Brine Shrimp Lethality Test and Acetylcholine esterase Inhibition Studies on Selected South African Medicinal Plants

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister of Scientiae in the South African Herbal Science and Medicine Institute at the University of the Western Cape.

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Co-supervisor: Prof. Quinton Johnson

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DEDICATION

To the loving memory of my late grand mother I dedicate this thesis. Also to my mother “Nanna”, thank you for your love and encouragement throughout my academic career.

DECLARATION

I Clarese Staley Jooste do hereby declare that the thesis entitled:

"Brine Shrimp Lethality test and Acetylcholine esterase Inhibition studies on selected South African medicinal plants"

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Is my own work and submitted in partial fulfilment of the degree Magister Scientiae in the South African Herbal Science and Medicine Institute, Faculty of Natural sciences at the University of the Western Cape and that all research resources I have used is in this thesis have been duly acknowledged by means of complete references.

Name : Clarese Staley Jooste

Signature : 

Date : 15 May 2012

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ABSTRACT

Research into traditional medicines is often conducted in a multidisciplinary approach as motivated by a desire to understand them in as complete a manner as possible, realizing their chemistry, biology and pharmacology. One biological approach involves monitoring the cytotoxicity of the extracts of subfractions against the nauplii, *Artemia salina* (brine shrimp). Organic and aqueous extracts of seven South African medicinal plants was investigated for biological activity. Selected plant extracts was also evaluated for AChE inhibitory activity. The objectives of this study was to look for any correlation between known biological activities of the investigated plants and BSLT lethality data and also to look for any correlation between AChEI activity and BSLT lethality data for selected plant extracts. The most active of the plants was the n-hex extract of *T.alliacea*, followed by the aqueous extract of *C.mellei* and the MeOH extract of *C.quadrifidus*; the MeOH and the DCM extracts of *A.afra*; the DCM extract of *P.undulatum* and the EtOAc extract of *A.annua*. The results from this study show a good correlation with antitumor, antimicrobial and anti-trypanocidal activity.

The various plants extracts investigated showed good inhibitory activity towards AChE using the TLC bioautography method. The results obtained from this study indicate that this type of activity is not only subject to plants containing alkaloids, but rather a diverse class of compounds may exhibit this kind of activity. The extracts that showed good AChE inhibitory activity also showed good cytotoxicity towards brine shrimp nauplii.

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CHAPTER 1

Introduction and Literature Review

1. Introduction.

Since the antiquity of time, plants have been used to treat a variety of ailments. In the 1800's pure compounds were isolated, which paved the way for modern pharmaceuticals. In 1805, morphine was isolated from opium poppy, followed by the isolation of salicylic acid, from the bark of the willow tree. Aspirin was synthesized by Felix Hoffman in 1897, from salicylic acid. **(Fan et al., 2006)**. Traditional medicine has and continues to play an important role in human history for disease prevention, alleviating symptoms and cure. It is still a widespread phenomenon in the developing world, with countries in Africa having a large percentage of the population still relying on traditional medicine for their health care needs **(Ferro and Gray, 2007)**.

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The vast majority of people still rely on traditional medicines for their everyday health care needs. According to the World Health Organisation a quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs, and 80% of the world's population, primarily those living in developing countries still rely on plant-derived medicines for their health care **(Gurib-Fakim,2006)**. In South Africa it is estimated that approximately 3000 plant species are used as medicines, of which 1032 species from 147 families are used by Zulu traditional healers (approximately 25% of the flora of Kwazulu-Natal), thus

indicating the remarkable plant and cultural diversity in South Africa. This also shows the great importance of medicinal plants within the traditional health care system in South Africa, as practiced not only by traditional healers and herbalists, but also by the South African population in general.

Between 60-85% of the South African population uses traditional medicine **(Popat et al., 2001),** and due to the cultural and floral diversity, it is not surprising that most South Africans prefer to use medicinal plants as medicines, as an alternative to visiting Western health care practitioners **(Thring and Weits, 2006)**. These medicines are more accessible and affordable, especially in poor rural communities, which is also a reason for the widespread use of these medicines **(Fennel et al., 2004)**. Many traditionally used medicinal plants are being sold in the market place, due to an increasing demand for cheaper medicine, high unemployment rates and an increase in HIV infections and HIV related infections **(Shai et al., 2008)**.

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Over the years there has been an increase in scientific research in the field of Ethnopharmacology, due to the use of medicinal plants as a source of Primary Health Care in developing countries and with the Western world realizing this, most of the pharmaceutical research has been focusing on an Ethnobotanical approach to drug discovery **(Light et al., 2005)**. Artemisinin, triptolide, celastrol, capsaicin, and curcumin are "poster children" for the power and promise of turning traditional medicines into modern drugs. Their stories highlight the ongoing interdisciplinary research efforts that continue to be necessary to realize the pharmaceutical potential of traditional therapeutics **(Heinrich, 2008)**. Other examples of plant derived drugs used in Western medicine are found in quinine, antimalarial from *Cinchona sp;*

digitoxin,cardiotonic from *Catharanthus roseus;* colchicines, anti-gout from *Colchicum autumnale*; reserpine, a hypotensive agent from *Rauwolfia sp*; atropine, anticholinergic from *Atropa belladona*; codein, antitusive/analgesic from *Papaver somniferum* **(Orwa, 2002)**. Thus a great deal of research has been done on plants that are traditionally used as medicines and some have resulted in the isolation of bioactive compounds for the direct use in medicine **(Ndlala et al., 2009)**. Given the advances in modern medicine and drug discovery research, and that most of the population is not able to afford pharmaceutical drugs, traditionally used medicinal plants have received considerable attention due to their expression of bioactive compounds, which may lead to new drug discoveries **(Amor et al., 2009)**.

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1.1 Laboratory-based assessment of traditional medicines

1.1.1 Antibacterial activity

1.1.1.1 Colorimetric method- Resazurin microtiter plate method

The resazurin microtiter plate method is a rapid and inexpensive colorimetric method based on the oxidation- reduction indicators, Alamar blue and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium]. Resazurin was identified as the main component of Alamar blue, hence the development of this type of microtiter plate assay for antibacterial assessment **(Martin et al., 2003)**. Microbial dehydrogenase enzymes are responsible for the reduction of blue resazurin to pink resorufin **(Brouwer, 2004)**. The minimum inhibitory concentration of antibacterial compounds against tested bacteria is determined based on a micro-dilution method in a 96 well microtiter plate. Resazurin indicator is added to each well containing the various diluted concentrations of the test compounds, to which Mueller broth as well as the bacterial suspension is also added to each of the

wells. Plates are incubated at 37^{o}C for 18-24hrs, where after a colour change is assessed visually. Any colour change from purple to pink or colourless is recorded as positive. The lowest concentration at which colour change is observed is taken as the MIC value **(Vukovic et al., 2010)**.

1.1.1.2 Agar and Broth dilution method

These can be applied in solid (agar dilution) or liquid (broth dilution) media. Several dilutions of the antimicrobial substance are incorporated into the solid or liquid media to determine the Minimum inhibitory concentration **(Silva et al., 2005)**, (MIC) i.e. the smallest concentration of the test substance at which bacterial strains are inhibited. The MIC is thus defined as the lowest concentration of an extract that inhibits more than 99% of the bacterial population **(Mativandlela et al.,**). This method of determining antimicrobial activity is a quantitative method based on the principle of contact with a test organism to a series of dilutions of test substance. Growth can be seen visibly or by measuring the turbidity (broth dilution) and plating the solutions out on agar plates, counting colonies after incubation distinguishes between micro biostatic and microbicidal activity **(Brouwer et al., 2004)**. The use of more quantifiable MIC assay techniques are the most preferred method of antimicrobial assessment and is supported by the majority of publications on South African medicinal plants, representing 56% in extract studies and 62% in essential oil studies **(van Vuuren, 2008)**.

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1.1.1.3 Agar well and Disc diffusion method

This technique is also known as the Kirby – Bauer method and was standardized by **Bauer et al. in 1966**.It is the test which is mostly used in clinical practice and is recommended by Clinical and Laboratory Standards Institute **(Silva et al., 2005)**. The agar diffusion technique includes the disc diffusion or the well diffusion method. These methods are popular and have been used in a number of studies. They involve inoculation of the surface of an agar plate with the test micro-organisms or pour molten agar inoculated with the test organism into a Petri dish. The compound to be evaluated can be applied to a paper disc or into a well made in the agar. After appropriate incubation, the appearance of zones of growth inhibition around the disc or the well is observed, the diameter of the zone being a good indicator of antimicrobial activity **(Palombo, 2009)**. An extract concentration exhibiting a 1mm zone of inhibition can be taken as the MIC value for the test substance, as a 1mm zone is the smallest zone that can be detected as growth inhibition of micro-organisms *in vitro* , using the agar diffusion method **(Thamburan et al., 2009)**.

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1.1.2 Antifungal activity

1.1.2.1 Colorimetric methods

The tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT), has been employed for antifungal testing of yeasts and resulted in clear cut end points for various antifungal agents. Tetrazolium salts can rapidly penetrate into intact cells and directly into subcellular membranes with dehydrogenase activity, where they are converted to colored formazan derivatives and can thus be used as indicators of reducing

systems. XTT is converted into a water soluble formazan, thereby avoiding the additional steps for the solubilization of formazan derivatives, but needs the presence of an electron coupling agent. The nature and the concentration of this agent are critical in order to obtain a good correlation between the formazaan production and the number of viable fungi **(Meletiadis et al., 2001)**.

The Fluorescein diacetate (FDA) assay is a more rapid assay, which requires less sample and with a more objective determination of the end point for the screening of novel antifungal agents. The assay depends on the hydrolyses of FDA to yellow-green fluorescent compound, fluorescein, by non-specific esterases present in actively metabolising microbes. The innoculum is diluted to a desired concentration and incubated along with the test substance and FDA. The fluorescence is then measured after incubation using a fluorescence plate reader. The MIC in the FDA assay is defined as the lowest concentration of an antifungal that causes a reduction of growth by 80%, compared to the growth in a drug free control **(Brouwer et al., 2006)**.

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1.1.2.2 Microtiter plate broth assay

The microtiter plate broth assay, developed by **Broekaert et al (1990)**, is the most simple and reliable quantitative assay for fungal growth inhibition. In this assay fungi are grown in wells of microtiter plates and their growth is monitored by measuring the turbidity of the microcultures with a microplate reader. By correlating culture absorbance with dry weight measurements Broekaert et al. (1990) showed that the microplate reader could be used as a reliable tool for the monitoring of fungal biomass of various filamentous fungi. The advantage of this

assay is the fast and easy handling of large numbers of samples, with the use of 96 well microplates; only small amounts of test substance and fungal spores are required and the assay can be adapted to non-sporulating fungi and is highly reproducible **(Pillay et al., 2011)**.

This liquid dilution method allows for the determination of whether a compound or extract has fungicidal or fungistatic action at a particular concentration. The serial dilution test was reported to give the most reproducible results on the minimal inhibitory concentration and was recommended as the general standard methodology for the testing of natural products. Serial dilution techniques have been recommended for working with lipophilic compounds from natural products. Microdilution techniques fully worked out, will gives a direct comparison with the activity of antifungal drugs and therefore appears to be appropriate for the examining the anti-yeast properties of plant derived compounds in general **(Benaducci et al., 2007)**.

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For dermatophyte strains incubations are performed at 28^{o}C while for yeasts and filamentous fungi, plates are incubated at 35-37^{o}C for 24, 48,72hrs. MIC value is defined as the lowest concentration of extract at which no fungal growth is observed after incubation **(Muschietti et al., 2005)**.

1.1.2.3 Agar- based disc diffusion method

The agar-based disc diffusion assay is widely used because of its simplicity and low cost. Absorbent disks or circular reservoirs containing various amounts of the substance to be examined are left in contact with an inoculated solid medium and the diameter of the clear zone around the disk or reservoir is measured at the end of the incubation period and compared with standard drugs. There has been much

research interest in agar based testing of antifungal susceptibility via the disc diffusion method, due to its relative ease and the lack of specialized equipment **(Benaducci et al., 2007)**.

1.1.3 Antioxidant activity.

1.1.3.1 ABTS method

This method was first developed by **Rice Evans and Miller in 1994** and was then modified by **Re et al. in 1999**. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, and its reduction in the presence of hydrogen donating antioxidants, is measured spectrophotometrically at 734nm. This decolourisation assay measures total antioxidant capacity in both lipophilic and hydrophilic substances. The effect of the antioxidant concentration and the duration of the inhibition of the radical cation's absorption are taken into account when the antioxidant activity is determined. Trolox a water soluble analog of vitamin E, is used as a positive control. The activity is expressed in terms of the Trolox-equivalent antioxidant capacity of the extracts (TEAC/mg). The modification is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS chromophore via the reaction of ABTS and potassium persulfate **(Krishnaiah et al., 2010)**.

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1.1.3.2 DPPH method

The 1, 1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was first described by **Blois in 1985** and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate

a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant solution is measured by the decrease in absorption at 515nm. In this method, a 0.1mM solution of DPPH in methanol is prepared, and 4ml of this solution are added to 1ml of the sample solution in methanol at varying concentrations. Thirty minutes later the absorbance is measured at 517nm. A large decrease in absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound **(Krishnaiah et al., 2010)**.

1.1.3.3 Online assays (HPLC-DPPH/ ABTS).

The online methods aim at rapid pinpointing of antioxidants as they are simple and easy to handle, compared to the assays using reactive oxygen species. A reduction of DPPH and ABTS by the antioxidant, either through hydrogen transfer or electron transfer leads to a significant shift in the UV-vis absorption spectrum of the compound. The HPLC separated analytes react with DPPH post column and the reduction is detected as a negative peak by an absorbance detector at 517nm. HPLC analytes also act post column with ABTS and the reduction is detected as a negative peak by a UV absorbance detector at 734nm. The HPLC-ABTS assay is more sensitive then the HPLC-DPPH assay as the ABTS radical is more water soluble than DPPH and is also more widely used for the evaluation of water-soluble antioxidants **(Niederlander et al., 2008).**

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1.1.4 Anti cancer activity

1.1.4.1 Tumor excision assay

Excision assays involves the removal of a tumor, bone marrow, and other tissues from the host after treatment to determine the effects of a therapy. Unlike standard in situ experimental designs such as increase in life span, tumor growth delay and local tumor control, excision assays require removal of the tumor or normal tissue from the environment in which the treatment was delivered. An advantage of this assay is that cell survival can be directly measured, important for both malignant and normal cells. The greatest disadvantage of excision assays is that extended treatment regimens cannot be studied, because cells that are killed by treatments will lyse and be lost to the environment. The survival of malignant cells from tumors treated *in vivo* and then excised is often determined by colony formation (CFU) in cell culture **(Teicher, 2009).**

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1.1.4.2 Caspase 3-like assay

Caspases, the cytoplasmic aspartate-specific cysteine proteases, have been shown to play a central role in the apoptotic signalling pathway. Caspase-3, a member of the caspase family has been shown to play an important role in apoptosis, induced by a variety of stimuli **(Paul et al., 2010)**. The activation of caspase-3 is detected by using a monoclonal antibody specific for the cleaved form of caspase-3. Tumor cell lines are fixed and stained with an anti active caspase-3 antibody. Cell fluorescence is measured by flow cytometry **(Thamburan, 2009)**. The production of the substrate caspase-3 Ac-Asp-Glu-Val-Asp-AMC in a different study is also monitored in a Fluostar Optima 96- well plate reader, using an excitation wavelength of 370nm and

an emission wavelength of 450nm. Relative Fluorescence Units (RFU) is calculated via the ratio of average rate of the fluorescence increase and protein concentrations **(Schmidt et al., 2009)**.

1.1.4.3 In vitro assay for cytotoxicity – MTT assay

The assay was first described by **Mosmann (1983)** and has been modified several times since then. The MTT assay protocol was initially developed to determine the viability of adherent animal cells and is now routinely used in biological and biomedical research. The assay utilizes 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a water soluble yellow dye that can be reduced to water-insoluble purple formazan crystals by the dehydrogenase system of active cells. The formazan crystals thus formed can be quantified spectrophotometrically by dissolution in an organic solvent, and the concentration is directly proportional to the number of metabolically active cells in a culture **(Wang et al., 2010)**. Detection and quantification of the formazan crystals are performed by a multiwell plate reader (Thermo spectra III Reader with software easy easyWIN-fitting , V6.0a, Tecan, Austria) at 570nm, with reference wavelength of 690nm **(ScherlieB, 2011)**. The conversion of MTT to formazan crystals by living cells determines mitochondrial activity and since for most cell populations, the total mitochondrial activity is related to the number of viable cells. The assay is thus used to measure the *in vitro* cytotoxicity of drugs on cell lines or primary patient cells **(van Meerloo et al., 2011).**

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1.2. The Brine Shrimp Lethality test (BSLT)

Research into traditional medicines is often conducted in a multidisciplinary approach as motivated by a desire to understand them in as complete a manner as possible, realizing their chemistry, biology and pharmacology. One biological approach involves monitoring the cytotoxicity of the extracts of subfractions against the nauplii, *Artemia salina*. The susceptibility of *Artemia salina* (*Artemiidae*), or brine shrimp larvae to treatment with medicinal plant extracts can be used as a measure of toxicity of chemicals as well as natural products **(Logarta et al., 2001)**.

The Brine Shrimp lethality test is a simple bench top bioassay used to screen plant extracts for biological activity and has yielded good results **(Ajaiyeoba et al., 2006)**. A wide variety of chemicals as well as natural products are toxic towards brine shrimp nauplii; the death of this organism when exposed to the various plant extract concentrations forms the basis of the toxicity test. Bioactive compounds are almost always toxic in high concentrations, and as toxicology can be described as pharmacology at higher doses, this premise has been applied to the screening of medicinal plant extracts in the BSLT **(Campos et al., 2007)**.

