The antioxidant properties of bufadienolides, analogous to the orbicusides of *Cotyledon orbiculata L. var orbiculata* (Haw.) DC.

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LIST OF ABBREVIATIONS

ACN	- Acetonitrile
AED	- Anti-epileptic drug
APC	- Allophycocyanin
ASE	- Accelerated solvent extraction
BHT	- Butylated hydroxytoluene
BSA	- Bovine serum albumin
Ca^{2+}	- Calcium ions
Cl^{-}	- Chloride ions
DCM	- Dichloromethane
dd H_2O	- Distilled water
DE	- Diatomaceous earth
DMAP	- 4-(Dimethylamino)pyridine
DMEM	- Dulbecco's modified eagle medium
EDCl	- N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
$FeCl_3$	- Ferric (III) chloride
GAA	- Glacial acetic acid
GABA	- Gamma amino butyric acid
GSH	- Glutathione
HCl	- Hydrochloric acid
H_2O_2	- Hydrogen peroxide
HPLC	- High performance liquid chromatography
H_2SO_4	- Sulfuric acid
IPSP	- Inhibitory post synaptic potentials
K^{+}	- Potassium ions
KCN	- Potassium cyanide

LDH - Lactate dehydrogenase

LOOH - Lipid peroxides

$LO^{\bullet}$ - Alkoxyl

$LOO^{\bullet}$ - Peroxyl

MDA - Malondialdehyde

Mg^{2+} - Magnesium ions

mtDNA - Mitochondrial DNA

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Na^{+} - Sodium ions

Na_2SO_4 - Anhydrous sodium sulfate

NBD - Nitro-blue diformazan

NBT - Nitro-blue tetrazolium

NMDA - N-methyl-D-aspartate

NO - Nitrogen oxide

$O_2^{\bullet-}$ - Superoxide anions

$OH^{\bullet-}$ - Hydroxyl radicals

PBS - Phosphate buffer

PTZ - Pentylenetetrasole

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RS - Reactive species

$SbCl_3$ - Antimony (III) chloride

SEM - Standard error of the mean

SOD - Superoxide dismutase

TBA - Thiobarbituritic acid

TCA - Trichloroacetic acid

TLC - Thin layer chromatography

WHO - World Health Organisation

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ABSTRACT

The use of traditional and natural medicines in primary healthcare or alternative therapy is on the increase. However, the safety and efficacy of these medicines have not yet been confirmed. Pharmacognosy, the study of the properties of drugs, potential drugs or drug substances of natural origin and the search for new drugs from natural resources, is therefore of extreme importance in today's healthcare environment. *Cotyledon orbiculata L. var. orbiculata (Haw.) DC.,* a succulent shrub that is widely distributed over the whole of southern Africa, is an example of a plant used in traditional medicine for its antiepileptic effects.

Oxidative stress can either be the cause of, or be secondary to epilepsy pathogenesis. Lipid peroxidation causes the disruption of cell membranes which leads to cell destruction and, in the case of neurological disorders, neurodegeneration. Reactive species have also been found to influence neurotransmission by affecting neurotransmitter metabolism and functions. Reactive species can therefore be responsible for the development of convulsions. Conventional anti-epileptics have shown to exert neuroprotective effects but information or research regarding their ability to prevent epilepsy from becoming chronic does either not exist or is not promising. Antioxidants have potential in the treatment of epileptic seizures as well as the prevention of chronic epilepsy by preventing the effects that oxidative stress has on neurotransmitter metabolism and functions that cause alterations in neuronal excitability and seizure threshold, ultimately leading to epileptic foci.

The aim of this study was to evaluate the potential of the bufadienolide orbicusides of *C. orbiculata* and analogues as anti-epileptic treatment through antioxidant activity.

Initially the isolation of novel antioxidants from *C. orbiculata* leaf juice was attempted. The antioxidant activity of the concentrated juice and fractions resulting thereof were evaluated with two assays. The thiobarbituric acid (TBA) assay was used to measure the extent of lipid peroxidation and nitroblue tetrazolium (NBT) assay was used to measure superoxide scavenging activity in rat brain homogenate. The low concentrations of orbicusides prompted the determination of the activity of two commercial bufadienolides (bufalin and cinobufotalin) and two bufadienolide analogues, synthesised by the esterification of trans-androsterone and androstanolone, respectively, using coumalic acid, producing Compound **1** and Compound **2**. The toxicity of the commercial bufadienolides and synthesised analogues were evaluated by using the MTT assay (a cell viability assay).

C. orbiculata juice showed significant pro-oxidant activity in both assays. Bufalin showed significant pro-oxidant activity in the TBA assay. Cinobufotalin showed no significant activity. Compound **1** showed pro-oxidant activity in the TBA assay and Compound **2** showed slight antioxidant activity in the NBT assay. The commercial bufadienolides showed low cell viability, indicating significant toxicity. The synthesised analogues showed a significant reduction in toxicity (despite Compound **2** being moderately toxic) when compared to the toxicity of the commercial bufadienolides.

The low concentrations of orbicusides in the plant material and the antioxidant assay results of the two commercial bufadienolides suggested that the orbicusides may not be involved in the antioxidant properties of *C. orbiculata*. However, the antioxidant activity of Compound **2** showed that altering the pyrone moiety of bufadienolides could possibly improve antioxidant activity. The reduced toxicity and slight antioxidant activity of the synthesised bufadienolide analogues motivates further investigation.

Keywords: *Cotyledon orbiculata*, orbicusides, antioxidant, anti-epileptic, toxicity.

OPSOMMING

Die gebruik van tradisionele en natuurlike medisyne as primêre gesondheidsorg of alternatiewe terapie neem toe, maar die veiligheid en effektiwiteit van hierdie medisyne moet nog bevestig word. Farmakognosie, die studie van die eienskappe van geneesmiddels of potensiële geneesmiddels van natuurlike oorsprong en die soektog na nuwe geneesmiddels vanaf natuurlike bronne, is dus van uiterse belang in vandag se gesondheidsorgsektor. *Cotyledon orbiculata L. var. orbiculata (Haw.) DC.,* 'n vetplant wat wydverspreid oor suider Afrika voorkom, word byvoorbeeld in tradisionele medisyne gebruik as 'n anti-epileptikum.

Epilepsie kan veroorsaak word of kan sekondêr wees tot oksidatiewe stres. Lipied peroksidasie kan lei tot die ontwrigting van selmembrane wat lei tot selvernietiging en, in die geval van neurodegeneratiewe siektetoestande, neuronale degenerasie. Dit is ook gevind dat reaktiewe spesies neurotransmissie kan beïnvloed deur neurotransmittermetabolisme en funksies te affekteer. Reaktiewe spesies kan dus verantwoordelik wees vir die ontwikkeling van konvulsies. Konvensionele anti-epileptiese middels toon neuronale beskermende effekte, maar hul vermoë om te voorkom dat epilepsie chronies word, is nie bekend nie of nie belowend nie. Antioksidante het die potensiaal om epileptiese aanvalle te behandel en chroniese epilepsie te voorkom deur die effek van oksidatiewe stres op neurotransmittermetabolisme en -funksies, wat kan lei tot veranderinge in neuronale eksitasie en epileptiese drempel met epileptiese foki as die gevolg, te voorkom.

Die doel van die studie was om die potensiaal van die bufadiënolied orbikosiede van *C. orbiculata* en analoë as anti-epileptiese behandeling, deur antioksidant aktiwiteit, te ondersoek.

Die studie het begin deur te poog om nuwe antioksidante vanuit *C. orbiculata* plant sap te isoleer. Die antioksidant aktiwiteit van die gekonsentreerde sap en fraksies daaruit verkry is geëvalueer deur gebruik te maak van die tiobarbituursuur (TBA) analise (wat die omvang van die inhibisie van lipiedperoksidasie kwantifiseer) en die nitrobloutetrasolium (NBT) analise (wat die omvang van die superoksiedanioonopruiming kwantifiseer) met rotbrein homogenaat. Weens die lae konsentrasie van orbikosiede in *C. orbiculata* is daar van twee kommersiële bufadiënoliede gebruik gemaak (bufalien en sinobufotalien), asook twee bufadiënoliedanaloë (gesintetiseer deur die esterifikasie van trans-androsteroon en androstanaloon, respektiewelik, met kumaliensuur om Verbinding **1** en Verbinding **2** te vorm) om die antioksidant aktiwiteit van bufadiënoliede te evalueer. Die toksisiteit van die

kommersiële bufadiënoliede en gesintetiseerde analoë is geëvalueer deur gebruik te maak van die MTT analise (‘n sel lewensvatbaarheid analise).

Die sap van *C. orbiculata* het betekenisvolle pro-oksidant aktiwiteit in beide analises getoon. Bufalien het betekenisvolle pro-oksidant aktiwiteit getoon in die TBA analise. Sinobufotalien het geen betekenisvolle aktiwiteit getoon nie. Verbinding **1** het pro-oksidant aktiwiteit getoon in die TBA analise en Verbinding **2** het geringe antioksidant aktiwiteit getoon in die NBT analise. Die kommersiële bufadiënoliede het lae sellewensvatbaarheid getoon wat aandui dat die verbindings merkbaar toksies is. Die gesintetiseerde analoë het ‘n merkbare afname in toksisiteit getoon (ten spyte van matige toksisiteit getoon deur Verbinding **2**) in vergelyking met die toksisiteit van die kommersiële bufadiënoliede.

Die lae konsentrasies van die orbikosiede in die plantmateriaal en die resultate van die antioksidant analise van die twee kommersiële bufadiënoliede dui daarop dat die orbikosiede moontlik nie betrokke is by die antioksidant aktiwiteit van *C. orbiculata* nie, maar die antioksidant aktiwiteit van Verbinding **2** dui aan dat die antioksidant aktiwiteit van bufadiënoliede wel moontlik verbeter kan word deur die piroongedeelte van die bufadiënoliede te verander. Die verlaagde toksisiteit en geringe antioksidant aktiwiteit van die gesintetiseerde bufadiënolied analoë dien as motivering vir verdere ondersoek.

Sleutelwoorde: *Cotyledon orbiculata*, orbikosiede, antioksidant, anti-epilepties, toksisiteit.

1 INTRODUCTION, RESEARCH AIM AND OBJECTIVES

1.1 Introduction

Cotyledon orbiculata L. var orbiculata (Haw.) DC., a small succulent shrub, is an example of a traditional medicine used for the treatment of epilepsy (Van Wyk *et al.*, 2005). Epilepsy is the second most common chronic neurological condition seen by neurologists next to headaches, affecting about 50 million people globally (Carpio & Hauser, 2009; Guberman & Bruni, 1999; Ono & Galanopoulou, 2012). There are three theoretical mechanisms that can be involved during the generation of epileptic seizures, namely the non-synaptic, synaptic (neurochemical) and oxidative stress mechanisms (Engelborghs *et al.*, 2000).

It is postulated that oxidative stress is the causative factor, mediator or byproduct in certain cases of neuropathology. Oxidative injury of the brain may play a role in the initiation and progression of epilepsy (Acharya *et al.*, 2008; Azam *et al.*, 2010). Increases in lipid peroxides in epilepsy models, elevation of antioxidant enzymes during or after epileptic activity in brain homogenates or blood, and temporal correlations between free radical overproduction and seizure development in certain pathological conditions (e.g. hypoxia, hyperoxia and trauma) suggest that free radicals are produced during epileptiform events in brain tissue (Frantseva *et al.*, 2000). There are many other theories concerning which pathways and reactive species are involved in the pathogenesis of epilepsy. These theories also differ due to the different types and causes of epileptic seizures.

The prevention of epilepsy from becoming a chronic disease has not yet been successfully promoted by current anti-epileptic drugs (Acharya *et al.*, 2008; Azam *et al.*, 2010). Antioxidant therapy can play a favourable role in the altering of the clinical course of epilepsy (Azam *et al.*, 2010) due to their potential neuroprotective properties and ability to prevent the progression of oxidative stress during epilepsy. The protective efficacy and importance of antioxidants depend on the type of reactive species generated, the place of generation, how it is generated and the severity of the damage (Halliwell, 1994; Halliwell & Gutteridge, 2007). As with the many different oxidative stress pathways involved in the pathophysiology of epilepsy, there are many different antioxidant pathways used by the human body or used in drug treatment and research for epilepsy. The antioxidant systems involved also differ according to the age of the patient, type of epilepsy or seizures and the pathological pathway of the disease.

Kabatende (2005) and Amabeoku *et al.* (2007) confirmed that *C. orbiculata* has anticonvulsant effects via a GABAergic mechanism. *C. orbiculata* extracts also showed

antioxidant activity (Louw, 2009; Roux, 2012). The rationale behind this dissertation is that in cases where oxidative stress is involved in epilepsy pathophysiology, the antioxidant activity of the *C. orbiculata* extracts, which could probably be linked to the orbicusides present in *C. orbiculata*, may enhance anticonvulsant effects observed by Kabatende (2005) and Amabeoku *et al.* (2007). Pharmacognostic analytical techniques will be used to determine the potential of *C. orbiculata* and its three bufadienolides (orbicusides A-C) as antioxidant epilepsy treatment.

1.2 Research aim and objectives

The aim of this study was to evaluate the potential of the bufadienolide orbicusides of *C. orbiculata* and analogues as anti-epileptic treatment through antioxidant activity.

The research objectives included:

1. The fractionation of *C. orbiculata* leaf juice
2. The evaluation of the antioxidant activity of *C. orbiculata* leaf juice and the fractions resulting there from
3. The identification of the optimal method of extraction for the orbicusides of *C. orbiculata* leaf pulp by comparing the following extraction methods:
    a. Microwave extraction
    b. Maceration
    c. Soxhlet extraction
    d. Accelerated solvent extraction
4. The isolation of the orbicusides using column chromatography, precipitation reactions and HPLC fractionation
5. The evaluation of the antioxidant activity of two commercial bufadienolides, bufalin and cinobufotalin, structurally similar to the orbicusides
6. The evaluation of the toxicity of bufalin and cinobufotalin
7. The synthesis of two bufadienolide compounds analogous to the orbicusides of *C. orbiculata*
8. The validation of the structures of the synthesised compounds using:
    a. Nuclear magnetic resonance
    b. Mass spectrometry

c. Fourier transform infrared spectroscopy
d. Melting points

9. The evaluation of the toxicity of the two bufadienolide compounds analogous to the orbicusides of *C. orbiculata*

10. The determination of whether changes to the aglycone's 2-pyrone moiety and its position on the steroid structure of the aglycone can improve a bufadienolide's antioxidant activity by comparing the antioxidant activity of the synthesised compounds with that of the commercial bufadienolides

11. To determine whether changes of the aglycone's 2-pyrone moiety and its position on the steroid structure of the aglycone can reduce a bufadienolide's toxicity by comparing the cell viability of the synthesised compounds with that of the commercial bufadienolides

2 LITERATURE REVIEW

2.1 Introduction: The development of phytochemistry and pharmacognosy

Pharmacognosy, the study of the properties of drugs or potential drugs substances of natural origin and the search for new drugs from natural sources, is not a modern concept (Phillipson, 2007). There is written evidence of the use of plants in the treatment of a wide variety of diseases in the great civilizations of the ancient Chinese, Indians and North Americans. The isolation of active compounds from medicinal plants began in the 19th century, which led to expeditions into the almost impenetrable jungles and forests of the New World in the quest for new medicines. These expeditions would last for years and it was not until the plants arrived at well equipped phytochemical laboratories that real discoveries could be made. Phytochemicals continued to be discovered and developed throughout history, from quinine from *Cinchona* bark, morphine and codeine from the opium poppy, digoxin from *Digitalis* leaves to the discovery of the antibiotic effects of natural products isolated from *Penicillium*, *Cephalosporurium* and *Streptomyces* during and after World War 2 (Phillipson, 2001).

