticity was undertaken by Mr Ngwenya from the South African National Biodiversity Institute, KwaZulu-Natal Herbarium. Author names of the plants were obtained from the international plant index (IPNI) (Table 2.1). All the plants were collected in and around the homesteads with the permission of the owners. None of the plants collected is on the red data species list as being rare or in danger of extinction. Only enough material was collected for the research project. Table 2.1 displays the 26 plants species collected with corresponding voucher numbers.

2.2.2 Preparation of plant extracts

The plant materials which were left to completely dry at room temperature (bark, seeds, leaves, corms and roots) and were thereafter chopped into smaller pieces and grinded into a fine powder using a Sciencetec RSA hammer mill. The grinder was rinsed thoroughly with water and alcohol between all samples grinded to

eliminate cross contamination of the plants. Two types of extracts (aqueous and organic) were prepared.

2.2.2.1 Aqueous extracts

Aqueous extracts were extracted in a similar manner in which people from Maputaland use their traditional remedies (De Wet et al., 2016). Two methods were considered, either a decoction where plant materials were boiled in water or a maceration where plant materials were soaked in water depending on traditional use. Ten grams of dried plant materials is equivalent to one handful of fresh material. For the decoction, 10 g was boiled in 200 ml of distilled water for 30 min. For the maceration, 10 g was soaked in 200 ml warm water. The extract was then left for 24 hrs in a platform shaker (Merck). Thereafter it was filtered and stored at -80°C before lyophilization in a freeze drier (Labcon). The freeze dried material were then stored at room temperature and protected from light until further analysis.

2.2.2.2 Organic extracts

For the organic extraction, the ground material (10 g) was extracted twice with 200 ml of a methanol and dichloromethane (1:1) mixture. Preparation of organic extracts was undertaken to ensure the extraction of both polar and non-polar compounds. The powdered material was left on a platform shaker for 24 hrs at 37 °C, and thereafter filtered through 90 mm filter paper. The filtrate was left in open bottles in a fume hood for the complete evaporation of the solvent, leaving behind the solid extract. After the evaporation, the solid extracts were stored at 4°C until further analysis.

2.2.3 Percentage yield

The percentage yield was calculated for all the aqueous and organic extracts. The percentage yield was calculated by dividing the total weight of the extract obtained by the total weight of plant material used in the preparation of the extract, which is known as the biomass. This value was then multiplied by one hundred to obtain a percentage weight per weight (w/w) (Table 2.1).

Table 2.1 Plant species which were investigated in this study.

Scientific names, author citations and family names	Plant part used	Voucher specimen	Extract yield in %	
			Organic	Aqueous
Acanthospermum hispidium D.C Asteraceae	Roots	M Ramulondi 8	5.9	8.4
Albertisia delagoensis (N.E.Br) Menispermaceae	Roots	S Nciki 25	12.9	6.3
Aloe marlothii A.Berger Asphodelaceae	Leaves	TYORK 42	2.1	8.9
Cannabis sativa L. Cannabaceae	Leaves	ZN Ngcobo	22.8	13.1
Carpobrotus dimidiatus L. Mesembryanthemaceae	Leaves	M Ramulondi 7	4.1	6.3
Catharanthus roseus (L.) G.Don Apocynaceae	Roots	M Ramulondi 4	10.9	9.4
Citrullus lanatus (Thunb.) Matsum. & Nakai Cucurbitaceae	Fruit	ZN Ngcobo 7	1.8	11.1
Citrus limon (L.) Osbeck Rutaceae	Peel	TYORK 7	13.5	9.4
Cladostemon kirkii (Oliv.) Pax & Gilg *Capparaceae*	Roots	SC Ngubane 17	5.3	3.3
Hyphaene coriacea Gaertn. Arecaceae	Roots	SC Ngubane 35	6.0	3.3
Hypoxis hemerocallidea Fisch & Ave-Lall Hypoxidaceae	Corm	SC Ngubane 15	23.7	9.4
Lippia javanica (burm.F.) Spreng. Verbenaceae	Leaves	TYORK 1	11.6	22.1
Momordica balsamina L. Cucurbitaceae	Leaves	M Ramulondi 6	11.7	9.3

Scientific names, author citations and family names	Plant part used	Voucher specimen	Extract yield in %	
			Organic	Aqueous
Musa acuminata Colla Musaceae	Flower bracts	ZN Ngcobo 2	11.9	13.4
Ozoroa engleri R.Ferm & A.Ferm Anacardiaceae	Roots	ZN Ngcobo 10	8.9	6.1
Psidium guajava L. Myrtaceae	Roots	ZN Ngcobo 12	5.9	8.3
	Leaves	ZN Ngcobo 12	18.1	13.1
Ptaeroxylon obliquum (Thunb.) Radlk Rutaceae	Roots	M Ramulondi 5	7.2	4.4
Pyrenacantha kaurabassana Baill Icacinaceae	Roots	ZN Ngcobo 1	5.4	4.1
Ricinus communis L. var. *communis* Euphorbiaceae	Leaves	ZN Ngcobo 23	17.6	14.7
Sarcophyte sanguine Sparm. sups. *Sanguinea* Balanophoraceae	Stem	ZN Ngcobo 29	11.1	9.6
Sarcostemma viminale (L.) B.r. Saps Apocynaceae	Whole plant	ZN Ngcobo 11	10.6	6.3
Senecio serratuloides DC. Asteraceae	Whole plant	ZN Ngcobo 16	22.4	13.7
Strychnos madagascariensis Poir Strychnaceae	Seeds	M Ramulondi 3	11.3	6.1
Tetradenia riparia (Hochst.) Codd Lamiaceae	Leaves	ZN Ngcobo 8	17.5	11.2
Trichilia emetica Vahl Meliaceae	Leaves	ZN Ngcobo 5	11.5	7.3
Vangueria infausta Burch. subsp. *Infausta* Rubiaceae	Leaves	ZN Ngcobo 6	7.5	8.1

2.2.4 Sample preparation for the brine shrimp assay

The organic extracts were dissolved in dimethylsulfoxide (DMSO) and diluted with sterile water. Aqueous extracts were dissolved in sterile water. Samples that were tested singularly were prepared to a concentration of 2 mg/ml. If a higher concentration (above 1 mg/ml) was necessary to exhibit a toxic effect against brine shrimp, then the sample is not considered toxic in nature (Bussmann, et al., 2011). The concentration of the DMSO was kept at 10% to ensure that the solvent does not exert a toxic effect itself. Extracts that were tested in combination, were prepared in two concentrations namely: 2 and 4 mg/ml. The first concentration (2 mg/ml) was done to validate if the concentration in combination would have the same effect as the single dosage. The second concentration (4 mg/ml) was done to validate/determine the accumulative effect of the sample as the concentration was doubled.

2.2.5 The brine shrimp assay

Artificial sea water was prepared by dissolving 16 g of Tropic Marine® Sea Salt in 500 ml of deionised water. A sealed bottomless plastic bottle was placed in an inverted position in a glass beaker. Dried, brine shrimp (*Artemia franciscana*) eggs (Ocean Nutrition™) (0.5 g) was then added to the plastic bottle along with the salt water. To ensure a high hatch rate, the salt water was aerated with a rotary pump (Kiho) and a constant source of light as brine shrimp cyst hatching is increased in light (220–240 V). Brine shrimp eggs were incubated at 25°C for 18–24 hrs. After the incubation period, the salt water was transferred to a shallow plastic container, and the container was tilted so that the solution lies on one side of the container and the light was placed over that side for approximately 30 min. This was done so that the brine shrimps move towards the light which increases the sample size of brine shrimp when collecting. After 30 min, 48-well micro-titre plates were prepared by adding 400 µl salt water containing 40–60 live brine shrimps to each well along with 400 µl of the test sample (positive control, solvent control, negative control or plant extract). The negative toxic-free control was prepared by adding 16 g Tropic Marine® Sea Salt in 500 ml of deionised water that mimics the natural environment for the brine shrimp to support growth and survive. The solvent control was DMSO which was kept at 10%. The positive control consisted of 1.6 mg/ml potassium

dichromate, which is known to be a highly toxic compound (Sigma-Aldrich). Dead brine-shrimp were counted after 24 and 48 hrs by viewing plates under a light microscope (Olympus) at 40× magnification. After counting at 48 hrs a lethal dose of acetic acid (Saarchem; 100% (v/v); 50 µl) was added to each well, and after 30 min a final count was undertaken such as to calculate the percentage mortality. In order to get the percentage mortality, the number of dead brine shrimp were divided by the initial number of the brine shrimp (40-60) and then multiplied by 100 (Mortality = no. of dead nauplii / initial no. of live nauplii x 100). A mortality percentage of 50% and above was considered to be toxic (Bussmann et al., 2011). Plant extracts (aqueous) which were toxic in the BSLA were further analysed by a dose response at the concentrations of 0.031, 0.063, 0.125, 0.25, 0.5 and 1 mg/ml.

Table 2.2 shows all the combinations to be investigated along with the frequency they were reportedly used. Plant species incorporated in the combinations were *A. marlothii, A. delagoensis, C. sativa, C. dimidiatus, C. roseus, C. limon, C. kirkii, H. coriacea, H. hemerocallidea, L. javanica, M. balsamina, M. acuminata, P. guajava, S. sanguinea, S. serratuloides* and *T. emetica*. The ratio combination was 1:1 if two plants are combined, 200 µl of each plant was used. These plants were reported to be used independently as well as in combination for the treatment of hypertension (De Wet et al., 2016). Toxicity of plant combinations (two up to four) were further assessed using the sum of the fractional inhibitory concentration (ΣFIC), which will allow the classification of the type of the toxicity. This method is commonly used for the classification of antimicrobial combinations. However, it has been adapted in this study to classify the types of toxicity which occurs when different plants are combined. The interaction of toxicity was defined as being considerably less toxic (in other words the extracts interact synergistically to produce a less toxic therapeutic outcome) if the ΣFIC index was $\leq$ 0.5. When ΣFIC index was between 0.5 and 1, it was regarded as having reduced toxicity (additive effects) and when the ΣFIC value was between 1 and 4, the combination was regarded not having any effect (non-interactive) on toxicity. When the ΣFIC value was above 4, the combination was considered as having increased toxicity, in other words an antagonistic response. Mortality percentage obtained when two or more plant samples are combined in equal ratio (1:1) of each sample was also used in the ΣFIC calculation. The ΣFIC

value was determined using the methods described by Van Vuuren and Viljoen (2011) and modified to calculate interactive toxicity using the following equation;

$$\Sigma FIC = FIC^{(i)} + FIC^{(ii).}$$

$$FIC(i) = \frac{\text{Toxicity (a) in combination with (b)}}{\text{Toxicity (a) in dependently}}$$

$$FIC\ (ii).= \frac{\text{Toxicity (b) in combination with (a)}}{\text{Toxicity (b) in dependently}}$$

(a) and (b) represent different plant samples

Table 2.2 Plant combinations investigated along with their frequency of use.

Plant combination	Number of times reported
Catharanthus roseus + *Momordica balsamina*	5
Aloe marlothii + *Hypoxis hemerocallidea*	4
Aloe marlothii + *Momordica balsamina*	3
Hypoxis hemerocallidea + *Senecio serratuloides*	3
Cannabis sativa + *Momordica balsamina*	2
Aloe marlothii + *Cannabis sativa*	1
Momordica balsamina + *Musa acuminata*	1
Aloe marlothii + *Cladostemon kirkii* + *Sarcophyte sanguinea*	1
Lippia javanica + *Momordica balsamina*	1
Momordica balsamina + *Senecio serratuloides*	1
Citrus limon + *Musa acuminata*	1
Aloe marlothii + *Catharanthus roseus*	1
Aloe marlothii + *Hypoxis hemerocallidea* + *Momordica balsamina*	1
Carpobrotus dimidiatus + *Psidium guajava*	1
Catharanthus roseus + *Hypoxis hemerocallidea*	1
Catharanthus roseus + *Musa acuminata*	1
Trichilia emetica + *Aloe marlothii* + *Hyphaene coriacea*	1
Senecio serratuloides + *Musa acuminata* + *Aloe marlothii* + *Hypoxis hemerocallidea*	1
Albertisia delagoensis + *Senecio serratuloides*	1

2.3 Results and discussion

2.3.1 Toxicity analysis of individual plant samples

Individual plant samples (both organic and aqueous) derived from 26 medicinal plants used for the treatment of hypertension were investigated for toxicity using the BSLA and the results are recorded in Table 2.3. Extracts were considered toxic if the mortality rate was 50% or more. Aqueous extracts were less toxic with only two extracts (*C. roseus and C. limon*) toxic out of the 27 extracts tested. *Catharanthus roseus* was the one which was the most toxic with the mortality rate of 92% after 48 hours of continuous exposure. Aqueous extracts which were the least toxic (less than 10% mortality rate) includes *A. marlothii*, *C. sativa, C. lanatus, C. kirkii, H. hemerocallidea, M. acuminata, O. engleri* as well as *P. kaurabassana*. For the organic extracts, 63% of the extracts tested were toxic. Organic extracts which were most toxic include *A. delagoensis, C. sativa, C. limon, P. obliquum, P. kaurabassana, R. communis* and *T. riparia* which killed all the brine shrimps within 48 hours of continuous exposure. Organic extracts which were least toxic (less than 10% mortality rate) includes *A. marlothii, C. lanatus, M. acuminata, P. guajava (leaves), S. sanguinea and S. viminale*. The negative control demonstrated 0% mortality rate in every plate. The solvent control demonstrated a maximum mortality rate of 2%. The positive control resulted in 100% mortality rate of brine shrimp on all the plates within 24 hrs of exposure (Table 2.3) which is observed by an absence of internal or external movement within a period of ten seconds (Carballo et al., 2002). Some of the extracts (*C. roseus, C. dimidiatus, P. guajava* and *S*. *madagascariensis*) showed to have extreme difference of mortality between 24 and 48 hrs. A possible explanation could be that the brine shrimp cease to survive only after 48 hrs, because the type of toxicity could be sub-acute toxicity and not acute toxicity. According to Carballo et al. (2002), explanation for the big difference in the mortality rate between 24 and 48 hrs is at 48 hrs stage the brine shrimp have reached their second and third instar (developmental stage) and they exhibit the greatest sensitivity to the test compounds.

In the current study, the aqueous and organic extracts of the roots of *A. hispidum* were tested for toxicity using the BSLA. The aqueous extracts demonstrated no toxicity at 2 mg/ml, however, the organic extract showed 67% and 92% mortality of

brine shrimps within 24 and 48 hrs of exposure respectively. These results may be because the aqueous extracts may not extract non-polar compounds whereas the organic solvent extracted both the polar and non-polar compounds. A similar effect on the organic extract using the BSLA was also reported on this species where the seed and the whole plant were extracted using ethanol (Suad, 2012). In another study done by Sanon et al. (2003), the alkaloids from the stem and the leaves which were extracted using petroleum ether and ethanol showed a weak cytotoxicity against the following three cell lines, monocytes (THPl), normal melanocytes (MCDB) and malignant melanoma cells (HTB-66). In a study done by Bero et al. (2009), the dichloromethane, methanol and water extracts of the leaves, twigs and the roots showed low cytotoxicity when evaluated using the MTT (3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide) assay on two cell lines which are macrophage like murine cells (J774) and human normal fibroblasts (W138) at the concentration of 20 mg/ml. Furthermore, the *in vivo* acute (2000 mg/kg) and sub-acute (1000 mg/kg) toxicity tests on the crude acidic water extract of the flower, leaves and the stem did not show any toxicity in mice (Ganfon et al., 2012). This study correlates with the current study where the aqueous extract was also found to be non-toxic.

Another plant sample showing the similar trend was the aqueous root extracts of *A. delagoensis*. The aqueous extract did not show any potential toxicity, while the organic extract indicated potential toxicity with the mortality percentages of 91% and 100% within 24 and 48 hrs of exposure respectively. According to a study which was done by De Wet et al. (2007), leaves and rhizome (methanol extracts) of *A. delagoensis* showed low cytotoxicity at 200 µg/ml against Graham cell line. Apart from the study done by De Wet et al. (2007), no further studies could be found in the literature regarding the toxicity of *A. delagoensis*.

In the BSLA, both organic and aqueous leaf extracts of *A. marlothii* were considered non-toxic after 24 and 48 hrs of continuous exposure (Table 2.3). These results correlate with other studies. This included the study done by Du Plessis and Hamman (2013) where the cell viability indicated no cytotoxicity against human hepatocellular cells (HepG2), human neuroblastoma cells (SH-SY5Y) and human adenocarcinoma epithelial cells (HeLa) for the leaf and the gel. A study done by Van

Zyl and Viljoen (2002) also indicated that methanol leaf extract of *A. marlothii* did not exhibit any toxicity towards the Graham cell line and GCT cell line at the concentration of 50 µg/ml. According to the study done by Naidoo et al. (2013), the cell viability indicated that this plant is non-toxic at 100 µg/ml against the human kidney epithelial cell line. The non-toxicity profile of this plant correlates with the high frequency of traditional use of this plant as it was documented to be the second most used plant for the treatment of hypertension (De Wet et al., 2016).

In the current study the organic and aqueous leaf extracts of *C. sativa* were tested and the aqueous extract was found to be non-toxic after 24 and 48 hrs of continuous exposure. The organic extract demonstrated 96% and 100% mortality of brine shrimp within 24 and 48 hrs of exposure respectively (Table 2.3). The use of *C. sativa* has been associated with lung, head, neck, larynx, prostate, cervix, testes and brain cancers (Sidney et al., 1997; Zhang et al., 1999; Hashibe et al., 2002; Daling et al., 2009). *Cannabis sativa* has also been associated with cardiovascular disorder, necrosis (death of the tissue) of the kidney and brain, and it is also known to affect bone metabolism and has teratogenic effects on the developing of the brain following perinatal exposure (Reece, 2009). Acute oral toxicity has also been reported from ethanol extracts at the concentrations between 225 and 1800 mg/kg. The death of rats was observed between 36 to 72 hrs after the treatment, regardless of the dose level. Mortality resulted from severe hypothermia and other central effects (Thompson et al., 1973). However, the toxicity assessment of the petroleum ether extract of *C*. *sativa* which was done on the Wistar white albino rats proved otherwise. The results showed hepatorenal lesions but no death at the daily dose of 0.01, 0.1 and 1 ml/kg. There were no changes in the physiological behaviour (Badwi et al., 2012). It has also been reported that *C. sativa* induced inhibition of hepatic drug metabolism, altering *in vitro* cell viability, decreased fertilization capacity and decrease activities of P-glycoprotein and other drug transporters (Bergamaschi et al., 2011). Another chronic study of cannabidiol (compound found in *C. sativa*) which was performed on humans revealed that oral administration of cannabidiol daily for 21 days did not induce neurological, clinical (including electrocardiogram), psychiatric, blood and urine changes (Mincis et al., 1973). Likewise oral administration of *C. sativa* in healthy participants did not show any sign of toxicity

and serious side effects which was observed on neurological, physical, blood and urine analysis (Cunha et al., 1980). This plant was recorded to be one of the most used plants in treating hypertension. Although the organic extracts seem to be toxic, the aqueous extract was non-toxic which is good since the traditional people use water for preparing the decoction.

The organic leaf extracts of *C. dimidiatus* showed toxicity at 65% mortality after 48 hrs of exposure (Table 2.3). The aqueous extract did not show any signs of toxicity. The aqueous extract results correlate with the results reported by Hurinanthan et al. (2009) where the aqueous extracts of the leaves and the flowers were found to be non-toxic at concentrations of 1, 10, 100, 1000 µg/ml towards BSLA. However, the methanol extract of this plant was reported to be non-toxic when tested in the BSLA which differs from the results obtained from the current study. The contradicting results could be due to the different concentrations used. Hurinanthan et al. (2009) tested this species in lower concentrations (1, 10, 100, 1000 µg) compared to the concentration which was used in the current study (2 mg/ml). The contradicting results could also be due to different solvents used for extraction. In the study done by Hurinanthan et al. (2009), methanol was used for extraction whereas the current study used dichloromethane:methanol (1:1). Apart from the study done by Hurinanthan et al. (2009), nothing could be found in the literature regarding the toxicity of *C. dimidiatus*.

Both organic (92% mortality) and aqueous (90% mortality) root extracts of *C. roseus* showed toxicity with the BSLA. The methanol extract of the leaves also shown cytotoxicity against the human lymphoma U-937 GTB cell line (El-Seedi et al., 2013). Dichloromethane:methanol (1:1) and aqueous extracts of the leaves and twigs have showed toxicity in the Chang liver and 3T3-L1 cell lines after 48 hours of exposure at a relatively low concentration of 12.5 µg/ml (Van de Venter et al., 2008). *Catharanthus roseus* has been previously reported to be extremely poisonous because of the presence of cardial glycosides (Van Wyk and Wink, 2015). It has also been reported that the plant can irritate the skin, respiratory tract and damage the cornea of the eye (Van Wyk et al., 2002; Govindasamy and Srinivasan, 2012). Furthermore it has been reported to have hypoglycaemia and neurotoxic effects (Van Wyk and Wink, 2015). The methanol leaves extract of the plant was also reported to have sub-acute toxicity on Sprague Dawley rats at the concentrations of

0.1, 0.5 and 1 g/kg (Kevin et al., 2012). Surprisingly on the frequency of traditional use, this plant was documented to be the eighth most used plant for the treatment of hypertension (De Wet et al., 2016) although it is extremely toxic. Regardless of the toxicity profile of *C. roseus*, this plant has been reported to be useful in the treatment of various other conditions including diabetes (Nammi et al., 2003), diarrhoea, as anthelminthic, for microbial infections (Gajalakshmi et al., 2013) and ulcers (Sain and Sharma, 2013). Traditional people use this plant because of its effectiveness (according to them) but they lack the knowledge of its long term effect on their health. Regarding medicinal plants there is a fine line between dosage and toxicity i.e. it can be a remedy at low concentration but toxic at higher concentration (Deichmann et al., 1986).

The aqueous and organic fruit extracts of *C. lanatus*, when tested in the BSLA demonstrated no toxicity at 2 mg/ml after 24 and 48 hrs of continuous exposure (Table 2.3). No information could be found in the literature on toxicity of this plant. This is not surprisingly as it is well-known edible fruit.

In the current study, both organic and aqueous extracts of *C. limon* peel were tested individually in the BSLA for potential toxicity. Both aqueous and organic extracts demonstrated toxicity; with the organic extract having 100% brine shrimp death after 48 hrs (Table 2.3). This is surprising because *C. limon* is a well-known edible fruit. The reason that the *C. limon* showed apparent toxicity can be due to PH effects. Brine shrimp nauplii are surprisingly hardy, but large PH changes can kill them. *Citrus limon* extracts would be expected to contain high levels of organic acids (e.g. ascorbic acid) and these may reduce the PH drastically. Thus, this may be a fallacious result, due to PH changes rather than toxicity. Another study has been done on the peel extract of *C. limon.* The peel extract (80% ethanol) was evaluated for genotoxicity effect in the root meristem cells of *Allium cepa* at the concentration of 25, 50 and 75 g/l. The extract showed genotoxicity effect such as break, bridges, stickiness, and pole deviation of micronuclei on the root meristem cells (Ali and Celik, 2007). The essential oils of *C. limon* peels has also been investigated for cytotoxicity potential effect. The essential oils were tested against the human colorectal carcinoma cell line LIMI863 and the results showed that the essential oils had the cytotoxicity effect on the cell line tested at the concentrations between 0.5-48 µg/ml (Jomaa et al., 2012). There has also been a report on possible central

nervous system depressant and anticonvulsant activities in mice caused by essential oils from the leaves of *C. limon* (Campelo et al., 2011). However, *C. limon* fruit extract was reported to possess hepatoprotective effect against carbon furan induced toxicity in wistar rats (Jaiswal et al., 2015).

The root extracts of *C. kirkii, H. coriacea and P. kaurabassana* (organic and aqueous) were also investigated for potential toxicity using BSLA. The aqueous extracts of all three plants were non-toxic towards brine shrimp after 48 hrs of continuous exposure. For the organic extracts, *C. kirkii* was non-toxic with the mortality rate of 30% whereas *H. coriacea* and *P. kaurabassana* were toxic with the mortality rate of 67% and 100% respectively after 48 hrs of continuous exposure (Table 2.3). No information could be found in the literature regarding the toxicity of these plants. These plants were documented for the first time to be used for the treatment of hypertension (De Wet et al., 2016). The results of these plants highlight the importance of assessing newly documented plants for their toxicity.

The aqueous extract *of H. hemerocallidea* corm demonstrated 5% mortality after 48 hrs of continuous exposure; however, the organic extract demonstrated 54% mortality after 48 hrs (Table 2.3). The cytotoxicity of organic extract was also supported by a study done by Jooste et al. (2012) where both aqueous and organic extracts (hexane, dichloromethane, ethyl acetate, and methanol) of the corm showed cytotoxicity effect against brine shrimp at 10, 100 and 1000 µg/ml. The cell viability test indicated that the organic (dichloromethane:methanol) and aqueous extract of the corm was non-toxic at 100 µg/ml against human kidney epithelial cell line (Naidoo et al., 2013). Chronic and acute infusions of *H. hemerocallidea* aqueous extract in male Wistar rats resulted in a decrease in the glomerular filtration rate, along with elevated plasma creatinine concentrations, indicating an impairment of kidney function (Musabayane et al., 2005). In another study involving guinea-pigs and rats, the aqueous extract of *H. hemerocallidea* corm indicated bradycardia (slow heartbeat) and brief hypotension (Ojewole, 2006). Cytotoxicity of the aqueous extract of *H. hemerocallidea* corm has also been investigated on three human cancer cell lines; prostate cancer cells (Du-145), breast cancer cells (MDA-MB-231 and MCF-7) and non-malignant breast cell line (MCF-12A). The extract was tested at the concentration of 50 µg/ml and the results showed that this plant species was non-toxic (Steenkamp and Gouws, 2006). Most of the studies (Steenkamp and Gouws,

2006; Naidoo et al., 2013) including the current study reported that the aqueous extract is non-toxic. The non-toxicity profile of this plant correlates well with the frequency of use, as it was reported to be the third most used plant to treat hypertension in northern Maputaland (De Wet et al., 2016).