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The assay is capable of detecting a broad spectrum of bioactivity present in crude extracts. The aim of the method is to provide a front line screen that can be backed up by more specific and expensive bioassays once the active compounds have been isolated **(Psitthanan et al., 2004)**. It has been reported to be useful in predicting biological activities such as cytotoxicity, phototoxicity, pesticidal and trypanocidal activities, enzyme inhibition and ion regulation **(Mackeen et al., 2000)**. The BSLT has also been reported to give good correlation with cytotoxicity against some tumour cell lines, including colon carcinoma cells **(Wagensteen et al., 2007)**.

Since its introduction in 1982, this *in vivo* lethality test has been successfully employed for bioassay-guided fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba*, *cis*-annonacin from *Annona muricata* and *ent-kaur*-16-en-19-oic acid from *Elaeoselinum foetidum* (Pisutthanan et al., 2004).

1.2.1. An overview on the methodology for the BSLT

The first description of the methodology was given by **Meyer et al, (1982)**. Brine shrimp eggs are placed in brine and hatched within 48hours. Each plant extract is dissolved in 20ml methylene chloride: methanol (1:1) to prepare a stock solution of 10mg/ml. From the stock solutions 500, 50, 5µg/ml aliquots are transferred in triplicate to vials, and the solvent is allowed to evaporate. After evaporation 5ml of brine is added to each vial to prepare concentrations corresponding to 1000, 100,10ppm. Ten nauplii are added to each vial (30 shrimps per concentration). The number of survivors at each concentration is recorded and the Lethal concentrations at 50% mortality (LC50) values are calculated using the Finney Computer programme.

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According to the methodology as described by **McLauglin. (1991)**, plant extracts are tested at three concentrations, 1000, 100, 10µg/ml and also evaluated in triplicate. Samples are dissolved in aqueous Dimethylsulfoxide (DMSO), the final concentration of which must not exceed 1%. Survivors are then counted after 24hours. The general toxicity is considered weak when the LC_{50} value corresponds to concentrations between 500 and 1000µg/ml, moderate between 100 and 500µg/ml and strong with an LC_{50} ranging between 0 to 100µg/ml.

Another method of conducting the BSLT was described by **Pisutthanan. (2004)**, and this was a modification of the assay as described by **Solis et al. (1993)**. Ten milligrams of plant extract is made up to 2mg/ml in artificial seawater, except for water insoluble compounds which are dissolved in 50µl of DMSO, not exceeding a final concentration of 0.05%, prior to adding the sea water. Serial dilutions are made in 96 well microtiter plates in triplicate in 120µl sea water. Control wells with DMSO are included in the experiment. A suspension of 10-15 nauplii (100µl) is added to each well. The plates are covered and incubated at room temperature (25-29^{o}C) for 24hours. Plates are then examined under the binocular microscope and the number of dead nauplii in each well is counted. One hundred microliters of methanol are added to each well to immobilize the nauplii and after 15minutes the total number of brine shrimp in each well is counted. Analysis of the data is performed by probit analysis on a Finney computer programme to determine the lethal concentration to half of the test organism.

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This *in vivo* lethality test is a great resource in developing and under developed countries, where research institutions lack the expensive laboratory equipment and resources to conduct biological assays. The assay is simple, inexpensive and reproducible.

1.2.2 Advantages of *Artemia salina* as a toxicity test

The cysts (fig1) are commercially and readily available, so that the test can be carried out world wide with the same original material. The quantity of the cysts required per test is very small, so that the price of the biological material is negligible and the cysts can be stored for years under dry conditions without losing their viability. The necessity of year round maintenance of stock cultures, with all the biological and technical difficulties and the

considerable economic repercussions, is completely eliminated. Large numbers of test organisms of exactly the same age and physiological condition can be easily obtained to start the test

1.2.3 List of criteria for the development of a standard *Artemia* toxicity test.

The nauplii have to be hatched out under strictly controlled conditions of temperature, pH, salinity, aeration, and light. The larvae (fig2) must be of exactly the same age at the start of every test and during the test the larvae may not molt into instar stage with a different sensitivity. The test has to be carried out with cysts from the same geographical origin and the experimental conditions must be standardised and followed in every detail. A control test with a reference toxicant chemical must be carried out each time in parallel to check both the sensitivity of the larvae and the conformity with the standard technological procedure **(Vanhaecke et al., 1981)**.

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Fig 1. *Artemia salina* (brine shrimp) cysts.

Fig2. Newly hatched brine shrimp nauplii (larvae)

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1.3. Acetylcholine esterase Inhibition

Acetylcholine esterase Inhibitors (fig3), have been used in the treatment of Alzheimer's disease (AD). The treatment targets the cholinergic system using anti-choline esterase compounds **(Oh et al., 2004)**. Acetylcholine (ACh) is a neurotransmitter found in the synapses of the cerebral cortex and a deficiency of this neurotransmitter is one of the major features seen in sufferers of AD. Therapeutic agents which inhibit Acetylcholine esterase (AChE) are known to occur in plants with a tradition of being used for failing memory and other cognitive diseases **(Oh et al., 2004)**. Acetylcholine esterase inhibitors (AchEI) form the basis of the newest drugs available for the management of AD **(Marston et al., 2002)**, and plants with this kind of activity are increasingly attractive targets for the development of new drugs **(Gomes et al., 2009)**.

AchE rapidly hydrolyzes the active neurotransmitter, Ach into its inactive compounds choline and acetic acid, resulting in low levels of Ach in the synaptic cleft **(de Jongh**

et al., 2006), thus the rational therapeutic approach to treat AD is to increase the amount of Ach in the synaptic cleft by inhibiting the enzyme AchE **(Markmee et al., 2006)**. Traditionally, plants are a rich source of AChEI. People from the Caucasus used bulbs of snow drops (*Galanthus sp.*) to treat forgetfulness. The active compound galanthamine has been isolated and is produced commercially from *Narcissus species*. It is used to treat AD and is marketed under the name Reminyl. Other AchEI include huperzine A from *Huperzia serratta* and Rivastigmine (Exelon) derived from physostigmine isolated from the calabar bean, *Physostigma venenosum* **(de Jongh et al., 2006)**.

There are a few synthetic medicines, e.g. tacrine, donepezil and the natural product based rivastigmine for treatment of cognitive dysfunction and memory loss associated with AD **(SatheeshKumar et al., 2010)**. Despite intensive advancements in research, available therapeutic options are limited, and there still remains a great demand for new drugs **(Ahmed &Gilani, 2009)**. Current efforts to identify new AchEI are mostly focused on alkaloids. Recently more studies are being conducted on non-alkaloidal compounds. Flavonoids, xanthones, chalcones, coumarins and terpenoids have been described to possess such activities **(Pueyo & Calvo, 2010)**.

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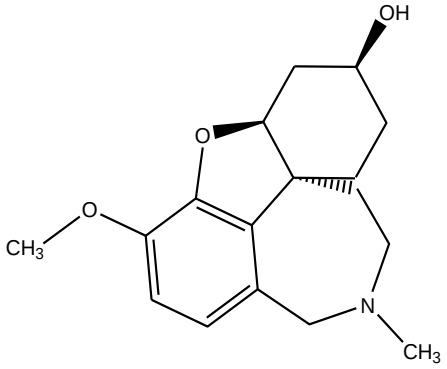
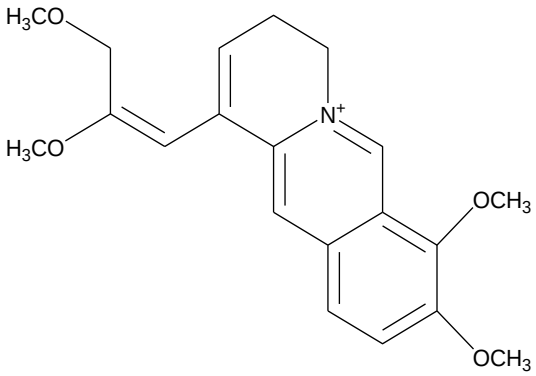
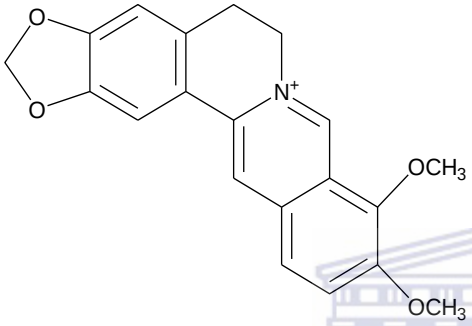
Fig3.Chemical structures of known Acetylcholine esterase inhibitors



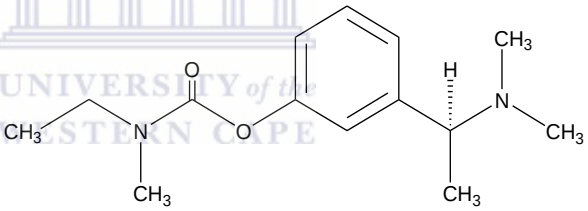
Galanthamine



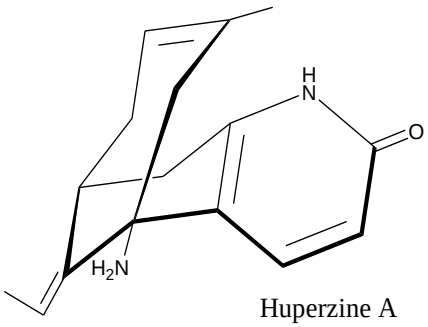
Palmatine



Berberine



Rivastigmine



Huperzine A

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1.3.1. An overview on the methodology of the AchE inhibition assay

1.3.1.1 Microtiter plate method

A method by **Ellman et al, (1961)** has been used to determine AchE inhibition, whereby a concentration of 0.037M Acetylcholine iodide solution is prepared in water. A concentration of 0.01M 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB), known as Ellmans reagent is dissolved in potassium phosphate buffer (pH 7) containing 0.15% (w/v) sodium bicarbonate. Human Recombinant Acetylcholinesterase (HuAchE) stock solution is prepared by dissolving 1000U in 0.1M phosphate buffer (pH8), containing Triton X-100 (0.1%). The enzyme is diluted first in order to reach an activity ranging between 0.130 and 0.100AU/min in the final assay conditions.

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Stock solutions of the test compounds (1mM) are prepared in water. Five different concentrations of each compound are used in order to obtain inhibition of HuAchE activity ranging between 20-80%.

The assay solution consists of a 0.1M phosphate buffer (pH8), to which is added 340µM DTNB, HuAchE and 550µM acetylthiocholine iodide. The final assay volume should be 1ml. Test compounds should be added to the solution and preincubated with the enzyme for 20minutes, before the addition of a substrate. A Jasco V-530 double beam spectrophotometer is used for initial rate assays and performed at 37^{o}C. The rate of increase in absorbance is followed at 412nm for 5minutes. Assays are run with a blank containing all of the components except HuAchE in order to account for a non – enzymatic reaction. The reaction rates are compared and the percent inhibition due to the presence of test compounds is calculated. Each concentration should be analysed in triplicate. The percent inhibition of the enzyme activity is calculated by the

following expression: 100-(v_i / v_o x 100) where v_i is the initial rate calculated in the presence of inhibitor and v_o is the enzyme activity. Inhibition curves are obtained for each compound by plotting the percent inhibition *vs.* the logarithm of inhibitor concentration in the assay solution. The linear regression parameters are determined for each curve and the IC_{50} extrapolated. The computer programme used to analyse these data is GraphPad Prism 3.0 **(Bartolini et al., 2003)**.

1.3.1.2 TLC bioautographic method

Extracts are applied to TLC plates and after development the plates are sprayed with 50mM *Tris*-HCl (pH8), containing 5mM Acetylthiocholine iodide (ATCI) and 5mM DTNB. The plates are sprayed until saturated. Thereafter the plates are sprayed with 3U/ml AchE dissolved in 50ml *Tris*-HCl, pH8 at 37^{o}C. After a few minutes a yellow background should appear, with white spots indicating AchE inhibiting compounds **(Anderson et al., 2007)**.

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1.3.1.3 *Ex-vivo* Acetylcholine esterase Inhibition assay

AchE activity in rat brain is measured as described by **Isoma et al., (2002)**. Different doses of the test compounds are administered intraperitoneally. Animals are sacrificed by decapitation under anaesthesia 1hour after drug administration. The frontal cortex and hippocampus are dissected out in ice cold 0.1M phosphate buffer saline (pH8.0). The tissues are homogenized in ice cold 0.1M phosphate buffer saline (pH8.0) using homogenizer. The homogenates are centrifuged at 1000xg for 10min at 4^{o}C and the supernatant is used as a source of enzyme in the AchE assay. Protein concentration in the supernatant is measured using **Bradford (1976)** method **(Ahmed & Gilani, 2009)**.

1.4 Overview on the traditional uses, chemistry and biological activities of the selected plants.

1.4.1 *Artemisia afra*

Artemisia afra **(*Asteraceae*),**(fig 4a) commonly known as wormwood is a medicinal plant commonly found in most areas of South Africa, where it has a reputation for its claimed healing properties and use in specific aliments **(Mukinda et al., 2007)**. It is a common species with a wide distribution from the Cederburg mountains in the Cape, northwards to tropical East Africa and stretching as far north as Ethiopia **(Van der Kooy et al., 2008)**.

The plant is an aromatic, erect, multi-stemmed, perennial shrub, which is one of the most widely used traditional medicines in South Africa. It is extensively used to treat colds, coughs and influenza. It is also common practice for leaves to be heated and the smoke inhaled for therapeutic purposes **(Braithwaite et al., 2008)**. The plant is also used in combination with other herbals as a remedy against headache, eye disease, tinea captitus, haematuria, stabbing pain and used alone as an infertility agent. In many parts of Africa the plant is used to treat sore throat, asthma, indigestion, colic, constipation, gout and intestinal worms **(Nibret & Wink., 2010)**.

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Artemisia afra is rich in terpenes and is thus likely to have valuable biological activities. Early in the 1920's **Goodsen (1922)** found that *A.afra* contained a wax ester, triacontane, scopoletin and quebrachitol. Other secondary metabolites include sesquiterpenes, guanolides, triterpenes, long chain alkanes, coumarins, organic acids, flavonoids and also volatile secondary metabolites of which artemisyl acetate, 1; 8-cineole, α-thujone, β-thujone, Artemisia ketone, α-copaene, camphor, santolina alcohol, borneol and camphene are the most commonly occurring **(Liu et al., 2009)**.

The traditional use of *A.afra* as an anti-infectious therapy has found scientific support in studies of its anti-pathogenic qualities and was shown to have antimicrobial as well as antifungal properties. The essential oil of *A.afra*, which contains 1,8 cineole, thujone, camphor and borneol specifically inhibited the growth of *Apergillus ochraceus, A.niger, A.parasiticus, Candida alabicuns, Alternaria alternate* and *Geotrichum candidum* **(Ntutela et al., 2009)**.

The flavones and sesquiterpene lactones, isolated by **Kraft et al., (2003)** from *A.afra* proved to possess *in vitro* anti-plasmodial activity to some extent, but not the same efficacy as was reported for artemisin from *Artemisia annua* **(Liu et al., 2010)**. Apart from exhibiting antimicrobial activity, *A.afra* has also demonstrated anti-oxidant, ant-malarial, cytotoxic and sedative effects **(van Wyk et al, 2008)**.

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1.4.2 *Artemisia annua*

Artemisia annua **(*Asteraceae*)**, (fig 4b), is a vigorously growing annual weedy herb, usually single stemmed reaching up to 2-3m in height **(Brisibe et al., 2009)**, with strong fragrance and is endemic to the Northern parts of Chahar and Suiyuan Provinces in China, where it is known as "qinghao" (green herb) **(Baraldi et al., 2008)**. The plant is widely known for its anti-malarial, anti-inflammatory, anti-tumour and allelopathic activity **(Juteau et al., 2002)** and has been used as a remedy for chills and fevers for more than 2000years ago **(Baraldi et al., 2008)**.

Flavonoids, coumarins, steroids, phenolics, monoterpenoids, triterpenoids and sesquiterpenoids are some of the bioactive compounds produced by this plant. This far the most important of the sesquiterpenoids seems to be artemisinin, dihydroartemisinic acid, artemisinic acid and arteannuin B **(Brisibe et al., 2009)**. The

active constituent artemisinin, is a sesquiterpene lactone with a rare endoperoxide bridge which is used to treat multi-drug resistant strains of falciparum malaria . It is also used a potent blood schizonticide, which has been found to be effective against other infectious diseases such as Hepatitis B. Artemisin has also been found to be effective against numerous types of tumours, including breast cancer, human leukaemia, colon, and small-cell lung carcinomas **(Baraldi et al., 2008)**.

Since its isolation in 1971, artemisinin and its derivatives have become the main weapon against *Plasmodium falciparum* and *Plasmodium vivax* that has build up resistance against chloroquine treatment **(Van der Kooy et al., 2008)**. Artemisinin is a powerful oxidant and the peroxide bridge in artemisinin and its derivatives are essential for its anti-malarial activity. Artemisinin and its water soluble derivatives also demonstrated immunosuppressive activity *in vitro* and *in vivo* **(Noori et al., 2004)**.

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1.4.3 *Sutherlandia frutescens*

Sutherlandia frutescens **(*Fabaceae*)**,(fig.4c), commonly known as cancer bush is a well known medicinal plant in Southern Africa and has enjoyed a long history of use by many cultures in South Africa as a tonic for a diverse range of health conditions **(Stander et al., 2007)**. The genus is restricted to Southern Africa, occurring mainly in South Africa, mostly along the west coast of the Western cape **(Chinkwo, 2005)**, Botswana, Namibia and Zimbabwe.