The importance of phytochemistry and pharmacognosy in pharmaceutical industries lessened during the 1950s (Phillipson, 2001; Phillipson, 2007). The analytical techniques available at the time were clinically obsolete and many plant species studied were claimed to contain no active compounds. The use of synthetic materials became popular and it was confidently anticipated that all drugs, including natural ones, would be produced synthetically. However the pharmaceutical industry's interest in higher plants as drug sources again grew when pharmacognostic studies led to major medicinal developments, e.g. the development of the alkaloids vinblastine and vincristine for cancer chemotherapy by the Eli Lilly Company, and the establishment of major research areas, such as antimalarial and anticancer properties of natural products. Medicinal plant research became interdisciplinary, forming international collaborations between different scientific principles, including botany, biochemistry, pharmacognosy, pharmacology, phytochemistry, medicine, microbial chemistry, chemotaxonomy, toxicology, biotechnology, etc. (Phillipson, 2007).

The vast majority of plants available provide great potential for the discovery and development of new drugs. South Africa has well over 30 000 species of higher plants, of which more than 3 000 species are used as medicines. Of these plant species 350 are medicinal plants most commonly used and traded worldwide (Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*)), along with local equivalents for many of the famous remedies used elsewhere (Van Wyk *et al.*, 2005). The use of plant material in traditional medicine continues to increase. This increase was noticed

in 2002 and caused a rise of concern among health practitioners and consumers in terms of the safety, policy, regulation, evidence and biodiversity of traditional medicines and the preservation and protection of traditional knowledge (World Health Organisation, 2011b). The World Health Organisation (WHO) released a global plan on 16 May 2002 providing a framework for a policy to assist countries to regulate traditional medicine to make its use safer, more accessible to populations and sustainable. By 2008 the WHO stated that approximately 80 % of the African and Asian countries' population relied on traditional medicine for primary healthcare. In many developed countries 70-80 % of the population had used some form of alternative or complementary medicine, of which herbal treatments were the most popular (World Health Organisation, 2011a).

The advances in phytochemical and scientific methods of analyses widened the horizons of pharmacognostic research. Where scientists were unable to identify all active compounds of many studied plants in the past, they now have the ability to study the effects of a single compound, even several, on a multitude of potential targets simultaneously by using high resolution instrumentation (Larsson *et al.*, 2008). But technological and scientific advances are not the only reasons for the potential that pharmacognosy holds. The vast majority of plant life available, the high level of traditional medicine use and the fact that there is little evidence of these medicines' safety or efficacy confirms or emphasizes the potential and importance of pharmacognostic research, especially in today's healthcare environment.

In this study pharmacognostic analytical techniques will be used to determine the medicinal potential of a plant commonly used in traditional medicine to treat epilepsy, namely *Cotyledon orbiculata L. var orbiculata (Haw.) DC.*

2.2 *Cotyledon orbiculata L. var orbiculata (Haw.) DC.*

C. orbiculata is a small succulent shrub, from the family Crassulaceae, with woody branches and thick, broad, rounded, fleshy leaves. The leaves are light to bright green, grey-green or grey in colour, often with a reddish margin and covered with a smooth waxy surface layer. Bell-like and pendulous light orange-red to dark purple-red flowers grow on long, slender stalks (Kellerman *et al.*, 2005; Tolken, 1978; Vahrmeijer, 1981; Van Wyk *et al.*, 2005). Common names in various languages for *C. orbiculata* (figure 2.1) include (Vahrmeijer, 1981; Van Wyk *et al.*, 2005):

- Plakkie, Hondeoor-plakkie, Kooltrie, Varkiesblaar (Afrikaans) or Kouterie (Afrikaans, Koi)
- Pig's ear (English)
- Imphewula (Xhosa)
- Seredile (Sotho, Tswana)

Figure 2.1: *Cotyledon orbiculata L. var orbiculata (Haw) DC.* (Steyn, 2011)

C. orbiculata can grow in a wide variety of soil-types but prefers sandy, well-drained spots. It is often found in the shelter (semi-shade) of other plants and stones, usually on hills or mountain slopes, but is sometimes found in open areas as well. The plant is drought resistant and is often grown in rockeries. *C. orbiculata* is widely distributed over the whole of southern Africa, but is usually confined to rocky outcrops in grassland, fynbos and Karoo regions (Harris *et al.*, 2010; Kellerman *et al.*, 2005; Vahrmeijer, 1981).

Medicinal uses of *C. orbiculata* include the following:

- The fleshy part of the leaf is applied to corns and warts to soften and remove them
- A single leaf is eaten daily to expel worms
- Fresh leaf juice is swallowed once daily for treating a sore throat
- Warmed leaf juice is used as eardrops for earache and drops for toothache
- Warmed leaves are used to treat boils, abscesses, earache or inflammation
- The cut leaf surface is applied to nappy rash
- The cotyledon toxin is said to have local anaesthetic effects
- The cotyledon toxin is also said to act as a central nervous system depressant
- The plant is used to treat epilepsy.

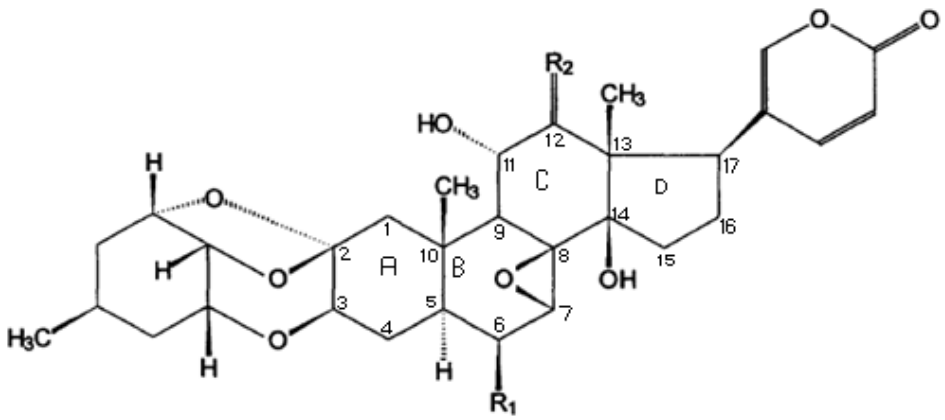
(Van Wyk & Gericke, 2003; Van Wyk *et al.*, 2005)

The plant contains toxic cardiac glycosides and it is ill advised to use this plant material orally.

In this study the potential or efficacy of *C. orbiculata* as epilepsy treatment via its antioxidant activity will be evaluated.

2.2.1 The cardiac glycosides of *C. orbiculata*

C. orbiculata contains three bufadienolides, namely orbicusides A-C (Steyn *et al.*, 1986; Van Wyk *et al.*, 2005). Bufadienolides are cardiac glycosides (polyhydroxy C_{24} steroids and their glycosides) that are characterized by the presence of a six-membered, di-unsaturated δ-lactone (α-pyrone) ring located at C- 17β (Dinan *et al.*, 2001). "Bufa" refers to the toad from which this aglycone is *inter alia* obtained. Over 250 bufadienolides have been identified of which about 160 were from plants. Six plant families, namely Crassulaceae, Hyacinthaceae, Iridaceae, Melianthaceae, Ranunculaceae and Santalaceae have species which contain bufadienolides (Dinan *et al.*, 2001). Bufadienolide glycosides with multiple ether bridges between the aglycone and carbohydrate moieties have only been isolated from members of the Crassulaceae family (Steyn *et al.*, 1986), of which the orbicusides are examples. The structures of orbicusides A ($C_{30}H_{36}O_{10}$), B ($C_{30}H_{38}O_{10}$) and C ($C_{30}H_{36}O_{11}$) are shown in figure 2.2.



COMPOUND	R1	R2
Orbicuside A	-H	-β-OH; -α-H
Orbicuside B	-H	=O
Orbicuside C	-OH	=O

Figure 2.2: The orbicusides of *C. orbiculata* (Bruneton, 1999; Krenn *et al.*, 1998)

The following structural elements are required for, or are favourable to, the orbicusides' activity (Bruneton, 1999):

- A lactone at C-17: A X=C-C= function (where X represents a heteroatom) is required, and it must be in the β configuration.

- Configuration of the rings: Activity will be maximised when the A, B and C rings are in the *cis*, *trans*, *cis* configuration. Activity will greatly decrease when the A and B rings are *trans* fused, but is maintained when the A ring is partially unsaturated. C and D rings must be *cis* fused.
- Substituents: Inversion of the configuration at C-3 decreases activity, but 3-deoxy compounds are not completely inactive.

2.2.1.1 Cardiac glycoside toxicity

Cardiac glycosides are collectively the single most important plant poison of southern Africa, annually accounting for about 33 % of all cattle deaths from plant poisonings (Botha, 2003; Botha *et al.*, 2007; Kellerman *et al.*, 1996; Kellerman *et al.*, 2005; Kellerman, 2009), resulting in annual losses of millions of Rands to the South African livestock industry. South Africa holds a large number of plants, introduced ornamentals and endemic species, containing cardiac glycosides which lead to serious toxicological problems (Naude, 1977). The large variety and wide distribution of these plants, extensive grazing due to droughts, unplanned fires and poor land management (forcing animals to eat plants that normally wouldn't be eaten) are probable reasons for the high incidence of stock losses due to cardiac glycoside poisoning. Accidental ingestion, e.g. contaminated hay, is also a probable cause for poisoning (Botha & Penrith, 2008).

Cardiac glycoside intoxication in people via the ingestion of plant material is relatively rare and can occur when poisonous plants are mistaken for edible plants, food is contaminated with poisonous plant material or when poisonous plant material is used for remedies (Botha & Penrith, 2008). Intoxication is more associated with therapy, in terms of an overdose, undue accumulation errors (Naude, 1977) or due to our lack of knowledge concerning the safe and efficient use of natural products in traditional medicine.

2.2.1.2 Krimpsiekte

Krimpsiekte is a form of poisoning which has long been known in Southern Africa. Also known as cotyledonosis or nenta-poisoning, krimpsiekte is a chronic, accumulative intoxication affecting the nervous and muscular systems. It is a different poisoning syndrome which once was never thought to be related to cardiac glycosides due to its presentation which differed greatly from known symptoms of cardiac glycoside poisoning. Krimpsiekte has several features unusual for cardiac glycoside poisoning, namely its paralytic effects, minimal cardiac involvement and the cumulative effect of bufadienolides from Crassulaceae. Krimpsiekte is also the only plant poisoning in South Africa with a potential danger of affecting people and other animals when the uncooked meat of an affected animal is

consumed. This is especially problematic in rural areas where people, due to financial constraints, tend to consume animals that die in the veld (Botha, 2003; Botha *et al.*, 2007; Kellerman *et al.*, 1996; Naude, 1977).

The first official record regarding krimpsiekte was made in 1864. In 1890 the cotyledons were suspected of toxicity when Weyer (of Darlington) discovered that *Cotyledon ventricosa* was responsible for krimpsiekte in goats, but questioned the correctness of the evidence as it was unusual for a member of the Crassulaceae family to be that poisonous. Studies by Soga, Borthwick and Hutcheon supported Mr. Weyer's findings (Botha, 2003; Kamerman 1926), although the matter was still open for further investigation. *C. orbiculata* was implicated in poisoning in 1908 by Burtt-Davy, the Government Agrostologist and Botanist, and his herbarium assistant. They reported an incident of suspected poisoning of fowls after thinning out *C. orbiculata* in a woman's garden and feeding the plants to her fowl (Botha, 2003; Kamerman, 1926). The relationship between cardiac glycosides and krimpsiekte was only suspected when toxicity trials were done on guinea-pigs at Onderstepoort, during the isolation of the toxic compounds from *Cotyledon wallichii*. (Naude, 1977).

Krimpsiekte mainly affects sheep and goats and there are two types:

- **"Opblaas" krimpsiekte (due to acute toxicity)**

Opblaas krimpsiekte occurs due to the ingestion of large quantities of plant material and is characterised by dullness, apathy, recumbency, evidence of severe pain, salivation, ruminal stasis and tympani ("opblaas"). The mandibles of the animal often droop, tongue may protrude slightly, excessive salivation may occur and the muscles of mastication and deglutition may undergo clonic spasms. The animals tire easily, showing signs of trembling and hyperaesthesia, especially when excited. Nervous signs are worsened when the animals are exposed to sunlight for long periods. The animals still have an appetite but are unable to swallow. Irregular respiration, polypnoea and tachycardia also occur and ewes may abort. Death occurs suddenly or within three days of the first clinical signs (Kellerman *et al.*, 1996; Naude, 1977; Vahrmeijer, 1981).

- **"Dun" krimpsiekte (due to chronic toxicity)**

Chronic toxicity occurs due to repeated ingestion of small quantities of plant material. The cardiac glycosides involved are extremely cumulative and very potent neurotoxins. Affected animals easily become exhausted and lag behind the flock. Once forced to move they tire quickly, drop down exhausted or stand with trembling muscles. The neck of the animal may be held in a peculiar, twisted fashion and can often dangle loosely as it walks. Tetaniform

convulsions may appear and eventually the animal becomes paralysed and dies. Paralysis resembles that of ordinary botulism and can last for weeks. Should the animal recover, it could suffer from the peculiarly twisted neck for months (Kellerman *et al.*, 1996; Naude, 1977; Vahrmeijer, 1981).

2.2.1.3 Mode of action of the cardiac glycosides

Cardiac glycosides act as neurotoxins (Wink, 2010) by affecting important ion channels (e.g. sodium ion (Na^+), potassium ion (K^+) and calcium ion (Ca^{2+}) channels) of neuronal cells, permanently activating or inactivating them. This stops neuronal signal transduction and blocks the activity of the central nervous system, as well as neuromuscular signalling, resulting in paralysis of both the striated and smooth muscles of the heart, lungs and skeleton.

The Na^+-K^+ ATPase ion pump is the most important ion pump in neuronal and other cells for maintaining the ion gradient needed for action potentials and transport mechanisms and is strongly inhibited by cardiac glycosides found in several plant families and toad skins (genus *Bufo*) (Joubert, 1981; Kellerman *et al.*, 2005). Toxins and poisons are classified according to their oral toxicity as determined in rat experiments. Because of this ion pump's importance, cardiac glycosides are considered to be toxins of class Ia, meaning extremely hazardous (5 mg or less per kg body weight) (Wink, 2010). Cardiac glycosides have an allosteric nature, binding extracellularly and affecting ATP intracellularly. Small doses of cardiac glycosides cause a positive inotropic effect on the myocardium, which coincides with the increase of intracellular Ca^{2+} and Na^+ and decrease in K^+ concentrations. These changes in intracellular ion concentrations continue to follow this path as the cardiac glycoside concentration increases, leading to toxicity. It is therefore evident that both the therapeutic and toxic levels of cardiac glycosides cause the same inhibition of the enzyme system, only on different levels (Joubert, 1981; Kellerman *et al.*, 2005).

2.2.1.3.1 Sodium ions

The Na^+-K^+ ATPase ion pump has a specific affinity towards Na^+. The pump requires Na^+ on the intracellular binding area for the activation of the enzyme system. Three Na^+ ions are carried actively out of the cell per ATP molecule that is hydrolysed. This active transport of Na^+ is inhibited by cardiac glycosides, leading to increased intracellular Na^+ (and Ca^{2+}), contributing to the strengthened contractions of e.g. cardiac muscle fibres (Joubert, 1981).

2.2.1.3.2 Potassium ions

Cardiac glycoside inhibition of the Na^+-K^+ ATPase ion pump causes lowering of the concentration of intracellular K^+, resulting in an increase in the strength of muscle fibre (and myocardial) contractility. Intravenous administration of K^+ can therefore be used to treat cardiac glycoside intoxication. Factors such as hyper- or hypokalemia can affect cardiac glycoside poisoning. Patients with hyperkalemia or kidney damage should not receive K^+ as treatment and regular ECG monitoring is vital. Patients with hypokalemia can experience an onset of cardiac glycoside poisoning at levels lower than normal. Other extracellular cations can also activate the Na^+-K^+ ATPase ion pump similarly to potassium, namely ammonia, rubidium, cesium and lithium (Joubert, 1981).