When the leaves extracts (organic and aqueous) of *L. javanica* were tested for potential toxicity using the BSLA, the aqueous extract was shown to be non-toxic while the organic extract had 52% mortality rate after 48 hrs of continuous exposure (Table 2.3). These results are supported by the results which were found in the study done by Hubsch et al. (2014a). The study reported that the essential oils and aqueous extract from the leaves displayed minimal inhibitory effect whereas the organic (dichloromethane:methanol) extract had a LC50 of 0.51 ± 0.03 mg/ml which was considered as weak/low toxicity according to the toxicity classification by Bussmann et al. (2011). Another study which validates the weak cytotoxicity of *L. javanica* was done by Ayuko et al. (2009). The study showed that an organic extract (methanol) of the roots of *L. javanica* has weak cytotoxicity when tested in the BSLA. The extract was tested with different concentrations (50, 100, 200, 400, 500, 1000 and 2000 µg/ml). Another study reported the non-toxicity profile of the leaf water extract towards BSLA at the concentration of 2 mg/ml (Cock and Van Vuuren, 2014). The aqueous extract of *L. javanica* was also tested for toxicity in mice and the results showed that within 48 hrs all the mice which were fed the extract of *L. javanica* at 12.5% - 37.5% v/v were lethargic and the overall mortality was 37.5%. It was suspected that the toxicity could be due to the presence of the xanthine compound. This compound is a derivate of caffeine which has pharmacological actions such as central nervous system stimulation, myocardial stimulation, peripheral vasoconstriction and diuresis if consumed at high dosages (Madzimure et al., 2011). *Lippia javanica* is also known to cause liver damage and photosensitisation in livestock due to the presence of triterpenoids and the prolonged use of the plant is potentially harmful (Van Wyk et al., 2014). According to Maroyi (2017), essential oils are not known to have acute toxicity properties. The triterpenoids compounds are not present in the essential oils and therefore this may explains why the essential oils are non-toxic.

Momordica balsamina leaves, when tested in the BSLA, demonstrated no toxicity for the aqueous extract. However, the organic extract showed a weak toxicity with the

mortality rate of 50% after 48 hrs of continuous exposure (Table 2.3). The difference between organic and aqueous extracts results could be that the aqueous extracts did not extract non-polar compounds where the organic solvents extracted both the polar and non-polar compounds. *Momordica balsamina* aqueous and organic leaf extracts (dichloromethane:methanol) were shown to be toxic against C2C12 muscle cells and hepatocytes cell lines when tested at the concentration of 12.5 µg/ml. This *in vitro* toxicity raises concern about the chronic use of this plant (Van de Venter et al., 2008). This can be potential dangerous to the lay people since this plant is used on a daily basis to treat chronic hypertension in northern Maputaland. *Momordica balsamina* stem bark of the aqueous extract has also been reported to be toxic at very high dosages (0.56 mg/100 g) on tissues and organs of rats (Geidam et al., 2007). The compound balsaminoside found in this plant tested toxic at a high dosage against human hepatoma (Huh-7) cell line (Ramalhete et al., 2014). Tritepenoids and balsominol compounds from the leaves of *M. balsamina* showed weak toxicity towards human breast cancer (Mcf-7) cell line (Ramalhete et al., 2010). However, an acute toxicity study which was done on rats, where aqueous extract of the leaves was orally administered for three weeks, showed no sign of any clinical toxicity. It was concluded that this plant is safe at a low dosage (Karumi et al., 2006). The results of the study done by Karumi et al. (2006) correlate with the current study where the aqueous extracts were found to be non-toxic. The toxicity of the organic extracts of *M. balsamina* raises concern as this plant was the most used plant for the treatment of hypertension in the study area. However, it should also be taken into consideration that traditional people use water for preparing a decoction and it could therefore be safe for consumption at lower dosage.

The aqueous and organic extract of the red flower bracts of *M. acuminata,* when tested individually in BSLA, demonstrated no toxicity at 2 mg/ml after 24 and 48 hrs of continuous exposure (Table 2.3). These results are confirmed by a study done by Sumathy et al. (2011) where the organic extracts (methanol) of the red flower bracts were shown to be non-toxic against brine shrimps with the LC50 value of 9.97 mg/ml. The viability assay of the organic (dichloromethane:methanol) and aqueous extracts of the roots of this plant also indicated that this plant is non-toxic at 100 µg/ml against human kidney epithelial cell line (Naidoo et al., 2013). All the results of

the two different plant parts (flower bracts and the roots) that has been subjected to toxicity testing confirm the fact that this plant is non-toxic.

The root extracts (aqueous and organic) of *O. engleri* were also investigated for potential toxicity using BSLA. The aqueous extract demonstrated no toxicity at 2 mg/ml, however, the organic extract showed 58% mortality of brine shrimps after 48 hrs of exposure (Table 2.3). Another study which validates the toxicity of the organic extract of *O. engleri* was done by Prozesky et al. (2001). The dichloromethane stem bark extract of *O. engleri* was reported to have cytotoxicity with the ID50 at 35 µg/ml against vervet monkey kidney cell line. However according to the study done by Naidoo et al. (2013), a cell viability assay of the organic (dichloromethane:methanol) and aqueous extracts of the leaves of this plant indicated that the plant was non-toxic at 100 µg/ml against the human kidney epithelial cell line. The aqueous extract results of Naidoo et al. (2013) correlate with the aqueous extract results obtained in the current study.

The aqueous and organic (dichloromethane:methanol) extracts of the leaves of *P. guajava* were found to be non-toxic after 48 hrs of continuous exposure. The aqueous extract of the roots was also non-toxic; however, the organic extract of the roots was toxic with the mortality rate of 59% after 48 hrs of continuous exposure (Table 2.3). Several studies have been done on the toxicity of *P. guajava*. A study done by Ajaiyeoba et al. (2006) confirmed the results found in the current study where the 90% methanol (organic) leaf extract was found to be non-toxic in BSLA with the LC50 value of 707 µg/ml. Ling et al. (2010) also reported that the aqueous and ethanol leaves extracts were non-toxic against 3T3 (mouse embryonic fibroblast cell) and 4T1 (mouse breast cancer cell) at 100 µg/ml. Acute toxicity studies of the water extract of the leaves has been done by Etuk and Francis (2003). In that study the rats were orally administrated with 10-50 mg/100g of the water extract and the results showed that there was no significant harmful effect in the rats after 72 hrs. Another study by Van de Venter et al. (2008) reported that the organic (dichloromethane:methanol) and aqueous extracts of leaves and roots were toxic against Chang liver and 3T3-L1 fat cell lines at the concentration of 12.5 µg/ml. The leaves have been reported to possess hepatoprotective properties at a lower dosage and hepatotoxic properties at a higher dosage (Sambo et al., 2009). Another study that reported the hepatoprotetive effect of *P. guajava* was done by Roy et al. (2006).

The aqueous extract of the leaves was studied on rat liver damage induced by carbon tetrachloride. The results showed that the leaf extract at the doses of 500 mg/kg produced significant hepatoprotection. However, the methanol extract of the leaves inhibited the pronounced cytotoxic effect at the concentration of 10 µg/ml on HeLa cell lines (Joseph and Priya, 2010). A 70% ethanol extract of the leaves of *P. guajava* also caused a decrease in cell viability and growth on human gingival fibroblast (Fernandes et al., 2010). Acute and sub-chronic toxicity studies of the root extracts has also been determined. Methanol extracts of the roots was orally administered at the concentrations of 100, 150, 500, 1000, 2000, 4000, 5000 mg/kg and 150, 300, 600 and 1200 mg/kg to test for both acute and sub-chronic toxicity. The results showed signs of anorexia, weakness and sluggishness. A significant reduction in the body weight also occurred. Mortality from the group receiving the highest dosage of the extract was observed (Onyekwe et al., 2011). The BSLA has also been done to determine the toxicity of the essential oil of *P. guajava* stem bark and the results showed that the oil was toxic at the LC50 value of 1.0009 µg/ml (Fasola et al., 2011). All the results involving organic root extracts correlate with each other showing that the root extracts are toxic. The non-toxicity profile of the aqueous extracts (roots and leaf) correlates with the traditional preparation of using water to prepare a decoction (De Wet et al., 2016).

The root of *P. obliquum* was also assessed for toxicity potential in the current study. The aqueous extract did not show any signs of toxicity, the organic extract after 24 and 48 hrs of exposure, showed that all the brine shrimp were killed (Table 2.3). The aqueous and organic (methanol) extracts of the leaves have also been tested for toxicity in the BSLA and the results did not show any signs of toxicity (Cock and Van Vuuren, 2014). Even though the same assay and concentration was used in both studies, the difference in results of toxicity potential in this case could be due to the fact that different plant parts were used. Apart from the study done by Cock and Van Vuuren (2014), nothing more could be found in literature regarding the toxicity of *P. obliquum*.

When the organic and aqueous extracts of the leaves of *R. communis* were tested for toxicity in the BSLA, the aqueous extract was found to be non-toxic towards the brine shrimp assay. The organic extract killed all the brine shrimp within the first 24

hrs of exposure (Table 2.3). The organic extract results validate a previous study done by (McGaw et al., 2007), where the leaves, which were extracted using hexane and methanol, at the concentrations of 0.1, 1.2 and 5 mg/ml were found to be toxic towards BSLA. An acute toxicity study was done on the aqueous extract of the leaves of *R. communis* by Sadashiv (2011). The extract was administered orally to Swiss mice with a dosage of 2, 4, 6, 8 and 10 g/kg. The results showed that there was no mortality even at the highest dose level of 10 g/kg (Sadashiv, 2011). Another acute toxicity study was done on the ricin (an alkaloid found in the seed) on albino mice. The results indicated damage to the kidney and liver and it was concluded that the ricin of the plant has hepatotoxicity and nephrotoxicity activities (Kumar et al., 2003). Another acute toxicity study done on the seed also confirms that the seed is toxic. Petroleum ether extracts of the seed were given to birds at the concentrations of 4, 9, 14, 19 and 24 g/kg and the results showed that after a few minutes of administration, dizziness was observed, which was followed by diarrhoea. After 48 hrs, mortality was experienced (Ukachukwu et al., 2011). The seeds are extremely poisonous but the oil is not toxic if used in moderate dosage (Van Wyk et al., 2014). *Ricinus communis* has been reported to be used to treat various conditions. The oil from the seed has been reported to be used as an agent for purging the bowel (Rana et al., 2012), has anti-oxidant activity, reduces fertility and has wound healing properties (Jena and Gupta, 2012). The plant has been reported to possess antioxidant activity, anti-inflammatory, antidiabetic, as a central analgesic, anti-tumour, larvicidal as well as anti-nociceptive activity (Rana et al., 2012; Jena and Gupta 2012). The toxicity of this plant is due to the presence of lectin and piperidine alkaloids (Van Wyk et al., 2014). The leaves of *R. communis* have also been reported to be toxic due to the presence of the alkaloid ricinine (Burgess et al., 1988). However, the root of this plant has been reported to be non-toxic. According to the acute and sub-chronic toxicity study done by Ilavarasan et al. (2011) where methanol and aqueous extracts of the roots were orally administered to the Wistar albino rats, it did not show any symptoms of toxicity or mortality at the dosages of 200 mg/kg (sub-acute toxicity) and 1000 mg/kg (acute toxicity). Regardless of the toxicity profile of this plant, few deaths have been reported in association with this plant most likely due to slow absorption of the toxin or instability in the digestive tract (Bruneton, 1999).

The aqueous and organic stem extracts of *S. sanguinea* in the BSLA demonstrated no toxicity with the mortality rate of 11% and 1% for the aqueous and organic extracts respectively after 48 hrs of continuous exposure (Table 2.3). Another cell viability assay (human kidney epithelial cell line) indicated that both the organic (dichloromethane:methanol) and aqueous extracts of this plant are non-toxic at 100 µg/ml (Naidoo et al., 2013). Apart from the study done by Naidoo et al. (2013), no further results could be found in the literature on the toxicity of this plant. This plant was one of the plants that were documented for the first time in literature to be used for the treatment of hypertension (De Wet et al., 2016).

In the current study, both the organic and aqueous extracts *of S. viminale* were non-toxic in the BSLA. However*, S. viminale* has been reported to be toxic in animals due to the presence of a steroid glycoside. The poisoned animals display symptoms similar of hypersensitivity, seizures and paralysis (Van Wyk et al., 2014). The leaves of this plant has also been reported to be poisonous to fish, however, people do not suffer any side effects when consuming the poisoned fish (Neuwinger, 2004). Except for the above anecdotal report (evidence based on hearsay rather than scientific data), no concrete evidence could be found in literature regarding the toxicity of this plant on humans.

The aqueous and organic extract of the leaves of *S. serratuloides* in the BSLA demonstrated no toxicity at 2 mg/ml after 24 and 48 hrs of continuous exposure (Table 2.3). This correlates with a previous cell viability assay of both organic (dichloromethane:methanol) and aqueous extracts which indicated that this plant is non-toxic at 100 µg/ml against the human kidney epithelial cell line (Naidoo et al., 2013). The non-toxic profile of this plant correlates with its various medicinal uses in northern Maputaland, such as the treatment for respiratory problems (York et al., 2011), sexual transmitted infections (De Wet et al., 2012) skin diseases (De Wet et al., 2013) women problems (De Wet and Ngubane, 2014) and hypertension (De Wet et al., 2016).

The aqueous extract of *S. madagascariensis* seeds was non-toxic and the organic extract was toxic with a mortality of 68% after 48 hrs of exposure (Table 2.3). The seeds of *Strychnos* species are known to be extremely bitter and toxic due to the

presence of strychnine (Van Wyk et al., 2014). The stem of *S. madagascariensis* has been reported to be used as a fish poisoning ingredient (Neuwinger, 2004). Although *S. madagascariensis* has been reported to be poisonous to fish, no side effect was reported on humans consuming the poisoned fish. No further information could be found in the literature regarding the toxicity of *S. madagascariensis* on humans.

The aqueous extract of *T. riparia* leaves did not show any sign of toxicity while the organic extract had a mortality rate of 81% after the 24 hrs of exposure. After 48 hrs all the brine shrimp were killed (Table 2.3). This could be that the water did not extract non-polar compounds whereas the organic solvent extracted both the polar and non-polar compounds. A compound from the essential oil from the leaves of *T. riparia* known as 9β, 13β-epoxy-7-abietene has shown high cytotoxicity when evaluated using the MTT test on the MDA-MB-435 (human breast carcinoma), HCT-8 (human colon), and SF-295 (human nervous system) cell lines (Gazim et al., 2014). Non-toxicity profile of the aqueous extract correlates with the traditional method of preparation, as the plant decoction was prepared with water (De Wet et al., 2016).

When the organic and aqueous leaf extracts of *T. emetica* were tested in the BSLA for toxicity, they showed a low toxicity profile with the mortality rate of 40% and 4% after 48 hrs of exposure for both organic and aqueous extracts respectively (Table 2.3). These results correspond with a study done where both aqueous and ethyl ether extracts of the root did not show toxicity (LC50 > 1000 g/ml) in the BSLA (Germano et al., 2005). The organic extracts (acetone, ethanol, dichloromethane, chloroform and petroleum ether) of the stem bark of this plant was also non-toxic against the monkey kidney cell line with the LD50 of 50 µg/ml (Prozesky et al., 2001). However, another study showed that the leaf extracts (dichloromethane, methanol and water) of *T. emetica* had low cytotoxicity when evaluated using the MTT test (3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) on macrophage like murine cells (J774) and human normal fibroblasts (Wi38) at the concentration of 20 mg/ml (Bero et al., 2009). The stem bark was reported to be poisonous to fish even though people who consume the poisoned fish do not experience any side effect (Neuwinger, 2004). The non-toxicity of this plant correlate with its traditional

uses as it is one of the plants which were reported to be used frequently for the treatment of different aliments like diarrhoea (De Wet et al., 2010), respiratory infections (York et al., 2011), sexually transmitted infections (De Wet et al., 2012) and hypertension (De Wet et al., 2016).

The aqueous and organic leaf extracts of *V. infausta* when tested in the BSLA demonstrated no toxicity at 2 mg/ml after 24 and 48 hrs of continuous exposure, respectively (Table 2.3). *Vangueria infausta* methanol leaf extract was reported to be moderately safe but should be used with caution according to the study done by Munodawafa et al. (2012) where the LC50 was 338 ± 23.4 µg/ml in the BSLA. The methanol root extract of this plant was found to be non-toxic in the MTT assay (Mthethwa et al., 2014). The results which were found in all the studies previously undertaken correlate with the traditional use of this plant to treat various conditions such as diarrhoea (De Wet et al., 2010), cough (McGaw et al., 2008), malaria, pneumonia (Venter and Venter, 1996) and hypertension (De Wet et al., 2016).

Table 2.3 Toxicity results of the individual plants expressed in percentage death of the brine shrimps.

Plant names and parts used	% (percentage) mortality			
	Organic		Aqueous	
	24 hrs	48 hrs	24 hrs	48 hrs
Acanthospermum hispidium (roots)	**67***	**92**	7	10
Albertisia delagoensis (roots)	**91**	**100**	22	35
Aloe marlothii (leaves)	2	10	6	6
Cannabis sativa (leaves)	**96**	**100**	4	6
Carpobrotus dimidiatus (leaves)	14	**65**	9	11
Catharanthus roseus (roots)	14	**90**	6	**92**
Citrullus lanatus (fruit)	7	16	5	6
Citrus limon (peel)	**72**	**100**	**60**	**63**
Cladostemon kirkii (roots)	0	30	4	4
Hyphaene coriacea (roots)	**54**	**67**	4	44
Hypoxis hemerocallidea (corm)	29	**54**	4	5
Lippia javanica (leaves)	46	**52**	6	8

Plant names and parts used	% (percentage) mortality			
	Organic		Aqueous	
	24 hrs	48 hrs	24 hrs	48 hrs
Momordica balsamina (leaves)	34	**50**	10	39
Musa acuminata (flower bracts)	0	4	5	6
Ozoroa engleri (roots)	28	**58**	1	7
Psidium guajava (leaves)	4	7	6	11
Psidium guajava (roots)	13	**59**	4	20
Ptaeroxylon obliquum (roots)	**100**	**100**	4	5
Pyrenacantha kaurabassana (roots)	**90**	**100**	4	4
Ricinus communis (leaves)	**100**	**100**	2	3
Sarcophyte sanguinea (stem)	0	1	2	11
Sarcostemma viminale (whole plant)	2	16	7	8
Senecio serratuloides (whole plant)	1	36	5	6
Strychnos madagascariensis (seed)	7	**68**	3	3
Tetradenia riparia (leaves)	**82**	**100**	3	4
Trichilia emetica (leaves)	38	40	2	4
Vangueria infausta (leaves)	5	26	1	5
DMSO (negative control)	2	2	1	1
Sea water (negative control)	0	0	0	0
Potassium dichromate (positive control)	**100**	**100**	**100**	**100**

*Results in bold are toxic.

Twenty one out of the 26 plants have previously been assessed for toxicity using various assays including *in vivo* assays. The five plants that have not been tested previously for any toxicity *are C. lanatus, C. kirkii, H. coriacea, P. kaurabassana* and *S. madagascariensis*. Even though most of the plants have been subjected to toxicity tests, most of the studies focused only on testing the organic extracts (Van Zyl and Viljoen, 2002; De Wet et al., 2007; Sumathy et al., 2011; Kevin et al., 2012; Suad et al., 2012; Sanon et al., 2013) instead of the aqueous extracts. The plant parts previously tested differ mostly from the current plant parts tested. The summary of all the plants that has been tested for toxicity is documented in Table 2.4 along with the types of assays used, the solvents used for preparing extracts, as well as the concentrations. Most of the organic extracts tested were shown to be toxic towards

BSLA. However, the aqueous extracts only *C. roseus* and *C. limon* were toxic. These results demonstrate that the primary method of water extraction by the lay people is the safer option. Traditional methods of preparation are often aimed at eliminating toxins of the plants (Van Wyk et al., 2015). Some organic extracts which were found to be highly toxic were *A. hispidium* and *A. delagoensis* while the water extracts were non-toxic. The plants with low toxicity in both organic and aqueous extracts were found to be: *A. marlothii, C. lanatus, C. kirkii, M*. *acuminata, P. guajava* (leaves) *S. sanguinea, S. viminale, S. serratuloides, T. emetica* as well as *V*. *infausta*. Although some of the potential toxicity of these medicinal plants has been reported in previous studies, it is worth noting that the reports are based on different assays, different solvents used for extraction and different plant part tested (Table 2.4). The results obtained in the current study highlight the importance of subjecting all newly documented medicinal plants for toxicity testing, as 26% of the plants extracts documented for the first time to treat hypertension were toxic at the concentration tested.

Table 2.4 Summary of toxicity studies done previously and correlation with BSLA.