The leaves are hairy and divided into small leaflets with large red flowers. The plant was first used by the "Khoi San" and "Nama people". Traditionally this plant has been used to treat a number of health conditions, which include relieving the

symptoms of colds and flu, for the treatment of chicken pox, poor appetite, constipation, heart failure, kidney and liver problems, hypertension **(Ojewole, 2008)**, stomach cancer, uterine diseases, eye infections, as a blood tonic **(Chinkwo, 2005)**, diabetes, internal cancers, rheumatoid arthritis, peptic ulcers, gastritis, osteoarthritis,anxiety, clinical depression and menopausal symptoms **(Mills et al., 2005)**.

Anecdotal reports from South African doctors and health care workers described a positive effect of a herbal preparation of this plant on HIV patients **(Tai et al., 2004)**. Chemical studies on this plant have identified a component canavanine, a non-protein amino acid, which possesses anti-tumour properties **(Fernandes et al., 2004)**. Chemicals isolated from this plant include pinitol, triterpenoid saponins, flavonoids, and several free amino acids, including L-canavanine and GABA. Canavanine has documented anti-viral activity, including inhibition of the influenza virus and retrovirus. It is also an inhibitor of nitric oxide synthase and has a potential for the treatment of septic shock **(van Wyk et al., 2008)**.

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Sutherlandia leaves contain all of the above mentioned chemicals, but no alkaloids. According too **Tai et al., (2004)**, canavanine and its major metabolite canaline are being developed as new anti-cancer drugs. All of the biologically active chemicals present in *Sutherlandia* have been linked to therapeutic applications, where in triterpenoids have been associated with corticomimetic activity, anti-inflammatory, anti-ulcer, anti-nociceptive and anti-tumour properties **(Prevoo et al., 2008)**.

1.4.4 *Hypoxis hemerocallideae*

Hypoxis hemerocallideae **(*Hypoxidaceae*)**, (fig4d), commonly known as African potato is a well known Zulu and Sotho medicinal plant **(Van Wyk, 2008)**, which usually grows in meanders, grassland and mountainous regions of South America, South Africa, Australia and coastal regions of Asia **(Laporta et al., 2007)**.

Weak infusions of this plant are taken as tonics against wasting diseases, tuberculosis and cancer. Traditionally it is used to treat benign prostatic hypertrophy (BPH) and urinary tract infections, as a laxative and vermifuge **(Van Wyk, 2008)** as well as for the treatment of rheumatoid arthritis and immune system disorders **(Steenkamp et al., 2006)**. It has been claimed to be effective against HIV/AIDS related diseases, hypertension, diabetes, asthma, and some central nervous system disorders **(Ojewole, 2006)**.

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The reported traditional use of this plant for the treatment of BPH and prostate edenoma has been attributed to the presence of β-sitosterol, whereas its anti-inflammatory activity has been associated with the presence of rooperol. Treatment of patients with BPH during a clinical trial study showed positive outcomes **(Steenkamp et al., 2003)**.

Anti- inflammatory, analgesic and antidiabetic effects of the aqueous extract of *Hypoxis* have been investigated, and it was found that the extract does possess anti-nociceptive, anti-inflammatory and anti-diabetic properties in mammalian laboratory animal models, while its safety profile was also demonstrated in mice **(Ojewole, 2006)**. Extracts of *Hypoxis*, which contain a high content of hypoxoside exhibit strong

antioxidant activity and the compound rooperol has been shown to inhibit lipid peroxidation **(Laporta et al., 2007)**.

Hypoxoside, the norlignan glycoside is reported to be the most important phytochemical with regards to the medicinal value of this plant. Additional chemical components include β-sitosterol, stigmasterol and stigmastenol. The aglycone of hypoxoside, rooperol has been reported to possess anti-cancer, anti-phologistic, bacteriostatic and bactericidal properties **(Nair et al., 2007)**.

1.4.5 *Pseudognaphalium undulatum*

Pseudognaphalium spp **(*Asteraceae*),** (fig.4e), is widely used in Chile, from Amira communities in the North to Mapuche communities in the South. *Pseudognaphalium* is represented by 14 species, all of which show a characteristic combination of glandular and non-glandular trichomes and the species are further characterised by the secretion of resinous exudates from twigs and leaves **(Mendoza et al., 1997)**. The plant is used in folk medicine as a wound healing anti-septic, for the treatment of colds and flu and different bronchial illnesses **(Rezende et al., 2000)**.

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Reported biological activities for *Pseudognaphalium spp* include antimicrobial, antiparasitic, insect antifeedent and anti-inflammatory activities. Such activities have been associated with different kauranes, with *ent*-16-kauren-19-oic acid exhibiting most of these biological activities **(Rezende et al., 2000)**. **Rangel et al., (2002)**, found that the acetone, ethanol and aqueous extract of *P.moritzianum* were active against bacterial strains such as *Staphallococus aureus*, *Enterococcus faecelis* and *Pseudomonas aeruginosa*. This activity has been attributed to the presence of flavonoids.

Mendoza et al., (1998) isolated the following constituents from the dichloromethane extract of *P.cheiranthifolium,* 5-hydroxy-3,6,7,8-tetramethoxyflavone, *ent*-3α-hydroxy-9(11),16-kauradien-19-oic acid and from *P.robustum,* 5,7-dihydroxyflavone (pinocembrin), *ent*-16-kauren-19-oic acid and from *P.vira vira,* 5-hydroxy-3,6,7,8-tetramethoxyflavone. The compounds 5,7-dihydroxy-3,8-dimethoxyflavone and 3β-hydroxy-kaurenoic acid, isolated from *P.robustum* and *P.vira vira*, respectively have been shown to possess some fungitoxic activity against *B.cinerea*. The exudates from *P.vira vira* present antibacterial and antifungal properties and contain mainly the diterpenoids, kaurenoic acid and 3β-hydroxy-kaurenoic acid **(Cotoras et al., 2004).**

1.4.6 *Tulbaghia alliaceae*

Tulbaghia alliacea **(*Alliaceae*)**, (fig.4f), common name wild garlic is a strongly aromatic plant which reaches about 15-30cm in height. The flowers are 6-10 on pedicles and 20mm long, brownish to green, with an orange corona **(http://fernkloof.com)**. *Tulbaghia alliacea* is indigenous to South Africa and is distributed in the Western and Eastern Cape Provinces, from Clanwilliam to the Cape Peninsula, east wards to Port Elizabeth and North into Kwazulu Natal, Mpumalanga and Gauteng **(Burbridge, 1978)**. Plants within the *Alliaceae* genus are known for medicinal, ornamental as well as nutritive values. Different plant parts such as roots, bulbs, leaves and flowers are used in the treatment of a variety of conditions. The bulbs of *Tulbaghia violacea* are used as a remedy for pulmonary tuberculosis as well as an anthelmintic **(Ngunge et al., 2010)**. Traditionally *Tulbaghia alliacea* is used as a remedy for fever, fits, rheumatism and paralysis **(Mackraj et al., 2008)**.

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Tulbaghia alliacea contains high levels of sulphur compounds that give an even more powerful garlic odour than commercial garlic (*Allium sativum*) **(Long, 2006)**. *Tulbaghia species* are known to have antifungal properties that may be beneficial in the treatment of both human and plant pathogens **(van den Heever et al., 2008)**. A study conducted by **Thamburan et al., (2006)** revealed that the aqueous and chloroform extracts exhibited anti-infective activity against *Candida species* and that the compound responsible for such activity was marasmicin. **Maoela (2005)** reported on the presence of four S-alk (en) yl cystein sulfoxides in a study conducted on *Tulbaghia alliacea*.

1.4.7 *Carpobrotus sp.*(*acinaciformis, quadrifidus, mellei*)

Carpobrotus sp **(*Aizoaceae*)**, (fig.4g-i), is well known in South Africa by the name "sour fig" in English and "vygies" in Afrikaans. Different *species* within this *genus* are used as traditional medicine, and their appearances are very similar. These plant *species* occur in abundance amongst communities and are widely distributed from the coastal towns to the inland **(Springfield et al., 2003)**. In addition, the *Aizoaceae genus* is considered as one of South Africa's most diverse and abundant plant families, but also the least studied.

The leaf juice of *Carpobrotus edulis* is more widely used than that of *Carpobrotus acinaciformis*, as a traditional remedy for a wide range of fungal and bacterial infections, the treatment of sinusitis, diarrhea, infantile eczema, tuberculosis and other internal chest conditions. The leaves also contain an astringent antiseptic juice which can be taken orally for treating sore throat and mouth infections. Decoctions of *C.murii* and *C.quadrifidus* have been used to treat various infections **(van der Watt & Pretorius, 2001)**.

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Martins et al., (2005) conducted an antimycobacterial study on *Carpobrotus edulis* and found that *C.edulis* extracts exhibited antimicrobial activity, suggesting that they may serve as a source of new antimicrobial agents that are effective against problematic drug resistant intracellular infections. Compounds reported to be responsible for the antimicrobial activity of *Carpobrotus* are flavonoids, which include rutin, neohesperidin, hyperoside, catechin, and the phenolic, ferulic acid **(Springfield et al., 2003)**.

1.5 Aims and Objectives

Aims:

The main aim of the study is to evaluate and compare the organic extracts as well as the aqueous extracts of the selected South African medicinal plants for biological activity, through investigation of their toxicity towards brine shrimp nauplii, as well as assessment of Acetylcholine esterase inhibitory activity of some of the plant extracts.

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Objectives:

To compare the biological activity/toxicity of the aqueous extracts and organic extracts.

To look for any correlation between BSLT lethality data and known biological data of the various extracts

To determine enzyme inhibitory activity of the most active extracts and to look for any correlations, if any between BSLT activity and AChE inhibitory activity.

Fig4. Images of the different plants used in the study

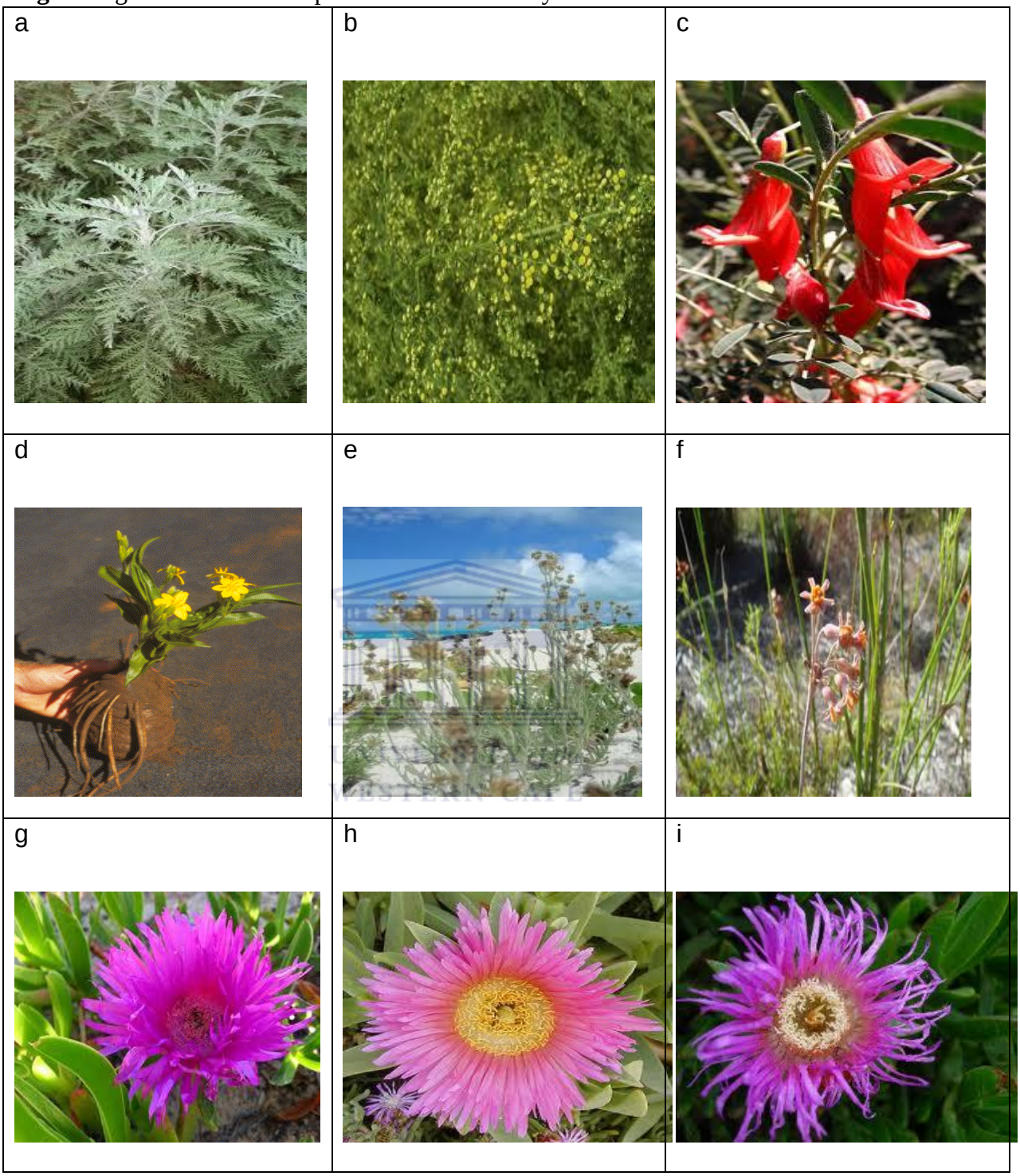

Fig.4 (**a**) *Artemisia afra* (**b**) *Artemisia annua* (**c**) *Sutherlandia frutescence* (**d**) *Hypoxis hemerocallideae* (**e**) *Pseudognaphalium undulatum* (**f**) *Tulbaghia alliaceae* (**g**) *Carpobrotus acinaciformis* (**h**) *Carpobrotus quadrifidus* (**i**) *Carpobrotus mellei*

Table 1. Summary of known chemical constituents from the various plants.

Plant	Chemical constituents
1. *Artemisia afra*	sesquiterpenes, guanolides, triterpenes, long chain alkanes, Coumarins, organic acids, flavonoids, and volatiles (arte-Misyl acetate, 1;8-cineole, α-thujone, β-thujone, Artemisia Ketone, α-copaene, camphor, santolina alcohol, borneol Camphene.
2. *Artemisia annua*	flavonoids, coumarins, steroids, phenolics, monoterpe-noids, triterpenoids, sesquiterpenoids (artemisinin, dihydroartemisinic acid, artemisinic acid, arteannuim B
3. *Sutherlandia frutescens*	pinitol, triterpenoids, saponins, flavonoids, GABA, free amino acids, L-canavanine, hexadecanoic acid, γ-sito sterol, stigmast-4-en-3-one, long chain fatty acids
4. *Hypoxis hemerocallideae*	the norlignan glycoside Hypoxoside, rooperol, β-sitoste-rol, stigmasterol, stigmastenol
5. *Pseudognaphalium sp.*	Flavonoids, kaurane diterpenoids, diterpenoids.
6. *Tulbaghia alliaceae*	S-alk (en) yl cystein sulfoxides, marasmisin
7. *Carpobrotus sp.* (*acinaciformis,quadrifidus,mellei*)	flavonoids, which include rutin, neohesperidin, hyperoside, Catechin and furelic acid

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CHAPTER 2

Materials and Methods

2.1Plant material and Chemicals

2.1.1 Plant collection and identification

The nine plants were purchased from the following places: *Artemisia afra* from Grassroots, *Artemisia annua* from Acupuncture & Herbs, Inc. Southern Oregon, Klamath Falls and *Sutherlandia frutescens* from Afriplex, *Tulbaghia alliacea* from Parceval pharmaceutics, *Hypoxis hemerocallideae* from a herbalist in Khayalitsha, *Pseudognaphalium undulatum* was collected in the Eastern Cape and authenticated by the taxonomist, Mr.Frans Weitz,Department of Biodiversity and Conservatio, University of the Western Cape and the three *Carpobrotus species* was collected from Saldanha. These plant material was available at The South African Herbal Science and Medicine Institute (SAHSMI), as well as at the Department of Chemistry, University of the Western Cape. The materials used in the study were obtained from batches which have been used before and therefore it was not necessary to verify the identity of the species and to deposit voucher specimens.

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2.1.2 Chemicals used

Brine shrimp eggs were purchased from INVE group, Grantsville, Utah 84029, U.S.A. Acetylcholine esterase, 1-naphthyl acetate, Fast Blue B salt, Tris, Chlorogenic acid and boldine were purchased from Sigma. All organic solvents were acquired as analytical reagent grade from KIMIX chemicals, South Africa and distilled before use.

2.1.3 Preparation of extracts

Aerial parts of *A.afra, A.annua, S.frutescens, P.undulatum, Carpobrotus sp* and underground parts of *T.alliacea* and *H.hemerocallidea* were used. Sequential extraction was conducted with all the plant species. Each of the plant materials (± 500g) was extracted with 500ml of hexane, dichloromethane, ethylacetate and methanol respectively. The suspensions were stirred for 2hours and allowed to stand overnight, where after it was stirred for 1hour. All four extracts per plant were concentrated to dryness under reduced pressure at 40-45^{o}C with the aid of a rotary evaporator. The extracts were stored in the fridge at 4^{o}C until further use.

An aqueous extract of each of the nine plants was also prepared. Original plant material (500g) was boiled in distilled water (500ml). The extract was then freeze-dried. These aqueous extracts were also stored in the fridge until further use. The organic as well as the aqueous extracts were used in the BSLT, and a select number of extracts were used in the AChEI assay.

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2.1.4 The Brine Shrimp Lethality Test (BSLT).

2.1.4.1 Hatching of Brine shrimp eggs

Artificial seawater was prepared using salt (40g) dissolved in dH_2O (1liter), supplemented with dried yeast (600mg). The eggs (1g) were then added to the artificial seawater. A pump was used to generate a stream of bubbles, which helped to keep the eggs in suspension, in order to facilitate the hatching process. The hatching set-up was placed under illumination with the aid of an electric lamp. The set-up was then left for 48hours, after which most of the eggs had hatched.