2.2.1.3.3 Calcium ions

Cardiac glycoside inhibition of the active transport of Na^+ and K^+ changes the membrane polarity, allowing Ca^{2+} to easily pass through cell pores. Low concentrations of Ca^{2+} have an inhibiting effect on the Na^+-K^+ ATPase ion pump via competition with Mg^{2+} (which act as intracellular cofactors to activate this enzyme system) (Bruneton, 1999; Joubert, 1981).

Cardiac muscles do not contract in the absence of Ca^{2+}, whereas increases in Ca^{2+} concentration causes muscle contraction. The higher the Ca^{2+} levels the stronger and longer the contractions are in comparison to normal amounts of Ca^{2+} concentrations. Very small concentrations of cardiac glycosides can mimic the effects of Ca^{2+} on cardiac muscle contraction. The positive inotropic effect will only take place once Ca^{2+} is administered, leading to stronger contractions. The addition of Ca^{2+} causes lowering of the threshold of the inotropic effect and toxicity of cardiac glycosides and vice versa (Bruneton, 1999; Joubert, 1981).

2.2.1.3.4 Magnesium ions

Mg^{2+} is needed as an intracellular cofactor for the activation of the Na^+-K^+ ATPase ion pump. Hypo-magnesia causes a decrease in the threshold value for cardiac glycoside poisoning. Magnesiumsulphate can therefore be used as treatment of cardiac glycoside poisoning if the patient is also hypo-magnesic. The activation effect of Mg^{2+} can be cancelled out via competition with Ca^{2+} (Joubert, 1981).

2.2.1.4 Effects of the orbicusides

As mentioned, krimpsiekte is characterised with minimal cardiovascular effects, as compared to the normal cardiac glycoside toxic syndrome. Norman Sapeika (1935) evaluated the cardiovascular effects of three *Cotyledon* species (*C. wallichii, C. paniculata and C.*

reticulata) in frogs and mammals. Significant changes in heartrate and amplitude was detected (vagal stimulation, initial increase in heartrate, followed by slow decrease until permanent ventricular systole occurs). Initial increases in blood pressure were also detected, followed by sudden drops to zero. Possible vasoconstrictor action in coronary vessels was induced, but required potent plant extract preparations in high concentrations. Increased contractions and tonus, also via high extract concentrations, was detected in the intestines and uterus muscles.

C. orbiculata is used in traditional medicine as an anti-inflammatory agent. The assumption is that *C. orbiculata* and, in effect, the orbicusides play a role in anti-inflammation via the scavenging and reduction of the reactive species of nitrogen oxide (NO) produced during inflammation, leading to the reduction of damage of cellular components (Polya, 2003). Studies regarding analgesic activity of the orbicusides were completed and indicated that these bufadienolides do have analgesic properties (Kabatende, 2005). *C. orbiculata* is also used in the treatment of epilepsy.

2.3 Epilepsy

Epilepsy is a seizure disorder with no apparent cause or trigger and which occurs repeatedly. Seizure disorders occur when the brain's electrical activity is periodically disturbed, resulting in a degree of brain dysfunction. Basic symptoms or experiences of a seizure disorder are unusual sensations just before a seizure begins, uncontrollable shaking and unconsciousness (in some patients). Otherwise, the patient will merely stop moving or become unaware of what is happening. Diagnosis is determined with brain imaging, blood tests and electroencephalography (which records the brain's electrical activity) to identify the cause. Drug therapy is usually only initiated should the seizures interfere with the patient's lifestyle or work environment (Epilepsy Foundation; Porter *et al.*, 2008).

Epilepsy is the second most common chronic neurological condition seen by neurologists next to headaches, affecting about 50 million people globally (Carpio & Hauser, 2009; Guberman & Bruni, 1999; Ono & Galanopoulou, 2012). It often begins in childhood or early adulthood, is often chronic and has a prevalence of approximately 1 %, 80 % of which is in developing countries. Active cases of epilepsy are defined as having had one or more seizures in the previous five years. The prevalence is fairly uniform in countries of similar socio-economic development and is also uniform at different ages (Carpio & Hauser, 2009; Guberman & Bruni, 1999). The incidence of epilepsy ranges between 40 and 70 per 100 000 in most developed countries and between 100 and 190 per 100 000 in developing countries. 50 to 60 % of epilepsy begins at the age of 16 years. The chance of acquiring epilepsy at

some time during life is 2 to 4 % and the chances of having at least one seizure during a lifetime are approximately 8 % (Guberman & Bruni, 1999).

Approximately 60 % of all epilepsies are idiopathic (etiology is unknown and can be presumed to be genetic) or cryptogenic (due to an acquired brain lesion that has not been identified or is of unknown cause). Virtually any type of brain pathology can cause seizures or epilepsy. Processes affecting the cerebral cortex also cause epilepsy and are more likely to do so (Guberman & Bruni, 1999).

Approximately 70 % of people with epilepsy achieve long-term remission, most of them within five years of diagnosis (Carpio & Hauser, 2009). The risk of recurrence in developing countries after a first unprovoked seizure is 33-37 %, similar to developed countries (Carpio & Hauser, 2009; Guberman & Bruni, 1999). Epilepsy secondary to underlying structural causes or with abnormal electroencephalogram readings produce the worst prognosis. The standardised mortality rates in epilepsy are two to four times higher than normal and are highest in the first ten years after diagnosis, especially in the first year (Guberman & Bruni, 1999). In developed countries the overall mortality is two to three times greater than mortalities found in the general population (Carpio & Hauser, 2009). Causes of death in epileptic patients include:

- Status epilepticus (in other words directly related to a seizure) – 10 %
- Accidents during a seizure – 5 %
- Suicide – 7 to 22 %
- Sudden unexpected death in epilepsy – more than 10 % (Guberman & Bruni, 1999)

2.3.1 Epilepsy pathophysiology

There are three theoretical mechanisms that can be involved during the generation of epileptic seizures, namely the non-synaptic, synaptic (neurochemical) and oxidative stress mechanisms. Epileptic seizures seldom occur due to one of these mechanisms, they more often result from interactions between them (Engelborghs *et al.*, 2000).

2.3.1.1 Non-synaptic theoretical mechanisms of seizure generation (Engelborghs *et al.*, 2000)

- Alterations in the ionic microenvironment (e.g. increased extracellular K^+ or decreased extracellular Ca^{2+}) affect neuronal excitability.
- Decreases in the size of extracellular space could lead to interactions between neurons (ephaptic interactions). Currents from activated neurons excite adjacent neurons which are not connected to each other via synaptic connections. The smaller the extracellular space, the more ephaptic interactions can occur.

- The activation of the Na^+-K^+ pump is important for the regulation of neuronal excitability during excessive neuronal discharges. The blocking or failure of the Na^+-K^+ pump to function (as seen in cases of hypoxia and ischemia) can induce epileptogenesis.
- A Cl^--K^+ co-transport mechanism controls the intracellular Cl^- concentration and the Cl^- gradient across the cell membrane which regulates the effectiveness of GABA-activated inhibitory Cl^- currents. Interference with this process can lead to a progressive decrease in the effectiveness of GABAergic inhibition, which in turn leads to increased excitability.
- There is a correlation between the amount of transmitter released and the depolarisation of presynaptic terminals. Abnormal bursts of action potentials (presynaptic terminal bursting) occur in the axons during epileptogenesis, affecting the excitability of the axons, and therefore affecting synaptic excitability.

2.3.1.2 Synaptic (neurochemical) theoretical mechanisms of seizure generation

- **The Gamma Amino Butyric Acid (GABA) hypothesis**

The GABA hypothesis of epilepsy implies that reducing GABAergic inhibition can result in epilepsy due to an increase in neuronal excitability. Repetitive activation of cortical circuits leads to the gradual decrease in inhibitory post synaptic potentials (IPSPs). This could be due to decreases in GABA release from terminals, desensitisation of GABA receptors coupled to increases in chloride ion conductance, or alterations in the ionic gradient because of intracellular accumulation of chloride ions. Where intracellular accumulation of chloride ions is involved, passive redistribution is ineffective. The Cl^--K^+ co-transport becomes less effective during seizures due to its dependence on the K^+ gradient. The Cl^--K^+ co-transport can also be affected by hypoxia or ischemia due to its dependence on metabolic processes. These mechanisms play a critical role in seizure generation (Engelborghs *et al.*, 2000; Ono & Galanopoulou, 2012).

The pathophysiology of epilepsy involves low GABA levels and glutamic acid decarboxylase (GAD) activity. Reduced GABA binding in brain tissue from epileptic patients were also noted, as well as reduced benzodiazepine receptor binding in epileptic foci. The degree of benzodiazepine receptor reduction shows a positive correlation with seizure activity (Engelborghs *et al.*, 2000; Meldrum *et al.*, 1999).

- **Glutamate**

The activation of both ionotropic and metabotropic postsynaptic glutamate receptors are proconvulsant. There is evidence of altered N-methyl-D-aspartate (NMDA) receptor function in epilepsy patients, e.g. increased sensitivity to glutamate which leads to an enhanced entry

of Ca^{2+} into neurons during synaptic activity (Engelborghs *et al.*, 2000; Meldrum *et al.*, 1999; Ono & Galanopoulou, 2012).

Patients with absence seizures showed significantly increased levels of plasma glutamate increasing neuron excitability. Interactions of glutamatergic and serotonergic mechanisms are also involved in the triggering and maintenance of epilepsy (Gerber *et al.*, 1998).

- **Catecholamines**

Central nervous system catecholamine abnormalities have been reported in several genetic models of epilepsy. Decreased levels of dopamine have been found in epileptic foci, lowering the threshold triggering seizures. Seizures can be exacerbated by dopamine antagonists and alleviated by dopamine agonists in absence epilepsy patients (Engelborghs *et al.*, 2000; Meldrum *et al.*, 1999).

2.3.1.3 Oxidative stress

Oxidative stress can cause direct oxidative cell injury (due to production of strong oxidants) and can also be involved in signal transduction and the regulation of gene expression via redox-sensitive mechanisms. Oxidative stress can influence many redox-sensitive processes in cells and can therefore act as second messengers which lead to the transactivation of genes (Boelsterli, 2007). Targets for oxidative damage are nucleic acids, proteins, carbohydrates and lipids, as well as small biomolecules (e.g. biogenic amines and ascorbic acid) (Boelsterli, 2007; Shacter, 2000).

- **Oxidative DNA damage**

The oxidation of nucleic acids by reactive oxygen species (ROS) is a normal process counterbalanced by antioxidants and repair systems. Estimates show that each day the DNA in a cell is theoretically “hit” by an oxidative event approximately 1.5×10^5 times, which culminates as a total of 1019 hits per individual (Boelsterli, 2007). In contrast to nuclear DNA, mitochondrial DNA (mtDNA) is far more prone to be hit by an oxidative event and to be permanently damaged. This increased susceptibility is due to the absence of protective histones in mtDNA, the close proximity to the production of ROS in mitochondria and inefficient repair mechanisms, leading to the accumulation of oxidatively damaged bases. Additionally mtDNA lacks non-coding sequences, which makes an oxidative event potentially more relevant (Boelsterli, 2007).

- **Oxidative Protein damage**

Oxidative stress causes amino acid residue side chain oxidation, protein-protein cross-link formation and protein fragmentation due to the oxidation of the peptide backbone. Defence

and repair mechanisms against protein damage include heat shock proteins (renatures damaged proteins or resolubilise aggregates of damaged proteins) and the major cytosolic protease system (proteasomes that recognise and readily degrade oxidatively damaged proteins) (Boelsterli, 2007; Jesberger & Richardson, 1991). If the oxidation of proteins cannot be prevented or the damage repaired, the major consequences for enzymes are the loss of catalytic function or the impairment of function of structural proteins (Boelsterli, 2007; Shacter, 2000). Oxidative damage to proteins also causes increased surface hydrophobicity due to the partial unfolding of the protein, causing possible formation of large protein aggregates, which are toxic if they accumulate in a cell.

- **Oxidative lipid damage**

Oxidative stress in the hydrophobic compartments of cells causes the deterioration of polyunsaturated lipids (lipid peroxidation) (Halliwell & Gutteridge, 2007). The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals and lead to peroxidation. Each of the formed lipid peroxides also act as free radicals and lead to a cascade of peroxidation as newly formed peroxides react with neighbouring fatty acids, yielding even more lipid peroxides and spreading damage to further areas and other organelles (Jesberger & Richardson, 1991). Transition metals, e.g. iron and copper, plays an important role in this cascade, catalysing the degradation of accumulating lipid peroxides (LOOH) to form alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) compounds which causes protein damage and the continuance of the lipid peroxidation cascade (Boelsterli, 2007; Halliwell, 2001). LOOH can also form toxic aldehydes (e.g. malondialdehyde (MDA) and α, β-unsaturated aldehyde, 4-hydroxynonenal) via oxidative fatty acyl degradation. Lipid peroxidation in biomembranes can lead to significant alterations in membrane function and structure (e.g. it can affect membrane fluidity, leading to alterations in calcium influx). Free radical associated lipid peroxidation can therefore be involved in membrane damage associated with normal aging and pathologies such as ischaemia and hypoxia (Jesberger & Richardson, 1991).

2.3.1.3.1 Oxidative stress and neuropathology

It is postulated that oxidative stress is the causative factor, mediator or byproduct in certain cases of neuropathology. The brain is especially sensitive to oxidative damage (Halliwell, 2001). The reasons for this:

- The brain contains a high content of easily peroxidisable polyunsaturated fatty acids (Floyd, 1997).
- The brain contains a low level of antioxidant protective enzymes, e.g. catalase.

- The brain has a high rate of oxygen utilisation. Oxygen is needed for the high ATP consumption of neurons, maintenance of membrane potentials and the release and storage of neurotransmitters (Floyd, 1997; Halliwell, 2001).
- The brain contains high iron content in certain regions (e.g. substantia nigra). Important iron-containing proteins in the brain include cytochromes, ferritin (which stores most of the total iron in a healthy brain), aconitases, mitochondrial non-haem iron proteins, cytochromes P450 and tyrosine and tryptophan hydroxylase enzymes. Damage of brain tissue leads to the release of iron and copper ions which readily catalyses hydroxyl radical formation, lipid peroxidation and autoxidation of neurotransmitters.
- The brain contains a high level of ascorbate that will cause peroxidative damage when the tissue becomes disrupted (Halliwell & Gutteridge, 2007).
- Many of the neurotransmitters are autoxidisable molecules, e.g. dopamine, levodopa and noradrenaline react with oxygen to generate superoxide anions ($O_2^{\bullet-}$) that can deplete glutathione and bind to protein –SH groups.
- Brain metabolism generates hydrogen peroxide (H_2O_2), e.g. dopamine oxidation by monoamine oxidase.
- The brain contains microglia, which produce $O_2^{\bullet-}$ and H_2O_2 upon activation and can secrete cytokines, which primes the microglia to produce more ROS and NO when activated.
- The brain contains cytochromes P450 in certain regions. Reactive species can leak away from the catalytic intermediates in the P450 cycle, which lead to $O_2^{\bullet-}$ and H_2O_2 generation.
- The brain extensively uses glutamate as a neurotransmitter, creating a very delicate balance with Ca^{2+}, NMDA receptor stimulation and NO synthesis which will be discussed later.
- The older brain is also found to be more sensitive to oxidative stress than a younger brain. This is due to the formation of age-related lesions in the brain mitochondria (Floyd, 1997).

The pathological processes of neurological disorders are not entirely understood and it is also difficult to determine whether the reactive species involved in these disorders are the cause or byproducts of these pathologies. Investigations regarding the pathogenesis of neurological disorders have made important contributions to one's understanding of the basic neurological events that take place in normally functioning and disordered brains (Jesberger & Richardson, 1991).