Plant species	Type of extract	Type of assay	Concentration	Correlation with current study	Reference
A. hispidium	ethanol	BSLA.	1, 10, 100,1000 ppm	Yes	Suad, 2012
	petroleum ether	Monocytes, normal melanocytes and malignant melanoma cell lines.	5.02 µg/ml	Yes	Sanon et al., 2003
	dichloromethane :methanol and water	MTT assay.	20 mg/ml 6.25-200 µg/ml	Yes	Bero et al., 2009
	water	Acute and sub-acute assays.	1000, 2000 mg/kg	Yes	Ganfon et al., 2012
A. delagoensis	methanol	Graham cell line.	200 µg/ml	No-different assay	De Wet et al., 2007
A .marlothii	leaf gel	Human hepatocellular human neuroblastoma cells and human adenocarcinoma epithelial cells	0.01 to 1000 mg/ml	Yes	Du Plessis and Hamman, 2013

Plant species	Type of extract	Type of assay	Concentration	Correlation with current study	Reference
	methanol	Graham cell line and GCT cell line	50 µg/ml	Yes	Van Zyl and Viljoen, 2002
	dichloromethane :methanol and water	Human kidney epithelial cell line	100 µg/ml	Yes	Naidoo et al., 2013
C. sativa	ethanol	Acute toxicity (rats)	225,180 mg/kg	Yes	Thompson et al., 1973
	petroleum ether	Acute toxicity (rats)	0.01, 0.1,1 ml/kg	No-different assay	Badwi et al., 2012
C. dimidiatus	water and methanol	BSLA	1, 10, 100, 1000 µg/ml	Yes	Hurinantha et al., 2009
C. roseus	methanol	Human lymphoma U-937 GTB	10, 100, 1000 µg/ml, 1 mg/ml	Yes	El-Seedi et al., 2013
	dichloromethane :methanol and water	Chang liver and 3T3-L1 cell lines	12.5 µg/ml	Yes	Van de Venter et al., 2008
	methanol	Sub-acute (rats)	0.1, 0.5, 1 g/kg	Yes	Kevin et al., 2012
C. limon	ethanol	Genotoxicity (root meristem cells of *Allium cepa*)	25, 50, 75 g/l	Yes	Ali and Celik, 2007
	essential oils	Human colorectal carcinoma cell line LIM1863	0.5-48 µg/ml	Yes	Jomaa et al., 2012
H. hemerocallidea	hexane, dichloromethane ethyl acetate, methanol and water	BSLA	10, 100, 1000 µg/ml	Yes	Jooste et al., 2012
	dichloromethane :methanol and water	Human kidney epithelia cell line	100 µg/ml	Yes	Naidoo et al., 2013
	water	Acute and sub-chronic toxicity (Wistar rats)	3 ml/kg	No-different assay	Musabayane et al., 2005
	water	Human cell lines (Du-145, MDA-MB-231, MCF-7 and MCF-12A	50 µg/ml	Yes	Steenkamp and Gouws, 2006
L. javanica	methanol	BSLA	50, 100, 200, 400, 500, 1000, 2000 µg/ml	Yes	Ayuko et al., 2009

Plant species	Type of extract	Type of assay	Concentration	Correlation with current study	Reference
	methanol	BSLA	2 mg/ml	Yes	Cock and Van Vuuren, 2014
	dichloromethane :methanol and water	BSLA	2 mg/ml	Yes	Hubsch et al., 2014
		Acute toxicity (mice)	12 g/kg	No-different assay	Madzimure et al., 2011
M. balsamina	dichloromethane :methanol and water	C2C12 muscle cells and hepatocytes cell lines	12.5 µg/ml	Yes	Van de Venter et al., 2008
	water	Acute toxicity (rats)	0.56 mg/100g	Yes	Geidam et al., 2007
	water	Acute toxicity (rats)	16 g/kg	Yes	Karumi et al., 2006
M. acuminata	methanol	BSLA	0.195, 100 mg/ml	Yes	Sumathy et al., 2011
	dichloromethane :methanol and water	Human kidney epithelia cell line	100 µg/ml	Yes	Naidoo et al., 2013
O. engleri	dichloromethane	Vervet monkey kidney cell lines	35 µg/ml	Yes	Prozesky et al., 2001
	dichloromethane :methanol and water	Human kidney epithelia cell line	100 µg/ml	Yes	Naidoo et al., 2013
P. guajava	methanol	BSLA	10-5000 µg/ml	Yes	Ajaiyeoba et al., 2006
	Methanol and water	3T3 (mouse embryonic fibroblast cell) and 4T1 (mouse breast cancer cell)	100 µg/ml	Yes	Ling et al., 2010
	water	Acute toxicity (rats)	10-50 mg/100g	Yes	Etuk and Francis, 2003
	dichloromethane :methanol and water	Chang liver and 3T3-L1 cell lines	12.5 µg/ml	Yes	Van de Venter et al., 2008
	methanol	HeLa cell lines	10 µg/ml	No-different assay	Joseph and Priya, 2010
	methanol	Acute and sub-chronic toxicity (rats)	100, 150, 300, 500, 600,1000, 1200, 2000, 4000, 5000 mg/kg	Yes	Onyekwe et al., 2011

Plant species	Type of extract	Type of assay	Concentration	Correlation with current study	Reference
	hexane (essential oils)	BSLA	10, 100, 1000 µg/ml	Yes	Fasola et al., 2011
	ethanol	MTT (human gingival fibroblast)	50 µg/ml	Yes	Fernandes et al., 2010
P. obliquum	methanol and water	BSLA	2 mg/ml	Yes	Cock and Van Vuuren, 2014
R. communis	methanol and hexane	BSLA	0.1, 1.2, 5 mg/ml	Yes	McGaw et al., 2007
	water	Acute toxicity (swiss mice)	2, 4 ,6 ,8, 10 g/kg	Yes	Sadashiv, 2011
	petroleum ether	Acute toxicity (birds)	4, 9, 14, 19, 24 g/kg	Yes	Ukachukwa et al., 2011
	methanol and water	Acute and sub-chronic toxicity (wistar albino rats)	200, 1000 mg/kg	No-different assay	Ilavarasan et al., 2011
S. sanguinea	dichloromethane :methanol and water	Human kidney epithelia cell line	100 µg/ml	Yes	Naidoo et al., 2013
S. serratuloides	dichloromethane :methanol and water	Human kidney epithelia cell line	100 µg/ml	Yes	Naidoo et al., 2013
T. emetica	ethyl ether and water	BSLA	1000 g/ml	Yes	Germano et al., 2005
	acetone, ethanol, dichloromethane chloroform and petroleum ether	Vervet monkey kidney cell line	50 µg/ml	Yes	Prozesky et al., 2001
	dichloromethane methanol and water	MTT(macrophage like murine cells and human normal fibroblast	20 mg/ml	No-different assay	Bero et al., 2009
V. infausta	methanol	BSLA	23.4 µg/ml	Yes	Munodawafa et al., 2012
	methanol	MTT	5 mg/ml	Yes	Mthethwa et al., 2014

2.3.2 Further analysis of plant extracts which were toxic at 2 mg/ml for dose response

When the aqueous extracts of *C. roseus* and *C. limon* were tested at the concentration of 2 mg/ml, the results showed a mortality percent of 92% and 63% respectively. Since the aqueous extracts are the main form in which the traditional

medicine were prepared, the two extracts were further analysed at different concentrations to determine if their toxicity profile is dosage dependant. The organic extracts of *C. roseus* and *C. limon* were included for comparison. The results of different concentrations tested are depicted at Figure 2.1. Mortality percent of 50% and above was considered to be toxic. The negative control used (sea salt water) had 1% mortality rate after 48 hours of exposure (not included in the graph). Positive control (potassium dichromate) killed 93% of the brine shrimp after 48 hrs of exposure. The positive control does not display in the graph as it was tested only at one concentration (2 µg/ml). When the plant extracts (organic and aqueous) were tested at the concentration of 1 mg/ml, all the extracts showed to be toxic. All the other concentrations tested show a non-toxic profile, thus the toxicity of *C. limon* and *C. roseus* is dosage dependant. These two plants are safe to use at the concentration of 0.5 mg/ml and lower.

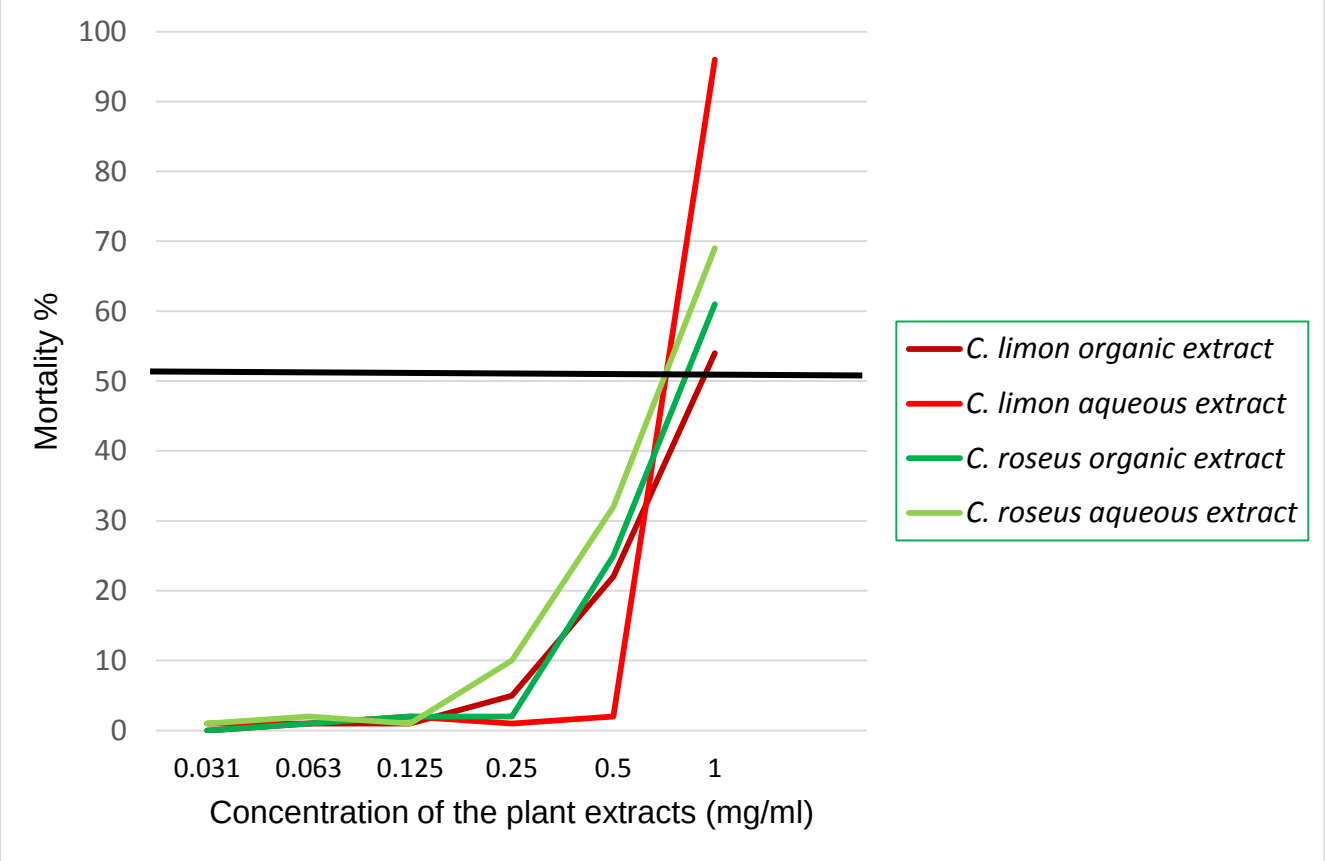

Figure 2.1 Dose response curves of the medicinal plants which were further analysed at lower concentrations.

2.3.3 Toxicity analysis of plant combinations

A total of 38 plant combinations made up of 16 plant extracts (organic and aqueous) were analysed for toxicity using the BSLA. The plant combinations were done in two concentrations (2 and 4 mg/ml) for dose comparison. Results with positive (potassium dichromate) and negative controls (sea salt water and DMSO) are presented in Figures 2.2 (organic) and 2.3 (aqueous). The line represent the cut off of 50%, thus everything above the line was considered to be toxic. *Aloe marlothii* was the most used species in combination (eight combinations) followed by *M. balsamina* (six combinations), *H. hemerocallidea* (five combinations) and *M. acuminata, C. roseus* and *S. serratuloides* in four combinations each. *C. sativa* was involved in three different combinations and the remaining plant species were involved in one plant combination (De Wet et al., 2016). The overall toxicity profile indicated that the organic extracts (13 plant combinations) had a mortality rate of 68% at 4 mg/ml and 47% at 2 mg/ml, while the aqueous extracts had a mortality rate of 31% (six plant combinations) at 4 mg/ml and 16% (three plant combinations) at 2 mg/ml. Combinations which were tested at the concentration of 4 mg/ml were more toxic than when tested at the concentration of 2 mg/ml, thus toxicity of combinations was dosage dependant. For the organic plant combination extracts, the highest noted toxicity was shown with the combinations between *A. delagoensis* and *S. serratuloides*, *A. marlothii* and *C. sativa*, *M. balsamina and C. roseus*, *M. acuminata* and *C. limon* with a mortality rate of 100% at the concentration of 4 mg/ml and 64%, 62%, 67% and 57 at the concentration of 2 mg/ml respectively. The aqueous extracts combinations did not kill all the brine shrimp. The highest noted toxicity was observed in the combinations between *M. balsamina* and *C. roseus* with a mortality of 99% at 4 mg/ml and 73% at 2 mg/ml, *A. marlothii* and *C. roseus* with the mortality of 98% at 4 mg/ml and 76% at 2 mg/ml as well as the combination between *C. roseus* and *M. acuminata* with the mortality rate of 89% at 4 mg/ml and 66% at 2 mg/ml. It is worth noting that all the aqueous extracts combinations with a higher toxicity involve *C. roseus.*

Some combinations showed a clear difference in the toxicity level between the two concentrations tested. For organic extracts, combinations which are worth noting with reduced toxicity at 2 mg/ml is a combination between *A*. *marlothii* and *C. roseus* (Figure 2.2). When this combination was tested at 4 mg/ml it was toxic with the

mortality rate of 93%. However, when tested at the concentration of 2 mg/ml, the combination was non-toxic with a mortality rate of 45%. Another combination which is worth noting is the combination between *A. marlothii, H. hemerocallidea* and *M. balsamina*. When tested at the concentration of 4 mg/ml, the combination was toxic with a mortality rate of 56% and at the concentration of 2 mg/ml it was reduced and non-toxic (33%). Another combination was between *C. roseus* and *M. acuminata* which was toxic (64%) at 4 mg/ml and non-toxic (44%) at 2 mg/ml as well as the combination between *A. marlothii, T. emetica* and *H. coriacea*, which was toxic (72%) at the concentration of 4 mg/ml and non-toxic (46%) at the concentration of 2 mg/ml.

For aqueous extracts, the combination which is worth noting is the combination between *A. delagoensis* and *S. serratuloides* (Figure 2.3). When the combination was tested at the concentration of 4 mg/ml it was toxic with the mortality rate of 57%. However, when tested at the concentration of 2 mg/ml it was non-toxic with the mortality rate of 45%. Another combination which resulted in a decreased toxicity at 2 mg/ml is the combination between *C. roseus* and *H. hemerocallidea*. At 4 mg/ml, the combination had 66% mortality whereas at 2 mg/ml, the combination showed non-toxicity profile with the 44% mortality.

However, some combinations resulted in reduced toxicity when plant species are combined, rather than used singularly. For the organic extracts, such combinations include a combination between *C. roseus* and *H. hemerocallidea*. Singularly both extracts were toxic, *C. roseus* was toxic with the mortality of 90% and *H. hemerocallidea* had mortality rate of 56%. However these two plants seemed to be working together (synergistic) since in combination showed a mortality rate was less with 38% at 4 mg/ml and 17% at 2 mg/ml after 48 hours of continuous exposure. This is a good example of using plants in combination. It is also worth noting that this is the only combination which involves *C. roseus* that was shown be non-toxic. Another combination which is noteworthy with a reduced toxicity for the combination of *P. guajava* and *C*. *dimidiatus* (organic extracts). Singularly, *P. guajava* (leaves) was non-toxic where *C. dimidiatus* was toxic with the mortality rate of 65% after 48 hours of continuous exposure. However, in combination the mortality rate was reduced to 4% at 4 mg/ml and 3% at 2 mg/ml. The results showed that *P. guajava*

extract was able to neutralize the toxicity effects produced by *C. dimidiatus*. For aqueous extracts, a combinations that resulted in reduced toxicity is the combination between *C*. *roseus* and *H. hemerocallidea*. Singularly, *C. roseus* was toxic with the mortality rate of 92% and *H. hemerocallidea* was non-toxic with the mortality rate of 5%. Their combination resulted in decreased toxicity of 66% at 4 mg/ml and 44% at 2 mg/ml. Another combination which resulted in decreased toxicity includes the combination between *C. limon* and *M. acuminata*. The combinations between *H. hemerocallidea* and *A. marlothii; P. guajava and C. dimidiatus* as well as *H. hemerocallidea* and *S. serratuloides* resulted in reduced toxicity in both extracts (organic and aqueous).

However, some of the combinations tested in the current study resulted in increased toxicity compared to when they are used singularly. For the organic extracts the combinations include a combination between *A. marlothii* and *M. balsamina* (Figure 2.2). *Aloe marlothii* was not toxic with the mortality rate of 10% where else *M. balsamina* was toxic with the mortality rate of 50%. However, their combination resulted in higher mortality rate of 78% at 4 mg/ml and 57% at 2 mg/ml compared to when they are used singularly. Another combination which is worth noting is the combination between *L. javanica* and *M. balsamina*. Singularly *M. balsamina* and *L. javanica* were toxic with mortality rate of 50% and 52% respectively. Although both these plants were toxic, their combination resulted in an increase of toxicity of 90% at 4 mg/ml and 66% at 2 mg/ml. Other combinations which resulted in increased toxicity include the combinations between *M. balsamina* and *M. acuminata*; *C. roseus and A. marlothii; M. acuminata* and *C. roseus* as well as the combination between *M. acuminata and C. limon.* For the aqueous extracts, the combination that resulted in an increased toxicity is the combination between *A. delagoensis* and *S. serratuloides*. Singularly, both plant extracts were non-toxic with the mortality rate of 35% and 6% for *A. delagoensis* and *S. serratuloides* respectively. However, their combination resulted in toxicity of 57% at 4 mg/ml and 45% at 2 mg/ml. These two plants were working against (antagonistic) each other and resulted in toxicity. Other combinations that resulted in increased toxicity are combinations between *A*. *marlothii* and *C. roseus; H. hemerocallidea and C. roseus; T. emetica, A. marlothii* and *H. coriacea* as well as the combination between *C. roseus* and *M. acuminata.*

Apart from the combination between *H. hemerocallidea* and *S. serratuloides* which was documented by De Wet et al. (2012) to be used for the treatment of internal and external sores caused by STI (sexual transmitted infections) as well as the combination between *A. marlothii and H. hemerocallidea* which was investigated for toxicity by Naidoo et al. (2013), no previous studies could be found on the toxicity of plant combinations which were investigated in the current study. Therefore this study is the first to document the toxicity of these plant combinations. However, *A. marlothii, H. hemerocallidea* and *S. serratuloides* has been reported to be used in combinations with other plants for the treatment of STI and respiratory infections. The combinations involve *Musa acuminata*, *Carica papaya* L., *Ozoroa engleri, Euphorbia hyperifolia* L., *Tabernaemontana elegans* Stapf, *Ranunculus multifidus* Forssk, *Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) kokwaro, *Senecio deltoideus* Less. as well as *Syzygium cordatum* Hochst. (York et al., 2011; Wet et al., 2011).

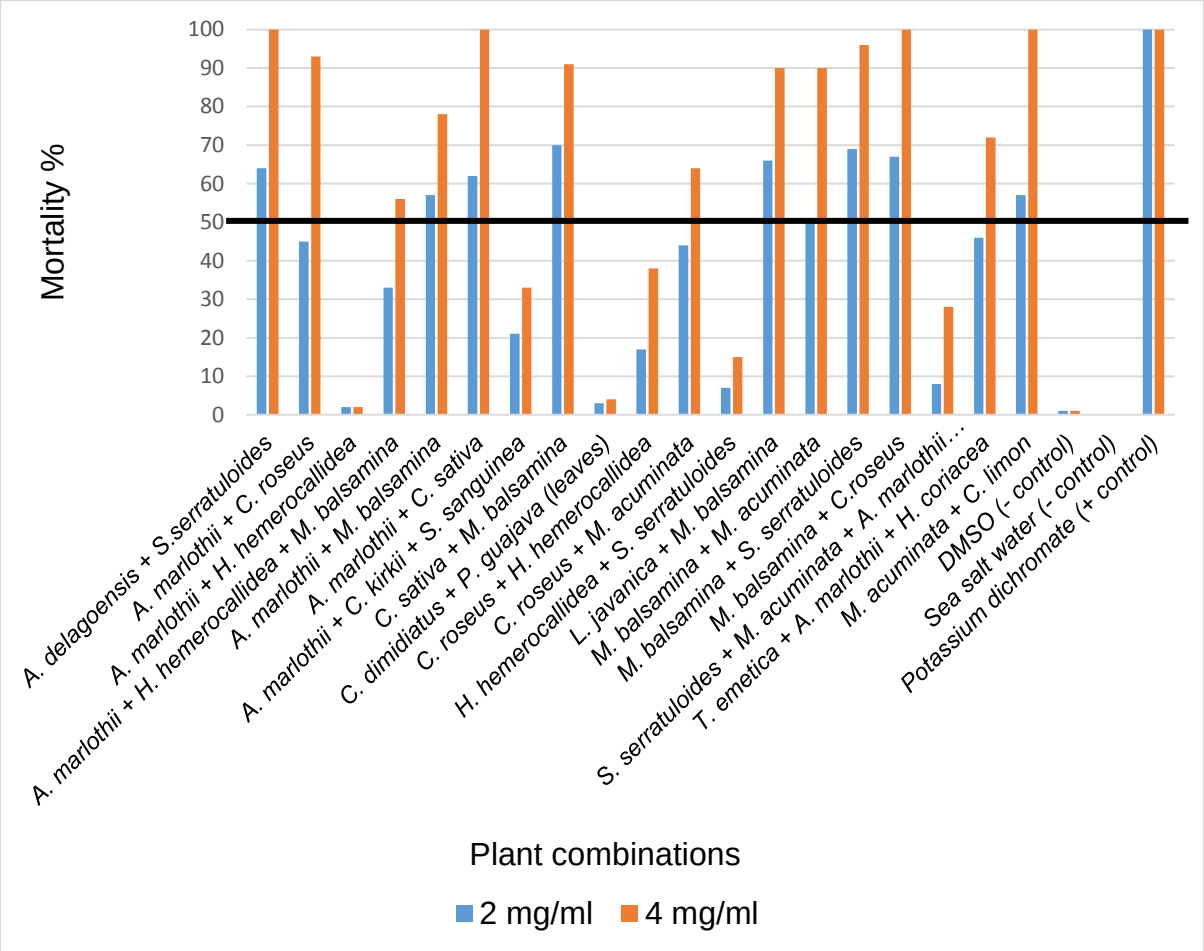

Figure 2.2 A comparison of results of the organic plant combinations extracts tested in BSLA at the concentration of 2 and 4 mg/ml.

The combination between *A. marlothii* and *H. hemerocallidea* were tested for toxicity against a human kidney epithelial cell line and shown to have no toxicity (Naidoo et al., 2013). This result correlates with the current study where the combination between *A. marlothii* and *H. hemerocallidea* was found to be non-toxic (both the organic and aqueous extracts). It should also be noted that the combination between *A. marlothii* and *H. hemerocallidea* was the most mentioned combination to be used for the treatment of hypertension (De Wet et al., 2016) and in all studies (organic and aqueous) demonstrated a synergistic profile, correlating with the safety in traditional use. In general, the plant combinations tested in the current study showed to be more toxic than when they are used singularly.

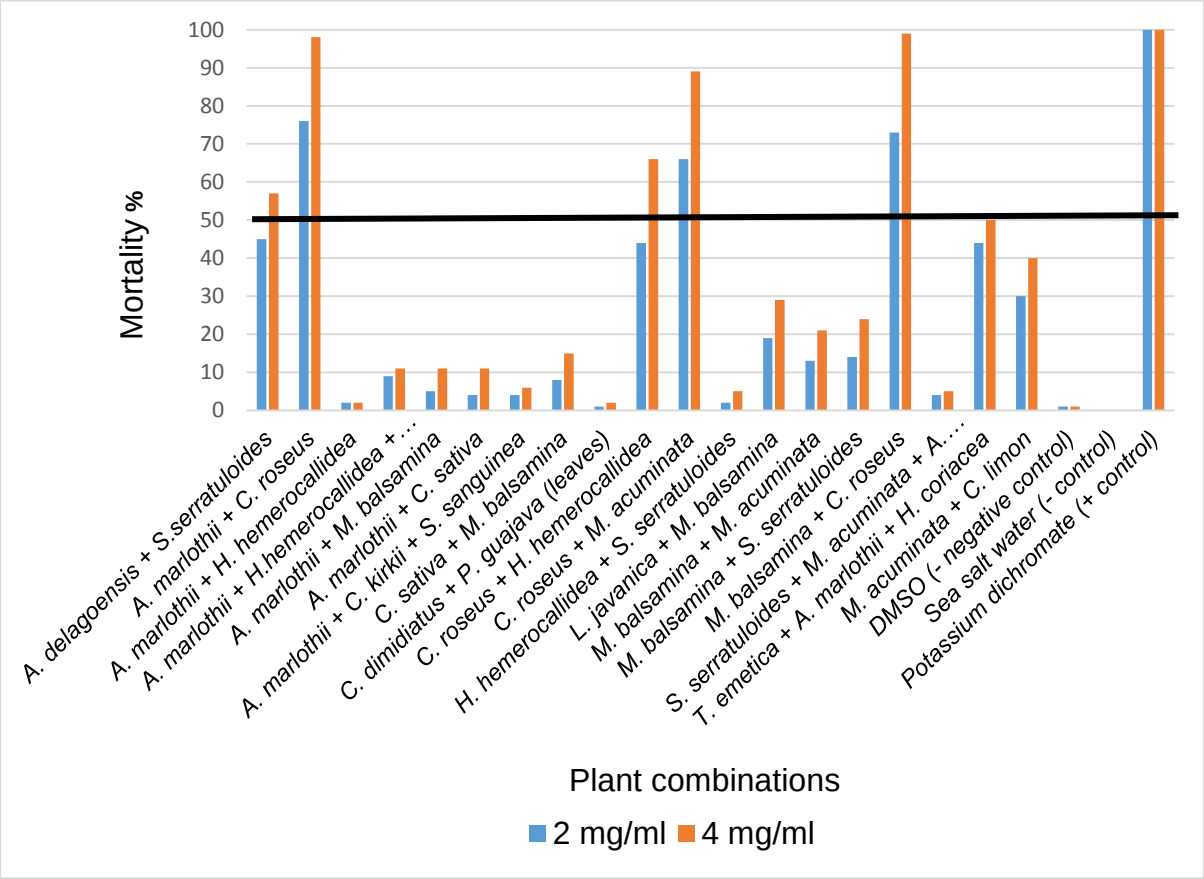

Figure 2.3 A comparison of the results of aqueous plant combinations extracts tested in BSLA at the concentration of 2 and 4 mg/ml.

Toxicity of plant combinations (both concentrations) were further analysed for their ΣFIC value and the results are depicted on Table 2.5 with their respective interpretations. When tested at the concentration of 2 mg/ml, for organic extracts,

53% (10 plant combinations) were shown to have non-interactive interactions whilst 21% (four plant combinations) had antagonistic interactions and 5% (one plant combination) had additive interactions while 21% (four plant combinations) showed synergistic interactions. For the aqueous extracts, 26% (five plant combinations) had non-interactive interactions, 32% (six plant combinations) had antagonistic interaction, 21% (four plant combinations) had additive interaction and 21% (four plant combinations) had synergetic interaction. When the plant combinations were tested at the concentration of 4 mg/ml, for organic extracts, 42% (eight plant combinations) had non-interactive interaction, 37% (six plant combinations) had antagonistic interactions, 5% (one plant combination) had an additive interactions and 16% (four plant combinations) had synergistic interactions. For the aqueous extracts, 53% (ten plant combinations) had non-interactive interaction, 26% (five plant combinations) showed antagonistic interactions, 11% (two plant combinations) showed additive interactions while another 11% (two plant combinations) had synergistic interactions. For the organic extracts the lowest ΣFIC values were 0.06 (2 mg/ml) and 0.12 (4 mg/ml) which were obtained when *H. hemerocallidea* was combined with *A. marlothii*. The highest ΣFIC values were 7.42 (2 mg/ml) and 13 (4 mg/ml) which were obtained when *C. limon* was combined with *M. acuminata*. For the aqueous extracts, the lowest ΣFIC values were 0.09 (2 mg/ml) and 0.18 (4 mg/ml) which were obtained when *P. guajava* (leaves) was combined with *C. dimidiatus*. The highest ΣFIC values were 6.71 (2 mg/ml) and 8.7 (4 mg/ml) which were obtained when *C. roseus* was combined with *A. marlothii*. Most of the plant combinations (organic and aqueous extracts) had different interactions such as the combination between *A. delagoensis* and *S. serratuloides* where the organic extract had non-interactive interaction while the aqueous extract had antagonistic interaction.