2.1.4.2 Brine shrimp nauplii exposure to extracts

The extracts were prepared at three concentration levels namely, 1000, 100, 10µg/ml. Ten newly hatched brine shrimp nauplii were used per test tube, in which they were then exposed to the various concentrations of the plant extracts. A negative control, DMSO not exceeding 0.05% was included for the organic extracts. For the aqueous extracts salt water was used as a negative control. After 24hours the number of surviving nauplii was determined in order to generate the lethality data. Each of the experiments was performed in triplicate for all four organic extracts as well as for the aqueous extracts. A computer statistical programme, SPSS or Finney probit computer programme may be used to determine the LC_{50} (Concentration at which 50% of the nauplii died), however for the data analysis of this study Microsoft Excell 2010 was used to determine the LC_{50} values.

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2.1.5 A rapid TLC bioautographic method (Acetylcholinesterase Inhibition assay).

2.1.5.1 Preparation of Enzyme solution

Acetylcholine (1000U) was dissolved in 150ml of *Tris*-Hydrochloric acid (pH7.8). Bovine serum albumin (150mg) was added to the solution in order to stabilize the enzyme during the assay. The enzyme stock solution was stored in the fridge at $4^{o}C$ until use.

2.1.5.2 Development of TLC plates

A select number of plant extracts were used in the AchEI assay, depending on the nature of the results from the BSLT. The TLC plates were eluted with Isopropanol in order to wash them and dried thoroughly before use. The reference compounds

boldine and chlorogenic acid were dissolved in methanol and applied to the plates in varying dilutions. Galanthamine was used as a positive control (1mg/ml). Stock solutions of plant extracts were prepared at 10mg/ml and applied to the plates (15µl). The plates were eluted with chloroform: methanol: water (10: 1.3: 0.1). After migration of the samples, the plates were thoroughly dried.

2.1.5.3 Application of enzyme

The plates were sprayed with the enzyme stock solution and dried again. They were placed in a plastic container with plugs to lay the plate on. The container was filled with a little water, such that the plate did not come into contact with the water, but enough to keep the atmosphere humid inside the container(90% humidity), when it was covered. Incubation was performed at 37^{o}C for 30minutes.

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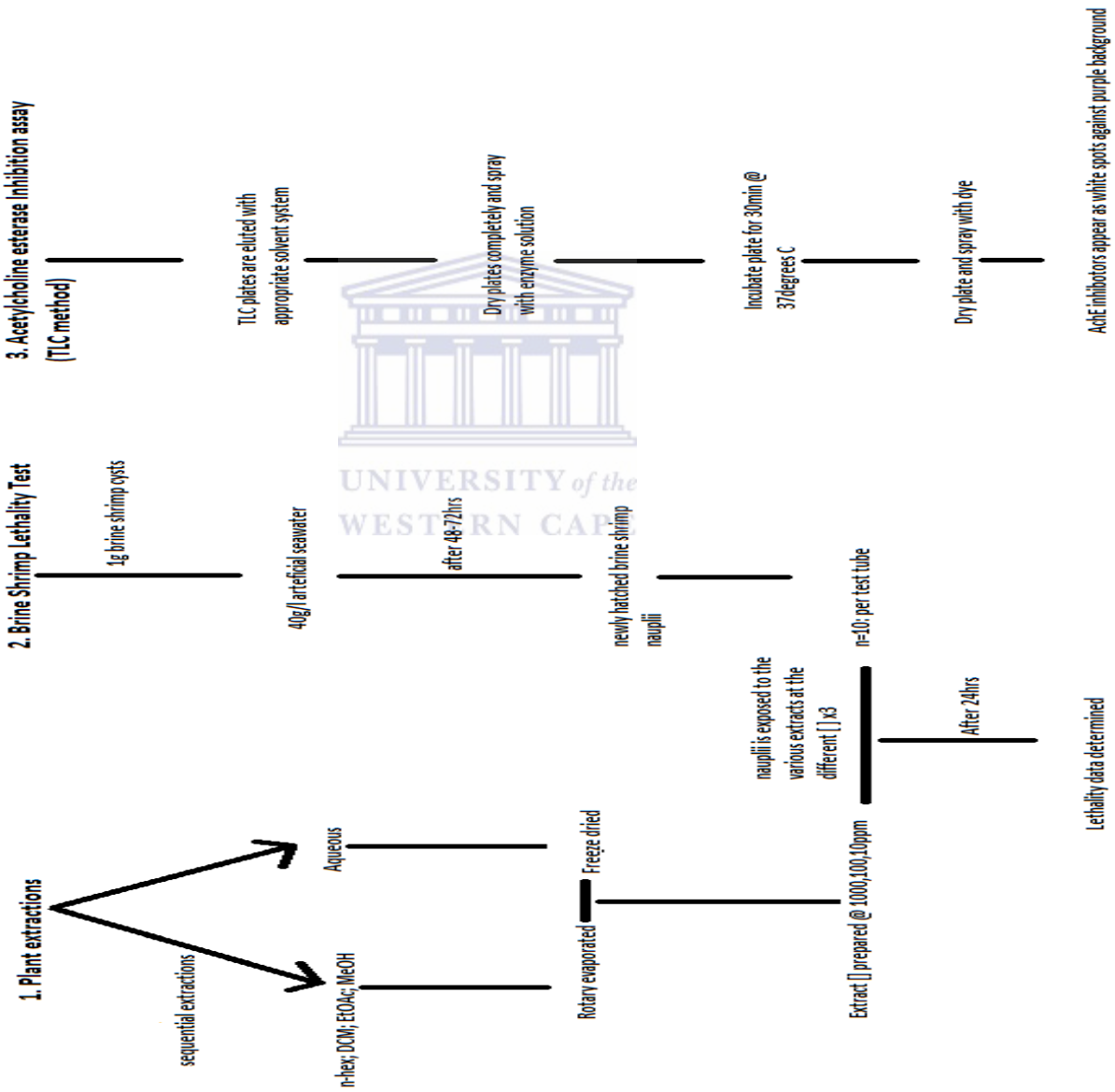
2.1.5.4 Preparation of detection solution

1-Naphthyl acetate (250mg) was dissolved in 100ml of ethanol and Fast Blue B salt (400mg) was dissolved in 160ml dH_2O. The naphthyl acetate solution (10ml) and 40ml Fast Blue B salt solution were mixed together. These solutions were prepared immediately before use to prevent decomposition. After incubation the plates were sprayed with the solution, where after 1-2minutes a purple coloration was observed. Inhibitors of AChE appeared as white spots against a purple colored background.

2.2 Experimental procedures

Fig 5. Schematic diagram of experimental procedures.



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CHAPTER 3

Results and Discussion

3.1 The Brine Shrimp Lethality Test

The lethality assay was conducted on nine South African medicinal plants. Crude extracts were obtained using hexane, dichloromethane, ethyl acetate, methanol and water. The Lethal concentration (LC_{50}) values were determined using Microsoft Excel, 2010. The percentage mortality vs. $\log_{10}$ of the various concentrations was plotted. The regression equation displayed on the charts was used to calculate the LC_{50} values. The inverse log of this value is determined as the LC_{50} value. Appendices **B**, page **(85-89)** show the different graphs for each of the extracts of the nine different plants. Results are indicated in table format **(table 2a-j)**.The level of toxicity is classified as follows:

- Very active: LC_{50} < 100µg/ml, Active : 100 < LC_{50} < 500µg/ml, Moderate : 500 < LC_{50} < 750µg/ml, Inactive : LC_{50} > 1000µg/ml

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Summary of BSLT results are shown in **table 3**, and a summary of the correlation studies are indicated in **table 4**.

3.1.1 *Artemisia afra*

Table 2(**a) Brine** shrimp death indicated as % mortality at the various concentrations tested.

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	10	100	6	60	1	10	51.79	Very active
DCM	10	100	6	60	1	10	51.79	Very active
EtOAc	10	100	7	70	2	20	37.28	Very active
Methanol	10	100	7.33	73.33	4.33	43.33	23.67	Very active
Aqueous	2.33	23.33	1	10	1	10	>1000	Inactive

The MeOH extract was the most active of the four extracts followed by the EtOAc, n-hex and DCM extract. Overall all four organic extracts of *A.afra* were very toxic towards the brine shrimp nauplii. The aqueous extract was inactive.

Artemisia afra has a history of being used in traditional medicine for the treatment of a wide variety of infections. Extracts of this plant have been reported to possess antimicrobial activities. **Ntulela et al (2009)** found that the DCM extract of *Artemisia afra* inhibited the growth of *Mycobacterium aurum* and *Mycobacterium tuberculosis* with an IC_{50} (Inhibitory Concentration) = 270µg/ml and 290µg/ml respectively. It was also found that most of the antimycobacterial activity of the DCM extract was associated with the isolate fraction C8 that contains sesquiterpene lactones such as artemin and arsubin as the most prominent molecules. The antiplasmodial activity of the apolar (chloroform extract) and the polar fractions of *Artemisia afra* was investigated against *P.falcipatum* and it was found that the chloroform extract was very effective with an IC_{50} ranging between 8.55µg/ml and 12.35µg/ml. The aqueous and methanol extracts showed no activity. In the same study three phenylpropanoids, caffeic acid, chlorogenic acid and 3,5-dicaffeoyl quinic acid were also identified from the apolar fractions of *Artemisia afra* **(Liu et al., 2010)**. Apolar compounds which are present in the essential oil of *Artemisia afra* include monoterpenes, sesquiterpenes and triterpenes, whereas flavonoids form the major part of polar compounds in this species. The DCM extract of this plant has also been reported to be very active against *T.b.brucei*, with an IC_{50} value of 25.27µg/ml **(Nibret & Wink, 2010)**. The essential oil component of this plant has been reported to exhibit toxic effects, and since it has been reported that the volatiles are easily lost during the traditional preparation **(Liu**

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et al., 2009), it could explain the inactivity of the aqueous extracts towards brine shrimp nauplii. Essential oils are also insoluble in water and is easily extracted in hexane, hence the toxicity of the hexane extract. Their low solubility may also be a contributing factor towards the inactivity of the aqueous extract. On the other hand, it should also be bare in mind that sufficient quantities of these bioactives are also taken via inhalation therapy by patients rather than oral administration in the traditional setup.

3.1.2 *Artemisia annua*

Table 2 (**b)** Brine shrimp death indicated as % mortality at the various concentrations tested.

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	9	90	5.33	53.3	1	10	74.58	Very active
DCM	8.67	86.7	4.33	43.3	1	10	102.62	Active
EtOAc	10	100	5	50	2.33	23.3	50.62	Very active
Methanol	10	100	5	50	1.67	16.7	56.21	Very active
Aqueous	2.33	23.33	2	20	1.33	13.3	>1000	Inactive

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The EtOAc extract of *Artemisia annua* was the most active followed by the MeOH, n-hex and DCM extract. All of the organic extracts were active where as the aqueous extract was not inactive.

Artemisinin from *Artemisia annua* is an antimalarial drug used for the treatment of malaria. It was structurally defined in 1972 in China as a sesquiterpene lactone with an endoperoxide bridge. Toluene, hexane or petroleum ether are the most widely used solvents for the extraction of artemisinin, with extraction times which can vary from a

few minutes to several hours **(Hao et al., 2002)**. It has been reported that flavonoids from this plant, are also involved in the antimalarial activity. In an investigation by **Bilia et al., (2006)**, it was found that hexane is more selective for the isolation artemisinin, while dichloromethane is more selective for the extraction of flavonoids, which include chrysosplenol, eupatin, cirsilineol, casticin, chrysoplenetin and artemetin. The flavone casticin was purified from an ethylacetate extract of *Artemisia annua*, using high-speed counter-current chromatography **(Han et al., 2007)**.

In addition to artemisinin, four flavonoids; chrysoplenetin, casticin, eupatin and artemetin, had been previously isolated and identified from the hexane extract of *A.annua* **(Baraldi et al., 2008)**. There is controversy regarding the efficacy of *Artemisia annua* in a tea form for the treatment of malaria. A study conducted by **Atemnkeng et al (2009)**, demonstrated that mice infected with *Plasmodium chabaudi* and treated with *A.annua* infusion, showed decreased parasataemia, but not to a level where it is curative.

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The pure compound, artemisinin and crude dichloromethane extracts of four *Artemisia species*, which included *Artemisia annua*, showed trypanocidal activity with IC_{50} value of 35.91µg/ml and 41.05µg/ml respectively (Nibret & Wink, 2010). A new sesquiterpene (Z)-7-acetoxy-methyl-11-methyl-3-methylene-dodeca-1,6,10-triene (AMDT) was isolated and identified from the methanol extract of *A.annua* and showed cytotoxicity against human tumor cell lines 95-D and Hela with LC_{50} values of 27.08 and 20.12µmol/l, respectively **(Zhai et al., 2010)**.

3.1.3 *Sutherlandia frutescens*

Table 2 (**c**). Brine shrimp death indicated as %mortality at the various concentrations tested. (powdered)

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	5.67	56.7	1.67	16.7	2.67	26.7	>1000	Inactive
DCM	3	30	1.33	13.3	1	10	>1000	Inactive
EtOAc	7.67	76.7	1.67	16.7	1	10	331.02	Active
Methanol	8.33	83.3	1.33	13.3	2	20	227.68	Active
Aqueous	5.33	53.3	2	20	1.33	13.3	>1000	Inactive

Table 2 (**d**). Brine shrimp death indicated as %mortality at the various concentrations tested. (tea cut)

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	10	100	2.33	23.3	1	10	107.71	Active
DCM	7.67	76.7	1.33	13.3	1.67	16.7	337.98	Active
EtOAc	7	70	2.67	26.7	1.33	13.3	321.48	Active
Methanol	10	100	1.67	16.7	1.67	16.7	113.50	Active
Aqueous	7.33	73.3	2	20	1	10	364.39	Active

The hexane and DCM extract for the powdered material was inactive, whereas the hexane and DCM extract of the Tea Cut material were active. The EtOAc extracts of the powdered and tea cut materials were active and the MeOH extracts of the powdered and tea cut material were active. The aqueous extract of the powdered

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material was inactive, compared to the aqueous extract of the tea cut material which was active.

Sutherlandia frutescens is a medicinal plant known to act on the immune system and for the treatment of various illnesses, including inflammatory conditions. The antioxidant activity of *S.frutescens* hot water extract was investigated by **Fernandes et al, (2004)** and it was found that this extract possessed superoxide scavenging activities at concentrations as low as 10µg/ml and hydrogen peroxide scavenging activities at 0.62µg/ml, which could account for some of the anti-inflammatory properties that have been reported for this plant. Phenolic compounds such as tannins and flavonoids were implicated in the activity observed in this extract. Tannins, a known class of compounds present in *Sutherlandia* leaves are also known for exhibiting HIV inhibitory activities. Aqueous and organic extracts of *Sutherlandia* have been shown to possess anti HIV activities. This indicates that the inhibitory effects observed in these extracts were probably due to the presence of tannins **(Harnett et al., 2005)**.

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Steenkamp & Gouws, (2006) determined the cytotoxicity of *Sutherlandia* aqueous extract against a prostate carcinoma cell line (DU-145), breast cancer cell lines (MCF-7 and MDA-MB-231) and a non malignant breast cell line (MCF-12A). The extract inhibited the growth of oestrogen dependent cancer cell lines and stimulated the growth of MCF-12A and MDA-MB-231 cells. Ethanolic extracts of *S.frutescens* have been reported to inhibit proliferation of both MCF-7 mammary adenocarcinoma cells.

A concentration of 1.5mg/ml of this extract was found to statistically significantly inhibit 50% of MCF-7 cell proliferation after 24hours, when compared to the vehicle-

treated control **(Stander et al., 2007)**. Canavanine, a natural L-arginine analogue and its metabolite canaline are known to possess antitumor activities and are likely to contribute to the antiproliferative and apoptotic effects of *Sutherlandia frutescens* extracts **(Ojewole, 2008)**. A cycloartane triterpenoid (SU3), isolated from a methanolic extract of *S.frutescens* by **Olivier et al, (2009)** was previously reported to exhibit inhibitory effects towards skin carcinomas.

The difference in lethality data observed for the two forms of *Sutherlandia* plant material, may be attributed to harvesting and processing factors since different batches of the material was used. The powdered material was shown to be less active than the tea cut material and this difference may probably be as a result of mechanical shearing forces prevailing during the milling process, which may have led to the selective destruction of certain molecules, since this process involves the generation of heat.

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3.1.4 *Hypoxis hemerocallidea*

Table 2 (**e**) Brine shrimp death indicated as %mortality at the various concentrations tested.

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	7.67	76.7	1	10	0	0	478.32	Active
DCM	9.33	93.3	2.67	26.7	1.33	13.3	117.10	Active
EtOAc	10	100	1.33	13.3	2.33	23.3	111.31	Active
Methanol	7.67	76.7	1.33	13.3	1.33	13.3	355.33	Active
Aqueous	9	90	2	20	3	30	112.77	Active

The EtOAc extract was the most active, followed by the aqueous extract. The DCM extract was active, while the hexane and MeOH extracts were less active.