Though there are multiple neurodegenerative diseases and other pathologies either caused or influenced by free radicals, only epilepsy will be discussed in this study. Epilepsy is not necessarily classified as a neurodegenerative disease, but can lead to cell loss and neuronal degeneration, which contribute to disease progression (Ercegovac *et al.*, 2010). The progression of epilepsy pathophysiology (e.g. increase in frequency, duration and severity of epileptic foci) and associated neurological dysfunction (e.g. impairment of cognitive, physical and behavioural dysfunction) tends to be similar to neurodegenerative diseases in certain patients (Ono & Galanopoulou, 2012). Neurodegenerative diseases can also result in epileptic seizures, e.g. dementia and Alzheimer's disease, indicating that pathways responsible for neurodegeneration and epileptogenesis can interact or converge.

2.3.1.3.2 Oxidative stress and epilepsy

The oxidative injury of the brain may play a role in the initiation and progression of epilepsy (Acharya *et al.*, 2008; Azam *et al.*, 2010). Increases in lipid peroxides in epilepsy models, elevation of antioxidant enzymes during or after epileptic activity in brain homogenates or blood, and temporal correlations between free radical overproduction and seizure development in certain pathological conditions, e.g. hypoxia, hyperoxia and trauma, suggest that free radicals are produced during epileptiform events in brain tissue (Frantseva *et al.*, 2000). There are many other theories concerning which pathways and reactive species are involved in the pathogenesis of epilepsy. These theories also differ due to the different types and causes of epileptic seizures.

Figure 2.3 is a schematic summary of the multiple oxidative stress pathways involved in epilepsy pathophysiology. It illustrates the involvement of oxidative stress in neurotransmitter function and how different reactive species and their effects interact with one another, producing a vicious cycle of damaging effects resulting in epileptic foci. The complexity and importance of the involvement of oxidative stress in epilepsy pathophysiology is also illustrated. Oxidative stress not only causes lipid, DNA and protein damage, but can also influence neurotransmitter release and metabolism, which affects normal neuronal functions, leading to epilepsy pathogenesis. Other neurodegenerative diseases, e.g. Parkinson's and Alzheimer's disease follow a similar pattern, starting with or progressing due to the effects of oxidative stress on neuronal function. For the purpose of this study epilepsy pathophysiology, due to oxidative stress, and the potential use of antioxidants as treatment will be the main focus.

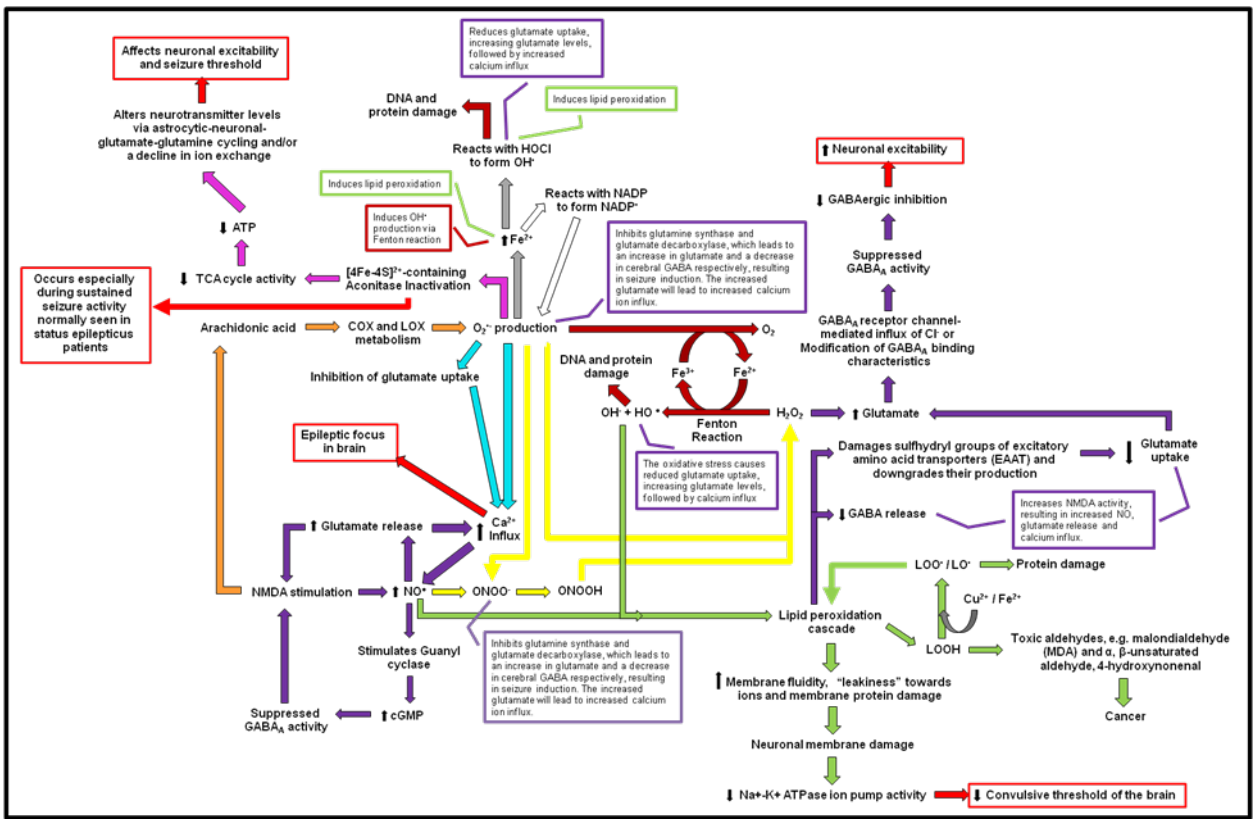


Figure 2.3: Schematic summary of oxidative stress pathways involved in epilepsy pathophysiology, their effects on neurotransmitters and their involvement in the development of epileptic foci (see Appendix A for the list of references used)

2.4 Antioxidant therapy

2.4.1 The balance between oxidative stress and antioxidant defences

Reactive species generation increases with age while some endogenous antioxidant defences decrease, leading to progressive damage of cellular structures and aging. Reactive species are needed for certain physiological functions, e.g. cell signalling and redox regulation. Increased reactive species production can occur due to endogenous reasons (inflammation, increased oxygen concentrations and increased mitochondrial leakage) and/or exogenous reasons (environmental factors, pollution, strenuous exercise, smoking, nutrition, chronic inflammation, psychological and emotional stress, etc.). Oxidative stress can also occur due to decreased antioxidant defences (decreased endogenous antioxidant enzyme functions and reduced antioxidant intake) (Poljsak *et al.*, 2013).

Inappropriate antioxidant functions can also cause physiological problems. High antioxidant intake leads to the neutralisation of both beneficial and damaging reactive species. This can decrease the immune system's ability to fight bacteria and remove damaged cells (including precancerous and cancerous cells). Large amounts of antioxidant nutrients can also act as

pro-oxidants (e.g. ascorbic acid), increasing oxidative stress. Antioxidant defence systems must therefore remove harmful levels of reactive species, while still permitting sufficient reactive species production for cell function. Cells can tolerate these mild levels of necessary oxidative stress, e.g. by upregulating cellular repair processes and other protective mechanisms (Poljsak *et al.*, 2013).

2.4.2 Antioxidants and epilepsy

Certain conventional and new generation anti-epileptic drugs are effective for primary neuroprotection, but their efficacy in preventing the development of chronic epilepsy after an initial seizure is either unknown or not promising (Acharya *et al.*, 2008; Azam *et al.*, 2010). Antioxidant therapy can therefore play a favourable role in the altering of the clinical course of epilepsy (Azam *et al.*, 2010) due to their potential neuroprotective properties and ability to prevent the progression of epilepsy. The protective efficacy and importance of antioxidants depend on the type of reactive species generated, the place of generation, how it is generated and the severity of the damage (Halliwell, 1994; Halliwell & Gutteridge, 2007).

As with the many different oxidative stress pathways involved in the pathophysiology of epilepsy, there are many different antioxidant pathways used by the human body or used in drug treatment and research for epilepsy. The antioxidant systems involved also differ according to the age of the patient, type of epilepsy or seizures and the pathological pathway of the disease. Only the antioxidant systems or pathways with the potential to prevent and treat epilepsy will be discussed here.

Figure 2.4 is a schematic summary of the antioxidants that have been studied in epilepsy research. It illustrates the many effects of various types of endogenous antioxidant systems (superoxide dismutase (SOD), catalase and glutathione (GSH)) and exogenous antioxidants (chain breaking antioxidants (α-tocopherol) and reactive species scavengers which directly prevents oxidative damage and indirectly acting antioxidants (e.g. metallothionein) which prevents the formation of reactive species) (Mukherjee *et al.*, 2009). Combination therapy of these antioxidants can also be beneficial, as seen in the case of lipoic acid which enhances SOD activity and regenerates ascorbic acid and GSH, whilst also being able to scavenge reactive nitrogen species (RNS), lipid peroxides and ROS and act as transition metal scavengers, preventing the cascade of lipid peroxidation and the formation of hydroxyl radicals via the Fenton reaction (Militao *et al.*, 2010; Packer *et al.*, 1997). The combinational function of SOD with other antioxidant systems (e.g. GSH and peroxiredoxins) to remove the H_2O_2 that forms during the dismutation reaction between two $O_2^{\bullet-}$s is also an example (Boelsterli, 2007; Halliwell, 2006).

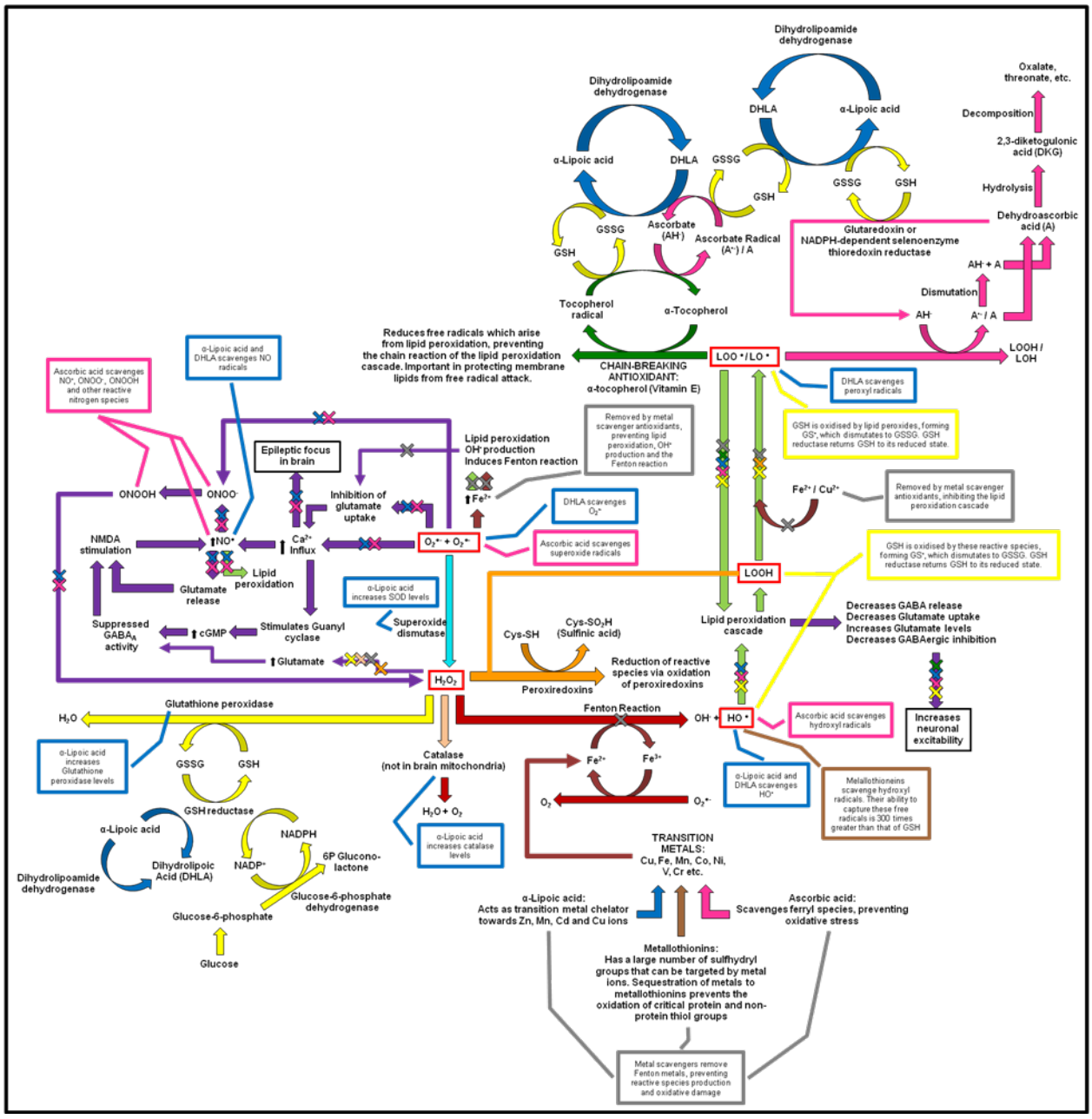


Figure 2.4: Schematic summary of the antioxidant pathways studied in epilepsy research for the prevention and treatment of epileptic foci by either removing reactive species or preventing reactive species production, leading to the restoration of redox equilibrium and normalisation of neurotransmitter metabolism and function (see Appendix B for the list of references used)

The hypothesis, that restoring redox equilibrium through the activation of intracellular signals, is also important in the antioxidation process. The trapping of excess free radicals could restore redox equilibrium in the initial states of cellular oxidative stress (Mukherjee *et al.*, 2009), preventing the development of epileptic foci.

2.4.3 Nutritional, synthetic and herbal antioxidants and the antioxidant effects of *C. orbiculata*

The human diet contains a large number of plants with antioxidant activity. Spices, condiments and many vegetables and fruits are particularly rich in natural antioxidants, e.g. vitamin C, tocopherols and carotenoids (Mukherjee *et al.*, 2009). This intake of natural antioxidants has been found to inversely correlate with the rates in incidence of many diseases (Mukherjee *et al.*, 2009). Contrary to the positive results of nutritional antioxidants, clinical trials show that intake of one or more synthetic antioxidants failed to show any benefit. Major clinical trials using mortality and morbidity as the end point found that supplementation with antioxidants, e.g. ascorbic acid and vitamin E, showed no positive results (Miller *et al.*, 2005; Omenn *et al.*, 1996; Poljsak *et al.*, 2013). Recent studies also indicated cases of increased mortality with synthetic antioxidant intake. The intake of only one antioxidant could alter the endogenous antioxidant defences of cells, modifying cell death rates. However, antioxidant supplements have been found to lower oxidative stress levels in cases where patients showed oxidative stress levels above normal. The antioxidant supplements, therefore, assist in the correction of elevated oxidative stress levels that cannot be controlled by endogenous antioxidants (Poljsak *et al.*, 2013).

Dosages of synthetic antioxidants are also considered as problematic. There are claims that the recommended daily allowance of ascorbic acid and vitamin E are too low to prevent oxidative stress and ingestion of large amounts of these supplements may have damaging or pro-oxidant effects. Determining an individual's oxidative stress levels before administering supplement therapy can therefore be beneficial when using antioxidants to treat diseases. Unfortunately, the reference values for a typical oxidative stress status of an individual have not yet been established and measuring oxidative stress is both difficult (reactive species have very short half lives) and expensive (Poljsak *et al.*, 2013).

There are a large number of medicinal plants which are claimed to be effective against diseases in which ROS are thought to play a role. Phenolic antioxidants such as flavonoids, tannins, coumarins, xanthenes and, more recently, procyanidins, scavenge radicals in a dose-dependent manner. They therefore have promising therapeutic potential (Mukherjee *et al.*, 2009).