Even though most of the plant combinations (aqueous and organic extracts) depicted different interactions, some of them had the same interactions such as the combination between *H. hemerocallidea* and *A. marlothii* where both the organic and aqueous extracts had synergistic interactions. Some of the combinations when tested at different concentrations (2 and 4 mg/ml) had different interactions. For example, when a combination between *M. balsamina* and *A. marlothii* (organic) was

tested at the concentration of 2 mg/ml it had a non-interactive interaction whilst at the concentration of 4 mg/ml the interaction was antagonistic.

The percentage (up to 37% at 4 mg/ml) of the combinations which showed antagonistic interactions raised a concern for the use of these plant combinations. These combinations include the aqueous combinations between *C*. *roseus* and *A. marlothii, H. hemerocallidea* and *C. roseus, M. acuminata* and *C. roseus, T. emetic, A. marlothii* and *H. coriacea* as well as the combination between *A. delagoensis* and *S. serratuloides*. Caution must be taken when using these combinations. On the other hand, some of the combinations were considered somewhat less toxic (synergistic) when combined i.e. plants working together in a synergistic manner in order to reduce toxicity. These are the combinations between *P. guajava* and *C. dimidiatus* (organic and aqueous extracts), *H. hemerocallidea* and *A. marlothii* (organic and aqueous extracts) as well as *H. hemerocallidea* and *S. serratuloides*. One combination resulted in reduced toxicity (additive effect). This combination is between *S*. *serratuloides, M. acuminate, A. marlothii* and *H. hemerocallidea*. The present study shows that when including toxic plants such as *C. roseus* and *C. sativa* in any combination, one must be very careful since it always remains toxic regardless of the fact that it could be combined with the plant which is non-toxic. These two plants go against the principle of plant combination to reduce toxicity. Another combination which is also worth noting is the aqueous extract combination between *A. delagoensis and S. serratuloides*. When *A. delagoensis* and *S. serratuloides* were tested singularly, they were both non-toxic towards BSLA with the mortality rate of 35% and 6% respectively. However, their combination resulted in an increased toxicity (antagonism).

Table 2.5 Plant combinations which were tested on BSLA along with their ΣFIC index and the respective interpretations.

Plant combinations	**Extracts**	**Mortality %**		**ΣFIC and interpretation**	
		2 mg/ml	**4 mg/ml**	**2 mg/ml**	**4 mg/ml**
A. delagoensis + *S. serratuloides*	Organic	64	100	1.21 (Non-interactive)	1.89 (Non-interactive)
	Aqueous	45	57	4.35 (Antagonistic)	5.56 (Antagonistic)

Plant combinations	Extracts	Mortality %		ΣFIC and interpretation	
		2 mg/ml	4 mg/ml	2 mg/ml	4 mg/ml
C. roseus + *A. marlothii*	Organic	45	93	2.50 (Non-interactive)	5.17 (Antagonistic)
	Aqueous	76	98	6.71 (Antagonistic)	8.70 (Antagonistic)
H. hemerocallidea + *A. marlothii*	Organic	2	2	0.06 (Synergistic)	0.12 (Synergistic)
	Aqueous	2	2	0.37 (Synergistic)	0.37 (Synergistic)
H. hemerocallidea + *M. balsamina* + *A. marlothii*	Organic	33	56	1.52 (Non-interactive)	2.60 (Non-interactive)
	Aqueous	9	11	1.18 (Non-interactive)	1.45 (Non-interactive)
M. balsamina + *A. marlothii*	Organic	57	78	3.24 (Non-interactive)	4.68 (Antagonistic)
	Aqueous	5	11	0.48 (Synergistic)	1.06 (Non-interactive)
C. sativa + *A. marlothii*	Organic	62	100	3.41 (Non-interactive)	5.50 (Antagonistic)
	Aqueous	4	11	0.66 (Additive)	1.84 (Non-interactive)
C. kirkii + *S. sanguinea* + *A. marlothii*	Organic	21	33	7.93 (Antagonistic)	12.47 (Antagonistic)
	Aqueous	4	6	0.67 (Additive)	1.01 (Non-interactive)
M. balsamina + *C. sativa*	Organic	70	91	1.05 (Non-interactive)	1.37 (Non-interactive)
	Aqueous	8	15	0.76 (Additive)	1.44 (Non-interactive)
H. hemerocallidea + *C. roseus*	Organic	5	9	0.25 (Synergistic)	0.56 (Additive)
	Aqueous	44	66	4.64 (Antagonistic)	6.96 (Antagonistic)
M. acuminata + *C. roseus*	Organic	63	79	5.74 (Antagonistic)	8.36 (Antagonistic)
	Aqueous	66	89	5.85 (Antagonistic)	7.98 (Antagonistic)
P. guajava + *C. dimidiatus*	Organic	3	4	0.23 (Synergistic)	0.32 (Synergistic)
	Aqueous	1	2	0.09 (Synergistic)	0.18 (Synergistic)
H. hemerocallidea + *S. serratuloides*	Organic	7	15	0.15 (Synergistic)	0.36 (Synergistic)
	Aqueous	2	5	0.37 (Synergistic)	0.92 (Additive)
L. javanica + *M. balsamina*	Organic	66	90	1.29 (Non-interactive)	1.77 (Non-interactive)
	Aqueous	19	29	1.4.3 (Non-interactive)	2.17 (Non-interactive)
M. balsamina + *M. acuminata*	Organic	51	90	6.89 (Antagonistic)	12.12 (Antagonistic)
	Aqueous	13	21	1.25 (Non-interactive)	2.02 (Non-interactive)

Plant combinations	Extracts	Mortality %		ΣFIC and interpretation	
		2 mg/ml	4 mg/ml	2 mg/ml	4 mg/ml
M. balsamina + *S. serratuloides*	Organic	69	96	1.65 (Non-interactive)	2.29 (Non-interactive)
	Aqueous	14	24	1.35 (Non-interactive)	2.31 (Non-interactive)
M balsamina + *C. roseus*	Organic	67	100	1.04 (Non-interactive)	1.56 (Non-interactive)
	Aqueous	73	99	1.30 (Non-interactive)	1.81 (Non-interactive)
M. acuminata + *C. limon*	Organic	57	100	7.42 (Antagonistic)	13.0 (Antagonistic)
	Aqueous	30	40	4.65 (Antagonistic)	3.62 (Non-interactive)
S. serratuloides + *M. acuminata* + *A. marlothii* + *H. hemerocallidea*	Organic	8	28	0.80 (Additive)	2.77 (Non-interactive)
	Aqueous	4	5	0.85 (Additive)	0.17 (Additive)
T. emetica + *A. marlothii* + *H. coriacea*	Organic	46	72	2.14 (Non-interactive)	3.36 (Non-interactive)
	Aqueous	44	50	7.36 (Antagonistic)	7.34 (Antagonistic)

2.4. Summary

- Five of the medicinal plants (*C. lanatus, C. kirkii, H. coriacea, P. kaurabassana* and *S. madagascariensis*) were investigated for the first time for any toxicity.
- For the toxicity of plant extracts tested singularly, the organic extracts of 17 of the plant species (63%) were toxic.
- For the aqueous extracts only two plant species [*C. roseus* (92%) and *C. limon* (63%] show toxicity.
- For organic extracts, 13 plant combinations tested at 4 mg/ml demonstrated toxicity and nine plant combination tested at 2 mg/ml showed toxicity.
- For the aqueous extracts six plant combinations tested at 4 mg/ml demonstrated toxicity and three plant combinations tested at 2 mg/ml showed toxicity.
- The FIC index for the plant combinations for the 2 mg/ml organic extracts resulted in 10 plant combinations with non-interactive interaction, four plant combinations with antagonistic interaction, one plant combination with additive interaction and four plant combinations with synergistic interactions.

- The FIC index at 4 mg/ml for the organic extracts resulted in eight plant combinations with non-interactive interaction, six plant combinations with antagonistic interaction, one plant combination with an additive interaction and four plant combinations with synergistic interactions.
- The FIC index for the plant combinations for the 2 mg/ml aqueous extracts resulted in five plant combinations with non-interactive interaction, six plant combinations with antagonistic interactions, four plant combinations with additive interactions and four plant combinations with synergistic interactions.
- FIC index at 4 mg/ml for the aqueous extracts resulted in 10 plant combinations with non-interactive interaction, five plant combinations with antagonistic interaction, two plant combinations with additive interaction and two plant combinations with synergistic interaction.

Chapter 3

Mutagenicity of medicinal plants (individual and combination) used for the treatment of hypertension in northern Maputaland

3.1 Introduction

Mutagens are chemicals or compounds that can cause chemical or physical modifications in the DNA arrangement, leading to mistaken duplication of a particular region of the genome. Mutations are linked to the development of most cancers and numerous degenerative ailments and genetic defects in offspring (.Cariño-Cortés et al, 2007). Mutagenicity is one of the toxicological effects that cause the highest anxiety in human health (Resende et al., 2012). Previous studies have demonstrated that some of the plants which are used for food and medicinal purpose have mutagenicity potential (Cardoso et al., 2006; Déciga-Campos et al., 2007; Mohd-Fuat and Kofi, 2007; Verschaeve and Van Staden, 2008). Several plants (*Catharanthus roseus, Cannabis sativa, Chaetacme aristata* E.Mey. ex Planch. *Plumbago auriculata* Lam. and *Ziziphus mucronata* Willd. subsp. *mucronata*) used in South African traditional remedies have been reported to have mutagenic potential (Busch et al., 1979; Elgorashi et al., 2003). Thus, the risk associated with the use of medicinal plants must not be ignored (Santo et al., 2013) and it is essential to continue screening traditional remedies for their mutagenicity potential, especially those that have not been previously tested for mutagenicity.

The Ames test is the most widely used method for testing the potential mutagenicity of medicinal plants (Bresolin and Ferrão Vargas, 1993; Mashele and Fuku, 2011; Liman et al., 2012; Resende et al., 2012; Santos et al., 2013; Eren and Ozata, 2014; Makhafola et al., 2014). The Ames *Salmonella*/microsome mutagenicity assay is an immediate bacterial reverse mutation assay intended to detect an extensive variety of chemical constituents that can yield genetic damage leading to gene mutations. This is a reliable and quick technique (Reid et al., 2006). A positive reaction in a single bacterial strain either in the presence or absence of metabolic activation (liver enzymes) is adequate enough to enable a constituent to be considered as a mutagen (Zeiger, 2001). This assay has been positively used to screen a wide range of South African remedies (Elgorashi et al., 2003; Reid et al., 2006; Luseba et al., 2007; Verschaeve and Van Staden, 2008; Fawole et al., 2009; Hurinanthan et al.,

2009; McGaw et al., 2010; Mashele and Fuku, 2011; Makhafola et al., 2014) and commercially used herbal mixtures (Ndhlala et al., 2011; Ngcobo et al., 2016).

The Ames test uses various strains of *Salmonella typhimurium* that are dependent on histidine, each carrying different mutations in different genes. The revertant bacteria are distinguished by their capacity of growing when there is no essential amino acid (histidine) (Tamokou and Kuete, 2014). Bacterial strains TA98 and TA100 are the ones that are mostly used because they identify the majority of mutagens. Strain TA98 gives an indication of frame modification mutation, whereas strain TA100 indicates base pair substitution (Mashele and Fuku, 2011). The Ames test can be done in the presence of metabolic liver enzymes known as S9 microsomal fraction or without the liver enzymes (using the phosphate buffer) (Déciga-Campos et al., 2007). Some mutagenic compounds, such as amines or polycyclic aromatic hydrocarbons are biologically inactive unless they are metabolised to active forms by the enzymes such as CYP. Since bacteria do not have metabolic capabilty, an exogenous mammalian organ activation system needs to be added to the petri plates together with the test sample and the bacteria (Mortelmans and Zeiger, 2000). For this purpose, a rodent metabolic activation system was introduced into the test system (Ames et al., 1973). Metabolic activation consists of S-9 microsomal supernatant fraction of liver homogenate, which is delivered to the test system in the presence of NADP and co-factors for NADPH-supported oxidation (S-9 mix) (Tejs, 2008).

Direct acting mutagens such as 4-nitroquinoline 1-oxide (4NQO) and sodium azide were used in the current study and are mostly used for strain TA98 and TA100 as positive controls respectively. 4-Nitroquinoline 1-oxide is an artificial water soluble mutagen and carcinogen which is a quinolone derivative. It applies intracellular oxidative pressure and its metabolic product binds to the DNA particularly at remains of guanine (Kanojia and Vaidya, 2006). It has been reported to induce alterations of DNA in bacteria, fungi and animals through the formation of bulky purine adducts and it has been widely used as a mutagen for genetic screening in both studies of DNA damage and DNA repair (Downes et al., 2014). Sodium azide is a common disinfectant, insecticide, and industrial nitrogen gas generator which is known to be highly mutagenic in some organisms (Sadiq and Owais, 2000). It has shown to be mutagenic towards several strains of *S. typhimurium* and *Eschericia coli.*

Mutagenicity in bacteria is characterised by the induction of base-change mutational lesions (Jones et al., 1980).

The aim of this chapter is to evaluate the potential mutagenic effect of 26 medicinal plants (organic and aqueous extracts) as well as the 19 plant combinations which were recorded to be used in northern Maputaland for the treatment of hypertension (Table 2.1 and Table 2.2).

3.2 Methods

For mutagenicity, the standard plate incorporation method (Ames test) established by Ames et al. (1973) was used. The aqueous plant extracts were prepared using distilled water. The organic plant extracts were made up in 10% DMSO. Plant extracts tested singularly were prepared at the concentration of 5 mg/ml and plant extracts tested in combinations were prepared at the concentration of 2.5 mg/ml. *Salmonella typhimurium* bacterial strains TA98 and TA100 were used. These strains were kindly donated by Prof Lyndy McGaw, University of Pretoria. On receipt all strains were plated and purity was confirmed. Bacterial stocks were incubated in 10 ml of nutrient broth for 24 hrs at 37°C. For the preparation of minimum glucose plates, 15 g of agar was dissolved in 930 ml of distilled water and autoclaved. After the agar had slightly cooled down, 20 ml of sterile 50 X Vogel-Bonner medium salts (VB salts) and 50 ml of sterile 40% glucose (prepared by dissolving 40 g of glucose in 100 ml of distilled water) was added to the agar and the agar was poured into the petri dishes. Vogel-Bonner medium salts (50 X VB salts) was prepared by dissolving 10 g of magnesium sulphate together with 100 g of citric acid and 500 g of potassium phosphate as well as 175 g of sodium ammonium phosphate in 670 ml of distilled water. The salts were added respectively and each salt was allowed to dissolve completely before adding another. The mixture was made up to 1000 ml using distilled water. The 50 X VB salts were then autoclaved.

A phosphate buffer was prepared by dissolving one tablet (containing sodium phosphate, sodium chloride, potassium chloride and potassium phosphate) in 200 ml of distilled water to yield 0.01 M phosphate buffer, 0.027 M potassium chloride and 0.137 M at the PH of 7.4. A histidine/biotin solution was also prepared. D-biotin (12 mg) was dissolved in 100 ml hot autoclaved water. When the solution has cooled down (50°C), L-histidine (10.5 mg) was also added and the solution was stored at

4°C. Top agar was prepared by dissolving 6 g of agar and 5 g of sodium chloride. The mixture was autoclaved and 100 ml was transferred to 250 ml glass bottles with screw caps. Ten millimetre of histidine/biotin solution was added to each 250 ml bottle containing 100 ml of top agar and maintained in a water bath which was heated to 50°C.

For the Ames test, 100 µl of the overnight culture (TA98 and TA100) was added together with 100 µl of the test sample [negative control, positive control (2 µg/ml) or plant extracts (5 mg/ml)] and 500 µl of phosphate buffer. This combination was then added to 2 ml of minimal top agar and then mixed using a vortex (Thermo scientific). This mixed solution was then poured over the surface of minimum glucose agar plates. The plates were inverted and incubated at 37°C for 48 hours. After 48 hrs, the colonies were counted manually and the reversion rate was compared to the colonies which were on the control plates. In order for a test constituent (plant extracts) to be classified as a mutagen, the number of revertant colonies on the plate containing the test extract should be double the number of colonies produced on the positive controls (Mashele and Fuku, 2011). For the negative controls, 10% DMSO, and water were used and for the positive control, 4-nitroquinoline N-oxide (4NQO) and sodium azide were used and both were prepared to the concentration of 2 µg/ml. Three replicates were undertaken and these assays were repeated twice.

3.3 Results and discussion

3.3.1 Mutagenicity analysis of individual plant samples

The mutagenicity properties of 54 (organic and aqueous) plant extracts derived from 26 medicinal plants used for the treatment of hypertension were determined. The results are recorded in Table 3.1. Positive controls allowed the growth of *S. typhimurium* in all the plates with number colonies of 500 or higher. No growth of *S. typhimurium* (TA98 and TA100) was evident with the negative control (10% DMSO and water) on minimum glucose plates. However, the growth of the bacterial strain TA98 and TA100 was evident when cultured in Tryptone Soya agar indicating viability of test strains. Overall, the mutagenic profile of all the tested extracts (organic and aqueous) showed that only two aqueous extracts were mutagenic (*C. roseus* and *O. engleri*) while for organic extracts, six extracts were mutagenic (*C.*

roseus, C. sativa, O. engleri, P. kaurabassana, S. sanguinea and *S. madagascariensis*).

Table 3.1 Medicinal plants investigated for mutagenicity potential using *S. typhimurium* bacterial strains, namely TA98 and TA100.

Plant species	**Extract**	**Number of colonies**	
		TA98	**TA100**
Acanthospermum hispidium	Organic	100	100
	Aqueous	28	18
Albertisia delagoensis	Organic	16	24
	Aqueous	87	71
Aloe marlothii	Organic	105	100
	Aqueous	87	78
Cannabis sativa	Organic	**1000**	**1000**
	Aqueous	97	102
Carpobrotus dimidiatus	Organic	39	44
	Aqueous	100	100
Catharanthus roseus	Organic	**1000**	**1000**
	Aqueous	**1000**	**1000**
Citrullus lanatus	Organic	36	41
	Aqueous	27	13
Citrus limon	Organic	52	63
	Aqueous	35	44
Cladostemon kirkii	Organic	200	200
	Aqueous	200	200
Hyphaene coriacea	Organic	200	200
	Aqueous	200	200
Hypoxis hemerocallidea	Organic	102	117
	Aqueous	100	105
Lippia javanica	Organic	100	200
	Aqueous	200	200
Momordica balsamina	Organic	109	99
	Aqueous	78	71

Plant species	**Extracts**	**Number of colonies**	
		TA98	**TA100**
Musa acuminata	Organic	35	44
	Aqueous	98	84
Ozoroa engleri	Organic	**1000**	**1000**
	Aqueous	**1000**	**1000**
Psidium guajava (roots)	Organic	72	84
	Aqueous	59	67
Psidium guajava (leaves)	Organic	91	99
	Aqueous	88	101
Ptaeroxylon obliquum	Organic	200	200
	Aqueous	100	100
Pyrenacantha kaurabassana	Organic	**1000**	**1000**
	Aqueous	200	200
Ricinus communis	Organic	200	200
	Aqueous	100	91
Sarcophyte sanguinea	Organic	**1000**	**1000**
	Aqueous	200	200
Sarcostemma viminale	Organic	200	200
	Aqueous	100	100
Senecio serratuloides	Organic	109	100
	Aqueous	71	88
Strychnos madagascariensis	Organic	**1000**	**1000**
	Aqueous	70	62
Tetradenia riparia	Organic	74	79
	Aqueous	69	61
Trichilia emetica	Organic	88	76
	Aqueous	100	109
Vangueria infausta	Organic	200	200
	Aqueous	200	200
Positive control (4NQO)	Organic	**500**	**500**
	Aqueous	**500**	**500**
Positive control (sodium azide)	Organic	**500**	**500**
	Aqueous	**500**	**500**

Plant species	Extracts	Number of colonies	
		TA98	**TA100**
Negative control (10% DMSO)	Organic	0	0
	Aqueous	0	0
Negative control (Water)	Organic	0	0
	Aqueous	0	0

*Results in bold are mutagenic.

In the current study, both the organic and aqueous extracts of *A. hispidium* were found to be non-mutagenic towards strains TA98 and TA100. These results correlate with the results reported by Hussain et al. (1990) where the aerial part of the plant was found to be non-mutagenic towards strain TM677. These previous studies were done in both the absence and presence of metabolic activation at the concentration of 0.31, 0.62, 1.25, 2.5 and 5.0 mg/ml.

When *A. delagoensis* (roots), *C. lanatus* (fruit), *C. limon* (peels), *C. kirkii* (roots), *H. coriacea* (roots), *L. javanica* (leaves) *and V. infausta* (roots) were tested for mutagenicity, all the extracts (organic and aqueous) were non-mutagenic towards both strains TA98 and TA100 at the concentration of 5 mg/ml. It is not surprisingly that *C. limon* and *C. lanatus* were found to be non-mutagenic as these plants are well known edible fruits. To the best of my knowledge, nothing could be found in the literature regarding the mutagenicity of *A. delagoensis*, *C. lanatus*, *C. limon*, *C. kirkii, H. coriacea, L. javanica* and *V. infausta*.

In the current study, the organic extract of *C. sativa* was found to be mutagenic whereas the aqueous extract was non-mutagenic towards both strains TA98 and TA100. A previous study has been done to determine the mutagenic potential of *C. sativa* smoke condensates. Acetone leaf extract was investigated for mutagenicity toward strains TA98, TA100, TA1535 and TA1537 with and without metabolic activation. The results showed that only strain TA98 was toxic in the presence of metabolic activation at the concentration of 1 mg/ml (Busch et al., 1979). These results differ with the results which were found in the current study as the organic extract of *C. sativa* was mutagenic in strains TA98 and TA100 without the metabolic activation. The contradiction could be due to different solvents used for extraction as in the previous study acetone was used and in the current study dichloromethane:methanol was used. Dichloromethane:methanol could have

extracted non-polar compounds that has mutagenic effect. Another factor could be the difference in the concentrations tested. More likely in the previous study 1 mg/ml was tested and in the current study 5 mg/ml of extract was tested, hence higher concentrations have high mutagenicity potential.

C. dimidiatus extracts (both organic and aqueous) were found to be non-mutagenic towards *S. typhimurium* strains TA98 and TA100 at the concentration of 5 mg/ml. These results correlate with the results which were previously reported by Hurinanthan et al. (2009), where the methanol and aqueous extracts of the leaves and the flowers were found to be non-mutagenic toward TA98 and TA100 strains at the concentrations of 5, 10, 20, 100 and 1000 µg/ml.

Both the organic and aqueous extracts of *C. roseus* roots were found to be mutagenic towards both strains TA98 and TA100. However, the dichloromethane and 90% methanol extracts of the leaf of *C. roseus* were reported to be mutagenic against strain TA98 in the presence of metabolic activation while being non-mutagenic towards strain TA100 both in the presence and absence of metabolic activation at the concentration of 0.05, 0.5 and 5.0 mg/ml (Elgorashi et al., 2003). In the literature this plant has been reported to be useful for cancer treatment (Van Wyk et al., 1997). However, according to the study done by Reid et al. (2006), *C. roseus* leaves did not have any anti-mutagenic properties when the dichloromethane and 90% methanol extracts of the leaves were tested at the concentrations of 0.05, 0.5.and 5.0 mg/ml against *S. typhimurium* strains TA98 and TA100 in both presence and absence of metabolic activation. There seems to be a difference in results obtained in the current study and a study done by Elgorashi et al. (2003). Although the same concentration (5 mg/ml) was tested, the differences are probably due to the fact that different plant parts were tested. In the current study, the roots were used whereas in the previous studies, the leaves were tested. The plant has been reported to have different alkaloids within the above ground and underground tissues (Shukla et al., 2006) and the root bark produces the most (Sandeep et al., 2014). The concentrations of the alkaloids can also be influenced by seasons. The concentration of some alkaloids such as vindoline and catharanthine are higher in leaves than the roots and vindoline is not found in the roots at all (Yu et al., 2010). The roots contain ajmalicine and serpentine which have been shown to be an important constituent for the management of blood pressure and other types of

cardio-vascular disorders (Shukla et al., 2006). The alkaloids which are found in *C. roseus* have not be associated with mutagenicity in literature. However, most of the alkaloids are used to treat various types of cancer such as “hodgkins diseases”, breast cancer, skin cancer and lymphoblastic leukemia (Sandeep et al., 2014).

In the current study, both the aqueous and organic extracts of *H. hemerocallidea* were found to be non-mutagenic when tested against the TA98 and TA100 strains. These results correlate with the results which were reported by Reid et al. (2006), where the dichloromethane and 90% methanol extracts of the corm and the leaves were non-mutagenic at the concentrations of 0.05, 0.5 and 5.0 mg/ml against the strains TA98 and TA100 both in the presence and absence of metabolic activation. *Hypoxis hemerocallidea* dichloromethane and 90% methanol corm extracts were also reported to be non-toxic against TA98 and TA100 at the concentration of 5.0, 0.5 and 0.05 mg/ml both with and without metabolic activation (Elgorashi et al., 2003). The results of this plant correlate with the traditional use as it was one of the most frequently mentioned plants used for the treatment of hypertension.