Hypoxis hemerocallidea is traditionally used for the treatment of BPH and urinary tract infections **(van Wyk, 2008)**, as well as for the treatment of rheumatoid arthritis and immune system disorders **(Steenkamp et al., 2006)**. Aqueous and ethanol extracts of *Hypoxis* have been shown to inhibit the growth of *E.coli* at concentrations of 62.5µg/ml. The same extracts were also investigated for inhibition of COX-1 and COX-2 catalysed prostaglandin biosynthesis. In the investigation, 250µg/ml of the ethanolic extract and aqueous extract inhibited COX-1 catalysed biosynthesis in a range between 88% and 98% and between 23-72%, respectively. The same extracts did show some inhibitory effects against COX-2 catalysed prostaglandin biosynthesis as well **(Steenkamp et al., 2006)**. Antioxidant activity against lipid peroxidation of isolated bioactive compounds from the corms of *Hypoxis* was investigated by **Laporta et al (2007)**. Rooperol was shown to inhibit lipid peroxidation showing an IC_{50} values as low as 2.6µM, in comparison to hypoxoside, which showed an IC_{50} value of 12.6µM. These compounds, derived from *Hypoxis* rhizomes may be used as potential strong antioxidants, especially rooperol, which has an outstanding capacity to inhibit lipid peroxidation. Hot aqueous extracts of *Hypoxis* are used to treat BPH and prostate adenoma. The biological activity as observed towards prostate adenoma is ascribed to β-sitosterol and the anti-inflammatory activity of *Hypoxis* extracts ascribed to rooperol **(Steenkamp, 2003).**

The antibacterial and antioxidant activities of the leaves and corms of *Hypoxis hemerocallidea* were investigated by **Katerere & Eloff, (2008)**. Ethanolic extracts of fresh leaves of *Hypoxis*, were consistently active against four bacterial strains with an $MIC_{50} \leq 0.63$mg/ml. Acetone extracts of the corms showed activity against *S.aureus* at a concentration of 0.31mg/ml. This study also showed that there is a clear chemical and biologically activity difference between the aerial and underground parts of

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Hypoxis hemerocallidea. The broad cytotoxicity displayed by the extracts is in agreement with the broad scope of activity reflected in the literature.

3.1.5 *Pseudognaphalium undulatum*

Table 2 (**f**) Brine shrimp death indicated as %mortality at the various concentrations tested.

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	10	100	4	40	3.33	33.3	52.19	Very active
DCM	10	100	4.67	46.7	3.67	36.7	43.04	Very active
EtOAc	10	100	4	40	3	30	55.20	Very active
Methanol	10	100	5.33	53.3	2.33	23.3	47.56	Very active
Aqueous	9	90	1	10	1	10	215.44	Active

The DCM extract was the most active, followed by the MeOH, n-hex, EtOAc and Aqueous extracts.

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Ethanol and Acetone extracts of *Pseudognaphalium moritzianum* have been reported to be active against *Staphyllococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* whereas the aqueous extract was active against *S.aureus* and *P.aeruginosa*. Previously isolated compounds from this genus were flavonoids **(Rangel et al., 2002)**. One such compound, 5, 7-dihydroxy-3-methoxyflavone has been shown to be active against *Bacillus cereus* with a MIC value of 125µg/ml. The most active extracts were those obtained from *P.vira vira* and *P.robustum*, which is in support of the practice where these two plants are the most commonly used in vernacular medicine.

The diterpenoids are reported to be the key compounds with regards to the antimicrobial activities exhibited by this genus **(Mendoza et al., 1997)**. A study conducted by **Mendoza & Urzua. (1998)**, reported on the presence of ent-3α-hydroxy-9(11), 16-kauradien-19-oic acid in addition to 5-hydroxy-3, 6, 7, 8-tetramethoxy flavone in *P.cheiranthifolium*, *ent*-16-kauren-19-oic acid in addition to 5, 7-hydroxy flavanone (pinocembrin) in *P.robustum* and from *P.vira vira* 5-hydroxy-3, 6, 7, 8-tetramethoxy flavone. All of these constituents were obtained from a chloroform extract of the various plants.

The resinous exudates of a chloroform extract of *Pseudognaphalium cheiranthifolium*, *P.heterotrichium*, *P.vira vira* and *P.robustum*, revealed the presence of sesquiterpenoids whereas the resinous exudates of *P.robustum* and *P.cheiranthifolium* showed the presence of monoterpenoids with manthanes being the most prevalent **(Urzua, 2004)**. Thus, although the subject species has not yet been investigated for biological/pharmacological activity, it may be fair to expect that it might display similar activity to the other species.

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3.1.6 *Tulbaghia alliacea*

Table 2(**g**) Brine shrimp death indicated as %mortality at the various concentrations tested.

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	10	100	8.33	83.3	5.67	56.7	15.62	Very active
DCM	9.67	96.7	5	50	3.33	33.3	46.02	Very active
EtOAc	10	100	3	30	2.33	23.3	75.97	Very active
Methanol	10	100	3.33	33.3	2	20	74.58	Very active

The hexane extract was the most active, followed by the DCM, MeOH and EtOAc extract. All four extracts were very active.

There is little published literature concerning the bioactivity of *T.alliacea*. The results of a study regarding the cytotoxicity and antiviral activity of *T.alliacea* showed that the aqueous extract was not very toxic towards HeLa or Vero cells, at any concentration used, but did exhibit cytotoxicity towards Jurkat E6.1, AA-2 and CEM-SS cells. Aqueous extracts were not found to inhibit replication of either Coxsackie B2 virus or HSV-1 **(Treurnicht, 1997)**.

Thamburan et al, (2006) investigated the anti infective effects of various extracts of *Tulbaghia alliacea.* Aqueous, MeOH and $CHCl_3$ extracts of *Tulbaghia alliacea*, *Tulbaghia violacea* and *Allium sativum* were assayed against *Candida albicans*. *Tulbaghia alliacea* aqueous extracts at concentrations 0.15% (w/v) and 0.30% (w/v) exhibited statistically bigger zones of inhibition than *Allium sativum* ($p<0.05$) and *Tulbaghia violacea* ($p<0.0001$). The MeOH extract of *T.alliacea* also showed statistically significant bigger zones of inhibition than *A.sativum* and *T.violacea*. The chloroform extract of *T.alliacea* inhibited *Candida albicans* at all concentrations, with the biggest zone of inhibition (10.67mm) at a concentration of 0.30% (w/v). Over all it was concluded that all extracts of *T.alliacea* at the various concentrations exhibited antifungal activities.

A lowest MIC value (minimum inhibitory concentrations) was obtained with a 0.024% (w/v) chloroform extracts of *T.alliacea*, for which a zone of inhibition of 1mm was observed. IC (Inhibitory concentrations) of the aqueous, MeOH and $CHCl_3$ against the growth of five fungal species, showed that the $CHCl_3$ extract was more

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potent than the aqueous and MeOH extracts, inhibiting the growth of the yeast strains, *Saccharomyces cereviseae, Candida albicans, Candida glabrata, Candida krusei* and *Cryptococcus neoformans*. NMR spectral analysis of the *T.alliacea* inhibiting fractions indicated the presence of the methyl and methylene groups of marasmicin. **Kubec et al, (2002)**, proposed that it is likely that marasmicin further decomposes, giving various sulphur-containing degradation products such as 2,4,5,7-tetrathiaoctane, 2,4,5,7-tetrathiaoctane-2,2-dioxide, 2,4,5,7-tetrathiaoctane-4,4-dioxide, of which many of these compounds have been associated with exhibiting strong antimicrobial, anti fungal as well as antithrombotic properties. The results seen from this study is somewhat in agreement with the activity of this plant observed for the BSLT activity.

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3.1.7 *Carpobrotus sp.*

Table 2 (**h**). Brine shrimp death indicated as %mortality at the various concentrations tested. *C.acinaciformis.*

Extracts	1000µg/ml	%Mortality	100µg/ml	%Mortality	10µg/ml	%Mortality	LC_{50} (µg/ml)	Remark
The average number dead counted after 24hours								
Hex	3.33	33.3	1.33	13.3	1	10	>1000	Inactive
DCM	2.67	26.7	1	10	1	10	>1000	Inactive
EtOAc	6.67	66.7	1	10	1	10	823.27	Moderate
Methanol	3.33	33.3	1.33	13.3	0	0	>1000	Inactive
Aqueous	4.67	46.7	4.33	43.3	4	40	525.25	Moderate

Table 2 (**I**) Brine shrimp results for *C.quadrifidus*

Extracts	**1000µg/ml**	**%Mortality**	**100µg/ml**	**%Mortality**	**10µg/ml**	**%Mortality**	**LC_{50} (µg/ml)**	**Remark**
The average number dead counted after 24hours								
Hex	3.67	36.7	3.67	36.7	3.67	36.7	>1000	Inactive
DCM	4.67	46.7	3.33	33.3	3.67	36.7	>1000	Inactive
EtOAc	10	100	5.33	53.3	5	50	29.70	Very active
Methanol	10	100	6.33	63.3	4.33	43.3	28.08	Very active
Aqueous	8.67	86.7	4	40	3	30	79.57	Very active

Table 2 (**J**). Brine shrimp results for *C.mellei*

Extracts	**1000µg/ml**	**%Mortality**	**100µg/ml**	**%Mortality**	**10µg/ml**	**%Mortality**	**LC_{50} (µg/ml)**	**Remark**
The average number dead counted after 24hours								
Hex	2	20	1.33	13.3	1	10	>1000	Inactive
DCM	2	20	1	10	1.33	13.3	>1000	Inactive
EtOAc	2	20	1.67	16.7	1	10	>1000	Inactive
Methanol	3	30	2.33	23.3	1	10	>1000	Inactive
Aqueous	8.33	83.3	7.67	76.7	5.67	56.7	22.15	Very active

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The hexane and DCM extracts of *C.acinaciformis, C.quadrifidus* and *C.mellei* were inactive. The EtOAc extract of *C.acinaciformis* was moderate; that of *C.quadrifidus* was very active and *C.mellei* was inactive. The MeOH extract of *C.acinaciformis* and *C.mellei* was inactive and that of *C.quadrifidus* was very active. The aqueous extract of *C.acinaciformis* and the aqueous extract of *C.quadrifidus* and *C.mellei* were very active.

Springfield et al., (2003) reported that the ethylacetate extract of *C.murri* and *C.quadrifidus* exhibited minimum inhibitory concentrations (MIC) of 7.5mg/ml against the bacteria *S.aureus*, and for *M.smegmatis* a MIC of 30mg/ml. The minimum bactericidal concentration was also determined against *S.aureus* and *M.smegmatis* for the ethylacetate extract of *C.murrii* and *C.quadrifidus*, which were 15mg/ml and 30mg/ml for the respective bacterial strains. It was proven in the study that the ethylacetate extracts were more potent then the aqueous extracts. The same researchers **(2006)** found that an ethylacetate extract of *C.mellei* showed antibacterial activity against *S.aureus* and *M.smegmatis* with MIC values of 7.5mg/ml and 15mg/ml, respectively. Phytochemical tests indicated the presence of flavonoids. Hydrolysable tannins, phytosterols and aromatic acids.

Flavonoids such as rutin, neohesperidan, hyperoside, cactichin and ferulic acid were isolated from a bioactive ethylacetate fraction, obtained from a methanol extract of *C.edulis*. Flavonoids are known to have antibacterial activity, thus the antibacterial activity observed for *C.edulis* can be attributed to the presence of different flavonoids as well as the presence of tannins **(van der Watt & Pretorius, 2001)**.

There is a significant difference observed for the activity of the aqueous extracts of dried plant material and that of fresh plant material. Water extracts of fresh material show better activity than those of dried material. It is a common phenomenon that certain plant chemical compounds tend to present difficulties regarding being taken back into solution after drying of the plant material and this could possibly explain the observed activity results. On the other hand, water-immiscible organic solvents extract dried material more efficiently than fresh, due to their ability to penetrate the plant matrix better in the former case than the latter.

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The assay is reported to have a good correlation with some tumour cell lines **(Wagensteen et al., 2007)**, and is said to be predictive of a number of biological activities such as cytotoxicity, phototoxicity, pesticidal and trypanocidal activity, enzyme inhibition and ion regulation **(Mackeen et al., 2000)**. **Babajide et al., (2010)**, found a good correlation between antimicrobial activity and brine shrimp lethality. **Mclaughlin et al., (1991)** interpreted that BSLT lethality results as such, that the lethality of substances may be linked to the probable ability of these compounds to kill cancer cells (antitumor activity) and possibly pesticidal and antimicrobial activity. Thus the BSLT provides a front line screen that can be backed up by more specific and expensive bioassays once the active compounds have been isolated **(Psitthanan et al., 2004)**. It is also speculated that compounds that slow down the activity of the naupllii may have an effect on the CNS; hence such observations may suggest possible AChE inhibition from such compounds.

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Table 3. Summary of BSLT results. (LC50) values.

Plant	n-hex	DCM	EtOAc	MeOH	Aqueous
A.fra	51.79	51.79	37.28	23.67	>1000
A.annua	74.58	102.62	50.62	56.21	>1000
***S.frutescens* (powdered)**	>1000	>1000	331.02	227.68	>1000
(tea cut)	107.71	337.98	321.48	113.50	364.39
H.hemerocallidea	478.32	117.10	111.31	355.33	112.77
P.undulatum	52.19	43.04	55.20	47.56	215.44
T.alliacea	15.62	46.02	75.97	74.58	
C.acinaciformis	>1000	>1000	823.27	>1000	525.25
C.quadrifidus	>1000	>1000	29.70	28.08	79.57
C.mellei	>1000	>1000	>1000	>1000	22.15

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Table 4. Correlation of very active extracts with known biological activity and active compounds.

Plants	Extractions	Biological activities	Active constituents
A.afra	DCM $CHCl_3$	antimicrobial antitrypanocidal	C8 fraction (artemin, arsubin) phenylpropanoids, caffeic Acid. Chlorogenic acid, 3, 5-dicaffeoyl acid
A.annua	n-hex DCM MeOH	antitrypanocidal antitumor	artemisinin, chrysosplenetin, casticin, eupatin, artemin, new sesquiterpene (Z)-7-Acetoxy-methyl-11-Methyl-3-methylene-Dodeca-1, 6, 10-triene (AMDT)
P.undulatum	$CHCl_3$ EtOH	antimicrobial	diterpenoids, monoterpenoids, sesquiterpenoids
T.alliacea	$CHCl_3$	antifungal antimicrobial antithrombotic	marasmicin, 2, 4, 5, 7-tetrathiaoc tane; 2, 4, 5, 7-tetrathiaoctane-2-dioxide, 2, 4, 5, 7-tetrathiaoc-tane-4, 4-dioxide
Carpobrotus sp.	EtOAc	antibacterial	flavonoids, hydrolysable tannins phytosterols, aromatic acids

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3.2 Acetylcholine esterase inhibition assay- TCL bioautography.

The AChEI assay was conducted on the extracts of *A.afra, A.annua, S.frutescens, H.hemerocallidea, P.undulatum, and T.alliacea*. Galanthamine (1mg/ml) and various plant extracts (10mg/ml) were spotted on TLC plates and developed in $CHCl_3$: MeOH: H_2O (10:1.3: 0.1). **Fig5**, illustrates AChEI mechanism of action and explains the formation of the purple dye and in the presence of AChEI, the formation of white spots. **Fig 6 (a – f)**, shows AChE results for the various extracts. White spots against a purple background show positive results for the inhibition of AChE. The plates were viewed under UV light @254nm **(i),** @360nm **(ii)**. TLC bioautography plates **(iii)**.

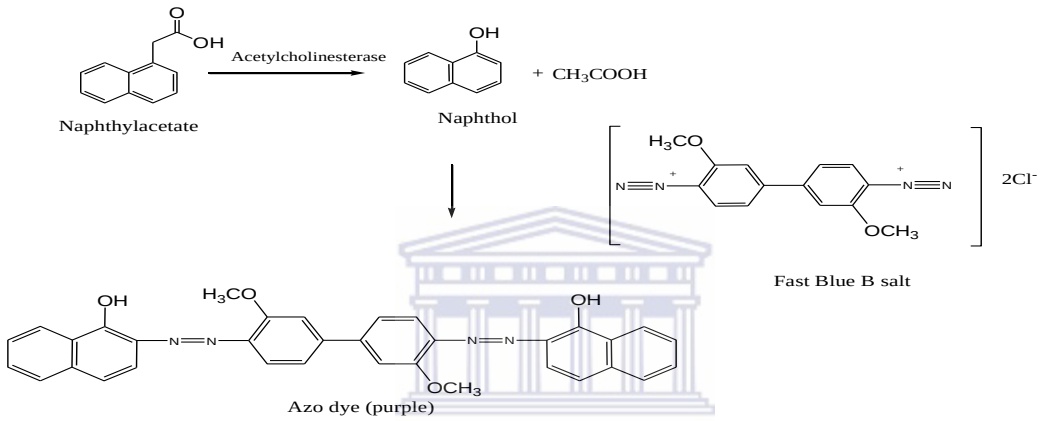

Fig 5. Reaction of acetylcholinesterase with naphthyl acetate and the formation of the purple dye (Marston et al., 2002)

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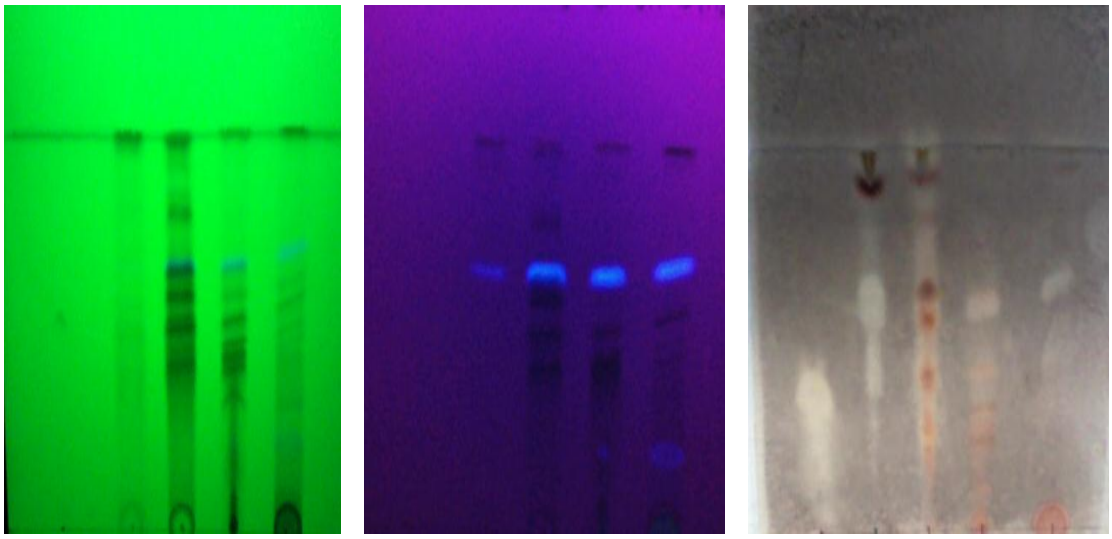
a) *A.afra*