C. orbiculata is an example of a medicinal plant used in traditional medicine with antioxidant activity. Louw (2009) and Roux (2012) confirmed via using a lipid peroxidation (thiobarbituritic acid (TBA)) assay and a nitroblue tetrazolium (NBT) assay that extracts of fresh and freeze dried *C. orbiculata* leaves showed significant antioxidant effects against lipid peroxidation and superoxide anions, respectively, in rat brain homogenate. This antioxidant

activity could explain why *C. orbiculata* is used in traditional medicine to treat epilepsy, seeing as epilepsy can be caused or progressed by oxidative stress.

2.5 The effects of *C. orbiculata* on epileptogenesis

2.5.1 The anticonvulsant effects of *C. orbiculata*

As previously mentioned *C. orbiculata* is used in traditional medicine for the treatment of epilepsy. Joseph Kabatende (2005) and Amabeoku *et al.* (2007) studied the anticonvulsant effects of *Cotyledon orbiculata* by inducing epileptic seizures in animals, administering plant extracts and observing the effects on the seizures.

GABA is a major inhibitory neurotransmitter in the brain and the inhibition of GABA is said to be an underlying factor in epilepsy (Amabeoku *et al.*, 2007; Kabatende, 2005):

- Pentylenetetrasole (PTZ) was used to induce epileptic seizures by inhibiting GABA neurotransmission.
- Picrotoxin was also used to induce seizures by inhibiting $GABA_A$ receptors and blocking chloride ion channels, preventing the influx of chloride ions into brain neurons.
- Bicuculline induces seizures by blocking GABA effects at central $GABA_A$ receptors.
- Anti-epileptic drugs (AEDs) antagonise epileptic seizures by enhancing GABA effects or increasing chloride influx, therefore enhancing GABA.
- The inhibition of the PTZ-, Picrotoxin- and bicuculline-induced seizures by the plant extracts indicate that *Cotyledon orbiculata* may have GABAergic enhancing effects.

Glutaminergic neurotransmission is also involved in epilepsy pathogenesis, where activation of NMDLA receptors leads to the activation of glutaminergic neurotransmission, exerting convulsant activity (Amabeoku *et al.*, 2007; Kabatende, 2005). *C. orbiculata* extracts delayed the onset of NMDA induced seizures, indicating that it may have an effect on the glutaminergic system.

2.5.2 The possible epileptogenic effects of the orbicusides

Though Kabatende and Amabeoku's results show that *C. orbiculata* does have potential anti-epileptic effects, the orbicusides pose a risk of being epileptogenic. The orbicusides' neurotoxicity is due to their ability to block the Na^+-K^+ ATPase ion pump (Joubert, 1981; Kellerman *et al.*, 2005), which regulates neuronal excitability (Engelborghs *et al.*, 2000). This also causes malfunction of the Cl^-K^+ co-transport mechanism, which regulates GABAergic inhibition, due to the changes in intracellular K^+ levels caused by the blockade of the Na^+-K^+ ATPase ion pump. Decreases in GABA-activated inhibitory Cl^- currents result in increased neuronal excitability, leading to epileptogenesis (Engelborghs *et al.*, 2000). Patients that use

C. orbiculata as epilepsy treatment therefore risk inducing epileptic seizures, rather than treating or preventing them.

2.6 The potential use of the orbicusides in the treatment of epilepsy

Kabatende (2005) and Amabeoku *et al.* (2007) confirmed that *C. orbiculata* has potential as epilepsy treatment. However, they did not confirm which compound(s) in the plant extracts is responsible for these possible GABAergic and glutaminergic effects. The exact mechanism(s) for these effects were also not identified. The rationale behind this dissertation is that oxidative stress may be involved. Oxidative stress can cause direct protein, DNA, carbohydrate and/or lipid damage and influences neurotransmission by affecting neurotransmitter release and metabolism, leading to epilepsy pathophysiology. *C. orbiculata* extracts have significant antioxidant activity (Louw, 2009; Roux, 2012), which could explain the GABAergic and glutaminergic effects of the plant extracts.

It is, however, also possible that the orbicusides of *C. orbiculata* are epileptogenic due to their neurotoxic activity. During Kabatende and Amabeoku's studies there was no mention of the detection of toxicity or the induction or worsening of epileptic seizures by the plant extracts used. There is also no indication of any human poisonings due to the use of *C. orbiculata* as a medicinal source in literature, possibly indicating that *C. orbiculata* does not contain sufficient concentrations of orbicusides to elicit toxicity or epileptic seizures when used in therapeutic doses.

The orbicusides of *C. orbiculata* hold potential as possible active compounds for the treatment of epilepsy. As bufadienolides the orbicusides are considered lipophilic, meaning that they can cross the blood-brain barrier and reach the central nervous system. Though the orbicusides are neurotoxic, they can be used to produce analogues capable of reaching the central nervous system with reduced toxicity and/or epileptogenic activity and increased antioxidant activity.

There are many cases where compounds isolated from natural sources can be used as a scaffold or precursor for the development of more effective, safer medicines. The podophyllin resin from the roots of *Podophyllum peltatum* (mayapple) contains the lignan podophyllotoxin that inhibits cell division. However this compound was considered too toxic to use medicinally other than as a wart removing agent. Despite its toxicity it held great potential as an anticancer chemotherapy due to its cell division inhibitory effects. By developing a semi-synthetic modified glucoside, namely etoposide, a new medicinal compound was synthesised that can be used to treat lung and testicular cancers by inhibiting topoisomerase II. Another example is artemisinin, a sesquiterpene endoperoxide isolated as the active compound of

Artemisia annua (a Chinese antimalarial herb). Artemisinin was found to be effective as an antimalarial and can be used to treat multi-drug resistant *Plasmodium falciparum* strain infections that cause malignant cerebral malaria. By developing a semi-synthetic derivative, e.g. arthemeter (methyl ether of dihydroartemisinin) the pharmacokinetic properties were improved (Phillipson, 2001).

The objective is to use the aglycone structures of the orbicusides to produce analogues with increased antioxidant activity and reduced toxicity to treat epilepsy (if oxidative stress is involved in the pathology). An orbicuside aglycone consists of a steroid structure and a 2-pyrone moiety (a six-membered cyclic unsaturated ester with physical properties similar to alkenes and aromatic compounds) connected on position 17 (McGlacken & Fairlamb, 2005). Pyrones are commonly found in many bacterial, microbial, plant, insect and animal systems and have various medicinal properties (e.g. can be used to treat Alzheimer's disease, high cholesterol, cancer, etc.). There are cases where the pyrones from natural sources showed significant antioxidant activity (Goel & Ram, 2009; Lee *et al.*, 2010; Thuong *et al.*, 2010). Thuong *et al.* (2010) determined that the pyrone ring of coumarins increases the free radical scavenging and anti-lipid peroxidation ability of the compounds by comparing the activity of the coumarins, aesculetin and scopoletin with that of phenolic acids complimentary to the two coumarins, caffeic acid and ferrulic acid. Steroids and steroid analogues have also been found to have antioxidant properties (Klinger *et al.*, 2002; Mohamed *et al.*, 2012; Ahmad *et al.*, 2013). In this study the 2-pyrone moiety of the orbicuside aglycone was targeted as a potential area where alterations could be made to improve the antioxidant activity of the compound.

2.7 Methods for the evaluation of antioxidant activity

To determine whether the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata* have potential as anti-epileptics, in epilepsy pathophysiology where oxidative stress is involved, the following assays can be used:

2.7.1 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is a kinetic assay used to measure the antioxidant capacity of samples. It is based on the oxidation of a fluorescein fluorescent probe by peroxyl radicals via hydrogen atom transfer. Peroxyl radicals are produced via the breakdown of 2,2'azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (ZenBio, 2013). There are various kits available to perform the ORAC assay (ZenBio, 2013; Cell Biolabs, 2013).

2.7.2 Ferric reducing ability of plasma (FRAP) assay

The FRAP assay is a simple and automated assay which measures ferric reducing ability. The reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) under low pH causes the formation of a ferrous-tripyridyltriazine complex (forms an intense blue colour) which can be measured spectrophotometrically at 593 nm (Benzie & Strain, 1996).

2.7.3 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH is a deep purple, stable free radical with an unpaired electron at one atom of the nitrogen bridge. DPPH is scavenged by antioxidants, reducing the purple colour measured spectrophotometrically at at 517 nm (Brand-Williams *et al.*, 1995; Sharma & Bhat, 2009; Deng *et al.*, 2011). The DPPH assay is an easy redox reduction reaction that can be used for antioxidant screening.

2.7.4 Lipid peroxidation (TBA) assay

Lipid peroxidation leads to the production of malondialdehyde (MDA), a toxic aldehyde, which can be used as a biomarker to measure lipid peroxidation (Boelsteri, 2007). The TBA assay involves the reaction of MDA with two TBA molecules in an acid catalysed nucleophylic addition reaction to form a pink MDA-TBA adduct (figure 2.5). This adduct is then isolated with butanol and measured spectrophotometrically (Ottino & Duncan, 1997a; Ottino & Duncan, 1997b; Hodges *et al.*, 1999). To induce lipid peroxidation ferric (III) chloride ($FeCl_3$), ascorbic acid and H_2O_2 is used.

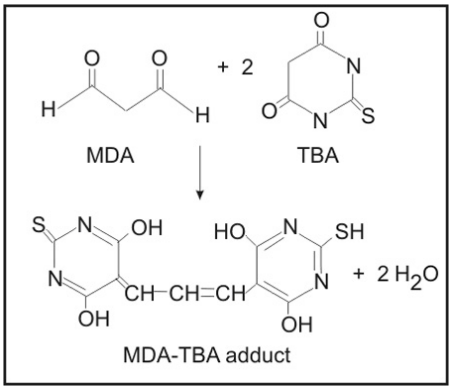


Figure 2.5: Formation of the pink MDA-TBA adduct in the TBA assay (Held, 2012)

2.7.5 Nitroblue tetrazolium (NBT) assay

The NBT assay involves the reduction of $O_2^{\bullet-}$ by yellow NBT to form a water insoluble blue dye, nitroblue diformazan (NBD). The resulting diformazan is then isolated with glacial acetic acid (GAA) and measured spectrophotometrically (figure 2.6). $O_2^{\bullet-}$ production is induced with potassium cyanide (KCN) (Ottino & Duncan, 1997a).

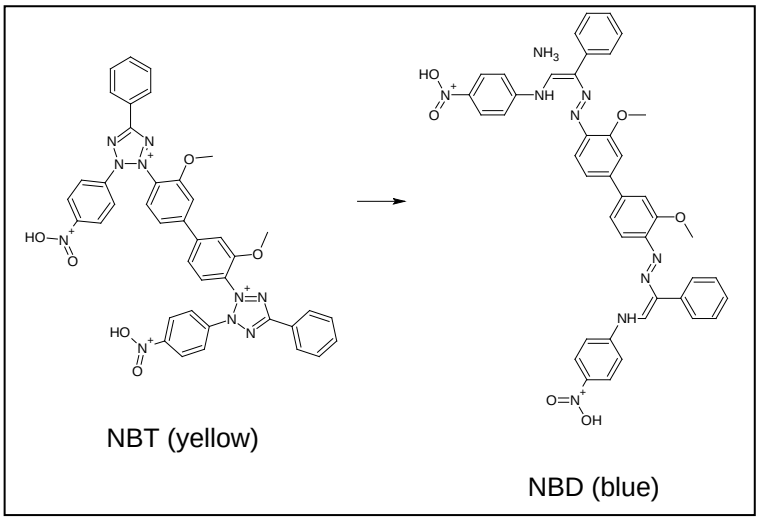


Figure 2.6: Reduction of NBT to form NBD (Kaur & Geetha, 2006)

2.7.6 Methods chosen for the evaluation of antioxidant activity during this study

To determine whether the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata* have potential as anti-epileptics in epilepsy pathophysiology where oxidative stress is involved, the lipid peroxidation (TBA) and nitroblue tetrazolium assays were chosen. The reasons for this included:

- The TBA and NBT assays evaluates the antioxidant activity of samples against specific oxidative stress mechanisms (lipid peroxidation and $O_2^{\bullet-}$ production respectively). The ORAC, FRAP and DPPH assays are simple and effective screening methods for antioxidant activity. However, for this study the identification of more specific antioxidant mechanisms of the samples were desired.

- The specific oxidative stress mechanisms targeted by the NBT and TBA assays correlates with the theoretical oxidative stress mechanisms involved in epilepsy pathophysiology.

  The brain's high content of easily peroxidisable polyunsaturated fatty acids (Floyd, 1997) makes it an easy target for lipid peroxidation, which can lead to a decrease in the convulsive threshold, increased glutamate levels and, in effect, increased neuronal excitability, resulting in epileptic foci (figure 2.3). The TBA assay can be used to determine whether the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata* have antioxidant effects against lipid peroxidation.

  $O_2^{\bullet-}$ production can affect neuronal excitability and seizure threshold by reducing brain energy metabolism (ATP production). It also inhibits glutamate uptake and increases Ca^{2+} influx which can result in epileptic foci. It is also involved in the production of more

potent reactive species (e.g. hydroxyl radicals) (see figure 2.3). The NBT assay can be used to determine whether the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata* have antioxidant scavenging effects against $O_2^{\bullet-}$.

- The TBA and NBT assays can evaluate the antioxidant effects of samples in a physiological environment. This can be used to not only to determine whether or not samples have antioxidant activity but can also show the extent or potency of the resulting activity of the samples (and different concentrations of the samples) in the physiological environment. The DPPH assay, for example, is a simple 'test tube' redox reaction that can only determine whether or not the sample has antioxidant activity. The effects that the sample would have in a physiological environment (the extent of the sample's activity) cannot be determined.

- The stability of the DPPH assay toward light, humidity, air, etc. and the assay's reproducibility was brought to question in several articles (Ozcelik *et al.*, 2003; Sharma & Bhat, 2009). The NBT and TBA assay is performed under controlled conditions (e.g. specific incubation temperatures and pH) to maintain the physiological environments in which the assay is performed (tissue homogenate), promoting stability and reproducibility.

2.8 Methods for the evaluation of toxicity

To determine the toxicity of the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata* the following assays can be used:

2.8.1 Apoptosis assay

The metabolic activity / annexin V / dead cell apoptosis kit with C_{12} resazurin, APC annexin V, and SYTOX® green for flow cytometry is a convenient apoptosis assay (Invitrogen™, 2010). It allows the visualisation of dead and live apoptic cells. SYTOX® green dye stains dead cells (by binding to cellular nucleic acids). Annexin V (conjugated to allophycocyanin (APC)) stains dead and apoptic cells by binding to phosphatidylserine. Viable cells reduce nonfluorescent C12 resacurin to orange-fluorescent C12 resorufin (Invitrogen™, 2010). Due to the cost of the kit other assays were considered during this study.

2.8.2 Evaluation of membrane integrity (the neutral red uptake and LDH leakage assay)

The neutral red uptake assay measures membrane integrity. Viable cells take up the neutral red dye, which then concentrates in the lysosomes of the cells (Fotakis & Timbrell, 2006). It is a cheap and effective method for the analysis of membrane damage.

The LDH (lactate dehydrogenase) leakage assay evaluates membrane damage by measuring the release of LDH from cells when the cellular membrane is damaged (Lobner, 2000; Fotakis & Timbrell, 2006). It can be used to measure neuronal cell death via necrosis and neuronal apoptosis. LDH activity is determined via an enzymatic test involving the reduction of NAD^+ to $NADH/H^+$ during the LDH-catalysed conversion of lactate to pyruvate. A diaphorase catalyst then promotes the reduction of a tetrazolium salt (3-(4-nitrophenyl)-5-phenyltetrazolium chloride) to a red formazan (Weyermann *et al.*, 2005).