The organic and aqueous extracts of the leaves of *M. balsamina* were non-mutagenic towards both strains at the concentration of 5 mg/ml. Non-mutagenic properties of this plant was also reported by Ndhlala et al. (2011), where petroleum ether, dichloromethane, 80% ethanol and water extracts of the leaves were found to be non-mutagenic toward *S. typhimurium* strain TA98 at the concentrations of 50, 500 and 5000 µg/ml with and without metabolic activation.

When *M. acuminata* flower bracts and *O. engleri* roots were investigated for mutagenicity potential, *M. acuminata* extracts (organic and aqueous) were found to be non-mutagenic where *O. engleri* extracts (organic and aqueous) were mutagenic towards strains TA98 and TA100. To the best of my knowledge, no information could be found in the literature regarding the mutagenicity of *M. acuminata* as well as *O. engleri*.

When the leaves and the roots of organic and aqueous extracts of *P. guajava* were tested for mutagenicity potential, both extracts were found to be non-mutagenic against strains TA98 and TA100. No previous studies have been recorded in literature on the mutagenicity of *P. guajava*. However, according to an earlier study done by Grover and Bala (1993), the water extract of the fruit of *P. guajava* was

reported to be a desmutagen, being able to inactivate the mutagenic potential of direct-acting mutagens such as 4-nitro-0-phenylenediamine, sodium azide, as well as S9 dependent mutagen, 2-aminofluorene, in *S. typhimurium* strains TA97, TA98, TA100 and TA1535.

In the current study, both the aqueous and organic extracts of *P. kaurabassana* and *P. obliquum* were investigated for mutagenic potential. The results showed that both organic and aqueous extracts of *P. obliquum* were non-toxic towards *S. typhimurium* strains TA98 and TA100. *Pyrenacantha kaurabassana* aqueous extract was also non-mutagenic towards strains TA98 and TA100 where else the organic extract was mutagenic towards both strains. Nothing could be found in the literature regarding the mutagenicity of *P. obliquum* and *P. kaurabassana*.

When *R. communis* organic and aqueous extracts of the leaves were tested for mutagenicity potential, the results showed that this plant was non-mutagenic at the concentration of 5 mg/ml against *S. typhimurium* strains TA98 and TA100. These results correlate with the results which were reported by Luseba et al. (2007), where the dichloromethane and 90% methanol extracts of the leaves and the stem of the plant were reported to be non-mutagenic against strain TA98 at the concentration of 50, 500 and 5000 µg/ml. Another study which confirms the non-mutagenic properties of *R. communis* was done by Elgorashi et al. (2003), where the dichloromethane and 90% methanol extracts of the roots were reported to be non-mutagenic at the concentrations of 0.05, 0.5 and 5.0 mg/ml against strains TA98 and TA100 both with and without metabolic activation. Two different plant parts (roots and leaves) have been tested and they both showed that this plant is non-mutagenic.

The leaf extracts (organic and aqueous) of *S. serratuloides* were found to be non-mutagenic towards *S. typhimurium* strains TA98 and TA100 at the concentration of 5 mg/ml. These results support the study which was previously done by Elgorashi et al. (2003), where the dichloromethane and 90% methanol extracts of the leaves were found to be non-mutagenic toward strains TA98 and TA100 at the concentrations of 0.05, 0.5 and 5.0 mg/ml both with and without metabolic activation.

When the leaves of *T. emetica* were tested, both the organic and aqueous extracts were found to be non-mutagenic towards both strains at the concentration of 5 mg/ml. The dichloromethane and 90% methanol extracts of the bark of this plant was

also reported to be non-toxic toward strains TA98 and TA100 at the concentrations of 0.05, 0.5 and 5.0 mg/ml both with and without metabolic activation (Elgorashi et al., 2003).

The whole plant extract (organic and aqueous) of *S. viminale* and the leaf of *A. marlothii* were found to be non-mutagenic towards strain TA98 and TA100 in the current study. These results correlate with a previous study where the dichloromethane and 90% methanol extracts of the stem of *S. viminale* and the leaves of *A. marlothii* were found to be non-mutagenic when tested against TA98 without the presence of metabolic activation at the concentrations of 50, 500 and 5000 µg/ml (Luseba et al., 2007).

When the stem of *S. sanguinea* was investigated for mutagenicity potential, the aqueous extract was non-mutagenic towards both strains where the organic extract was mutagenic towards both strains. Nothing could be found in the literature regarding the mutagenicity of *S. sanguinea*.

In the current study, both the aqueous and organic extracts (dichloromethane: methanol) of the leaves of *T. riparia* were found to be non-mutagenic towards strains TA98 and TA100. These results correlate with an earlier study done where the dichloromethane and 90% methanol extracts of the leaves of *T. riparia* were tested for mutagenicity against *S. typhimurium* TA98 strain. The results showed that the plant is non-mutagenic both in the presence and absence of metabolic activation at the concentrations of 0.05, 0.5 and 5.0 mg/ml (Elgorashi et al., 2003). Another study which validates the non-mutagenic properties of *T. riparia* was done by Ndhlala et al. (2011), where petroleum ether, dichloromethane, 80% ethanol and water extracts of the leaves of *T. riparia* were reported to be non-mutagenic towards *S. typhimurium* TA98 at the concentrations of 50, 500 and 5000 µg/ml with and without metabolic activation.

Thirteen of the 26 medicinal plants (50%) have been previously assessed for mutagenicity using various *Salmonella* strains. The strains include TA98, TA100, TA1535, TA1537 and TM677. A summary of all the medicinal plants that have been tested for mutagenicity are displayed in Table 3.2 along with the type of extracts tested, types of strains used, and concentrations tested as well as their correlation to the current study. The 13 plants that have not been investigated for mutagenicity are

A. delagoensis, C. lanatus, C. limon, C. kirkii, H. coriacea, L. javanica, M. acuminata, O. engleri, P. obliquum, P. kaurabassana, S. sanguinea, S. madagascariensis and *V. infausta.* Water was recorded to be used for the extraction of all the plants used to treat hypertension, so it was essential to determine mutagenicity of the aqueous extracts. Among the plants that have never been subjected to mutagenicity testing, approximately 31% (four plant species) were found to be mutagenic. These results further emphasize the importance of screening all the medicinal plants that have not been subjected to mutagenicity testing, especially the newly documented plants. Although mutagenic studies have been reported on some of the plants being investigated, very few of the studies focused on the aqueous extracts. Only studies done by Hurinanthan et al. (2009) and Ndhlala et al. (2011) included water extracts. Most studies have focused only on the mutagenicity of the organic extracts (Busch al., 1979; Hussain et al., 1990; Elgorashi et al., 2003; Reid et al., 2006; Luseba et al., 2007). In general the frequency of the plants mostly taken for hypertension correlated with their non-mutagenic profile (*A. marlothii, H. hemerocallidea, M. balsamina, M. acuminata and S*. *serratuloides*). Results from this study also revealed that some of the less frequently used plants for hypertension treatment (De Wet et al., 2016) such as *A. hispidum, A. delagoensis, H. coriacea,* and *V. infausta* were also found to be non-mutagenic. Although most of the plant extracts tested were non-mutagenic, it does not mean that they should be considered safe. It only means that the plant extracts is non-mutagenic towards the particular strains (TA98 and TA100) tested. Thus some of the plant extracts may be mutagenic towards other strains such as TA97, TA104, TA1535, TA1537 and TA1538 among others since all the strains are genetically different. Overall the results obtained support the non-mutagenicity of the anti-hypertension plants used by lay people in northern Maputaland. However, it should also be noted that some mutagenic compounds are only biologically active when they are metabolised to the active form (Déciga-Campos et al., 2007). Plants that show clear mutagenic effect should be considered unsafe and require further analysis before being endorsed to be used (Verschaeve and Van Staden, 2008). In literature, only two of the relevant plants were reported to be useful in cancer treatment namely: *C. roseus* and *H. hemerocallidea* (Hutchings et al., 1996; Van Wyk et al., 1997). However in the current study *C. roseus* was found to have mutagenic potential. This is contradicting as some mutagens have been associated with the development of cancer (Goldman and Shields, 2003).

Table 3.2 A summary of the medicinal plants that have been previously tested for mutagenicity.

Plant species	Type of extract	*S. typhimurium* strain	Concentration	Correlation with current study	References
A. hispidium	80% methanol	TM677	0.31, 0.62, 0.125, 2.5, 5 mg/ml	Yes	Hussain et al., 1990
A. marlothii	Dichloromethane, 90% methanol	TA98	50, 500, 5000 µg/ml	Yes	Luseba et al., 2007
C. dimidiatus	Methanol, water	TA98, TA100	5, 10, 20, 100, 1000 µg/ml	Yes	Hurinantha et al., 2009
C. sativa	Acetone	TA98, TA100, TA1535, TA1537	1 mg/ml	No - lower concentration tested	Busch et al., 1979
C. roseus	Dichloromethane, 90% methanol	TA98, TA100	0.05, 0.5, 5.0 mg/ml	No - different plant part tested	Elgorashi et al., 2003
H. hemerocallidea	Dichloromethane, 90% methanol	TA98, TA100	0.05, 0.5, 5.0 mg/ml	Yes	Reid et al., 2006
	Dichloromethane, 90% methanol	TA98, TA100	5.0, 0.5, 0.05 mg/ml	Yes	Elgorashi et al., 2003
M. balsamina	Petroleum ether, dichloromethane, 80% ethanol, water	TA98	50, 500, 5000 µg/ml	Yes	Ndhlala et al., 2011
P. guajava	Water	TA98, TA100	50, 500, 5000 µg/ml	Yes	Ngcobo et al., 2016
R. communis	Dichloromethane, 90% methanol	TA98	50, 500, 5000 µg/ml	Yes	Luseba et al., 2007
	Dichloromethane, 90% methanol	TA98, TA100	0.05, 0.5, 5.0 mg/ml	Yes	Elgorashi et al., 2003
S. serratuloides	Dichloromethane, 90% methanol	TA98, TA100	0.05, 0.5, 5.0 mg/ml	Yes	Elgorashi et al., 2003
S. viminale	Dichloromethane, 90%methanol	TA98	50, 500, 5000 µg/ml	Yes	Luseba et al., 2007
T. riparia	Dichloromethane, 90% methanol	TA98	0.05, 0.5 and 5.0 mg/ml	Yes	Elgorashi et al., 2003
	Petroleum ether, dichloromethane, 80% ethanol, water	TA98	50, 500, 5000 µg/ml	Yes	Ndhlala et al., 2011
T. emetica	Dichloromethane, 90% methanol	TA98, TA100	0.05, 0.5, 5.0 mg/ml	Yes	Elgorashi et al., 2003

3.3.2 Mutagenicity analysis of plant combinations

A total of 38 plant extracts (organic and aqueous) from 19 plant combinations were analysed for mutagenicity using the Ames test and the results are depicted in Table 3.3. The positive controls (4NQO and sodium azide) results were also included as well as the negative control results (water and 10% DMSO). The overall mutagenic profile of the plant combinations (organic and aqueous) indicated that 21% of the organic extracts had a mutagenic effect (combination between *C. roseus* and *M. acuminate*; *C*. *roseus* and *H. hemerocallidea*; *C. sativa* and *M. balsamina*, *A. marlothii* and *C. sativa*) while 11% of the aqueous extracts demonstrated a mutagenic effect (*M. balsamina* and *C*. *roseus* and *C. roseus* and *H. hemerocallidea*). The combinations which were mutagenic were combinations involving *C. sativa* and *C. roseus*. It is worth noting that these plants were mutagenic when tested singularly and when used in combination with plants that were non-mutagenic there was no reduction of mutagenicity. The combination *C. roseus* and *H*. *hemerocallidea* was mutagenic in both aqueous and organic extracts.

Plant combinations which resulted in reduced mutagenicity effect are *A. delagoensis and S. serratuloides* (aqueous*)*, *H. hemerocallidea*, *A. marlothii* and *M. balsamina* (organic*)*, *C. roseus* and *M. acuminata* (aqueous), *M. balsamina* and *A. marlothii* (organic and aqueous), *P. guajava* and *C. dimidiatus* (organic), *H. hemerocallidea* and *S. serratuloides* (aqueous and organic), *M. balsamina* and *M. acuminata* (aqueous and organic), *M. balsamina* and *S. serratuloides* (organic), as well as the combination between *S. serratuloides, M. acuminata, A. marlothii* and *H. hemerocallidea* (aqueous). The combination which is worth highlighting is the combination between *A. marlothii* and *C. roseus*. *Catharanthus roseus* was mutagenic towards both strains in the organic and aqueous extracts while *A. marlothii* was non-mutagenic in both extracts. The combination of the two plants, however, resulted in reduced mutagenic potential in both extracts (organic and aqueous), thus *A. marlothii* was able to neutralize the mutagenic effect of *C. roseus* in both extracts. Some of the combinations did not neutralize the mutagenic potential of the plants which were mutagenic when tested singularly. Instead they resulted in increased toxicity. Such combinations are the combinations between *C. sativa* and *A. marlothii* (organic), *M. balsamina* and *C. sativa* (organic), *H. hemerocallidea* and

C. roseus (organic and aqueous), *M. acuminata* and *C. roseus* (organic) as well as the combination between *M. balsamina* and *C. roseus* (organic and aqueous).

Table 3.3 Plant combinations which were tested on Ames test with two types of *S. typhimurium* strains used along with their ΣFIC index and their respective interpretations.

Plant combinations	**Extracts**	**Number of colonies**		**ΣFIC and interpretation**	
		TA98	**TA100**	**TA98**	**TA100**
A. delagoensis + *S. serratuloides*	Organic	20	29	0.72 (Additive)	0.75 (Additive)
	Aqueous	34	27	0.43 (Synergistic)	0.34 (Synergistic)
C. roseus + *A. marlothii*	Organic	85	90	0.44 (Synergistic)	0.49 (Synergistic)
	Aqueous	200	200	1.25 (Non-interactive)	1.38 (Non-interactive)
A. marlothii + *H. hemerocallidea*	Organic	21	24	1.05 (Non-interactive)	1.07 (Non-interactive
	Aqueous	29	40	1.23 (Non-interactive)	1.27 (Non-interactive)
A. marlothii + *H. hemerocallidea* + *M. balsamina*	Organic	37	33	0.35 (Synergistic)	0.31 (Synergistic)
	Aqueous	58	49	0.66 (Additive)	0.60 (Additive)
M. balsamina + *A. marlothii*	Organic	21	23	0.19 (Synergistic)	0.24 (Synergistic)
	Aqueous	35	31	0.42 (Synergistic)	0.42 (Synergistic)
C. sativa + *A. marlothii*	Organic	***1000**	**1000**	5.26 (Antagonistic)	5.50 (Antagonistic)
	Aqueous	150	200	1.63 (Non-interactive)	2.26 (Non-interactive)
C. kirkii + *S. sanguinea* + *A. marlothii*	Organic	200	200	1.04 (Non-interactive)	1.07 (Non-interactive)
	Aqueous	200	200	1.43 (Non-interactive)	1.52 (Non-interactive)
M. balsamina + *C. sativa*	Organic	**1000**	**1000**	5.09 (Antagonistic)	5.55 (Antagonistic)
	Aqueous	200	200	2.31 (Non-interactive)	2.39 (Non-interactive)
H. hemerocallidea + *C. roseus*	Organic	**1000**	**1000**	5.40 (Antagonistic)	4.77 (Antagonistic)
	Aqueous	**1000**	**1000**	1.0 (Non-interactive)	5.26 (Antagonistic)
M. acuminata + *C. roseus*	Organic	**1000**	**1000**	14.79 (Antagonistic)	11.86 (Antagonistic)
	Aqueous	100	100	0.56 (Additive)	0.64 (Additive)
P. guajava + *C. dimidiatus*	Organic	27	39	0.5 (Synergistic)	0.63 (Additive)
	Aqueous	200	200	2.14 (Non-interactive)	1.99 (Non-interactive)

Plant combinations	Extracts	Number of colonies		ΣFIC and interpretation	
		TA98	TA100	TA98	TA100
H. hemerocallidea + *S. serratuloides*	Organic	23	19	0.22 (Synergistic)	0.17 (Synergistic)
	Aqueous	40	45	0.48 (Synergistic)	0.46 (Synergistic)
L. javanica + *M. balsamina*	Organic	200	200	1.92 (Non-interactive)	1.51 (Non-interactive)
	Aqueous	100	100	0.89 (Additive)	0.95 (Additive)
M. balsamina + *M. acuminata*	Organic	19	27	0.36 (Synergistic)	0.45 (Synergistic)
	Aqueous	29	34	0.34 (Synergistic)	0.44 (Synergistic)
M. balsamina + *C. roseus*	Organic	200	200	9.27 (Antagonistic)	1.11 (Non-interactive)
	Aqueous	**1000**	**1000**	6.91 (Antagonistic)	7.54 (Antagonistic)
M. balsamina + *S. serratuloides*	Organic	33	39	0.44 (Synergistic)	0.31 (Synergistic)
	Aqueous	92	100	1.54 (Non-interactive)	1.64 (Non-interactive)
M. acuminata + *C. limon*	Organic	25	21	0.60 (Additive)	1.41 (Non-interactive)
	Aqueous	100	100	1.94 (Non-interactive)	1.73 (Non-interactive)
S. serratuloides + *M. acuminata* + *A. marlothii* + *H. hemerocallidea*	Organic	105	111	1.5 (Non-interactive)	1.43 (Non-interactive)
	Aqueous	30	25	0.36 (Synergistic)	0.29 (Synergistic)
T. emetica + *A. marlothii* + *H. coriacea*	Organic	100	100	0.87 (Additive)	0.94 (Additive)
	Aqueous	200	200	1.77 (Non-interactive)	1.80 (Non-interactive)

*Results in bold are mutagenic.

The toxicity of plant combinations (both concentrations) were further analysed for their ΣFIC value and the results are depicted on Table 3.3 with their respective interpretations. For the organic extracts, the highest percentage (37%) of the combinations tested in the current study has synergistic interactions. These combinations were considered less toxic when combined i.e. plants working together in a synergistic manner in order to reduce toxicity. Such combination includes the combinations between *H. hemerocallidea* and *S. serratuloides, M. balsamina* and *A. marlothii* as well as *H. hemerocallidea* and *A. marlothii.* Twenty one percent (four plant combinations) tested demonstrated a non-interactive interaction i.e. their combination did not have any increased effect on toxicity. Such combinations include the combination between *C. kirkii* and *A. marlothii* and *S. sanguinea*, *L. javanica* and *M. balsamina*. Twenty six percent (five plant combinations) of the combinations

resulted in an increased mutagenicity (antagonistic) interaction. These combinations should be used with caution. Such combinations include the combination between *C. roseus* and *A. marlothii, A. marlothii, H. hemerocallidea* and *M. balsamina, M. balsamina* and *A. marlothii, H. hemerocallidea* and *S. serratuloides* as well as the combination between *M. balsamina* and *M. acuminata.* A further 16% (three plant combinations) of the combinations were shown to have an additive effect. Such combinations include the combinations between *T. emetic, A. marlothii* and *H. coriacea* (organic extract), *L. javanica* and *M. balsamina* (aqueous extract) as well as *M. acuminata* and *C. roseus*.

For the aqueous extracts, 16% (three combinations) had an additive interaction, 5% (one plant combination) had an antagonistic interaction, and 32% (six plant combinations) had synergistic interactions while 47% (nine plant combinations) showed a non-interactive interaction. It is worth noting that for aqueous extracts which is the traditional way of preparing these plants, the only two combinations which resulted in increased mutagenicity (antagonistic effects) were the combinations between *M. balsamina* and *C. roseus* as well as *H. hemerocallidea* and *C. roseus*. Singularly, *M. balsamina* was non-mutagenic while *C*. *roseus* was mutagenic, thus *M. balsamina* did not neutralize the mutagenic effect of *C. roseus* instead these plants were working against each other resulting in an increased mutagenic effect. This combination should be used with caution as it was documented to be one of the combinations which are frequently used.

Some of the combinations had the same FIC index results for both aqueous and organic extracts. Such combinations include the combination between *H. hemerocallidea* and *A. marlothii* (synergistic interaction), *M. balsamina* and *A. marlothii* (synergistic interaction) as well as *C. kirkii*, *S. sanguinea* and *A. marlothii* (non-interactive interaction). While other combinations had different interactions for the aqueous and organic extracts. Such combinations include combinations between *P. guajava* and *C*. *dimidiatus, L*. *javanica* and *M. balsamina* as well as *M. acuminata* and *C. roseus*. Four of the combinations tested had different interactions between the *Salmonella* strains (TA98 and TA100). This could be because the two bacterial strains used are genetically different. These combinations includes *P. guajava* and *C*. *dimidiatus* (organic extract), the combination had synergistic interaction towards TA98 while having an additive interaction towards strain TA100. Another

combination which is worth noting is the combination between *H. hemerocallidea* and *C. roseus* (aqueous extract). This combination had non-interactive effect towards strain TA98 while having an antagonistic effect towards strain TA100. This could be because the two strains are genetically different, thus this combination resulted in increased frame shift mutations reversion with no increase in substation of DNA base. A similar effect on *C. roseus* singularly was also reported by Elgorashi et al. (2003), where the leaf extracts of the plant was mutagenic towards strain TA98 while being non-mutagenic towards strain TA100. Other combinations include the combination between *M. acuminata* and *C. limon* (organic extract) as well as *M. balsamina* and *C. roseus* (organic extract) as well as *H. hemerocallidea* and *C. roseus* (aqueous extract).

None of the combinations investigated in the current study have been previously tested for mutagenicity. This encourages further investigation into evaluating the mutagenicity potential of plant combinations.

3.4 Summary

- Thirteen (50%) of the plants being investigated in the current study have been previously tested for mutagenicity potential using various *S. typhimurium* strains.
- Four of the plant extracts which were tested for the first time for mutagenicity were found to have mutagenic potential.
- For the plant extracts tested singularly, only two aqueous extracts (*C. roseus* and *O. engleri*) had a mutagenic potential while six plant species of the organic extracts had a mutagenic effect.
- None of the combinations in the current study have previously been tested for mutagenicity potential.
- For the aqueous plant combinations extracts two plant combinations had a mutagenic effect while the organic plant combinations four plant combinations had a mutagenic effect.
- For the organic extracts, four plant combinations demonstrated a non-interactive interaction, five plant combinations are antagonistic, three plant combinations are additive and seven plant combinations demonstrated a synergistic interaction.
- For the aqueous extracts three plant combinations have additive interaction, six plant combinations have synergistic effect, nine plant combinations have non-

interactive interaction, and one plant combination resulted in increased toxicity (antagonistic interaction).

- The two aqueous extract combinations which resulted in increased mutagenicity (antagonistic effects) was the combination between *M*. *balsamina* and *C. roseus* as well as the combination between *H. hemerocallidea* and *C. roseus*.
- The combination between *M. balsamina* and *A. marlothii*, *H. hemerocallidea* and *S. serratuloides* as well as the combination between *M. balsamina* and *M. acuminata* showed the most synergistic interactions.
- These combinations were frequently used with *M. balsamina* and *C. roseus* being the third most used, *H. hemerocallidea* and *S. serratuloides* being the fourth most used and the combination between *M. balsamina* and *M. acuminata* being the 7th most used combination to treat hypertension.

Chapter 4

In vitro herb-drug interaction potential of 13 medicinal plants used in northern Maputaland for the treatment of hypertension

4.1 Introduction

When drugs enter the body they undergo several pathways. They can be changed into other compounds, eliminated unchanged by the body or they can be metabolised by different enzymes into different compounds to speed up their excretion process. However, most of the drugs are metabolised to facilitate excretion. Drug metabolism (drug biotransformation) is the conversion of pharmacologically inactive compounds to pharmacologically active metabolites (Kebamo et al., 2015). This process can be divided into two phases namely, phase one (hydrolytic, oxidative and reductive) and phase two (conjugation) (Anders, 1980). Phase one serves to increase hydrophilicity of a parent drug (Ansede and Thakker, 2004). Phase two conjugate the drug or phase one metabolite with substrates such as carbohydrates, amino acid and acetic acid to yield high polarity compounds (Anders, 1980). Biotransformation reaction is facilitated by a broad class of enzymes such as CYP, esterases, glucuronic acid and sulfate among others (Kebamo et al., 2015).