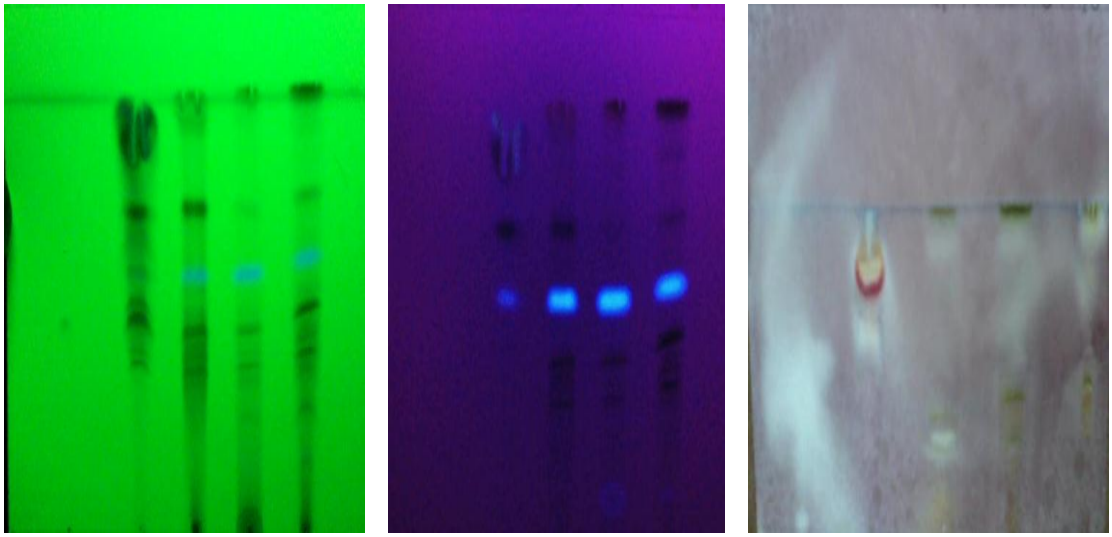


1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (n-hex), lane 3(DCM), Lane 4 (EtOAc), lane 5 (MeOH)

b) *A.annua*

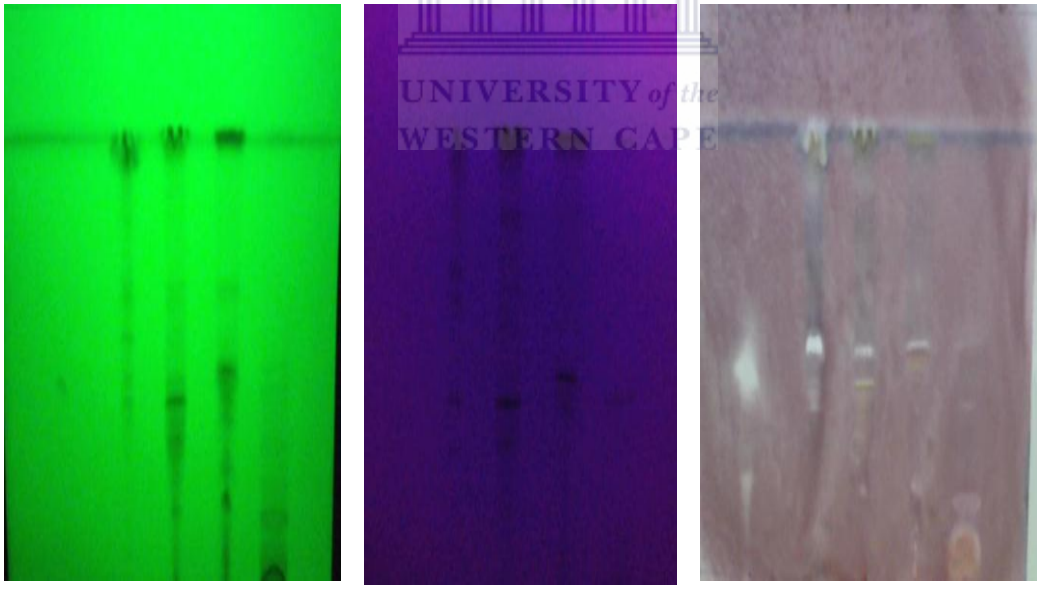


1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (n-hex), lane 3 (DCM), lane 4(EtOAc), lane 5 (MeOH)

c) *S.frutescens*



1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (n-hex), lane 3 (DCM), lane 4(EtOAc), lane 5 (MeOH)

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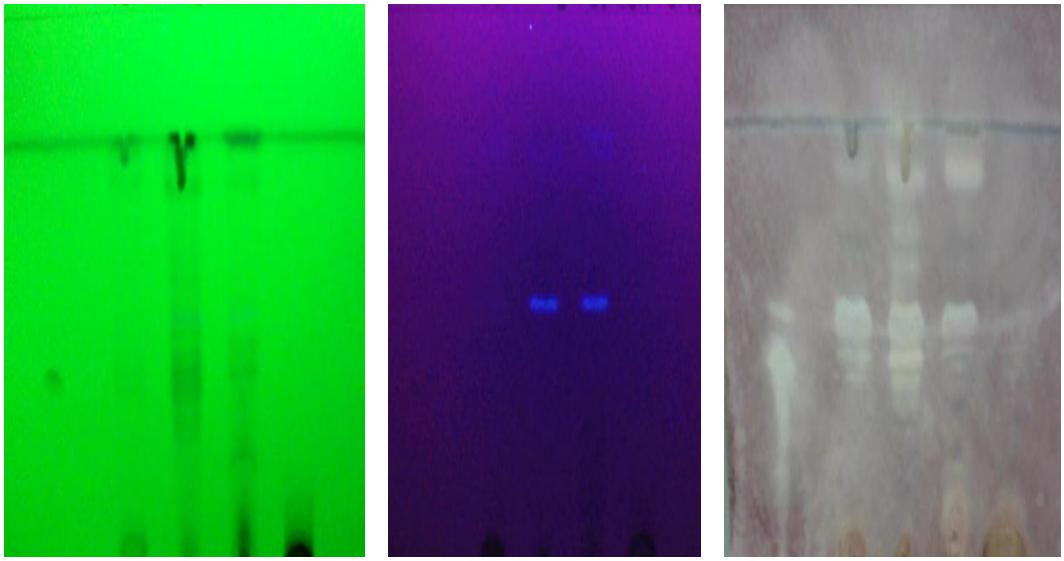
d) *H.hemerocallidea*



1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (n-hex), lane 3(DCM), lane 4 (EtOAc), lane 5 (MeOH)

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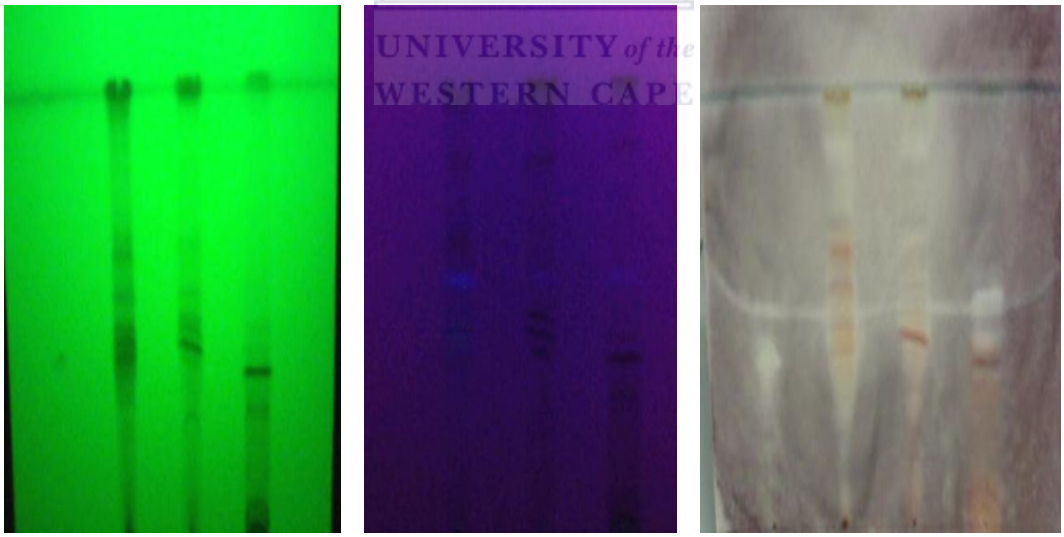
e) *P.undulatum*

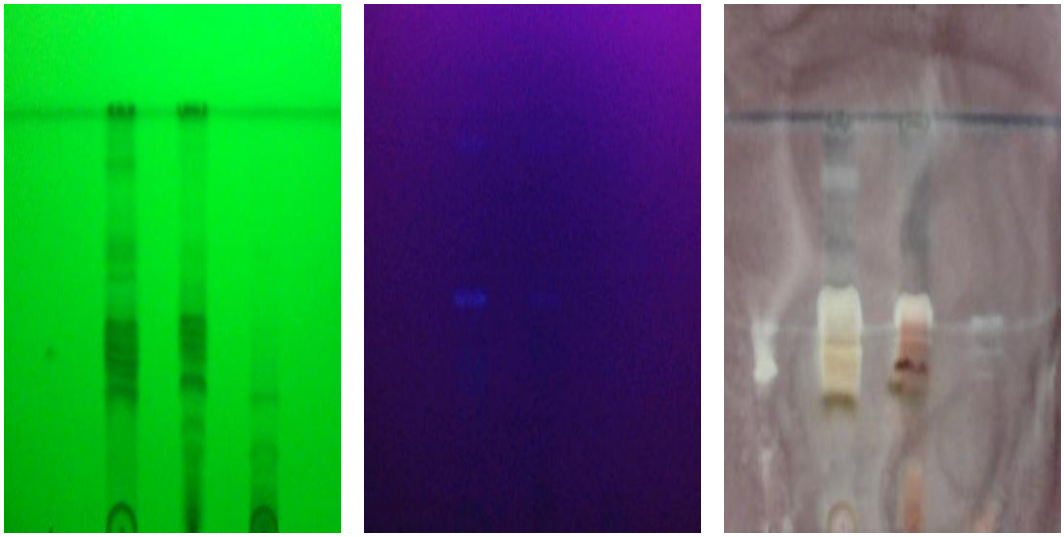


1 2 3 4 1 2 3 4 1 2 3 4

(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (DCM), lane 3 (EtOAc), lane 4 (MeOH)

f) *T.alliacea*



(i) **(ii)** **(iii)**

Lane 1 (galanthamine), lane 2 (DCM), lane 3 (EtOAc), lane 4 (MeOH)

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The search for plant derived inhibitors of AChE has increased due to the benefits of these drugs in the treatment of AD, senile and vascular dementia, ataxia, myasthenia gravis and Parkinson's disease **(Mukherjee et al., 2007)**. Inhibitors of AChE form the basis of the newest drugs available for the management of AD **(Marston et al., 2002)**, and the search for molecules that act on the CNS are increasingly attractive targets for the development of new drugs **(Gomes et al., 2009)**. Alkaloids such as galanthamine and Huperzine A have already proven to be promising sources of useful AChE inhibitors, and recently other classes of natural compounds such as terpenoids, sesquiterpene glycosides and coumarins have been studied for such activity, with the aim of discovering less toxic compounds compared to alkaloidal ones **(Dall'Acqua et al., 2010)**.

The plants under investigation are not known for containing any alkaloids, which is the major class of compounds known to exhibit inhibitory activity towards AChE. The n-hex, DCM, EtOAc and MeOH extracts of *A.afra* showed inhibitory activity; the

n-hex extract of *A.annua* showed inhibitory activity, whereas the DCM, EtOAc and MeOH extracts showed very faint spots of inhibition. *S.frutescens* n-hex, DCM and EtOAc, showed spots of inhibition, whereas the MeOH extract showed a faint spot of inhibition. The n-hex, DCM and EtOAc extracts of *H.hemerocallidea* showed inhibitory activity, whereas the MeOH extracts did not show any activity. The MeOH extract of *P.undulatum* showed inhibitory activity, whereas the DCM and EtOAc extracts did not show any activity. The DCM and EtOAc extracts of *T.alliacea* showed inhibitory activity, whereas the MeOH extract exhibited a faint spot of inhibition. Essential oils, which in general terms occur in many herbs, are widely studied for their large therapeutic potential. In particular, some of them possess CNS properties **(Nobrega de Almeida et al., 2011)**. The essential oil component of *A.annua*, camphor has been found to produce excitation of the CNS, while essential oils like linalool, 1,8-cineol, *p*-cymene and thujone depresses the CNS, reduces spontaneous activity and increases the hypnotic action of pentobarbital **(Perazzo et al., 2003)**. The AChE inhibitory activity observed for the apolar extracts of *A.afra* and *A.annua* can be attributed to the presence of essential oils in these extracts. In a clinical trial, where *Sutherlandia* was administered to HIV positive patients, it was found that the neurotransmitter GABA, present in *Sutherlandia* possibly contributes to mood improvement and general well being experienced by HIV positive patients **(Tai et al., 2004)**. The AChE inhibitory activity observed for the extracts of this plant, may explain a possible mechanism in which the *Sutherlandia* may exert an effect on the CNS. Flavonoids are one of the largest classes of compounds found in herbal sources, with a wide variety of biological activities. Flavonoids as polyphenolic substances have been known to have strong antioxidant activity, which is an advantage in the treatment of AD. Amongst the chemically diverse flavonoids,

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quercetin has been found to be very effective in inhbiting AChE **(Khan et al., 2009)**. Bioactivity guided fractionation of *F.capensis* extracts, yielded the isolation and characterization of nine compounds, three coumarin derivatives, three daucane derivatives, two phenol ester derivatives and 9-epoxyfalcarindiol 9, all of which were able to inhibit AChE as demonstrated by both in the TLC and spectrophotometrical methods, where scopoletin and scopolin were identified as potential new model compounds for the discovery of new AChEI **(Dall'Acqua et al., 2010)**.

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CHAPTER 4

Conclusion

Due to the simplicity of this type of biological bench-top bioassay, the aim of the study was to screen a number of plant extracts for cytotoxicity towards the brine shrimp nauplii and to correlate these results with the findings of more sophisticated and expensive biological assays. Most of the plant extracts evaluated showed good cytotoxicity towards brine shrimp nauplii. The most active of the plants was the n-hex extract of *T.alliacea*. The DCM extract also exhibited significant activity and was found to have a positive correlation with a $CHCl_3$ extract of this plant, which exhibited antifungal and antimicrobial activity, which is to be expected since DCM and $CHCl_3$ have similar solvent properties

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The aqueous extract of *C.mellei* and the MeOH extract of *C.quadrifidus* showed the highest activity amongst the *Carpobrotus species* tested. The EtOAc extract of these to species have been found to exhibit antibacterial activity. The MeOH and the DCM extract of *A.afra* showed the highest activity and have been found to exhibit antimicrobial activity. A $CHCl_3$ extract was shown to have antitrypanocidal activity. The DCM extract of *P.undulatum* showed the highest activity. The $CHCl_3$ and EtOH extract of this plant species have been found to exhibit antimicrobial activities. The EtOAc extract of *A.annu* showed the highest activity. The n-hex and the MeOH extract, showed good correlation with antitrypanocidal and antitumor activity, respectively.

The results from this study show a good correlation with antitumor, antimicrobial and antititrypanocidal activity.

The objective of the study was also to compare the activity of organic extracts and aqueous extracts. The organic extracts showed higher activity towards brine shrimp nauplii than aqueous extracts. Organic extracts of the tested material were done in the dry state, where as the *Carpobrotus species* was tested in the fresh state. Dried plant materials are free from water and easily penetrated by organic solvents, allowing efficient extraction of organo-soluble actives. The low activity observed for the organic extracts of the Carpobrotus *species* may be as a result of high water content which did not allow for the effective penetration of organic solvents. Such solvents may not be immiscible in water and therefore result in the inefficient extraction of organo-soluble actives. This can possibly explain why most organic extracts show higher activity then the aqueous extracts in this study.

The various plants extracts investigated showed good inhibitory activity towards AChE using the TLC bioautography method. The results obtained from this study indicate that this type of activity is not only subject to plants containing alkaloids, but rather a diverse class of compounds may exhibit this kind of activity. The extracts that showed good AChE inhibitory activity also showed good cytotoxicity towards brine shrimp nauplii.

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Concluding remarks and recommendations:

The simplicity of the assays enables it to be easily deployed. It would be advantageous to be used in laboratories, lacking specialized equipment needed for quality control of herbal plants. Traditional medicine practitioner's i.e. traditional healers who serve mostly poor communities may find the BSLT as a very cost effective method, in terms of material needed and technical staff, to ensure the quality control of their herbal preparations.

To give a clear correlation between BSLT and AChE inhibitory activity, a quantifiable method needs to be conducted for the AChEI activity of the investigated plant extracts.

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Appendix A

BSLT data was collected in triplicate. The tables indicate the Mean ±SE of the % mortality.