2.8.3 Evaluation of mitochondrial function (ATP and MTT assay)

The ATP assay is a bioluminescent assay used to measure ATP produced by viable cells. Luciferase catalyses the formation of light from ATP and luciferin and the intensity of the emitted light correlates with the amount of viable cells present (Weyermann *et al.*, 2005).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) follows principles similar to that of the NBT assay. MTT (yellow crystals) are reduced to formazan (purple) in the mitochondria of viable cells (figure 2.7). The formazan is extracted from the cells with isopropanol and measured spectrophotometrically. The amount of viable cells can be determined using the spectrophotometric data (the amount of formazan formed is proportional to the amount of viable cells) (Mosmann, 1983; Lobner, 2000).

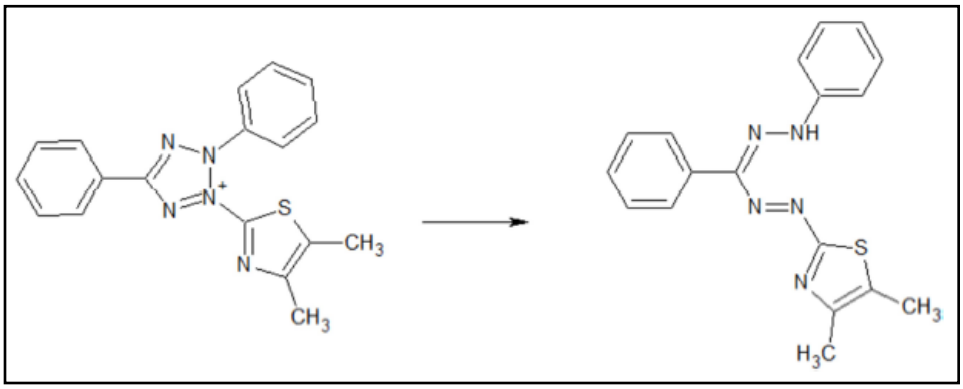


Figure 2.7: Reduction of MMT to form purple formazan (Mosmann, 1983)

To determine the cell viability or toxicity of the plant juice, fractions and bufadienolides analogous to the orbicusides of *C. orbiculata*, the MTT assay was chosen. It is a commonly used assay for the determination of mitochondrial integrity and is currently the established toxicity assay used by the Department of Pharmaceutical Chemistry.

3 SCREENING OF THE ANTIOXIDANT ACTIVITY OF THE JUICE OF *C. ORBICULATA*

The use of antioxidants may be the first step in chronic epilepsy prevention, due to their potential to prevent neurodegeneration (by preventing oxidative damage, therefore acting as neuroprotective agents) and the effects of oxidative stress on neurotransmitter function. Antioxidants can also be used in the treatment of other neurodegenerative diseases, e.g. Alzheimer's disease and Parkinson's disease. Previous studies on *C. orbiculata* focused on the antioxidant properties of various extracts (Louw, 2009; Roux, 2012), but not the plant as a whole. The juice is mostly used in traditional medicine e.g. for treating earache, toothache, etc. (Van Wyk *et al.*, 2005) and it was for this reason that the juice of *C. orbiculata* was screened for antioxidant activity.

3.1 Collection and preparation of plant material

The roots, leaves, stalk and flowers of *C. orbiculata* were collected at the Botanical Gardens of the North West University, Potchefstroom Campus, and an exemplar prepared (PUC 11553) and stored by the Department of Botany.

10 kg of *C. orbiculata* leaves were collected at the Botanical Gardens. The leaves were washed, chopped and juiced using a Breville Ikon juicer. The pulp was processed with the juicer three times to remove the excess water from the plant material. During the study various extraction methods were evaluated and the most effective method was used for bulk extraction. The juice was used to evaluate the antioxidant activity of the plant and the pulp was used for the extraction processes during the study. The juice and pulp were stored separately at -20 °C.

The frozen juice of *C. orbiculata* was thawed, filtered (to remove any leaf pulp remaining in the juice) and concentrated using a rotary vacuum evaporator (BUCHI Rotavapor RII) at 80-100 °C. The resulting concentrate was then stored at -20 °C.

3.2 Fractionation

The concentrated juice of *C. orbiculata* was fractionated by high performance liquid chromatography (HPLC) to afford Fraction 1 and Fraction 2 (figure 3.1).

The concentrated juice was dissolved in distilled water (dd H_2O) and HPLC fractionation performed on an Agilent 1200 series HPLC connected to an Agilent 1200 Series fraction collector (model G1364C, Agilent Technologies, Palo Alto, CA) using a Fusion®-RP 4 µm semi-preparative column (250 x 10 mm) protected by a 10 x 10 mm Fusion RP Security Guard cartridge (Phenomenex, Torrance, CA). The mobile phase was a gradient of 30 %

acetonitrile (ACN) and 70 % water at a flow rate of 3 ml/min. An injection volume of 100 µl and detection via a variable wavelength UV detector at 210 nm was used. A gradient system was increased to 85 % ACN after 5 minutes, held until 10 minutes and re-equilibrated to 15 minutes.

The resulting chromatogram (figure 3.1) shows the two distinct groupings of peaks that were separated to form Fraction 1 and Fraction 2. The antioxidant properties of these two crude fractions were evaluated before further fractionation was considered. Fraction 1 was dried, using the Rotavapor at 80 – 100 °C, and stored at -20 °C. Fraction 2 was concentrated but not successfully dried when the Rotavapor (at 80 – 100 °C) was used. The concentrated fraction was freeze dried with a VirTis SP Scientific Sentry 2.0 and stored at -20 °C.

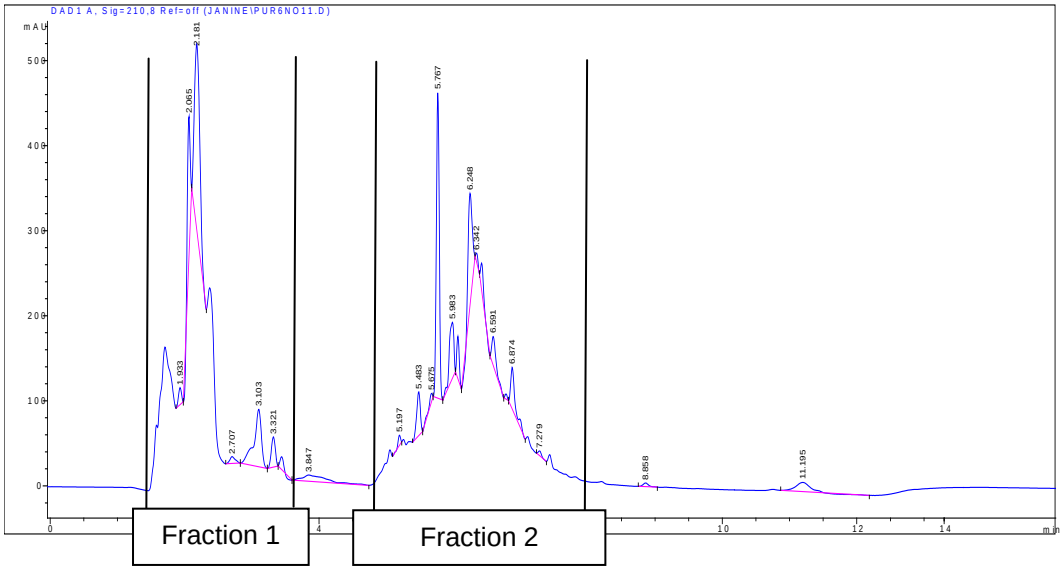


Figure 3.1: HPLC chromatogram of the concentrated juice of *C. orbiculata* and the fractions (Fraction 1 and Fraction 2) obtained via HPLC fractionation of the juice

3.3 Antioxidant screening

To evaluate the antioxidant properties of the concentrated juice, Fraction 1 and Fraction 2, two antioxidant assays were used, the lipid peroxidation or thiobarbituric acid (TBA) assay and the nitroblue tetrazolium (NBT) assay (refer to paragraph 2.7).

3.3.1 Lipid peroxidation (TBA) assay

3.3.1.1 Method

A modified version of the TBA assay of Ottino and Duncan (1997a; 1997b) was used. Because this study focused on treating epilepsy, a neurological disease, rat brain homogenate was used during this assay to evaluate the antioxidant activity of *C.orbiculata* in a neurological environment.

3.3.1.1.1 Animals

Sprague-Dawley rats weighing 200 – 250 g, from the experimental animal centre at the NWU, Potchefstroom campus, were used (approved by the North-West University Animal Ethics Committee). The rats were kept in windowless, well ventilated environments with light cycles of 12 hours. The temperature was maintained at 21 ºC and the humidity at 55 %. The animals received standard nutrition and water *ad lib*.

3.3.1.1.2 Chemicals and reagents

The chemicals used were of the highest chemical purity and purchased at Sigma Aldrich and Merck Chemicals.

Phosphate buffer (PBS buffer) was prepared by dissolving NaCl (8 g, 137 mM), KCl (0.2 g, 2.7 mM), Na_2HPO_4 (1.44 g, 10 mM) and KH_2PO_4 (0.24 g, 2 mM) in dd H_2O (800 ml). The pH of the buffer was adjusted to 7.4 and the volume made up to 1 L with dd H_2O. The PBS buffer was stored at 2 – 4 °C.

A 10 % trichloroacetic acid (TCA) solution was prepared by dissolving TCA (10 g) in dd H_2O (100 ml). The solution was stored at 2 – 4 °C.

Butylated hydroxytoluene (BHT) solution was prepared by dissolving BHT (0.05 g) in methanol (100 ml). The solution was stored at 2 – 4 °C.

A 0.33 % thiobarbituric acid (TBA) solution was prepared by dissolving TBA (0.132 g) in dd H_2O (40 ml). This solution must be freshly prepared when the assay is performed and covered in foil because the solution is light sensitive.

A standard curve was drawn using a 50 nmol/L malondialdehyde (MDA/TEP) solution which was prepared by making two dilutions. Two test tubes were filled with 10 ml PBS buffer. In one test tube 82 µl PBS was removed and 82 µl MDA added. In the second test tube 10 µl PBS was removed and 10 µl of test tube one's solution added. The second test tube's solution (at room temperature) was used in the calibration curve.

For the positive control a 10 mM Trolox solution was prepared by dissolving Trolox (0.005 g) in ethanol (2 ml).

A 5 mM H_2O_2 solution was prepared by diluting H_2O_2 (27.7 µl) with dd H_2O (9.9723 ml).

A 4.88 mM $FeCl_3$ solution was prepared by dissolving $FeCl_3$ (0.013 g) in dd H_2O (10 ml).

A 1.4 mM ascorbic acid solution was prepared by dissolving ascorbic acid (0.00246 g) in dd H_2O (10 ml).

Liquid nitrogen (for the storage of rat brain homogenate) was purchased at Afrox.

3.3.1.1.3 Rat brain homogenate

Rat brain homogenate was pre-prepared and stored at -80 °C until needed. According to Pothiwong *et al.* (2007) brain homogenate can be stored for up to six months at -20 °C before significant degradation or auto-oxidation occurs, which can interfere with assay results. Sprague-Dawley rats (200 – 250 g) were decapitated (by the centre's trained personnel) and the brains removed and placed in PBS buffer. The brains were homogenised in PBS buffer with a homogeniser blender to form 10 % brain homogenate. The homogenate was aliquotted to make multiple 8 ml and 6 ml aliquots (sufficient for performing two TBA and NBT assays respectively) and were snap frozen separately with liquid nitrogen. The homogenate aliquots were stored at -80 °C.

3.3.1.1.4 Preparation of samples

The juice concentrate, Fraction 1 and Fraction 2 were each dissolved in dd H_2O and 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml samples prepared.

3.3.1.1.5 Preparation of the standard curve

Eppendorf tubes containing known MDA concentrations were prepared (table 3.1) for the standard calibration curve. The Eppendorf tubes were vortexed, BHT (100 µl), TBA (200 µl) and TCA (100 µl) added, again vortexed and incubated at 60 °C for 60 minutes. The Eppendorf tubes were then cooled on ice before butanol (400 µl) was added. The Eppendorf tubes were centrifuged for 5 minutes at 2 000 x g. 200 µl of the supernatant of each Eppendorf tube was collected and placed into a 96 well culture plate. Butanol was used as a blank sample. The plate was scanned with a multiplate reader at 530 nm and the absorbance results documented. The absorbance results were plotted against the MDA concentrations. A straight line equation of $y = 0.02x + 0.0039$ with $R^2 = 0.9997$ was obtained (figure 3.2).

Table 3.1: Preparation of MDA standard samples

Concentration (nmol/L)	Volume of MDA (µl)	Volume of PBS (µl)
0	0	100
5	20	80
10	40	60
15	60	40
20	80	20
25	100	0

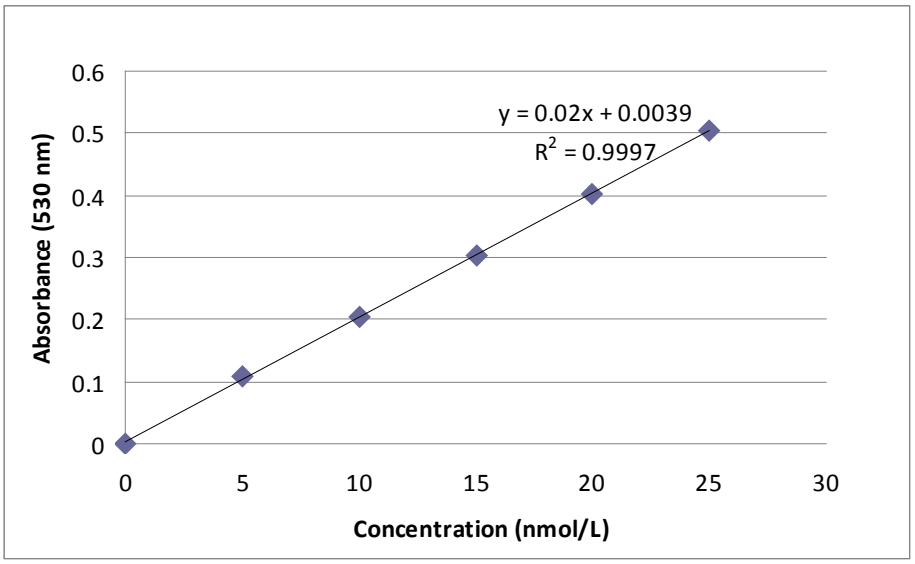


Figure 3.2: MDA standard curve

3.3.1.1.6 Assay

The samples were prepared in Eppendorf tubes according to table 3.2 on ice. To induce lipid peroxidation, the Eppendorf tubes were vortexed and incubated in an oscillating water bath at 37 °C for 60 minutes. To remove insoluble proteins, the Eppendorf tubes were then centrifuged for 20 minutes at 2000 x g. The supernatant of each Eppendorf tube was removed and placed in new Eppendorf tubes. BHT (100 µl) was added to stop the lipid peroxidation reaction, TCA (200 µl) added to precipitate DNA and proteins and TBA (100 µl) added to serve as the colouring reagent to detect lipid peroxidation levels. The Eppendorf tubes were vortexed and incubated at 60 °C for 60 minutes, cooled on ice and butanol (400

µl) added. The Eppendorf tubes were then vortexed and centrifuged for 10 minutes at 2000 x g. 200 µl of each Eppendorf tubes supernatant was collected and placed in a 96 well culture plate. Butanol was used as a blank sample. The absorbance was measured spectrophotometrically with a multiplate reader at 530 nm and the absorbance results documented.