There are several factors which have been described in the literature which affect the drug biotransformation. These factors include age, gender, genetic polymorphism, enzymes induction as well as enzymes inhibition (Pelkonen et al., 1998). Induction of the enzyme is when the exposed substrate results in accelerated metabolism of the drug. Inhibition of the enzymes occurs when two xenobiotics which are metabolised by the same enzyme compete for the enzyme receptor site (Ogu and Maxa, 2000). The induction of the enzyme results in increased amount and activity of the enzyme while the inhibition results in the exaggeration of the effect of the drug (Pelkonen et al., 1998). Thus, the induction and inhibition of the enzymes can lead to a decreased therapeutic effect, giving rise to an adverse reaction (Fasinu et al., 2013,a). Several studies has been conducted on various xenobiotics on enzyme mediated biotransformation which has resulted in the identification of several enzyme inducers and inhibitors (Benedetti, 2000; Sekikawa et al., 2002;

Singh et al., 2005; Chavez et al., 2006). The xenobiotics that have been described include drugs, food and medicinal plants. According to De Wet et al. (2016), people from northern Maputaland were using medicinal plants together with conventional drugs to treat hypertension. Three enzymes involved in the metabolism of hypertension drugs (Table 4.2) were investigated for potential inhibition by 13 medicinal plants documented (Table 4.1). The enzymes which were investigated were CYP3A4 (Cytochrome P450), carboxylesterase and beta-glucuronidase enzymes. These three enzymes were specifically chosen for investigation because they are involved in the metabolism of the hypertension drugs which were documented to be used concurrently with the medicinal plants in northern Maputaland.

Cytochrome P450 (CYP) enzymes are a family of hemoproteins that perform an important role in the breakdown of xenobiotics (including medicinal plants and drugs) (Coon, 2005). A number of CYP enzymes are expressed in mammalian species as well as in humans (Nelson et al., 1993). These enzymes are positioned in the smooth endoplasmic reticulum of the liver and other extra hepatic tissues. Various conventional drugs are metabolised by cytochrome P450 enzymes (Ionescu and Caira, 2005). These enzymes are the leading enzymes that are involved in phase one metabolism (Zhou et al., 2007). Various CYP enzymes (CYP2A6, CYP2C9, CYP2C19, CYP1A2, CYP2D6, CYP2E1, CYP2B6 and CYP3A4) have shown to play an important part in the absorption of xenobiotics (Cupp and Tracy, 1998). They are involved in the absorption of about 70% of the conventional drugs and endogenous compounds (Shimada et al., 1994). Cytochrome P450 enzyme has been shown to be involved in several interactions between drugs and food, medicinal plants and other conventional drugs (Delgoda and Westlake, 2004). Enzyme CYP3A4 has also been recognised as an important human CYP isozyme involved in the metabolism of most conventional drugs (Nelson et al., 1996; Nerbert and Russell, 2002; Basheer and Kerem, 2015). This enzyme is found in the prostate, breast, gut, colon and small intestine. It is highly expressed in the liver (Shimada et al., 1994; Lown et al., 1997; Guengerich, 1999). Several studies have demonstrated that food and medicinal plants can inhibit the CYP3A4 enzyme. The first incident was reported in 1989 when grapefruit juice was described to cause an increased plasma concentration of felodipine, which is used as an anti-hypertensive drug (Bailey et al., 1998). Later it

was found that this was caused by the inhibition of intestinal CYP3A4 enzyme (Bailey et al., 2000; Goosen et al., 2004). The *in vitro* inhibition of CYP3A4 has also been reported on plants such as *Ginkgo biloba* L.*, Glycyrrhiza glabra* Linn.*, Panax ginseng* (C.A Meyer) and *Uncaria tomentosa* (Willd.) DC. (Kupiec and Raj, 2005; Lopez et al., 2008; Tu et al., 2010; Basheer and Kerem, 2015).

Pharmacokinetic interactions (inhibition) have also been observed with drug metabolizing enzymes esterases (Li et al., 2007). These enzymes are involved in the breakdown of 10% of conventional drugs. Esterases hydrolyse the compounds/chemicals that comprise of carboxylic acid ester, amide, and thioester bonds, which cause pro-drug activation or detoxification. Among esterases, carboxylesterases are well acknowledged to be involved in the metabolism of a variation of drugs (Fukami and Yokoi, 2012). They are classified as phase one drug-metabolizing enzymes (Wang et al., 2011). If these enzymes are inhibited it could lead to greater instability of esters in the lumen and gut enterocytes, resulting in an increased absorption of the esters and therefore higher exposure of the active acids, through quick metabolism in plasma, for the pro-drugs (Li et al., 2007). In the current study, the inhibition of human carboxylesterase one (CES1) was investigated. Carboxylesterase one is expressed in the liver, with smaller quantities being produced in the intestine, kidneys, lungs, testes, heart, monocytes (white blood cells) and macrophages (cells that remove dying or dead cells) (Satoh et al., 1998; Satoh et al., 2002). Enzyme CES1 hydrolyses compounds that yield a smaller alcohol group and a larger acyl group (Imai et al., 2006). This has 47% similarity with the human intestinal carboxylesterase two (CES2) (Schwer et al., 1997). The CES2 enzyme is found in several hepatic tissues, commonly in the digestive tract while being expressed in lesser quantities in the liver (Xu et al., 2002). Enzyme CES2 hydrolyses compounds that yield a smaller acyl group and a larger alcohol group (Imai et al., 2006). Numerous conventional drugs are hydrolysed by CES1 including, enalapril, cocaine, heroin, pharmapress, meperidine, demerol and lidocaine (Kamendulis et al., 1996; Zhang et al., 1999, a; Alexson et al., 2002). There are studies that have demonstrated that some medicinal plants can inhibit the activity of carboxylesterases. According to Mai et al., (2015), some protostane triterpenoids found in the rhizome of *Alisma orientale* (Sam.) have been found to inhibit the carboxylesterase. The inhibition of carboxylesterase enzyme has also been reported

in medicinal plants such as *Lonicera japonica* Thunb. (jin yin hua), *Radix isatidis* L. and *Hydrastis canadensis* L. (Liu et al., 2011; Yang et al., 2012).

The third enzyme which was investigated in the current study was β-glucuronidase. The detoxification mechanism can be described as the process of chemically binding of a constituent to glucuronic acid through glycosidic bonds. This process is also known as glucuronidation. The process is used for the excretion of xenobiotics since they are poorly absorbed by the blood stream. β-glucuronidase enzymes are widely distributed in animals, plants and bacteria. They catalyse the hydrolysis of glucuronide to liberate xenobiotics (Sekikawa et al., 2002). The inhibition of this enzyme was investigated in the current study because according to De Wet et al., (2016), the lay people were using nonsteroidal anti-inflammatory (NSAIDs) drugs (aspirin) together with the angiotensin-converting-enzymes inhibitors (Ace inhibitors) drugs (enalapril and pharmpress). The NSAIDs class of drugs are known to lessen the effectiveness of the Ace inhibitors, diuretics and beta-blockers (Polonia, 1997; Opie, 2012). NSAIDs blocks prostaglandin production, where the Ace inhibitors tend to increase it. Studies have also suggested that the combination of NSAIDs and Ace inhibitors may have harmful effects on the renal function (Riegger et al., 1991; Mahe et al., 2001). NSAIDs drugs are known to be the substrate of beta-glucuronidase enzymes. Reabsorption of the liberated xenobiotics can cause toxicity in the intestine and decrease the excretion rate of the drugs (Sekikawa et al., 2002). It has been reported that the elimination of glucuronide moieties from drug metabolites by β-glucuronidase in the gastro intestinal lumen can considerably damage the abdominal epithelium (Roberts et al., 2013). If medicinal plants inhibit the activity of bacterial β-glucuronidase, it will result in a decrease of the reactivation of the NSAIDs thereby altering plasma levels. Thus the inhibition of the enzyme will reduce the negative effect of NSAIDs leading to a greater antihypertensive efficacy and the risk which accompany the removal of the glucuronide moieties from the pro drugs. Some of the beta-glucuronidase inhibitors have been isolated from medicinal plants [*Ganoderma lucidum* (Curtis) P. karst, *Glycyrrhiza uralensis* Fisch and *Paeonia emodi* Wall. ex Royle] and they are classified as terpenoids and their derivatives (Kim et al., 1999; Nawaz et al., 2000; Shim et al., 2000), flavonoid (silymarin) and its derivatives (Kim et al., 1994) as well as glucuronic acid and its derivatives [*Baphia racemosa* (Hochst.) Baker] (Cenci di Bello et al., 1984). Another medicinal plant which is known

to have β-glucuronidase inhibitory effect is *Chondia crassicaulis* Harvey ex J. Agarrdh (Sekikawa et al., 2002).

The rate of metabolism is an important factor which determines the duration and intensity of the pharmacological action of drugs. Inhibition of drug metabolizing enzymes may result in elevated drug bioavailability by decreasing the first-pass effect or by hindering the excretion of the drug which increases the side effect. This could be a serious case for a drug with narrow therapeutic index, thus the inhibition could be critical if a drug is metabolised through a single pathway (Liu et al., 2011). Some of the conventional hypertension drugs documented in northern Maputaland require metabolic activation through CYP3A4, beta-glucuronidase and carboxylesterase enzymes for biotransformation for successful management of hypertension. Thus the aim of this chapter was to determine if medicinal plants documented to be used for the treatment of hypertension in northern Maputaland can prevent the biotransformation of hypertension conventional drugs by inhibiting the enzymes involved (CYP3A4, beta-glucuronidase and carboxylesterase) in the metabolism of conventional hypertension drugs. Aqueous extracts were used because it is the common way in which all plants were prepared (De Wet et al., 2016). Several *in vitro* assays targeting drug metabolism have been proposed as appropriate methods to assess the potential for drug interaction thereby providing a strategy to evaluate the safety of medicinal plant extracts. *In vitro* methods were chosen over the *in vivo* methods because they are simple, easy to carry out, dependable and provide evidence about potential interactions (Patil et al., 2014).

Table 4.1 Medicinal plants which were documented to be used for the treatment of hypertension along with conventional drugs as recorded by De Wet et al. (2016).

Plant species	Conventional drugs
Aloe marlothii	Ridaq Aspirin Amlodipine Pharmapress
Cannabis sativa	Pharmapress Aspirin

Plant species	Conventional drugs
Catharanthus roseus	Aspirin Pharmapress Ridaq
Carpobrotus dimidiatus	Aspirin Ridaq
Hypoxic hemerocallidea	Ridaq Aspirin Pharmapress
Musa acuminata	Ridaq Amlodipine Aluminum hydroxide Adcon napamol
Momordica balsamina	Ridaq Aspirin Amlodipine Enalapril Pharmapress
Psidium guajava	Aspirin Ridaq
Pyrenacantha kaurabassana	Ridaq Aspirin Pharmapress Enalapril
Senecio serratuloides	Aspirin Ridaq Pharmapress
Sarcophyte sanguinea	Pharmapress Enalapril Amlodipine
Strychnos madagascariensis	Amlodipine Ridaq Pharmapress Aspirin

Plant species	Conventional drugs
Ricinus communis	Aspirin Ridaq Amlodipine

Table 4.2 Conventional drugs which were reportedly used by the people surveyed by De Wet et al. (2016), along with their drug class and the enzymes involved in their metabolism.

Conventional drugs	Drug class	Process of elimination by the body	Enzymes involved
Adcon napamol	Anti-pyretic and anti-analgesics	Absorbed and rapidly eliminated by kidneys	No enzymes involved
Amlodipine	Calcium channel blockers	Metabolised in liver	CYP3A4
Aluminum hydroxide	Anti-acids	Absorbed and rapidly eliminated by kidneys	No enzymes involved
Aspirin	Platelet aggregation inhibitors	Metabolised in stomach, intestinal mucosa, blood and the liver	UDP-glucuronosyltransferase 1A6, CYP2C9, N-acetyl transferase 2 and Carboxylesterase 2
Enalapril	Angiotensin 11 receptors blocker(Ace) inhibitors)	Metabolised in the liver	Carboxylesterase 1
Pharmapress	Non-sulfhydryl angiotensin converting enzyme inhibitors (Ace inhibitors)	Metabolised in the liver	Carboxylesterase 1
Ridaq	Diuretics, thiazide and derivatives	Not metabolised but rapidly eliminated by the kidneys	No enzymes involved

4.2 Methods

Thirteen of the medicinal plants (Table 4.1) reported to be used along with conventional hypertension drugs and these were investigated for potential *in vitro* herb-drug interactions. The following three *in vitro* assays were used: CYP3A4 inhibition assay, β-glucuronidase inhibition assay and the carboxylesterase inhibition assay.

4.2.1 CYP3A4 inhibition assay

The Vivid® CYP450 fluorescent screening kit was used according to the manufactured instructions (Life Technologies) to evaluate the potential CYP3A4 drug inhibition. The ability of plant extracts to inhibit the activity of human cytochrome P450 enzyme was determined as described within the kit protocol. The aqueous plant extracts were prepared to the concentration of 10 and 100 µg/ml using dimethyl sulfoxide (DMSO). The positive control used was ketoconazole [230 nanometre (nM)] which is a known inhibitor. The CYP3A4 baculosomes and regeneration system were mixed gently after thawing. The test compounds, solvent control and positive control were prepared at 2.5 times the test concentration within the 1x CYP450 reaction buffer. The substrate was reconstituted using acetonitrile while the fluorescent standard was reconstituted using DMSO. The CYP3A4 reaction buffer was diluted with nano-pure water to a desired concentration of 100 nM. This buffer was then used for the preparation of standards, inhibitors, master pre-mix and substrate solutions. Forty microliters of the plant extract, solvent control and positive control (ketoconazole) was added to a 96 well plate in triplicates.

The master pre-mix was prepared by adding 4850 µl of reaction buffer along with 100 µl of regeneration system as well as 50 µl of CYP3A4 baculosomes. This solution was made up to a concentration of 10 nM. Fifty microliter of master pre-mix was added to each well. The plate was then incubated at room temperature for 10 min to allow the compounds to interact with CYP3A4 in the absence of enzyme turnover (substrate). While the plate was incubated, 10x mixture of a vivid substrate and $NADP^+$ was prepared. This was prepared by adding 885 µl of reaction buffer and 15 µl of reconstituted substrate as well as 100 µl of vivid $NADP^+$. After 10 min of

incubation, the reaction was initiated by adding 10 µl of 10x substrate and $NADP^+$ mixture to each well. The plate was then incubated again at room temperature for 30 min. After 30 min, the reaction was terminated by adding 10 µl of STOP the reagent known as 0.5 Tris base. The fluorescence was measured using a fluorescent plate reader (Biotech® Synergy MX fluorimeter) at an emission of 590 following excitation at 550 NM.

4.2.2 β-glucuronidase inhibition assay

Plant extracts (aqueous) were tested at concentrations of 10 and 100 µg/ml. The positive control used was amoxapine at the concentrations of 1 µM. One microliter of the plant extract was added to 999 µl of PBS (phosphate buffer). The plates were prepared by adding 10 µl of the control (amoxapine), 10 µl of plant extract (diluted in buffer), 10 µl of buffer as well as 40 µl of the enzyme. The plates were incubated for 30 min at 37°C. After 30 min, the reaction was initiated by adding of 50 µl of a 5 mM p-NPG solution and the plates were incubated again at 37°C for 30 min. The reaction was stopped by adding 5 µl of 2 N sodium hydroxide solution to each well and the absorbance was read at 405 nm. Negative control was not used in the current assay.

4.2.3 Carboxylesterase inhibition assay

Plant extracts (aqueous) were tested at the concentrations of 10 and 100 µg/ml. The positive control used was Bis [p-nitrophenly] phosphate (BNPP) at the concentrations of 100 µM. Ten microliters of the test sample (diluted in a buffer 50 mM tris-HCl with pH8), 10 µl of buffer, 10 µl of positive control (BNPP) and 50 µl of recombinant human CES1 (20 µg/ml) were pipetted into the 96 well plates. The plates were then incubated for 10 min at room temperature. After incubation, the reaction was initiated by adding 50 µl 2 mM p-nitrophenyl acetate solution. The absorbance was measured in kinetic mode at 410 nm for 10 min at one minute intervals. Progress curves were constructed and the linear portion of the curve was used to calculate the reaction rate. Negative control was not used in the current assay.

4.3 Results and discussion

Results from the 13 aqueous plant extracts are summarised in Figures 4.1, 4.2 and 4.3. All three assays were done in triplicate. Plant samples with inhibition less than

10% were considered weak inhibitors as the sensitivity of the assays at such low inhibition is poor (represented by the black line). Inhibition between 11% and 49% were considered as moderate inhibitors of the enzymes where inhibition of 50% and above was considered to be strong inhibitors. The extracts were tested in two concentrations (10 and 100 µg/ml) for the comparison of dosages. The results of the two concentrations tested indicated that in most of the plant extracts tested, the inhibition of the enzymes by the plant extracts was dosage dependent. When the concentrations of the plant extracts were 100 µg/ml, the inhibitory percentage was higher compared to the percentage obtained when the concentration was 10 µg/ml. An exception is *M. balsamina* and *S. sanguinea* towards the enzyme beta-glucuronidase. It showed no dosage dependence (Figure 4.2) as the inhibitory effect was higher at lower concentration (10 µg/ml) compared to a higher concentration (100 µg/ml). None of the extracts tested for beta-glucuronidase inhibition showed to have negative values (no inhibition of the enzyme). *Momordica balsamina*, *S. serratuloides, P. kaurabassana* and *H. hemerocallidea* revealed negative values in the carboxylesterase and CYP3A4 inhibition assays, thus these plant extracts did not demonstrate meaningful inhibition of the enzymes.

4.3.1 CYP3A4 inhibition assay

The results of the 13 plant samples that were investigated for potential inhibition of CYP3A4 are depicted on Figure 4.1. The positive control ketoconazole is a clinical drug that has been widely used for the treatment of fungal infections (Heeres et al., 1979). This drug has been reported to inhibit cytochrome P450 enzymes especially the CYP3A family which consist of CYP3A4, CYP3A5 and CYP3A7 (Allqvist et al., 2007). The inhibition of the enzyme with the positive control was 88% at 10 µg/ml and 93% at 100 µg/ml. *Sarcophyte sanguinea* (97%) and *P. guajava* (97%) have inhibitory effects which was higher than the positive control (93%) at the concentration of 100 µg/ml, and thus are regarded as strong inhibitors of the CYP3A4 enzyme. *Ricinus communis* extract showed the least inhibitory activity (<10%) at both concentrations, and is considered to be a weak inhibitor. *Strychnos madagascariensis, C. sativa, P. kaurabassana, C. dimidiatus, P. guajava, A. marlothii* and *S. sanguinea* samples have noticeable higher inhibition activities at 100 µg/ml (Figure 4.1). When these seven plant extracts were tested at a lower concentration (10 µg/ml), *S. sanguinea* (82%) still had a high inhibitory effect. The

other six plants are dosage dependant. *Momordica balsamina, S. serratuloides* and *H. hemerocallidea* had negative values at the lower concentration tested (10 µg/ml). Negative values may indicate that the catalytic activity is increased. One should keep in mind that the inhibition of the activity of the enzyme is determined as the difference between the activity of the enzyme in the presence and absence of the plant extract. Thus when the differences are very small, the difference extends below sensitivity limits of the assay and one cannot determine an accurate response. Therefore the negative values are the indication that there is no meaningful inhibition of the enzyme.

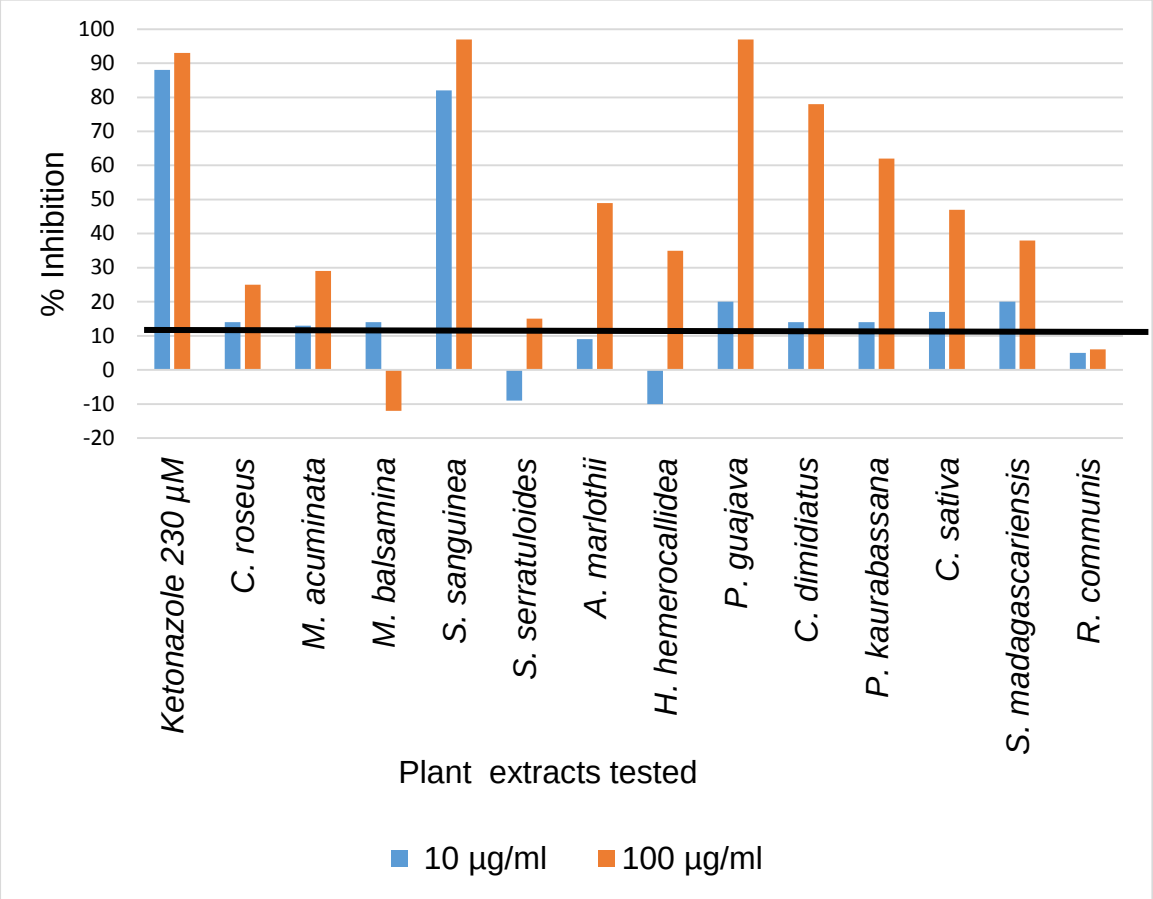

Figure 4.1 Inhibitory percentage of test samples on CYP3A4 activity.

4.3.2 β-Glucuronidase inhibition assay

The results of the 13 medicinal plants which were investigated for potential beta-glucuronidase inhibition assay are depicted in Figure 4.2. Amoxapine was used as the positive control. This is a tricyclic debenzoxazepine compound which is used as

an antidepressant drug (Ban et al., 1980). This drug has been reported to inhibit the activity of β-glucuronidase enzymes by binding at the active site of β-glucuronidase enzymes (Kong et al., 2014). All the plant extracts in this study were investigated for the first time for β-glucuronidase inhibitory effects. The positive control (Amoxapine) had an inhibitory effect of 55% at 10 µg/ml and 82% at 100 µg/ml. The following inhibitory activity (>10%) of the plant extracts was observed in *A. marlothii* (43%), *H. hemerocallidea* (25%), *P. guajava* (32%) and *R. communis* (26%) at the concentration of 100 µg/ml. Though, when *P. guajava* and *R. communis* were tested at a lower concentration (10 µg/ml) their inhibitory effect reduces drastically to less than 10%. *Cannabis sativa, P. kaurabassana, M. balsamina* and *M. acuminata* had an inhibitory effect of less than 10% on the enzyme at both concentrations, and therefore regarded as weak inhibitors. *Sarcophyte sanguinea* revealed an inhibitory effect of 15% at 10 µg/ml and 12% at 100 µg/ml. No explanation could be found in the literature to explain why the lower concentration (10 µg/ml) appears to have a stronger inhibitory effect on the enzyme than the higher concentration (100 µg/ml). Since most of the extracts inhibited beta-glucuronidase, this support their traditional use concurrently with hypertension conventional drugs as the inhibition of this enzyme improves the antihypertensive efficacy.

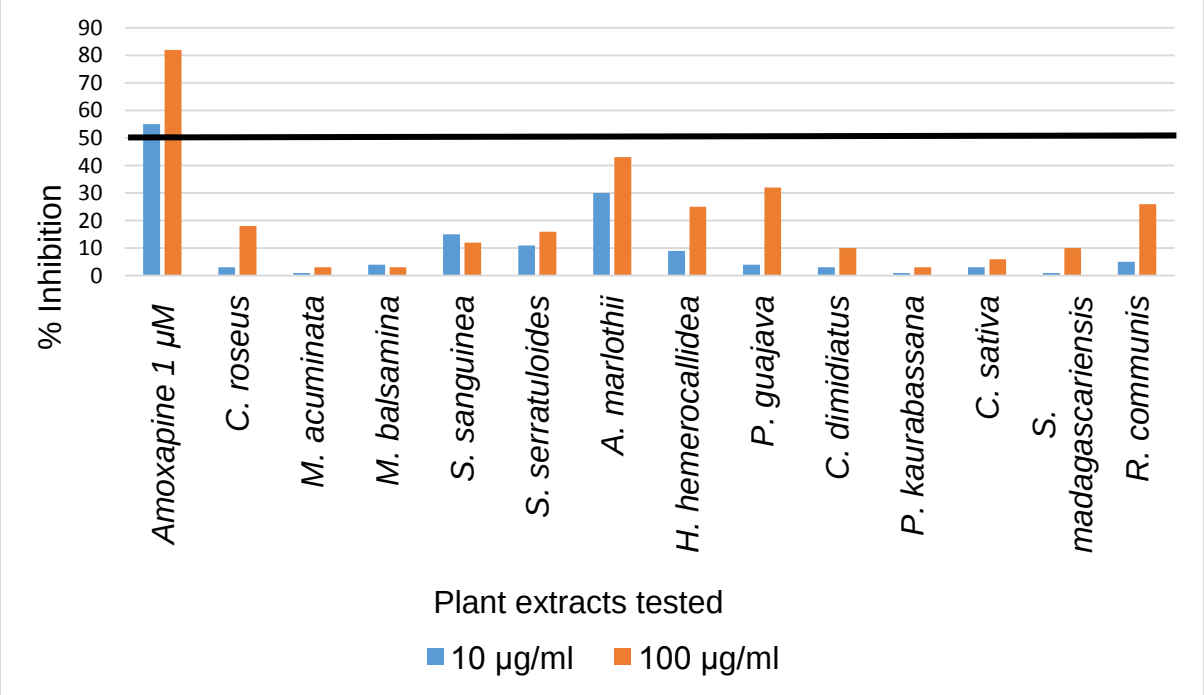

Figure 4.2 Inhibitory percentage of test samples on β-glucuronidase activity.