Artemisia afra

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	10	10	10	10	1	
1000	10	10	10	10	2	
1000	10	10	10	10	4	
Average	**10**	**10**	**10**	**10**	**2.33**	
%Mortality	**100**	**100**	**100**	**100**	**23.3**	
±SE	**0**	**0**	**0**	**0**	**0.88**	
100	5	6	7	8	1	
100	7	7	5	8	2	
100	6	5	9	6	0	
Average	**6**	**6**	**7**	**7.33**	**1**	
%Mortality	**60**	**60**	**70**	**73.3**	**10**	
±SE	**0.58**	**0.58**	**1.15**	**0.66**	**0.58**	
10	1	1	2	5	0	
10	2	1	2	4	1	
10	0	1	2	4	2	
Average	**1**	**1**	**2**	**4.33**	**1**	
%Mortality	**10**	**10**	**20**	**43.3**	**10**	
±SE	**0.58**	**0.58**	**0**	**0.34**	**0.58**	

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Artemisia annua

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	10	9	10	10	3	
1000	9	9	10	10	2	
1000	8	8	10	10	2	
Average	**9**	**8.67**	**10**	**10**	**2.33**	
%Mortality	**90**	**86.7**	**100**	**100**	**23.3**	
±SE	**0.58**	**0.34**	**0**	**0**	**0.34**	
100	5	5	5	5	1	
100	6	4	6	5	0	
100	5	4	4	5	5	
Average	**5.33**	**4.33**	**5**	**5**	**2**	
%Mortality	**53.3**	**43.3**	**50**	**50**	**20**	
±SE	**0.34**	**0.34**	**0.58**	**0**	**1.54**	
10	1	1	3	2	4	
10	1	0	2	1	0	
10	1	2	2	2	0	
Average	**1**	**1**	**2.33**	**1.67**	**1.33**	
%Mortality	**10**	**10**	**23.3**	**16.7**	**13.3**	
±SE	**0**	**0.58**	**0.34**	**0.34**	**1.34**	

Sutherlandia frutescens: Tea Cut

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	10	8	7	10	8	
1000	10	7	7	10	7	
1000	10	8	7	10	7	
Average	**10**	**7.67**	**7**	**10**	**7.33**	
%Mortality	**100**	**76.7**	**70**	**100**	**73.3**	
±SE	**0.00**	**0.34**	**0.00**	**0.00**	**0.34**	
100	4	1	3	2	2	
100	2	1	3	1	2	
100	1	2	2	2	2	
Average	**2.33**	**1.33**	**2.67**	**1.67**	**2**	
%Mortality	**23.3**	**13.3**	**26.7**	**16.7**	**20**	
±SE	**0.88**	**0.34**	**0.34**	**0.34**	**0.00**	
10	1	1	1	1	1	
10	1	1	3	4	1	
10	1	3	0	0	1	
Average	**1**	**1.67**	**1.33**	**1.67**	**1**	
%Mortality	**10**	**16.7**	**13.3**	**16.7**	**10**	
±SE	**0.00**	**0.66**	**0.88**	**1.20**	**0.00**	

Sutherlandia frutescence: Powdered

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[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	6	5	6	8	5	
1000	5	5	9	8	5	
1000	6	2	8	9	6	
Average	**5.67**	**3**	**7.67**	**8.33**	**5.33**	
%Mortality	**56.7**	**30**	**76.7**	**83.3**	**53.3**	
±SE	**0.34**	**1.00**	**1.00**	**0.34**	**0.34**	
100	2	1	3	3	2	
100	2	2	2	3	2	
100	1	1	0	1	2	
Average	**1.67**	**1.33**	**1.67**	**1.33**	**2**	
%Mortality	**16.7**	**13.3**	**16.7**	**13.3**	**20**	
±SE	**0.34**	**0.34**	**0.88**	**0.66**	**0.00**	
10	4	1	1	3	2	
10	3	1	1	2	1	
10	1	1	1	1	1	
Average	**2.67**	**1**	**1**	**2**	**1.33**	
%Mortality	**26.7**	**10**	**10**	**20**	**13.3**	
±SE	**0.88**	**0.00**	**0.00**	**0.58**	**0.34**	

Hypoxis hemerocallideae

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	9	10	10	7	9	
1000	6	9	10	8	10	
1000	8	9	10	8	8	
Average	**7.67**	**9.33**	**10**	**7.67**	**9**	
%Mortality	**76.7**	**93.3**	**100**	**76.7**	**90**	
±SE	**0.88**	**0.34**	**0.00**	**0.34**	**0.58**	
100	1	4	1	1	1	
100	2	3	1	2	4	
100	0	1	2	1	1	
Average	**1**	**2.67**	**1.33**	**1.33**	**2**	
%Mortality	**10**	**26.7**	**13.3**	**13.3**	**20**	
±SE	**0.58**	**0.88**	**0.34**	**0.34**	**1.00**	
10	0	0	0	2	3	
10	0	4	4	1	4	
10	0	0	3	1	3	
Average	**0**	**1.33**	**2.33**	**1.33**	**3.33**	
%Mortality	**0**	**13.3**	**23.3**	**13.3**	**33.3**	
±SE	**0.00**	**1.34**	**1.20**	**0.34**	**0.34**	

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Pseudognaphalium undulatum

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	10	10	10	10	10	
1000	10	10	10	10	9	
1000	10	10	10	10	8	
Average	**10**	**10**	**10**	**10**	**9**	
%Mortality	**100**	**100**	**100**	**100**	**90**	
±SE	**0.00**	**0.00**	**0.00**	**0.00**	**0.58**	
100	5	5	4	6	1	
100	3	4	3	5	0	
100	4	5	5	5	2	
Average	**4**	**4.67**	**4**	**5.33**	**1**	
%Mortality	**40**	**46.7**	**40**	**53.3**	**10**	
±SE	**0.58**	**0.34**	**0.58**	**0.34**	**0.58**	
10	3	8	3	2	1	
10	4	1	3	2	2	
10	3	2	3	3	0	
Average	**3.33**	**3.67**	**3**	**2.33**	**1**	
%Mortality	**33.3**	**36.7**	**30**	**23.3**	**10**	
±SE	**0.34**	**2.20**	**0.00**	**0.34**	**0.58**	

Tulbaghia alliacea

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	10	9	10	10		
1000	10	10	10	10		
1000	10	10	10	10		
Average	**10**	**9.67**	**10**	**10**		
%Mortality	**100**	**96.7**	**100**	**100**		
±SE	**0.00**	**0.34**	**0.00**	**0.00**		
100	8	5	5	4		
100	9	5	6	3		
100	8	5	4	3		
Average	**8.33**	**5**	**3**	**3.33**		
%Mortality	**83.3**	**50**	**30**	**33.3**		
±SE	**0.34**	**0.00**	**0.58**	**0.34**		
10	6	3	3	2		
10	6	3	2	2		
10	5	4	2	2		
Average	**5.67**	**3.33**	**2.33**	**2**		
%Mortality	**56.7**	**33.3**	**23.3**	**20**		
±SE	**0.34**	**0.34**	**0.34**	**0.00**		

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Carpobrotus acinaciformis

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	3	3	4	3	4	
1000	3	2	9	4	5	
1000	4	3	7	3	5	
Average	**3.33**	**2.67**	**6.67**	**3.33**	**4.67**	
%Mortality	**33.3**	**26.7**	**66.7**	**33.3**	**46.7**	
±SE	**0.34**	**0.34**	**1.46**	**0.34**	**0.34**	
100	1	1	1	1	3	
100	1	1	1	2	7	
100	2	1	1	1	6	
Average	**1.33**	**1**	**1**	**1.33**	**5.33**	
%Mortality	**13.3**	**10**	**10**	**13.3**	**53.3**	
±SE	**0.34**	**0.00**	**0.00**	**0.58**	**1.20**	
10	1	1	1	0	2	
10	1	1	1	0	5	
10	1	1	1	0	5	
Average	**1**	**1**	**1**	**0**	**4**	
%Mortality	**10**	**10**	**10**	**0**	**40**	
±SE	**0.00**	**0.00**	**0.00**	**0.00**	**0.82**	

Carpobrotus quadrifidus

[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	5	3	10	10	9	
1000	3	4	10	10	9	
1000	3	7	10	10	8	
Average	**3.67**	**4.67**	**10**	**10**	**8.67**	
%Mortality	**36.7**	**46.7**	**100**	**100**	**86.7**	
±SE	**0.66**	**1.20**	**0.00**	**0.00**	**0.34**	
100	3	4	6	6	6	
100	3	2	5	5	3	
100	5	4	5	8	3	
Average	**3.67**	**3.33**	**5.33**	**6.33**	**4**	
%Mortality	**36.7**	**33.3**	**53.3**	**63.3**	**40**	
±SE	**0.66**	**0.66**	**0.34**	**0.88**	**1.00**	
10	3	6	7	3	2	
10	3	7	4	3	5	
10	5	6	4	7	2	
Average	**3.67**	**5.67**	**5**	**4.33**	**3**	
%Mortality	**36.7**	**56.7**	**50**	**43.3**	**30**	
±SE	**0.66**	**0.34**	**1.00**	**1.34**	**1.00**	

Carpobrotus mellei

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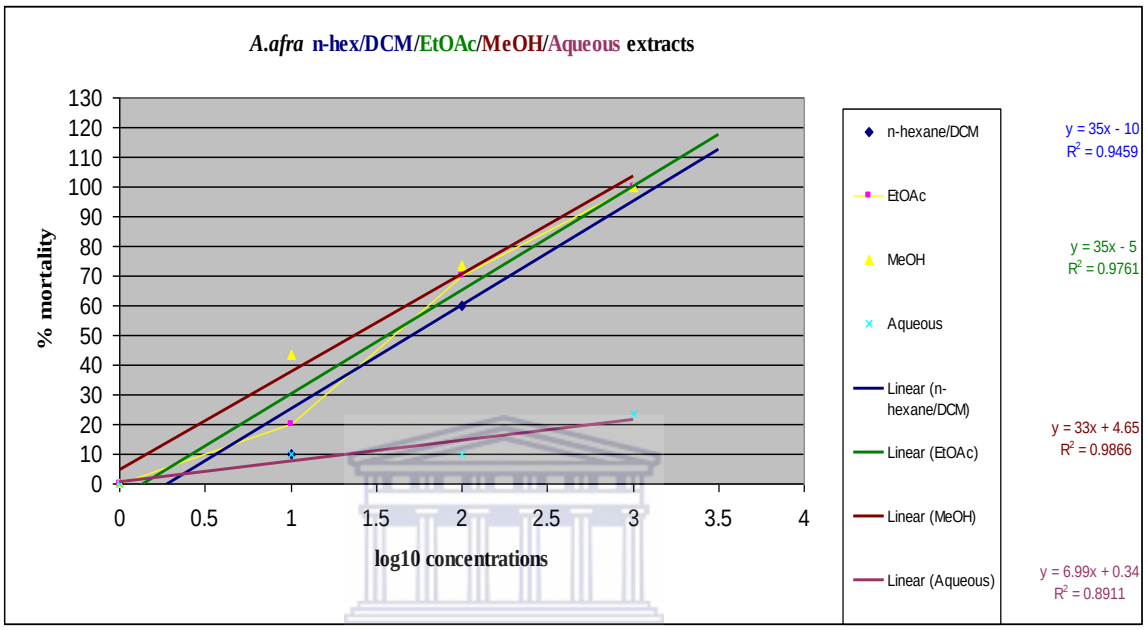
[ ] µg/ml	Hexane	Dichloromethane	EthylAcetate	Methanol	Aqueous	Control
1000	2	2	2	3	8	
1000	2	2	2	3	9	
1000	2	2	2	3	8	
Average	**2**	**2**	**2**	**3**	**8.33**	
%Mortality	**20**	**20**	**20**	**30**	**83.3**	
±SE	**0.00**	**0.00**	**0.00**	**0.00**	**0.27**	
100	1	1	1	3	9	
100	2	1	2	2	7	
100	1	1	2	2	7	
Average	**1.33**	**1**	**1.67**	**2.33**	**7.67**	
%Mortality	**13.3**	**10**	**16.7**	**23.3**	**76.7**	
±SE	**0.34**	**0.00**	**0.34**	**0.34**	**0.66**	
10	2	1	2	1	5	
10	1	1	1	1	5	
10	0	2	0	1	7	
Average	**1**	**1.33**	**1**	**1**	**5.67**	
%Mortality	**10**	**13.3**	**10**	**10**	**56.7**	
±SE	**0.58**	**0.34**	**0.58**	**0.00**	**0.66**	

Appendix B

Graphical demonstration for the determination of LC_{50} values.

1. *Artemisia afra*



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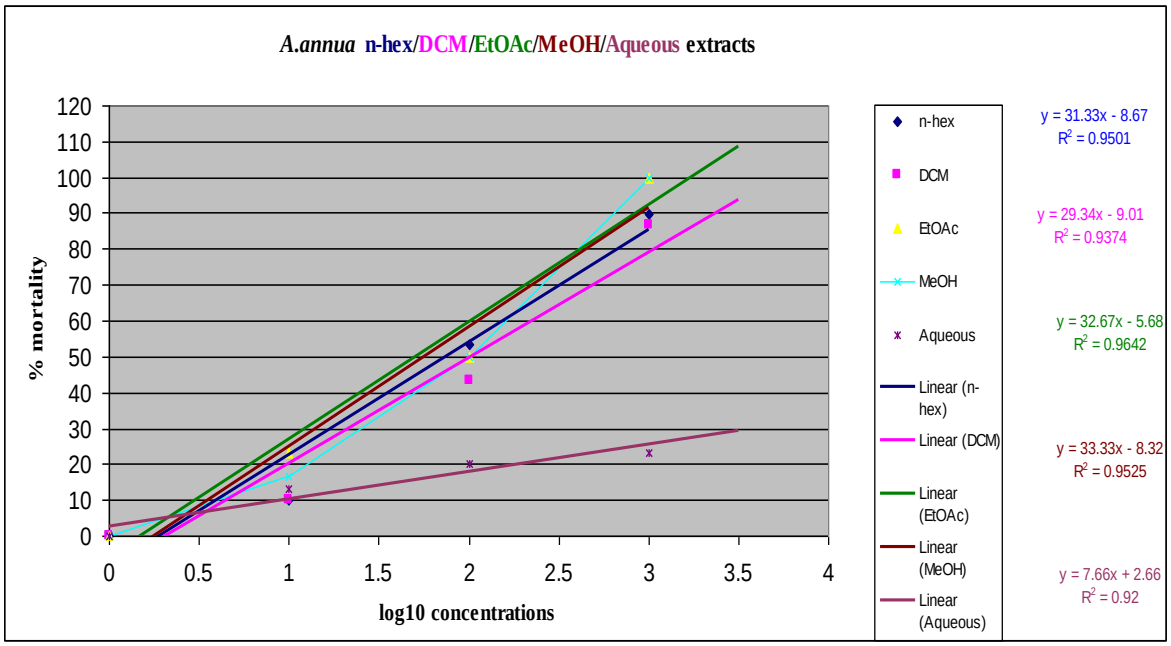
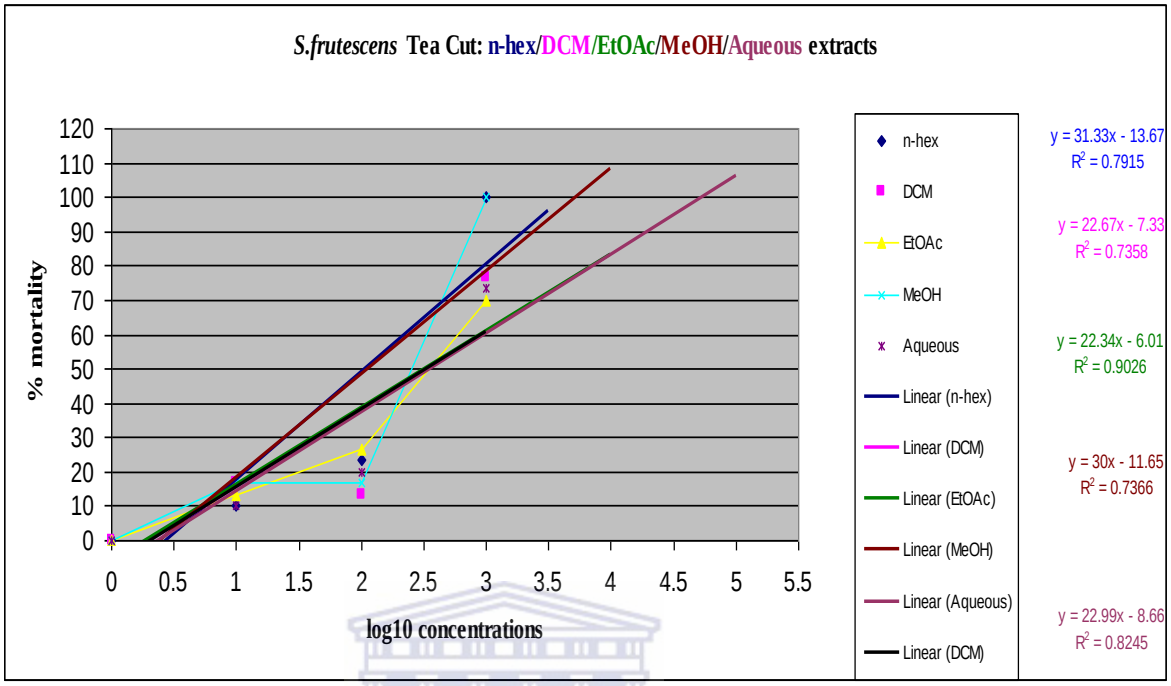
2. *Artemisia annua*