Table 3.2: Sample preparation for the TBA assay of *C. orbiculata* juice, Fraction 1 and Fraction 2

	Brain Homog. (µl)	**PBS (µl)**	**H_2O_2 (µl)**	**$FeCl_3$ (µl)**	**Vit C (µl)**	**Trolox 10mM (µl)**	**Solvent of Sample (dd H_2O) (µl)**	**Sample (µl)**
Negative Control	160	20	-	-	-	-	20	-
Toxin*	160	-	10	5	5	-	20	-
Trolox (Positive Control)	160	-	10	5	5	20	-	-
Sample	160	-	10	5	5	-	-	20

***Toxin: A combination of $FeCl_3$, H_2O_2 and vitamin C was used to induce lipid peroxidation in the rat brain homogenate**

3.3.1.2 Results

The levels of MDA formed (in nmol/mg tissue) in the tested samples were determined and summarised in tables 3.3, 3.4 and 3.5 and figures 3.3, 3.4 and 3.5. Statistical analysis of the results was performed with the Student-Newman-Keuls multiple comparisons test and one-way analysis of variance (ANOVA) via GraphPad software. The results of the samples were compared statistically with that of the Toxin.

Table 3.3: The effect of *C. orbiculata* juice on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	0.390	0.0156
Toxin	0.501	0.02235
Trolox (Positive Control)	0.120	0.008424
Juice 2.5 mg/ml	0.646	0.02132
Juice 1.25 mg/ml	0.691	0.01595
Juice 0.625 mg/ml	0.660	0.0161

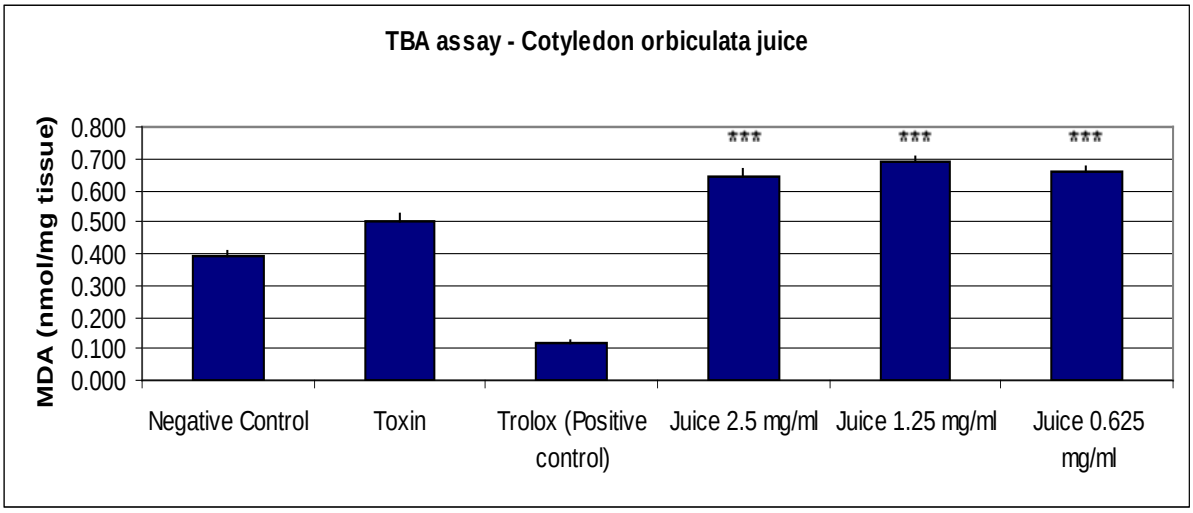


Figure 3.3: The effects of *C. orbiculata* juice on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). *** $p < 0.001$ vs Toxin.

The concentrated juice of *C. orbiculata* showed significant pro-oxidant activity (figure 3.3).

Table 3.4: The effect of Fraction 1 on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	± Standard error of the mean (SEM) n = 5
Negative Control	0.390	0.0156
Toxin	0.501	0.02235
Trolox (Positive Control)	0.120	0.008424
Fraction 1: 2.5 mg/ml	0.650	0.006904
Fraction 1: 1.25 mg/ml	0.664	0.01623
Fraction 1: 0.625 mg/ml	0.652	0.01658

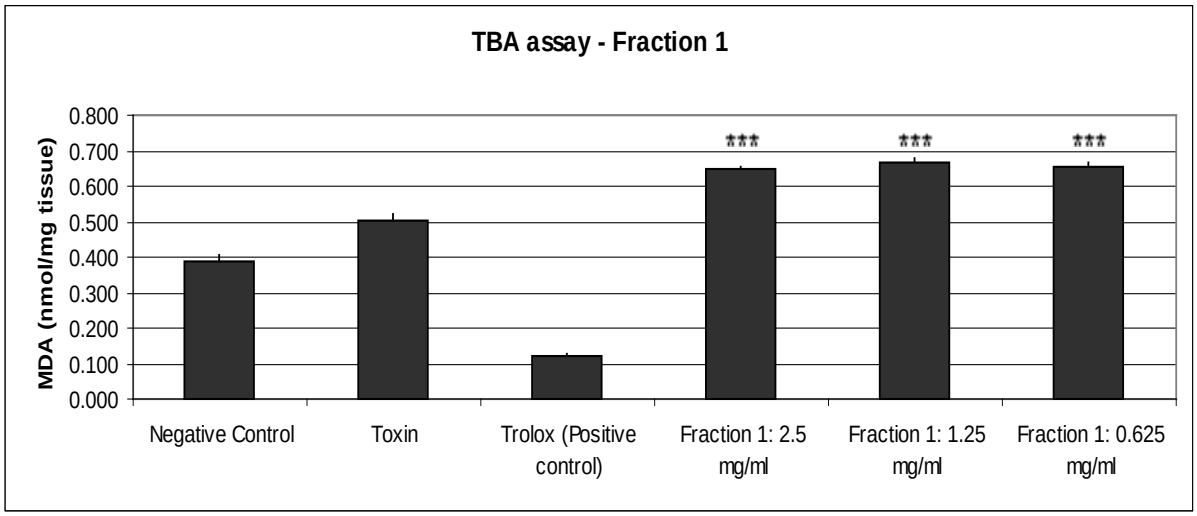


Figure 3.4: The effects of Fraction 1 on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). *** $p < 0.001$ vs Toxin.

Fraction 1 also showed significant pro-oxidant activity (figure 3.4).

Table 3.5: The effect of Fraction 2 on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	± Standard error of the mean (SEM) n = 5
Negative Control	0.306	0.01068
Toxin	0.628	0.007971
Trolox (Positive Control)	0.081	0.00799
Fraction 2: 2.5 mg/ml	0.562	0.008037
Fraction 2: 1.25 mg/ml	0.610	0.002728
Fraction 2: 0.625 mg/ml	0.621	0.003247

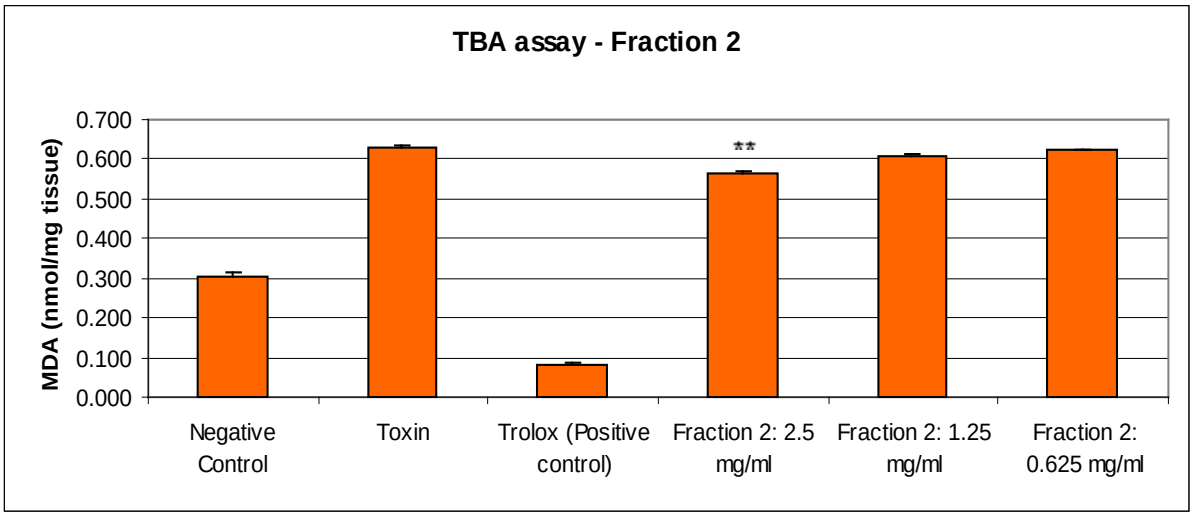


Figure 3.5: The effects of Fraction 2 on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM. ** $p < 0.01$ vs Toxin.

The 2.5 mg/ml Fraction 2 sample showed slight antioxidant activity (figure 3.5).

3.3.1.3 Discussion

The concentrated *C. orbiculata* juice and Fraction 1 showed significant pro-oxidant activity when statistically compared to the Toxin (a combination of $FeCl_3$, H_2O_2 and vitamin C). Fraction 2 showed only slight antioxidant activity, with MDA levels lower than that of the Toxin (where lipid peroxidation was induced in rat brain homogenate) but not near or lower than the levels of the negative control (which contained only rat brain homogenate),

indicating that Fraction 2 contained a potential antioxidant (which could slightly reduce the induced lipid peroxidation) but was not potent enough to be considered as an effective therapeutic antioxidant (could not reduce the induced lipid peroxidation levels back to the levels of that of the healthy or uninduced rat brain homogenate or negative control).

3.3.2 Nitroblue tetrazolium assay

3.3.2.1 Method

A modified version of the NBT assay of Ottino and Duncan (1997a) was used. Because this study focused on treating epilepsy, a neurological disease, rat brain homogenate was used during this assay to evaluate the antioxidant activity of *C.orbiculata* in a neurological environment.

3.3.2.1.1 Chemicals and reagents

The chemicals used are of the highest chemical purity and purchased at Sigma Aldrich and Merck Chemicals.

The pre-prepared and frozen aliquots of rat brain homogenate (paragraph 3.3.1.1.3) were used during the NBT assay.

The PBS buffer prepared for the TBA assay (paragraph 3.3.1.1.2) was used.

The 5.6 mM KCN solution used to induce $O_2^{\bullet-}$ production was prepared by dissolving KCN (0.036 g) in dd H_2O (100 ml). Gloves, a lab coat, safety goggles and a particulate respirator was worn during the weighing process and all processes in handling the KCN powder and solution was performed in a fume hood.

NBT was prepared by dissolving NBT (0.005 g) in ethanol (0.1 ml) and dd H_2O (4.9 ml). The solution must be freshly prepared when performing the assay and must be covered in foil (light sensitive).

For the positive control a 5.6 mM Trolox solution was prepared by dissolving Trolox (0.0056 g) in ethanol (1 ml).

For the standard curve a 400 µM/L stock solution of NBD was prepared by dissolving NBD (0.0037 g) in GAA (12.5 ml).

For the Bradford protein assay Bradford reagent (from Sigma Life Sciences) and bovine serum albumin (BSA) was used. A BSA stock solution was prepared by dissolving BSA (2 mg) in PBS buffer (1 ml).

3.3.2.1.2 Preparation of samples

The juice concentrate, Fraction 1 and Fraction 2 were each dissolved in dd H_2O and 0.625 mg/ml, 1.25 mg/ml and 2.5 mg/ml samples prepared.

3.3.2.1.3 Preparation of the standard curve

Eppendorf tubes containing known NBD concentrations were prepared according to table 3.6 to draw up the standard calibration curve.

Table 3.6: Preparation of NBD standard samples

Concentration (µmol/L)	Volume of NBD (µl)	Volume of GAA (µl)
0	0	255
100	63.75	191.25
200	127.5	127.5
300	191.25	63.75
400	255	0

The Eppendorf tubes were vortexed and 255 µl of each Eppendorf tube was collected and placed into a 96 well culture plate. GAA was used as a blank sample. The plate was scanned with a multiplate reader at 560 nm and the absorbance results documented. The absorbance results were plotted against the NBD concentrations. A straight line equation of $y = 0.001x + 0.0055$ with $R^2 = 0.9992$ was obtained (figure 3.6).

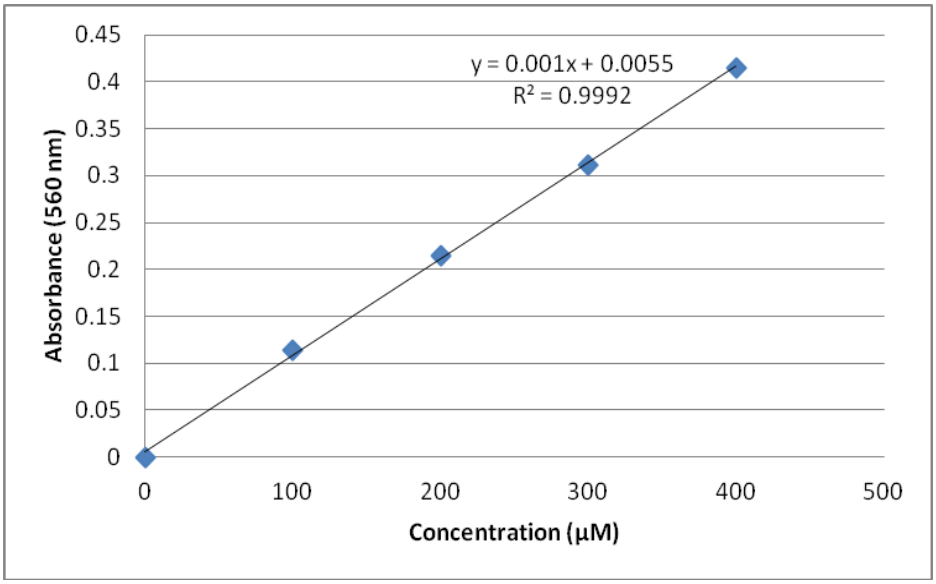


Figure 3.6: NBD standard curve

3.3.2.1.4 Assay

The samples were prepared in Eppendorf tubes according to table 3.7.

Table 3.7: Sample preparation for the NBT assay of *C. orbiculata* juice, Fraction 1 and Fraction 2

	Brain Homog. (µl)	**PBS (µl)**	**KCN (µl)**	**Trolox 1mM (µl)**	**Solvent of Sample (dd H_2O) (µl)**	**Sample (µl)**
Negative Control	100	50	-	-	50	-
***Toxin**	100	50	50	-	-	-
Trolox (Positive Control)	100	-	50	50	-	-
Sample	100	-	50	-	-	50

***Toxin: KCN was used to induce $O_2^{\bullet-}$ production in the rat brain homogenate**

The Eppendorf tubes were vortexed, NBT solution (80 µl) added, vortexed again and incubated for 60 minutes at 37 °C in a oscillating water bath. The Eppendorf tubes were then centrifuged at 3 000 x g for 10 minutes. The supernatant was removed and discarded and 400 µl of GAA added to the resting pellet of each Eppendorf tube. The Eppendorf tubes were vortexed and centrifuged at 4 000 x g for 5 minutes. 255 µl of each Eppendorf tube was

collected and placed in a 96 well culture plate. GAA was used as a blank sample. The absorbance was measured spectrophotometrically with a multiplate reader at 560 nm and the absorbance results documented.

3.3.2.1.5 Bradford Protein Assay

The Bradford protein assay was performed during the NBT assay's incubation period to measure protein quantities in the rat brain homogenate used to determine possible interference of proteins in the assay. Protein values higher than 1.4 mg/ml in rat brain homogenate will result in unreliable data.

Eppendorf tubes containing known BSA concentrations were prepared according to table 3.8 for the standard calibration curve. The Eppendorf tubes were vortexed and three samples of 5 µl of each Eppendorf tube placed in a 96 well culture plate.

Table 3.8: Preparation of the BSA standard samples

Protein Concentration (mg/ml)	Volume of BSA (µl)	Volume of PBS (µl)
0	0	100
0.1	5	95
0.4	20	80
0.7	35	65
1.0	50	50
1.4	70	30

Rat brain homogenate was prepared according to table 3.9. Three 5 µl samples of each Eppendorf tube were collected and placed in the 96 well culture plate.