4.3.3 Carboxylesterase inhibition assay

The results of the 13 medicinal plants which were investigated for potential carboxylesterase inhibition are depicted in Figure 4.3. Bis [p-nitrophenly] phosphate (BNPP) was used as the positive control. Bis [p-nitrophenly] phosphate has been known for its inhibitory effect of carboxylesterases. After the splitting off of the P-nitrophenol, the mono-p-nitrophenyl phosphate residue binds to the serine hydroxyl at the active site of carboxylesterases (Block and Wassermann, 1978). This organophosphate acts as an irreversible inhibitor of the carboxylesterase enzymes resulting in the generation of a steady phosphate ester covalently joined to the catalytic serine reside available within the enzyme active site (Hatfield and Potter, 2012). All the extracts that were investigated for potential carboxylesterase inhibition have not been recorded previously for any inhibition of carboxylesterase enzyme in the literature. The inhibitory effect of the positive control was 75% at 10 µg/ml and 80% at 100 µg/ml. The plant extract which inhibited the enzyme the most was *S. sanguinea* with the inhibitory percentage of 59% at 100 µg/ml and 38% at 10 µg/ml. The lowest inhibitory effect was observed on *C. roseus* with the inhibition of 9% at 100 µg/ml and 2% at 10 µg/ml. *Catharanthus roseus* and *M. acuminata* had less than 10% inhibitory activity at the concentration of 100 µg/ml (Figure 4.3), they were then considered weak inhibitors. *Sarcophyte sanguinea* showed a strong inhibition at both concentrations, while *P. guajava*, *C. dimidiatus*, *C. sativa* and *R. communis* also revealed inhibition at 100 µg/ml but not much inhibitory activity at 10 µg/ml and are thus considered dosage dependant. While a number of plant extracts (*M. balsamina, S. serratuloides, A. marlothii, H. hemerocallidea* and *P. kaurabassana*) yielded negative values the majority of them were only marginally greater than the mean standard deviation and are thus taken to represent no inhibition. The negative values obtained for samples may indicate activation of the enzyme, however, further studies are needed to confirm this. *Hypoxis hemerocallidea, M. balsamina* and *P. kaurabassana* have negative values in both concentrations, thus these extracts did not inhibit beta-glucuronidase at both concentrations. There seems to be a relationship between concentration and inhibitory activity in the majority of plant extracts tested against this enzyme.

Some of the plant extracts tested in the current study have been previously investigated for herb-drug interaction; however, most of the studies concentrated on

the pharmacodynamic interaction and have focused on different targets (enzymes and transporters). To the best of my knowledge, *H. hemerocallidea*, *C. roseus* and *R. communis* are the only three medicinal plants that were found in the literature that has been evaluated for CYP3A4 inhibition. Water and methanol extracts of *H. hemerocallidea* was reported to inhibit up to 86% of the normal CYP3A4 isoform activity at the concentration of 100 µg/ml with an increased inhibition on methanol extracts (Mills et al., 2005). Another study done by Mogatle et al. (2008) has evaluated the pharmacokinetic effect of *H. hemerocallidea* on efavirenz (antiretroviral drug). The study was done on ten healthy volunteers. From day one they were given a 600 mg efavirenz tablet and from day 16 the volunteers were given *H. hemerocallidea* aqueous extract at the concentration of 15 mg/kg until day 30. On day 29, volunteers were given a single dose of efavirenz. The results showed that the *H. hemerocallidea* extract did not significantly alter the pharmacokinetic of efavirenz.

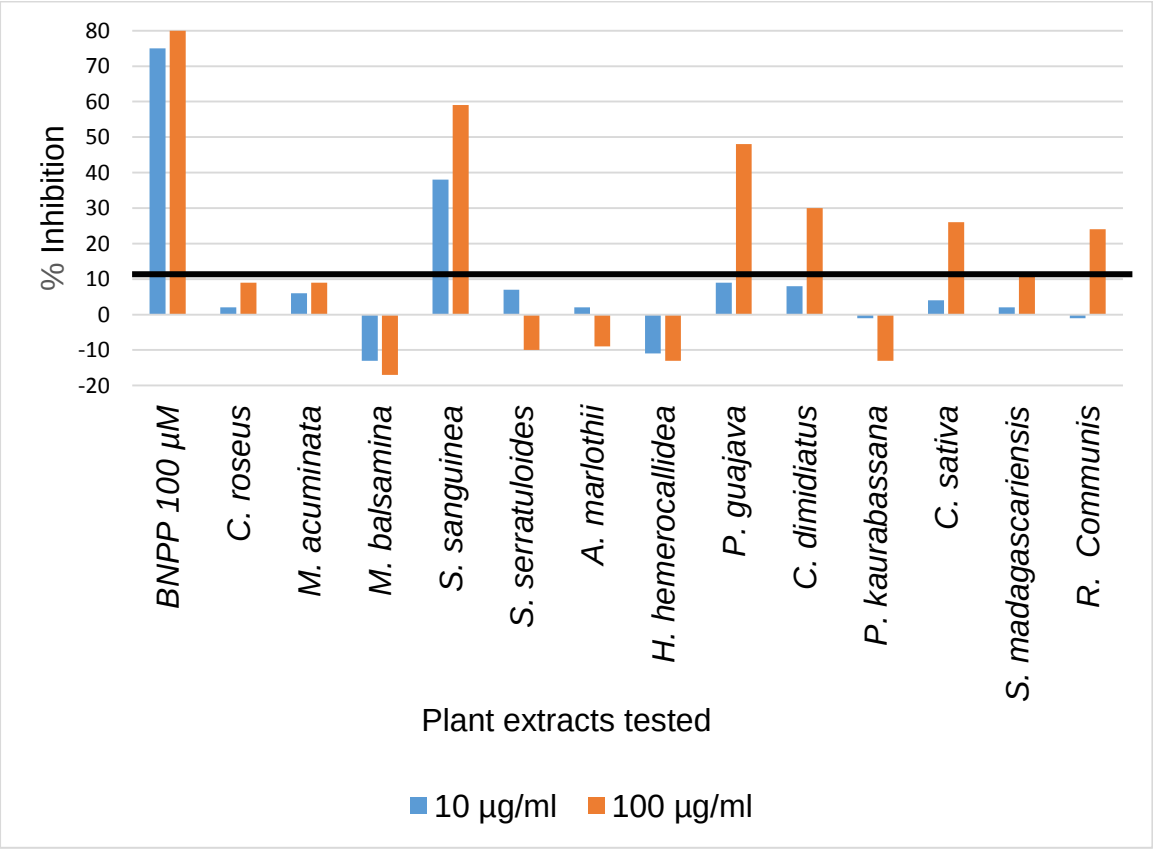

Figure 4.3 Inhibitory percentage of test samples on carboxylesterase activity.

Nair et al. (2007) has done a study on the pharmacokinetic interaction of *H. hemerocallidea* extract and its compounds (norlignan glycoside, hypoxoside, sterols,

beta-sitosterol, stigmasterol, stanol and stigmastanol) on the inhibition of cytochrome P450 enzymes (CYP3A4, CYP3A5, CYPA9 and CYP19). The study also examined the potential effect on drug transporting proteins (P-glycoprotein). The study was assessed using the fluoremetric microtitre plate assay and caco-2 cell line at the concentration of 10 µl/ml. The results showed that the extracts of *H. hemerocallidea* and some of its compounds (stigmasterol and norlignans) inhibited CYP3A4, CYPA9 and CYP19. The extract of *H*. *hemerocallidea* and the compound hypoxoside showed a high induction of P-glycoprotein. *Hypoxis hemerocallidea* aqueous extract was also reported to have strong inhibition on the enzymes CYP1A2 and CYP3A4 (Fasinu et al., 2013a).

According to the study done by Usia et al. (2006), the aerial parts of *C*. *roseus was* extracted with methanol and water and tested at the concentration of 1.65 mg/ml with the radiometric assay (CYP inhibition assay). The extract showed an inhibition of 50% on CYP3A4 and an inhibition of 94% on the CYP2D6 enzyme. In another study done by Usia et al. (2005), the two compounds isolated from *C. roseus* (ajmalicine and serpentine) showed very strong inhibitory activity against CYP2D6. However, these compounds did not show inhibitory activity against CYP3A4. The third plant that has been previously investigated for CYP3A4 inhibition was *R. communis*. A fluorometric enzyme inhibition assay was undertaken to predict the inhibitory effect of CYP3A4 by the methanol extract of *R. communis*. The results showed that the plant had a minimal inhibitory effect on the enzyme (Lee et al., 2007). A herbal mixture known as Canova, which is used in homeopathy comprises of *Ricinus communis*, *Aconitum napellus* L., *Bryonia alba* L., *Apis mellifica* (from the honeybee), *Lycopodium clavatum* L., *Pulsatilla nigricans* Mill., *Rhus toxicodendron* Mill., *Conium maculatum* L., *Veratrum album* L., *Carapichea ipecacuanha* (Brot.) L. Andersson, *Lachesis muta* L. and *Thuja occidentalis* L. has also been investigated for inhibitory effects against indinavir (antiretroviral drug) in terms of the efflux and metabolism modulation. The results showed that there was a pronounced inhibition of metabolism of indinavir which may result in increased bioavailability of indinavir after concurrent oral administration (Calitz et al., 2015).

Other medicinal plants that have been previously investigated for pharmacodynamic and pharmacokinetic interaction with different targets are *A. marlothii, C. sativa* and *P. guajava*. The gel of *A. marlothii* has been reported to inhibit the efflux transporter,

cimetidine. The study was conducted on rat intestinal tissue, where 50 mg of *A. marlothii* gel was dissolved together with 5 mg of internal standard (nicotini acid amide) in 1 ml of deuterium oxide (Beneke et al., 2013). An *in vitro* pharmacokinetic study was done on the compound delta 9-tetrahydro cannabinol, which is the main component in *C. sativa*. This compound induced the enzyme CYP2C9 resulting in increased metabolism of phenytoin (anti-epileptic drug) (Bland et al., 2005). A pharmacodynamic interaction study was done to evaluate if the aqueous leaf extract of *P. guajava* interact with muscarinic, serotonergic and adrenergic receptor systems. The results showed that the extract had agonistic actions on these systems (Mahaseth et al., 2015). *Psidium guajava* extract has also been reported to show high inhibitory effect on P-glycoprotein which mediated efflux of Rhodamine-123 in caco-2 cells. This may alter the pharmacokinetic of the drug (Junyaprasert et al., 2006).

The six medicinal plants (aqueous extracts) that have not been previously investigated for herb-drug interactions are *C. dimidiatus, H. hemerocallidea, P. kaurabassana, S. sanguinea, S. serratuloides* and *S. madagascariensis*. The herb-drug interaction of the medicinal plants which were frequently used was also analysed. For the CYP3A4 inhibition assay, all the frequently used medicinal plants (*M. acuminata, M. balsamina, A. marlothii, H. hemerocallidea, S. serratuloides* and *S. madagascariensis*) demonstrated an inhibitory effect. For the beta-glucuronidase assay, *M. acuminata, M. balsamina* and *S. madagascariensis* showed weak inhibition while *S. serratuloides, A. marlothii* and *H. hemerocallidea* showed moderate inhibition. For the carboxylesterase inhibition assay, *M. acuminata, S. serratuloides* and *A. marlothii* were shown to be weak inhibitors. *Strychnos madagascariensis* had moderate inhibitory effect, while *M. balsamina* and *H. hemerocallidea* did not inhibit the enzyme. None of the most frequently used medicinal plants demonstrated strong inhibitory effects against all the enzymes tested. Some of the frequently used medicinal plants work against the metabolism of the enzymes (CYP3A4 and Carboxylesterase) which break down prescribed hypertension drugs before they can be absorbed. However, the frequency of the use of medicinal plants tested for beta-glucuronidase inhibition correlate with the traditional uses, as the inhibition of the enzyme by the plant extracts improved the efficacy of hypertension prescribed drugs. Although *S. sanguinea* and *P. guajava*

were strong inhibitors of some enzymes, it should be noted that these medicinal plants were among the plants which were reported to be least used by the people in northern Maputaland to treat hypertension. Plants that show strong herb-drug interaction effects should be considered unsafe and thus they require further analysis before being recommended to be used concurrently with hypertension prescribed drugs.

The overall inhibitory results showed that all of the plant extracts tested had an inhibitory effect on either of the three enzymes. The highest inhibitory effect of the enzymes by the plant extracts was observed in CYP3A4 inhibition assay, with *S. sanguinea* and *P. guajava* having an inhibitory effect of 97%. Even though, the inhibition of beta- glucuronidase by the plant extracts works in favour for the efficacy of antihypertensive drugs, it should be noted that these medicinal plants also inhibited other enzymes (CYP3A4 and carboxylesterase) which are essential for antihypertensive drugs to be metabolised. The daily use of medicinal plants for the treatment of hypertension concurrently with hypertension prescribed drugs should not be encouraged based on inhibitory results found in this study. However, the clarifications of *in vitro* data are not straight forward. Pharmacokinetic factors such as dose, bioavailability of active compounds, dispersal as well as clearance need to be taken into account. It should also be noted that the full domain of drug interaction was not investigated, thus these medicinal plants may have positive effect on other aspects of drug metabolism.

4.5 Summary

- The majority of the plant extracts tested, yielded a strong inhibitory effect on all parameters measured, raising the possibility that these extracts may have negative influence on drug metabolism at various levels.
- The inhibitory effect of enzymes was mostly dosage dependant.
- The highest inhibitory effect of the enzymes by the plant extracts was observed in CYP3A4 inhibition assay, with *S. sanguinea* and *P. guajava* having both an inhibitory effect of 97% which may have negative effect on drug metabolism.
- *Aloe marlothii* (43%), *H*. *hemerocallidea* (25%), *P. guajava* (32%) and *R. communis* (26%) demonstrated inhibitory effect on beta-glucuronidase which

support the concurrent use with hypertension prescribed drugs as the inhibition of the enzyme improves the efficacy of antihypertensive conventional drugs.

Chapter 5

Hepatotoxicity of some medicinal plants used for the treatment of hypertension in northern Maputaland

5.1 Introduction

The liver performs a major role in the maintenance and performance of bile secretion, regulation of homeostasis, storage of vitamins as well as detoxification of the body (Saleem and Naseer, 2014). This organ is one of the most important parts of the body and appears to be a sensitive target site for substrate modulating biotransformation (Roy et al., 2014). During the process of metabolism of xenobiotics the reactive metabolites such as peroxides, epoxides and other radicals can cause damage to the liver (Dey et al., 2013). Hepatotoxicity symptoms may include dark urine, severe abdominal pains, light coloured stools, severe fatigue, skin rashes, swelling of the feet and continuous bleeding (Singh et al., 2011). Some of the xenobiotics have been implicated to cause liver toxicity and abnormalities (Manfo et al., 2014). Hepatotoxicity due to conventional drugs is widely acknowledged and studied; therefore patients prefer alternative natural treatments (such as medicinal plants) believing that the side effects are less (Stickel et al., 2000). However, previous studies have demonstrated that some medicinal plants [*Tithonia diviersifolia (*Hemsl.) A. gray, *Crossopteryx febrifuga (*Afzel. ex G. Don) Benth., *Pteleopsis hylodendron* Mildbr*., Microglossa pyrifolia* DC., *Gentiana scabra* Bunge and *Vernonia lasiopus O. Hoffm.*] have hepatotoxic potential (Rucker et al., 1994; Elufioye and Agbedahunsis, 2004; Elufioye et al., 2009; Mukazayire et al., 2010; Nana et al., 2011). Thus, hepatotoxicity associated with the usage of medicinal plants cannot be completely ignored. It is important to evaluate all medicinal plants for hepatotoxic potential.

Various model systems have been developed to evaluate hepatotoxicity. These models include microsomes, cancerous/immortalised cell lines, isolated primary human liver cells, liver slices and humanised rodents as well as *in vivo* assays (Lin and Khetani, 2016). The assay which was employed to evaluate the potential hepatotoxicity of medicinal plants in the current study is the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay using HepG2/C3A cells. Hepatic cell lines are the closest representation of human liver physiology (Lin and Khetani,

2016). The assay has the advantage of being reliable, well-controlled variables and it is accepted as an effective method for safety evaluation (Saad et al., 2006). HepG2 cells retain many of the morphological characteristics of the liver parenchymal cells and these cells also contain various enzymes that are responsible for the activation of various xenobiotics (Senthilraja and Kathiresan, 2015). The data obtained can be used to determine the appropriate doses for *in vivo* studies (Singh et al., 2011). The MTT assay (Mosmann, 1983), is a quantitative, sensitive and reliable colorimetric assay which measures proliferation, viability and activation of the cells. This assay is based on the ability of mitochondrial dehydrogenase enzymes in the cells to convert the yellow water-soluble substrate into a dark blue formazan product which is not soluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis. The cells that are dead because of toxic damage cannot transform MTT. The quality of formazan is measured by recording changes in absorbance at 560 nM using a plate reading spectrophotometer. When cells are dead they lose viability to convert MTT into formazan, thus the formation of colour serves as a useful and convenient marker of the viable cells (Fotakis and Timbrell, 2006; Senthilraja and Kathiresam, 2015). However, metabolic activity may be changed by different conditions or chemical treatments which can cause considerable variation in results reported from these assays (Wang et al., 2010).This one assay on one cell line, albeit a liver cell line with many enzyme functionalities retained, but does not provide a compressive representation of hepatotoxicity of test samples.

The aim of this chapter is to evaluate the potential hepatotoxic effect of medicinal plants which were recorded to be used in northern Maputaland concurrently with hypertension prescribed drugs. The thirteen medicinal plants which were investigated for herb-drug interactions were further analysed for hepatotoxicity because the metabolism of most conventional drugs reported are metabolism in the liver. Thus it was necessary to evaluate if the medicinal plants can damage the liver, without any drug-herb interaction involved.

5.2 Methods

Thirteen medicinal plants (aqueous extracts) that were investigated for herb-drug interactions were further evaluated for their hepatotoxicity potential. For hepatotoxicity, the MTT assay (human hepatocarcinoma cell line HepG2/C3A) was used. This method has been successfully used by Saad et al. (2006), Senthilraja and Kathiresan (2015) and Tukappa et al. (2015). The plant extracts were prepared to the concentration of 100 000 µg/ml using 10% of dimethyl sulfoxide (DMSO). The human tumour cell line HepG2/C3A was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum. For a trial experiment, cells were seeded into 96 well microtiter plates at a density of 6 000 cells/well using a volume of 100 µl in each well. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO_2, and 100% relative humidity for approximately 24 hrs prior to addition of test samples. Before the test samples were added, each sample was diluted to double the desired final maximum test concentration with complete medium and additional dilutions were made as indicated in the results. Aliquots of 100 µl of the dilution (100 µg/ml) was added to the appropriate microtiter wells already containing 100 µl of medium, resulting in the required final extract concentrations. Following the adding of test samples the plates were incubated for a further 48 hrs at 37°C, 5% CO_2, in a humidified incubator. At the end of this incubation period the medium was removed from the adherent cells and replaced with fresh culture medium containing MTT at a final concentration of 0.5 mg/ml. The 96-well plates were returned to the incubator and incubated for an additional 3 hrs after which the medium was removed and the MTT crystals solubilised in DMSO. The absorbance was measured at 560 NM using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). The negative control was the untreated HepG2/C3A cells and the positive control was melphalan which was prepared to the concentration of 70 µM. Three replicates were undertaken and these assays were repeated twice.

5.3 Results and discussion

5.3.1 Hepatotoxicity analysis of 13 individual plant samples

Hepatotoxicity potential of 13 medicinal plants which were documented to be used concurrently with hypertension prescribed drugs to treat hypertension was

determined. The assay was done to gain insight into whether or not the plant extracts used individually for the treatment of hypertension are toxic to the human liver cells irrespective of the drug-herb effect. The results are recorded in Figure 5.1. Cell viability was determined using the MTT assay and results expressed relative to the untreated control (negative control). Positive control, melphalan resulted in 100% cell death after 1 hr of exposure. While the negative control (cells which were not treated) resulted in 1% cell death. A cell death which was less than 10% was considered as negligible, while between 11% and 49% was regarded as moderately cytotoxic and 50% and above was considered to be relatively toxic at the concentration of 100 µg/ml (Kadan et al., 2013). The overall hepatotoxicity profile indicated that *S. sanguinea* caused a 69% cell death when tested at the concentration of 100 µg/ml, and thus had a potential to be toxic and therefore dosages must be given with caution. *Catharanthus roseus* (42% cell death), *P. kaurabassana* (40%), *H. hemerocallidea* (30%), *C. sativa* (20%) *M. acuminata* (18%) and *S. madagascariensis* (12%) extracts demonstrated moderate cytotoxicity. The plants [*A. marlothii* (7%), *C. dimidiatus* (9%) *S. serratuloides* (9%), *P. guajava* (7%), *M. balsamina* (2%) and *R. Communis* (1%)] have a cell death less than 10% and were considered to be non-cytotoxic to the liver.

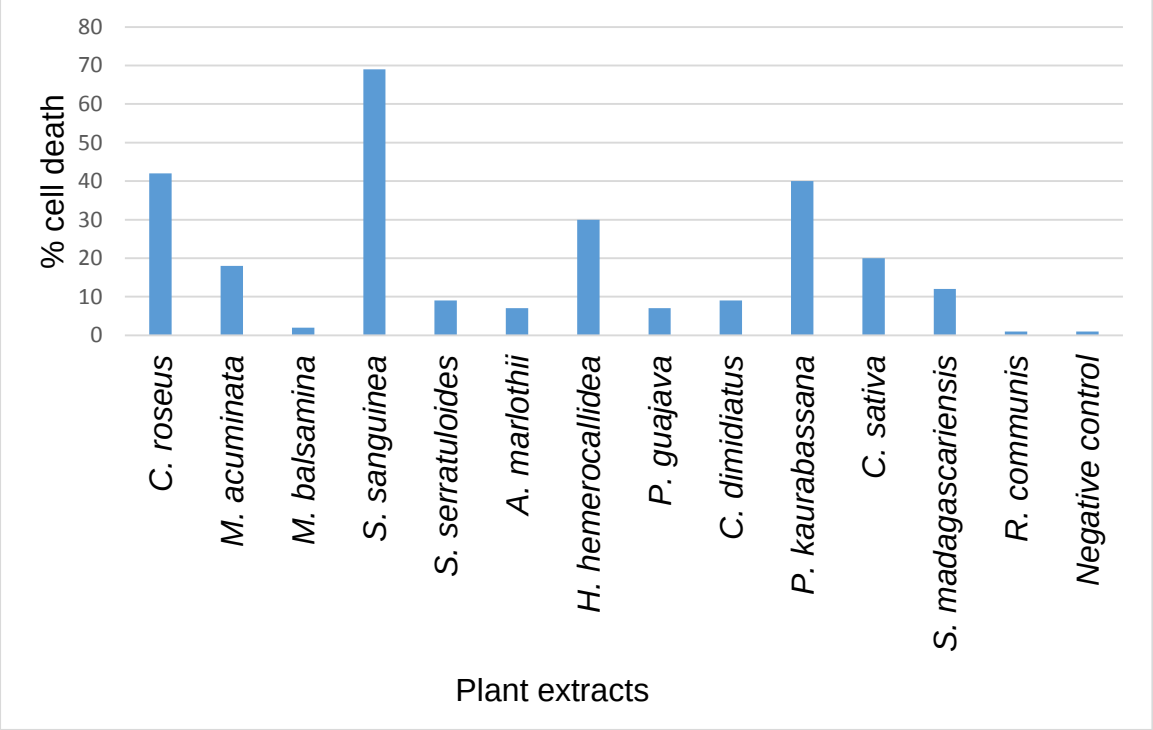

Figure 5.1 Cytotoxicity in HepG2/C3A cells treated for 48 hrs.