3. *Sutherlandia frutescens*



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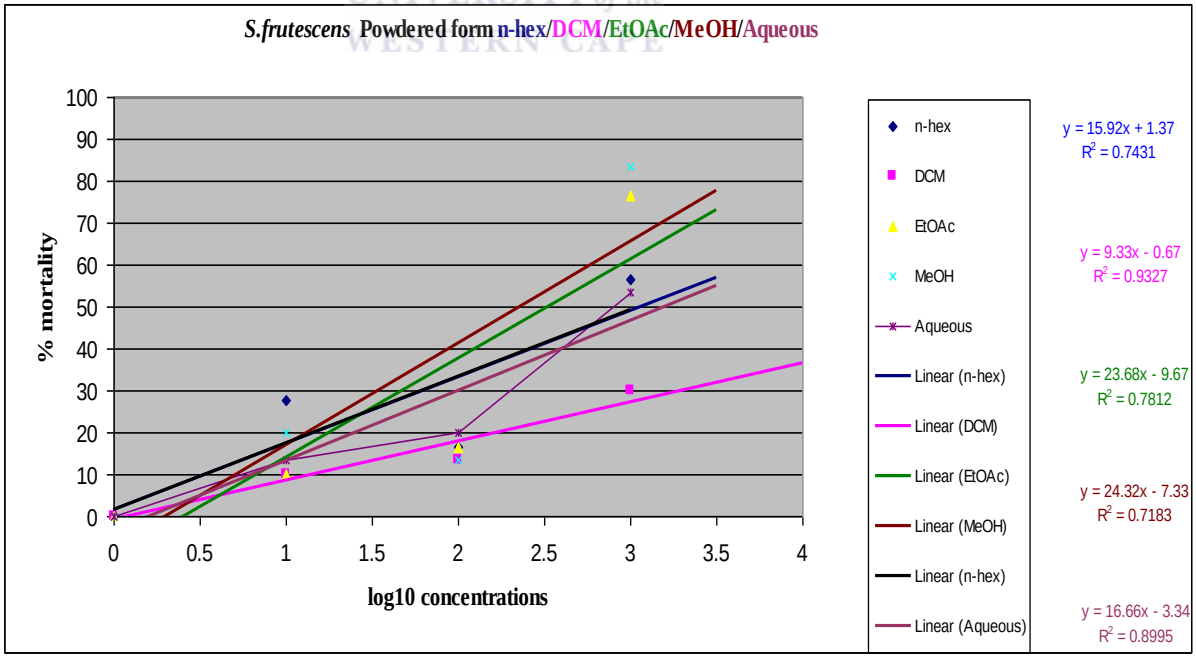
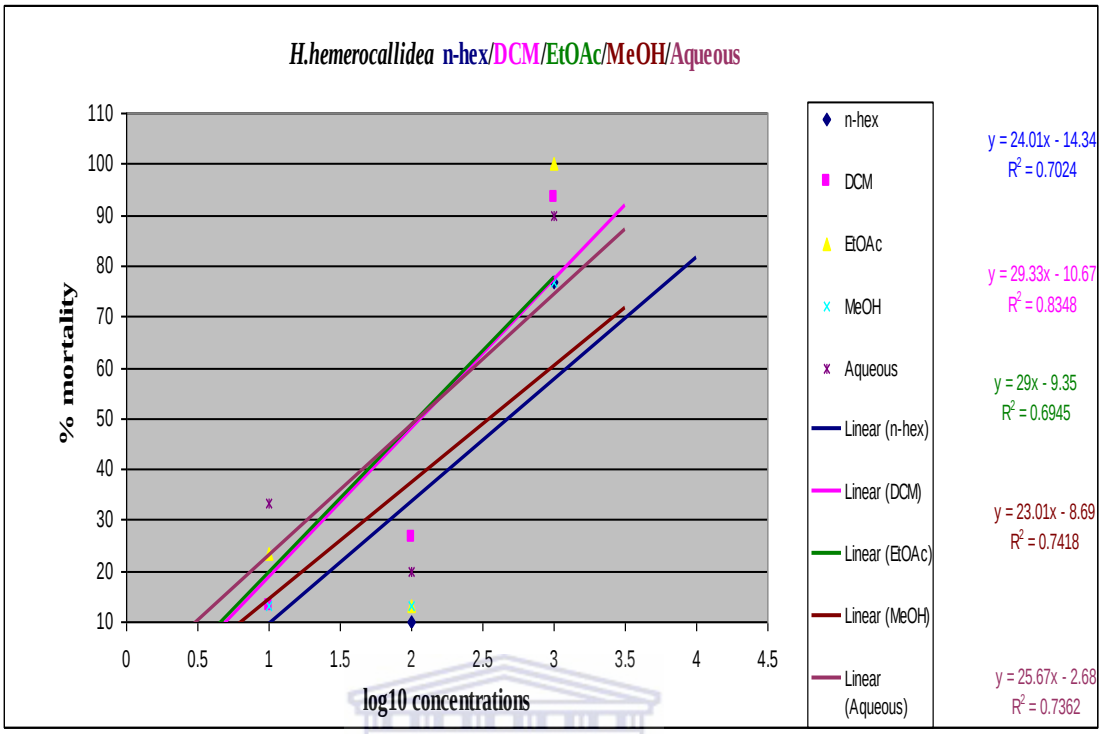


4. *Hypoxis hemerocallideae*



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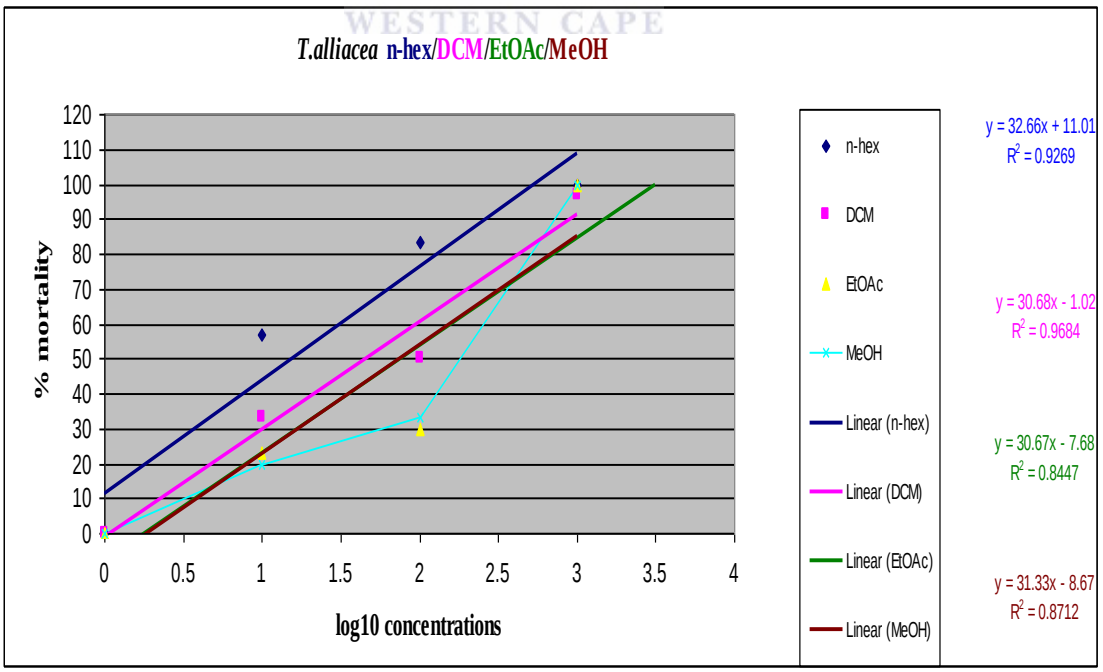
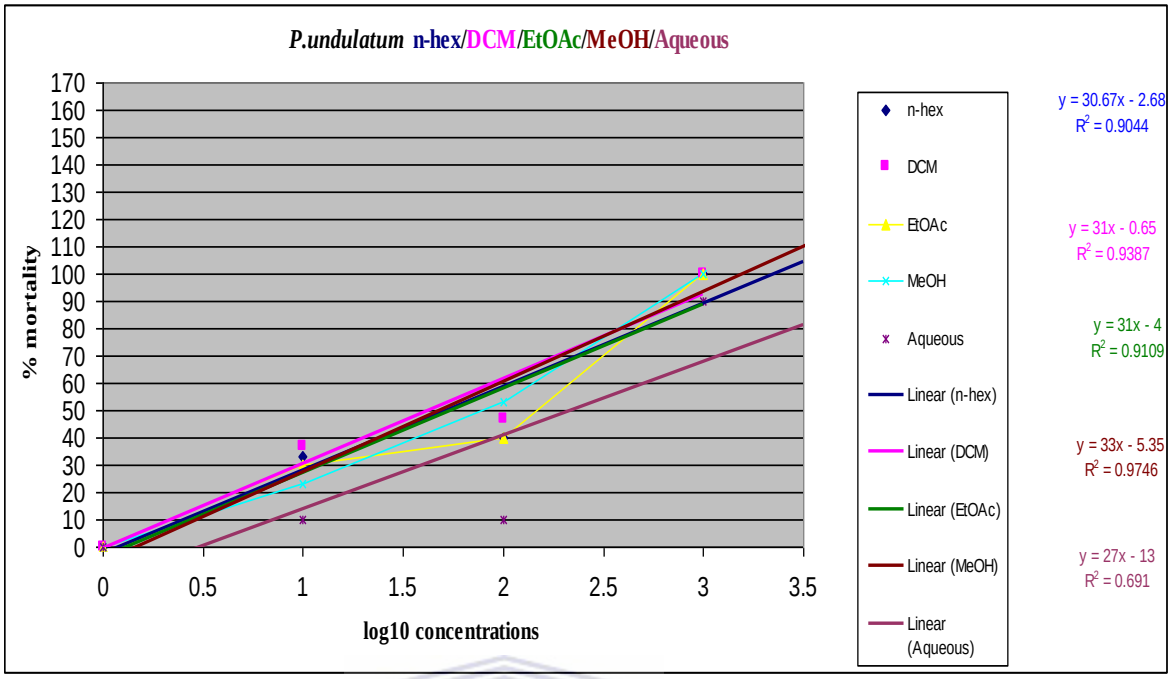
5. *Tulbaghia alliacea*



6. *Pseudognaphalium undulatum*



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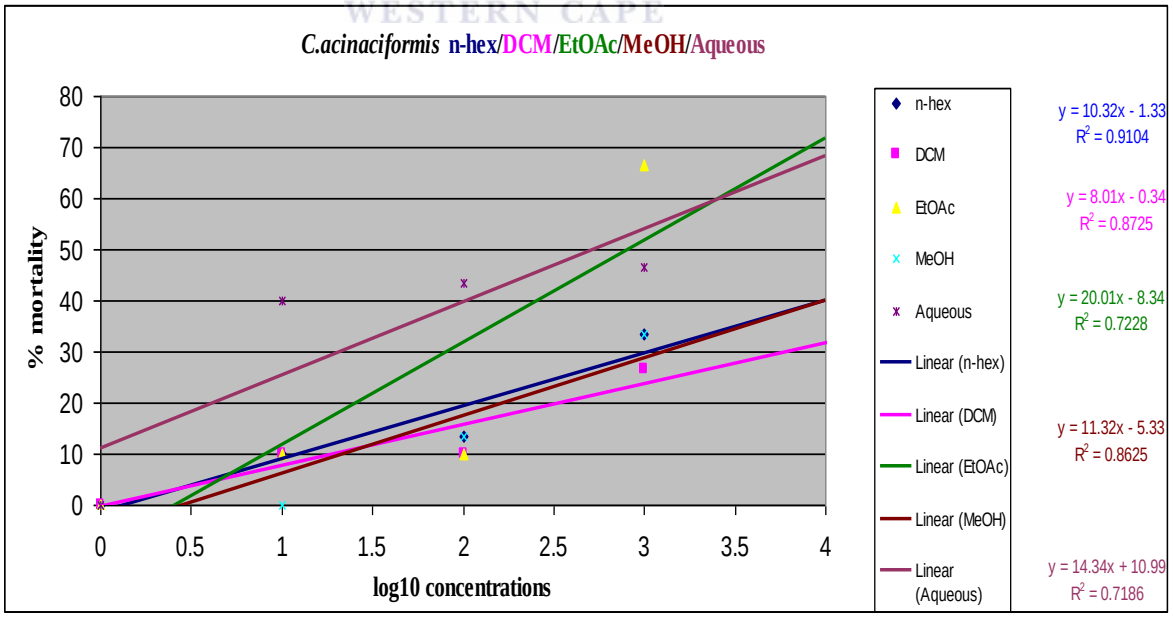
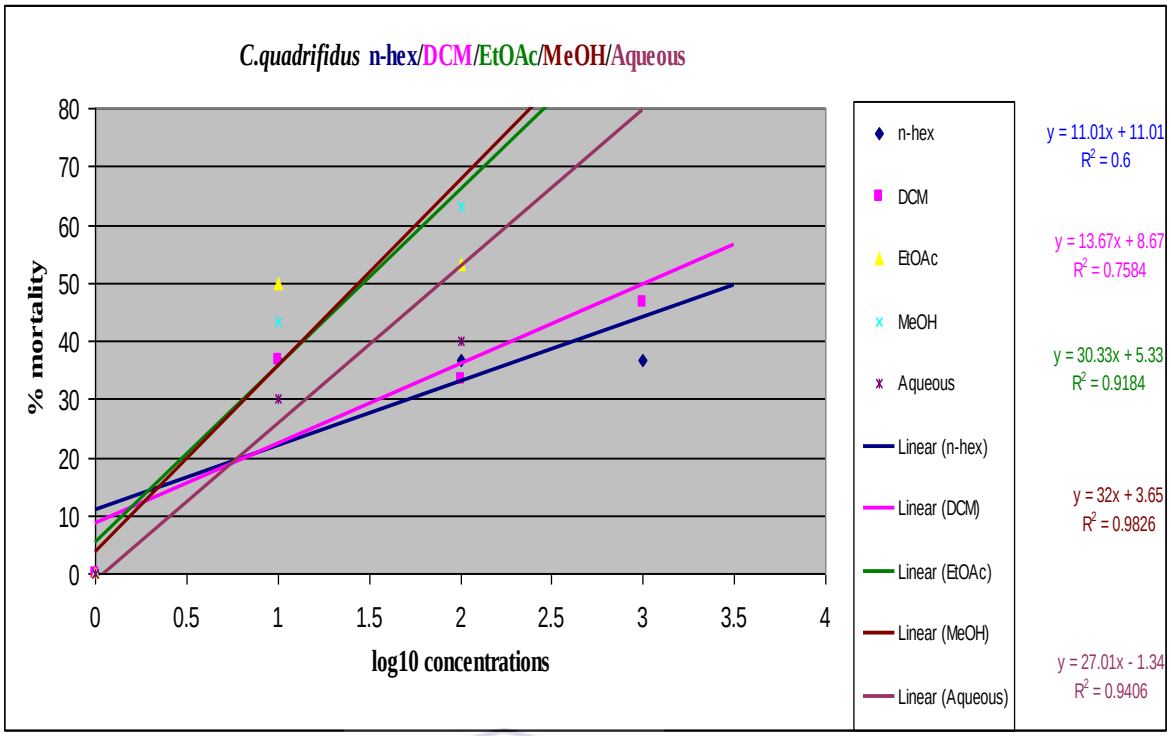
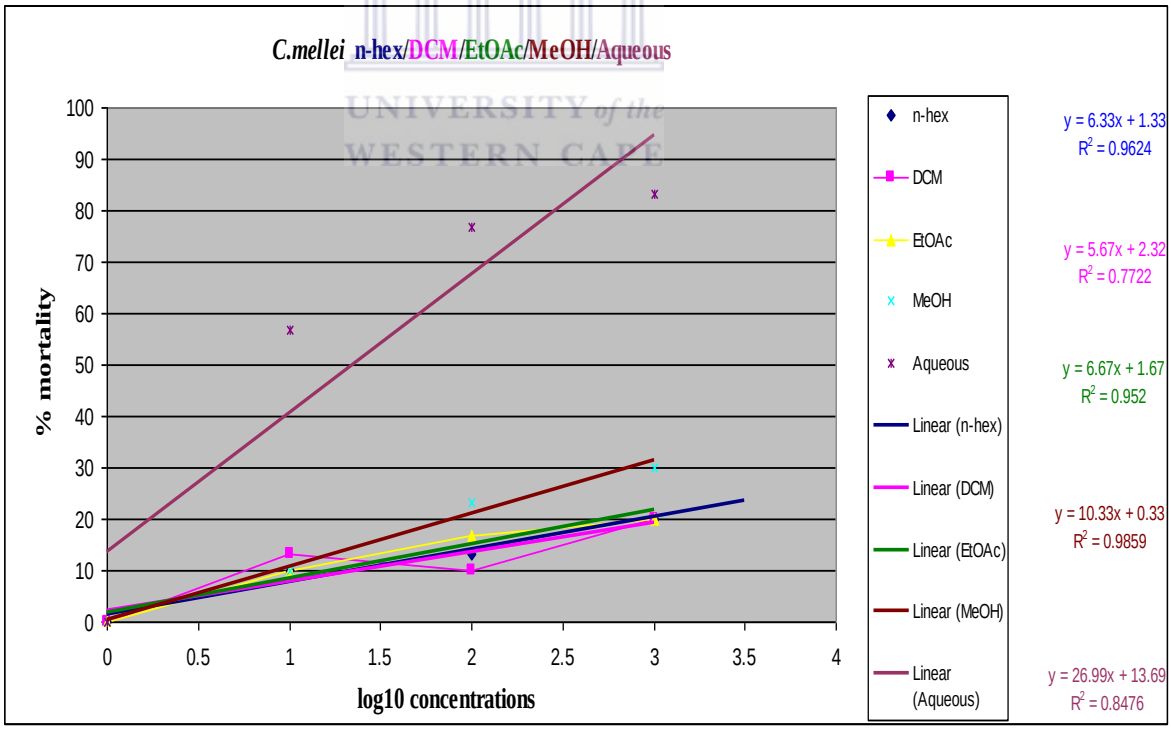
7. *Carpobrotus species.*







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