Some of the plant extracts investigated in the current study have been previously investigated for hepatotoxicity and hepatoprotective potential. To the best of my knowledge, nothing could be found in literature regarding the hepatotoxicity or hepatoprotective effect of *C. dimidiatus*, *H. hemerocallidea*, *M. acuminata*, *S. sanguinea*, *S. madagascariensis*, *S*. *serratuloides* and *P. kaurabassana*. Medicinal plants from this study that have been previously investigated for hepatotoxicity and hepatoprotective potentials are *C. roseus, M. balsamina, P. guajava, C. sativa* and *R. communis*. The difference between hepatotoxicity and hepatoprotective is that hepatotoxicity is the injury to the liver caused by the xenobiotics whereas hepatoprotective is the ability of xenobiotics to prevent damage to the liver (Mohit et al., 2011). The ethanolic leaves extract of *C. roseus* has been investigated for hepatoprotective activity against the drug simvastation which induced hepatotoxicity. The study was done on albino Wistar rats at the plant concentration of 300 and 500 mg/kg. The results showed that there were a significant decrease of the elevated serum marker enzymes and reversed the altered total protein to normal levels, indicating a hepatoprotective effect of *C. roseus* extract (Rao and Fazil, 2013). However, even though the ethanolic leaf extract of *C. roseus* was reported to have hepatoprotective effect, in the current study, the aqueous root extract was found to have the moderate hepatotoxicity. The different results are possible because of the different plant part tested and the use of different solvents. Thus caution must be taken when using *C. roseus* roots.

The ethanol extract of *M. balsamina* leaves have also been evaluated for hepatoprotective activity in wistar albino rats. The study was done on hepatotoxicity rats which were induced by carbon tetrachloride (CCl4). The rats showed a significant increase of transaminases and alkaline phosphate level due to hypocytes damage. When *M. balsamina* was administered at the concentration of 250-500 mg/kg the results showed a decrease in transaminases indicating a hepatoprotective effect against CCl4 (Alqasoumi et al., 2009). In the current study, *M. balsamina* showed no hepatotoxicity, which is good as it is the most used plant to treat hypertension in northern Maputaland.

Several studies have also been done on the hepatotoxicity and hepatoprotective effect of *P. guajava*. This effect of *P. guajava* leaves was evaluated in clone nine liver cells. For hepatotoxicity the clone nine liver cells were treated with *P. guajava*

extracts (acetone, ethanol and water) at the concentration of 50, 100, 200, 400, 500 and 600 µg/ml for 24 hrs. After 24 hrs, ethanol and acetone extracts in higher concentration showed cytotoxic effects on the clone nine liver cells which were shown by reduced cell viability. No hepatotoxicity effect was observed on the aqueous extract at the concentration of 600 µg/ml or lower. These results correlate with the results which were found in the current study, as the aqueous extract of *P. guajava* leaves was found to be non-hepatotoxic at the concentration of 100 µg/ml. The alanine aminotransferase (ALT) was also measured. The results showed that ALT increases as the concentration of the extracts increases. Thus the damage of the cells was dosage dependent. For the hepatoprotective effect on alcohol induced clone nine cells, the cells were treated with *P. guajava* leaves extracts for 24 hrs. After 24 hrs, 5% of ethanol was added for 30 min to induce injury. The results revealed the hepatoprotective effect of *P. guajava* at the concentration of 100 µg/ml and lower concentrations for the ethanol extracts. While for the aqueous extracts, all the concentrations had hepatoprotective effect (Chen et al., 2011). Another hepatoprotective study of *P. guajava* was done by Mello and Rana (2010). The ethanolic extract of *P. guajava* leaves was evaluated for hepatoprotective effect on paracetamol induced hepatic damage in albino rats by measuring the serum parameters. The results showed a hepatoprotective effect of *P. guajava* which was observed by the decrease of the level of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alanine aminotransferase (ALT) and bilirubin at the concentration of 200 and 400 mg/kg. A hepatoprotective study of *P. guajava* on liver injury induced by carbon tetrachloride was also reported by Roy et al. (2006). The aqueous extract was tested at the concentration of 500 mg/kg in rats. These results showed reduced elevated serum levels of aspartate aminotransferase, alanine amino transferase, alkaline phosphate and bilirubin. These are the enzymes which increase in levels due to the release of the enzymes from damaged hepatocytes. The histological examination of the liver tissues also supported the hepatoprotective effect of *P. guajava*. Hepatoprotective potential of *P. guajava* was also reported in the study done by Sambo et al. (2009). The aqueous extract leaf was tested at the concentration of 100 mg/kg on albino rats with erythromycin-induced liver damage. The results showed that the extract of *P. guajava* leaves possesses hepatoprotective properties at lower dose and hepatotoxic properties at higher concentrations (300 and 450 mg/kg). Another study

which confirmed the hepatoprotective effect of *P. guajava* was done by Taju et al. (2011). The aqueous extract of *P. guajava* leaves was examined on the Wistar albino rats which were induced by acetaminophen. The administration of 500 mg/kg resulted in a decrease in the level of aspartate amino transferase, alanine aminotransferase, alkaline phosphate and bilirubin which were increased as a result of the administration of acetaminophen. The results of all the studies that have been done on *P. guajava* leaves correlate with the frequency of its use as the plant was non-toxic to the liver and also has a hepatoprotective effect.

Another plant that has been evaluated for hepatotoxicity and hepatoprotective potential is *C. sativa* (seed oil). The study was conducted on female Wistar albino rats. For hepatotoxicity, a daily dose of 0.01, 0.1 and 1 ml/kg was administered orally for four weeks. The results showed hepatorenal lesions generalised by fatty changes in the liver, however, there was no elevated serum levels. For the hepatoprotective potential, the oil was administered at the concentration of 0.2 ml/kg for 10 days. The results indicated a decrease level of serums AST, ALT, ALP and bilirubin, suggesting hepatoprotective properties of *C. sativa* oil (Musa et al., 2012). Another study by Abdel-Salam et al., (2014) showed the hepatotoxicity and hepatoprotective potential of *C. sativa*. For hepatotoxicity, the seed extract was given at the dose of 10 and 20 mg/kg for two weeks. The results showed that the administration of *C. sativa* to the rats did not have any effect on the liver serum enzymes. However, the extract caused histological liver damage and fibrosis. When *C. sativa* was administered to rats which were hepatotoxicity induced by thioacetamide, serum protein and paraoxonase 1, activity increased. Thus the damage caused by thioacetamide was enhanced by *C. sativa*. Although different plant parts were tested, in the current study, the aqueous leaves extract of *C. sativa* was found to have moderate hepatotoxicity, thus caution must be taken when using this plant as a medicine.

The hepatotoxicity and hepatoprotective effect of *R. communis* has also been evaluated. The hepatotoxicity study was done on *R. communis* seed (extracted with distilled water) on the Wistar albino rats at the concentration of 3.8, 7.6 and 11.4 mg/kg. The extract was administered orally for 30 days. The results showed that the activity of ALT, AST and ALP only increased at higher concentration (11.4 mg/kg). The histological analysis of the liver after three months revealed no cellular death, necrosis or inflammation. Thus this plant was only considered to be hepatotoxic at

higher concentrations (Muhammad et al., 2015). Although different plant parts were tested, the results documented by Muhammad et al. (2015) correlate with the results which were obtained in the current study, as the leaves were non-hepatotoxic at the concentration of 100 µg/ml. Various hepatoprotective studies of *R*. *communis* have also been reported. Ethanol extract of the leaves were administered in carbon tetrachloride-induced liver damage rats at the concentration of 250-500 mg/kg. The results showed that the activities of serum transaminases, liver lipid peroxidation, protein, glycogen and activities of acid and alkaline phosphate in liver induced by carbon tetrachloride were significantly reduced by the treatment of *R. communis* ethanol extract. Histological examination further confirmed the hepatoprotective activity of *R. communis* when compared with carbon tetrachloride induced rats (Sabina et al., 2009). Another study which confirmed the hepatoprotective activity of *R. communis* was done by Padmapriya et al. (2012). The ethanol leaves extract was administered on ketoconazole induced liver damage mice at the concentration of 100 mg/kg. The degree of protection was measured by performing various liver functioning tests. The results showed a moderate reduction in hepatic enzymes, suggesting hepatoprotective properties. Another hepatoprotective study was done by Naveen et al. (2003). Aqueous leaves extract was tested for hepatoprotective effect on wistar rats where liver damage was induced by carbon tetrachloride. The results showed a moderate protective effect with lower serum levels (AST and ALT) which were increased as a result of hepatotoxic effect of carbon tetrachloride. The results obtained correlate with its traditional use, as the plant was found to be non-toxic and hepatoprotective.

Even though some of the plants have been subjected to hepatotoxicity and hepatoprotective tests, most of the studies focused only on testing the organic extracts (Roy et al., 2006; Alqasoumi et al., 2009; Sabina et al., 2009; Padmapriya et al., 2012; Rao and Fazil, 2013) instead of the aqueous extracts. The previous studies also focused on different plant parts and with different assays. Among the plants that has never been tested for hepatotoxicity potential, *P. kaurabassana, S. madagascariensis* and *H hemerocallidea* were found to have moderate hepatotoxicity effect while *S. sanguinea* showing to have a relative high hepatotoxicity effect. This emphasises the importance of screening all the medicinal plants that have not been subjected for hepatotoxicity testing. Some of the medicinal

plants (*M. balsamina, S. serratuloides* and *A. marlothii*) which were frequently used by the lay people were found to be non-hepatotoxic. The frequency of use of *M. acuminata, H. hemerocallidea* and *S. madagascariensis* which showed moderate hepatotoxicity could induce long term health problems. *Sarcophyte sanguinea* was the only medicinal plant that showed relatively high hepatotoxicity. However, this plant was documented to be one of the medicinal plants which are not used by many people (3 out of 100 interviewed) to treat hypertension. To prevent serious damage to their liver people should not use this plant at all as a remedy.

5.4 Summary

- Seven medicinal plants (*S. sanguinea, S. serratuloides, M. acuminata C. dimidiatus, S*. *madagascariensis, P. kaurabassana and H. hemerocallidea*) were evaluated for hepatotoxicity for the first time.
- The extracts of *A. marlothii, C. dimidiatus, M. balsamina, P. guajava, S. serratuloides* and *R. communis* were non-hepatotoxic at the concentration of 100 µg/ml.
- *Musa acuminata*, *C. roseus*, *H. hemerocallidea*, *P. kaurabassana*, *C. sativa* and *S. madagascariensis* extracts showed to have moderate hepatotoxicity.
- *Sarcophyte sanguinea* have a 69% cell death when tested at the concentration of 100 µg/ml, and thus had potential to damage the liver and therefore dosages must be given with caution.

Chapter 6

General conclusions and future recommendations

6.1 Summary

Various ethnobotanical studies done in northern Maputaland have documented that this rural community still rely greatly on medicinal plants for the treatment of various ailments including hypertension. A recent study done on the use of medicinal plants to treat hypertension (De Wet et al., 2016) revealed that some individuals prefers to use medicinal plants concurrently with hypertension prescribed drugs to enhance efficacy and reduce side effects caused by conventional drugs. However, various studies have raised a concern of the interactions which may occur when conventional drugs are used together with the medicinal plants. The focus of the current study was to validate the safety of medicinal plants individually, in combinations, as well as when they are used concurrently with prescribed drugs to treat hypertension by this rural community.

The extracts were investigated for toxicity potential using the BSLA and Ames test. Plant extracts which were found to be toxic in the BSLA were further analysed in lower concentrations for a dose response. Five of the medicinal plants (*C. kirkii, C. lanatus, H. coriacea*, *P. kaurabassana* and *S. madagascariensis*) investigated in the current study have not been previously documented for any toxicological studies. Individual plant samples were then combined into 19 different combinations as used by the people from northern Maputaland for the treatment of hypertension. These combinations were tested (in equal ratio) in the BSLA and Ames test, and further analysed for toxicity interaction using the ΣFIC index. Thirteen of the individual plant samples which were documented to be used concurrently with hypertension prescribed drugs were further analysed for herb-drug interactions. Three assays were employed, which are CYP3A4 inhibition assay, beta-glucuronidase inhibition assay and carboxylesterase inhibition assay. The thirteen medicinal plants which were investigated for herb-drug interaction potential were further analysed for potential hepatotoxicity. The assay which was employed for hepatotoxicity was the MTT assay (human hepatocarcinoma cell line HepG2/C3A).

6.1.1 Toxicity of the individual medicinal plants

When the plant samples were tested individually for toxicities, it was observed that organic extracts (dichloromethane:methanol) were more toxic than the aqueous extracts. In the BSLA, 17 of the organic extracts were toxic while only two of aqueous extracts were toxic. In the Ames test, six of the organic extracts were mutagenic, while two of the aqueous extracts were mutagenic toward strains TA98 and TA100. Aqueous extracts that demonstrated toxicity are *C. roseus*, *C. limon* and the combination of *O. engleri* with *C. roseus* being toxic in both assays. When the organic and aqueous extracts of *C. roseus* and *C. limon* were further analysed at lower concentrations (0.031, 0.063, 0.125, 0.25, 0.5 and 1 mg/ml), the results showed that the toxicity was dosage dependant. As the concentration decreases, the toxicity also decreased. The two plants were found to be toxic only at the concentration of 1 mg/ml while the other five concentrations tested were non-toxic. People from northern Maputaland should be more cautious in using *C. roseus* as it was also involved in various plant combinations and was also found to be toxic. The results of the 13 aqueous extracts that were further analysed for hepatotoxicity showed that *S. sanguinea* (69% cell death) had hepatotoxicity potential while *M. acuminata* (18%), *C. roseus* (42%), *H. hemerocallidea* (30%), *P. kaurabassana* (40%), *C. sativa* (20%) and *S. madagascariensis* (12%) extracts have moderate hepatotoxicity at the concentration of 100 µg/ml. When analysing the toxicity profile of the most frequently used medicinal plants in treating hypertension (*H. hemerocallidea, A. marlothii, M*. *balsamina, M. acuminata, S. serratuloides* and *S. madagascariensis*), the results showed that all of them were non-toxic when the aqueous extracts were tested. These results support their safety as a water extract remedy. However, the organic extracts of *H. hemerocallidea, M. balsamina* and *S. madagascariensis* were toxic towards BSLA. The aqueous extracts of *M. acuminata, H. hemerocallidea* and *S. madagascariensis* were found to have moderate cytotoxicity in the MTT assay, thus caution must be taken when using these medicinal plants. According to De Wet et al., (2016), *C. limon*, *O. engleri* and *S. sanguinea* were less frequently used which show a relationship between the toxicity results and frequency of usage.

6.1.2 Toxicity of the plant combinations

Nineteen plant combinations were tested for toxicity potential in equal ratios (1:1) using the BSLA and Ames test. For the BSLA, the overall toxicity profile indicated that the organic extracts had a mortality rate of 48% (13 plant combinations) at the concentration of 4 mg/ml and 33% (nine plant combinations) at the concentration of 2 mg/ml. The aqueous extracts had mortality rate of 22% (six plant combinations) at the concentration of 4 mg/ml and 11% (three plant combinations) at the concentration of 2 mg/ml. The overall results of the two tested concentrations showed that toxicity was dosage dependent, the higher the concentration of the plant extracts the more toxic they are. The aqueous extract combinations which were toxic in BSLA are the combinations between *A. delagoensis* and *S. serratuloides, A. marlothii* and *C. roseus*, *H. hemerocallidea* and *C. roseus, M. acuminata* and *C. roseus, M. balsamina* and *C. roseus* as well as the combination between *T. emetica*, *A. marlothii* and *H. coriacea* at the concentration of 4 mg/ml. At 2 mg/ml the combinations which were toxic are the combinations between *C. roseus* and *A. marlothii, C. roseus* and *M. acuminata* as well as the combination between *C. roseus* and *M. balsamina*. The aqueous extract combination between *A. delagoensis* and *S. serratuloides* resulted in an increased of toxicity. Singularly, both extracts were non-toxic but when they are used in combination they were toxic, thus the two plants were working against each other (antagonistic). This is one of the combinations that should be discouraged to use. For the combinations tested in the Ames test, the overall toxicity profile indicated that four plant combinations of the organic extracts had a mutagenic effect while two plant combinations of aqueous extracts had mutagenic effect towards strains TA98 and TA100. The aqueous extracts combinations which were mutagenic are the combinations between *C. roseus* and *H. hemerocallidea* as well as the combination between *M. balsamina* and *C. roseus*. Apart from the combination between *T. emetica*, *A. marlothii* and *H. coriacea*, all the combinations which were toxic involved *C. roseus*. Thus this plant should be discouraged to be used in combination with any other medicinal plant. The aqueous plant extract combinations which were toxic in both assays (BSLA and Ames test) are the combinations between *C. roseus* and *H. hemerocallidea* and the combination between *C. roseus* and *M. balsamina*, and should be discouraged to treat any ailment.

Plant combinations tested were further analysed for a toxicity interaction using the ΣFIC index. In the BSLA, six plant combinations (aqueous) resulted in increased toxicity (antagonistic) and those were the combinations between *A. delagoensis* and *S*. *serratuloides, C. roseus* and *A. marlothii*, *H. hemerocallidea* and *C. roseus, M. acuminata* and *C. roseus*, *M. acuminata* and *C. limon* as well as the combination between *T. emetica*, *A. marlothii* and *H. coriacea*. In the Ames test, *Momordica balsamina* and *C. roseus* as well as *H. hemerocallidea* and *C. roseus* were the only two combinations which had increased toxicity (antagonistic interaction) with the aqueous extracts. Some combinations resulted in reduced toxicity. For the BSLA, four plant combinations (aqueous) which resulted in reduced toxicity are combinations between *H. hemerocallidea* and *A. marlothii*, *M. balsamina* and *A. marlothii*, *P. guajava* and *C. dimidiatus* as well as the combination between *H. hemerocallidea* and *S. serratuloides*. For the Ames test, five combinations which resulted in increased toxicity were the combination between *A. delagoensis* and *S*. *serratuloides, M. balsamina* and *A. marlothii, H. hemerocallidea* and *S. serratuloides*, *M. balsamina* and *M. acuminata* as well as the combination between *S*. *serratuloides, M. acuminata, A. marlothii* and *H. hemerocallidea*. When analysing the toxicity profile of the most frequently used combinations the combination between *C. roseus* and *M. balsamina* were found to be toxic in both assays and therefore the use of this combination should be discouraged. The other most frequently used plant combinations (*A. marlothii* and *H. hemerocallidea*, *A. marlothii* and *M. balsamina* as well as the combination between *H. hemerocallidea* and *S. serratuloides*) were non-toxic in both assays, providing evidence for safety.

6.1.3 Herb-drug interactions

Thirteen medicinal plants which were documented to be used concurrently with conventional hypertension drugs were further analysed for herb-drug interaction potential. Seven of the extracts tested for herb-drug interaction were investigated for the first time. The extracts were tested in two concentrations (10 and 100 µg/ml) for dosage comparison. The results showed that all the plant extracts tested, inhibited either of the three enzymes tested. The results revealed that the inhibition of the enzymes was mostly dosage dependent, when the concentration was higher (100 µg/ml) the inhibition was higher. A higher percentage of the extracts tested in the current study inhibited the CYP3A4 enzyme. When analysing the herb-drug

interaction potential of the most frequently used medicinal plants, for the CYP3A4 inhibition assay, they all showed a moderate inhibition effect (*M. acuminata, M. balsamina, A. marlothii, H. hemerocallidea, S. serratuloides* and *S. madagascariensis*). For the beta-glucuronidase assay, *M. acuminata, M. balsamina* and *S. madagascariensis* showed weak inhibition while *S. serratuloides, A. marlothii* and *H. hemerocallidea* showing moderate inhibition. For the carboxylesterase inhibition assay, *M. acuminata, S*. *serratuloides* and *A. marlothii* showed to be weak inhibitors, while *S. madagascariensis* showed to have a moderate inhibition effect, and *M. balsamina* and *H. hemerocallidea* did not inhibit the enzyme. None of the most frequently used medicinal plants showed to have strong inhibitory effects against any of the enzymes. Those anti-hypertension plants used by the people that inhibited enzymes (CYP3A4 and carboxylesterase) responsible for the metabolism of the hypertension prescribed drugs are thus a health risk to use. However, the medicinal plants tested positive for beta-glucuronidase inhibition correlate with the traditional uses, as the inhibition of the enzyme by these plant extracts improves the efficacy of hypertension prescribed drugs. Although *S. sanguinea* and *P. guajava* were strong inhibitors of some enzymes and can cause a health risk, it should be noted that they are only used by a few individuals interviewed. The findings of this study showed that most of the plant extracts had a negative effect on one or more drug metabolizing enzymes, thus these medicinal plants have potential to affect the efficacy of conventional drugs.

6.2 Future recommendations

Poisonous plants can have an adverse effects on the entire spectrum of the organ systems in humans, thus it is recommended that all the singular plants and combinations tested in the current study should be further evaluated for other types of toxicities such as cardiotoxicity, neurotoxicity and nephrotoxicity among others. There should be an interrelationship of pharmacological and toxicological properties of South African medicinal plants. Of importance is that these medicinal plants should be further analysed for sub-acute toxicity since some of the alkaloids found in medicinal plants are not toxic, but their accumulation can lead to toxicity in the human system. According to the ethnobotanical survey done by De Wet et al. (2016), 54% of the interviewees were elderly people (50 years and above). Elderly people with hypertension are at risk of cardiovascular disease (Abdelhafiz, 2002;

Lionakis et al., 2012). None of the plant extracts investigated in the current study have been tested for any cardiotoxicity. Cardiotoxicity can be defined as the toxicity that affects the heart (Brana and Tabernero, 2010). A significant number of alkaloid and cardiac glycoside containing plants with cardio-activity have been described in the literature. Medicinal plants containing alkaloids that have been implicated in cardiotoxicity includes *Datura ferox* L. (EFSA, 2008) and *Atropa belladonna* L. (Caksen et al., 2003) amongst others. Medicinal plants containing cardiac glycoside that has been implicated in cardiotoxicity includes *Digitalis purpurea* L., *Bowiea volubilis* Harv. ex Hook.f. subsp *volubilis* and *Drimia sanguine* L. among others (Van der Bijl and Van der Bijl, 2012).

Further research should also focus on the concentration level where the toxicity effect starts, thus determining the dosage for clinical use, as all natural products can be potentially toxic if ingested in large quantities. All the substances have an LD_{50} which is referred to as the dose. To evaluate toxicity, the LD_{50} is compared to the therapeutic dose. If the LD_{50} is less than the average therapeutic dose, then toxicity occurs before the therapeutic effects and the substance should be avoided. If the LD_{50} is much lower than the therapeutic dose, then the substance is safe and can be administered in large quantity with no fear of toxicity. Among the plants that have been previously investigated for toxicity, most of them focused on testing the organic extracts. However, aqueous extracts should be the first preference as most of South African traditional medicines are prepared using water. Even though the safety of medicinal plants can be identified *in vitro*, it is essential to further investigate these medicinal plants with *in vivo* studies to validate their safety for human consumption.

Medicinal plants which were investigated for mutagenicity using the Ames test should be further investigated in the presence of metabolic activation (S9). Since some mutagenic compounds are biological inactive unless they are metabolised into an active form. Other types of bacterial strains should also be included since each strain detects different types of mutations. Plant extracts that showed clear mutagenic effect should be considered potentially unsafe. The fact that medicinal plants are complex mixture of phytochemicals makes it difficult to speculate about the compound causing toxicity, which warrant further analysis of the compounds responsible for toxicity. In future, the analysis of the results of the Ames test should be in standard deviations instead of the calculation of averages only. For

hepatotoxicity assay (MTT assay), it would be interesting to include non-cancerous, non-hepatic cell line for a comparison. Results should also be reported in IC_{50} values and an error bar should be included in the bar chart.

Clarifications of the *in vitro* herb-drug interaction data are not straight forward. This can be addressed by *in vivo* assays. It is recommended that half the maximal inhibitory concentration (IC_{50}) values be determined for the most active extracts so that the strength of the inhibition can be quantified. Many drugs have multiple pathways, thus the enzyme pool system such as human liver microsome pool should be used in further inhibition assays instead of only a few enzymes. Induction of drug metabolising enzymes should also be done, as some medicinal plants can act as inducers of the enzymes instead of the inhibitors. Examination of the interaction between medicinal plants and drug-transporter proteins should also be considered. The combinations between *C. roseus* and *M. acuminata*, *A. hispidium* and *S. serratuloides*, *S*. *serratuloides* and *H. hemerocallidea*, *C. limon* and *M. acuminata*, *H. hemerocallidea*, *M. balsamina* and *A. marlothii*, *A. marlothii* and *M. balsamina* as well as the combination between *P. guajava* and *C. dimidiatus* should also be analysed for herb-drug interaction as they were documented to be used concurrently with hypertension prescribed drugs.

6.3 Final conclusion

This study has scientifically supported the safety of most of the medicinal plants used in northern Maputaland for the treatment of hypertension. The safety of the medicinal plants was justified by its non-toxicity profiles demonstrated by most of the aqueous extracts tested individually and in combination. However, the *in vivo* studies are needed to validate this assumption. The results of the toxicity studies showed that the toxicity of medicinal plants was dosage dependant, thus as the concentration increases, the toxicity also increases. Some of the plant combinations resulted in increased toxicity (antagonistic interactions), thus they should not be recommended to be used in combination. Although all medicinal plants tested inhibited beta-glucuronidase enzyme (thus enhanced efficacy), they cannot be recommended to be used concurrently with hypertension prescribed drugs since they also inhibited the other two enzymes which are essential for the metabolism of hypertension prescribed drugs. To be safe, no medicinal plants should be taken concurrently with

any prescribed drugs. The most frequently used medicinal plants were non-toxic in all toxicological assays done in the current study which is very positive for the individuals who depend on medicinal plants for their primary health care.

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