Bradford reagent (250 µl) was added to the wells containing the standard curve samples and the rat brain homogenate samples. The well plate was then immediately placed on the plate reader's shaker for 30 seconds. The samples were incubated for 15 minutes at room temperature and the absorbance measured spectrophotometrically at 560 nm.

The standard curve was drawn up by plotting the absorbance results against the BSA concentrations. A straight line equation of y = 0.2601x + 0.0066 with R^2 = 0.992 was obtained (figure 3.7). The results obtained from the Bradford protein assay of the rat brain homogenate were used to determine the samples' NBD concentrations per milligram protein obtained during the NBT assay.

Table 3.9: Preparation of the rat brain homogenate samples

Volume of rat brain homogenate (µl)	Volume of PBS (µl)
100	0
10	90
5	95
2	98
0	100

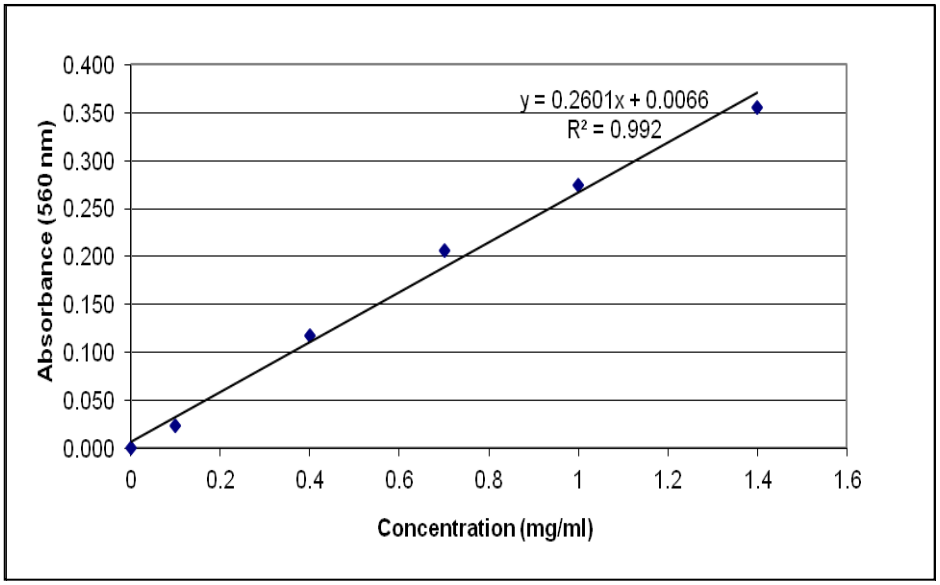


Figure 3.7: BSA standard curve

3.3.2.2 Results

The levels of NBD formed (in µmol/mg protein) in the tested samples were determined and summarised in tables 3.10, 3.11 and 3.12 and figures 3.8, 3.9 and 3.10. Statistical analysis of the results was performed with the Student-Newman-Keuls multiple comparisons test and

one-way analysis of variance (ANOVA) via GraphPad software. The results of the samples were statistically compared with that of the Toxin.

Table 3.10: The effect of *C. orbiculata* juice on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	79.072	0.4773
Toxin	174.630	6.161
Trolox (Positive Control)	66.134	5.846
Juice 2.5 mg/ml	202.455	7.579
Juice 1.25 mg/ml	205.417	6.604
Juice 0.625 mg/ml	197.623	7.152

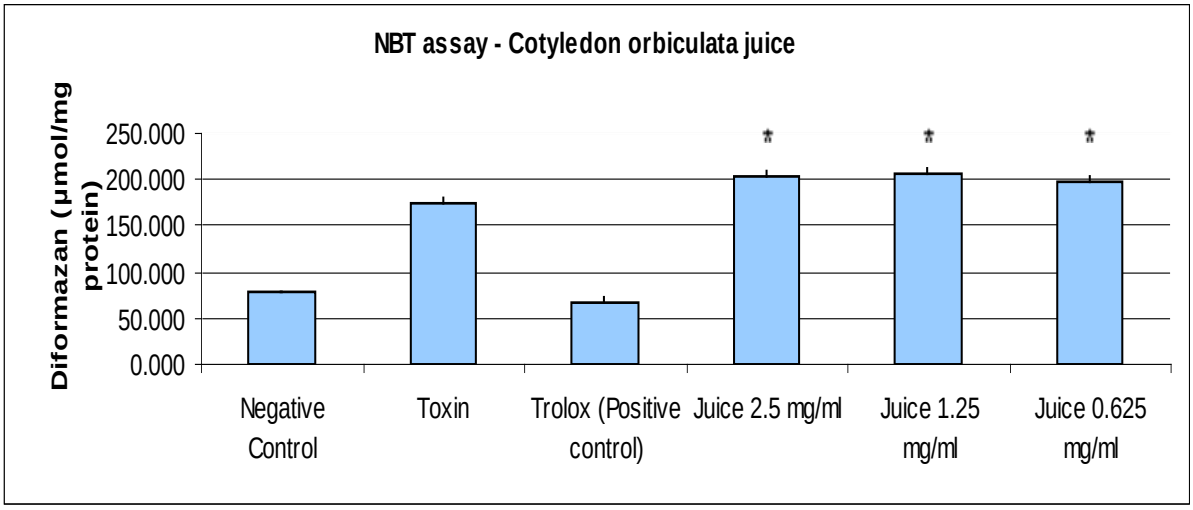


Figure 3.8: The effects of *C. orbiculata* juice on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM. * $p < 0.05$ vs Toxin.

The concentrated juice of *C. orbiculata* showed slight pro-oxidant activity (figure 3.8).

Table 3.11: The effect of Fraction 1 on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	76.938	9.701
Toxin	176.046	7.58
Trolox (Positive Control)	58.068	5.094
Juice 2.5 mg/ml	184.808	5.054
Juice 1.25 mg/ml	207.326	10.697
Juice 0.625 mg/ml	126.091	4.315

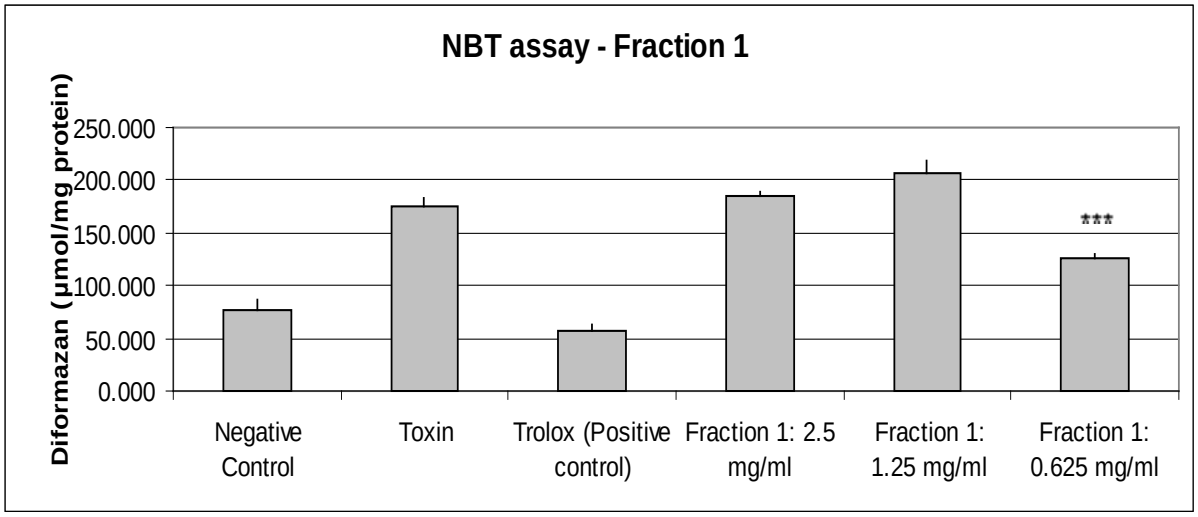


Figure 3.9: The effects of Fraction 1 on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM. *** $p < 0.001$ vs Toxin.

The 0.625 mg/ml concentration of Fraction 1 showed antioxidant activity (figure 3.9).

Table 3.12: The effect of Fraction 2 on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	± Standard error of the mean (SEM) n = 5
Negative Control	31.372	1.469
Toxin	109.102	3.518
Trolox (Positive Control)	41.664	1.3
Juice 2.5 mg/ml	85.045	1.686
Juice 1.25 mg/ml	113.400	1.659
Juice 0.625 mg/ml	116.571	1.961

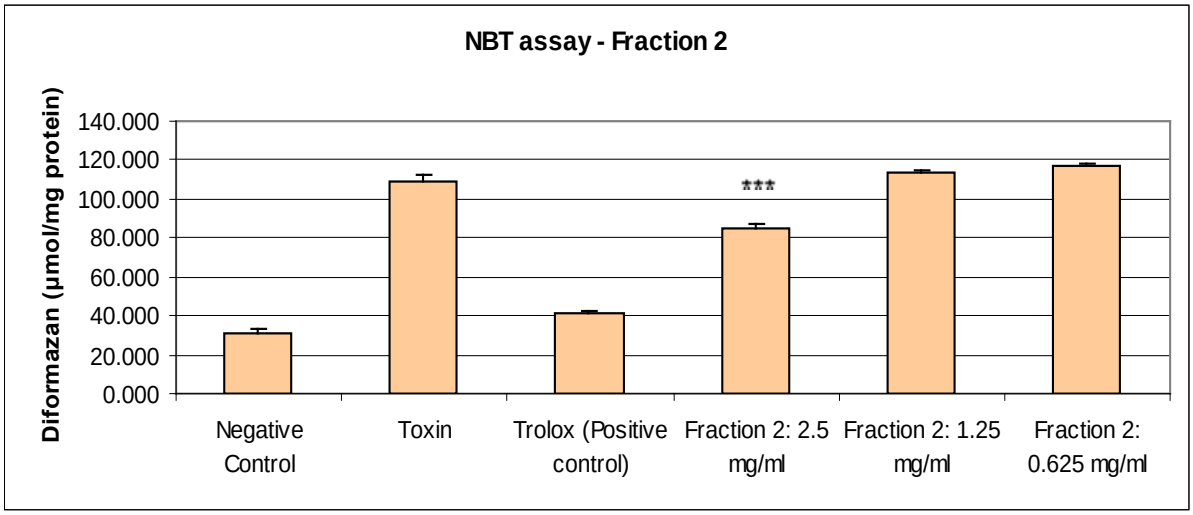


Figure 3.10: The effects of Fraction 2 on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM. *** $p < 0.001$ vs Toxin.

The 2.5 mg/ml Fraction 2 sample showed antioxidant activity (figure 3.10).

3.3.2.3 Discussion

The concentrated *C. orbiculata* juice showed slight pro-oxidant activity, whereas 0.625 mg/ml of Fraction 1 and 2.5 mg/ml of Fraction 2 showed slight antioxidant activity when statistically compared to the Toxin (KCN). The lowest concentration tested for Fraction 1 showed slight antioxidant activity, whereas the higher concentrations showed no significant activity possibly due to the reaction reaching an antioxidant threshold, where higher sample concentrations of

antioxidants could lead to negative, pro-oxidant activity due to the antioxidants influencing important cellular functions that use or require reactive species (e.g. signal transduction pathways). It is also possible that the samples of the higher concentrations did not dissolve properly (the solution became saturated) which could also affect the assay results.

3.4 Conclusion

The MDA level of 2.5 mg/ml of Fraction 2 in the TBA assay and diformazan levels of 0.625 mg/ml of Fraction 1 and 2.5 mg/ml of Fraction 2 were lower than that of the Toxin (where oxidative stress was induced in rat brain homogenate) but not near or lower than the levels of the negative control (which contained only rat brain homogenate), indicating that the fractions contained potential antioxidants (which could slightly reduce oxidative stress) but were not potent enough to be considered as effective therapeutic antioxidants (could not reduce the induced lipid oxidative stress levels back to the levels of that of the healthy or uninduced rat brain homogenate or negative control).

Due to the pro-oxidant activity of the juice and Fraction 1 and the low level of antioxidant activity of Fraction 2 in the TBA assay and the slight pro-oxidant activity of the juice and the slight antioxidant activity of Fraction 1 and Fraction 2 in the NBT assay further fractionation of these samples were not motivated. The extraction and isolation of the orbicusides generally accepted as the active compounds of *C. orbiculata* followed. The optimal method of extraction for the orbicusides was determined and the isolation thereof using column chromatography, precipitation reactions and HPLC fractionation was performed.

4 EXTRACTION OF THE ORBICUSIDES OF *C. ORBICULATA*

4.1 Evaluation of extraction methods for the optimal extraction of the orbicusides

To date the bufadienolide compounds, orbicuside A-C, are the only compounds isolated and characterised from *C. orbiculata* (Steyn *et al.*, 1986). The orbicusides are lipophilic, a beneficial characteristic when considering treatments for central nervous system diseases like epilepsy, as these compounds would be able to move across the blood brain barrier and reach the central nervous system. The likelihood of the orbicusides to have antioxidant activity is promising due to there being multiple cases of both steroids and 2-pyrones having antioxidant acivity (Ahmad *et al.*, 2013; Goel & Ram, 2009; Klinger *et al.*, 2002; Lee *et al.*, 2010; Mohamed *et al.*, 2012; Thuong *et al.*, 2010). Should the orbicusides have significant antioxidant activity, these compounds could have good potential as anti-epileptic therapy (in epilepsy pathophysiology where oxidative stress is involved).

Multiple extraction methods were evaluated to determine an optimal method of extraction for the orbicusides. Maceration, microwave extraction, Soxhlet extraction and accelerated solvent extraction (ASE) were performed. The standard range of solvents normally used during extraction (e.g. methanol, petroleum ether, etc.) was not used during the extraction of the orbicusides due to previous studies of the plant that have already used these solvents (Louw, 2009; Roux, 2012). Four solvents of increasing polarities were used during the evaluation of the optimal method of orbicuside extraction, namely cyclohexane, toluene, 1,4-dioxane and ethanol with a polarity index of 0.2, 2.4, 4.8 and 5.1 respectively.

4.1.1 Extraction methods

4.1.1.1 Maceration

Four solvents with increasing polarities were used consecutively, namely cyclohexane, toluene, 1,4-dioxane and ethanol. Fresh leaf pulp was placed in an Erlen Meyer flask and 125 ml cyclohexane added. The flask was agitated every two hours and kept at room temperature until extraction was complete (24 hours). The cyclohexane was drained and toluene added. The extraction process was repeated, followed by 1,4-dioxane and ethanol. The extracts obtained were concentrated using the BUCHI Rotavapor, weighed and stored separately at -20 °C until needed.

4.1.1.2 Microwave Extraction

Three solvents with increasing polarities were used consecutively, namely cyclohexane, toluene and 1,4-dioxane. Microwave extraction was performed with a CEM Discover

Focused Microwave Synthesis System. Fresh leaf pulp was added to 25 ml of cyclohexane and microwave extraction performed at 150 Watt and 70 °C until extraction was complete (5-10 minutes). The resulting extract was filtered and concentrated using the BUCHI Rotavapor. The filtered pulp is then added to 25 ml toluene and microwave extraction performed at 150 Watt and 90 °C until extraction was complete (5-10 minutes). The resulting extract was filtered and concentrated and the filtered pulp added to 25 ml 1,4-dioxane. Microwave extraction was performed at 150 Watt and 80 °C until extraction was complete (5-10 minutes). The resulting extract was filtered and concentrated and the three extracts stored at -20 °C until needed.

4.1.1.3 Soxhlet Extraction

Cyclohexane, toluene, 1,4-dioxane and ethanol was used consecutively during Soxhlet extraction. Fresh leaf pulp was placed in a 29 x 100 mm extraction thimble and extracted using cyclohexane until the extraction was complete (3 days). The process was repeated using the same plant material with toluene (3 days), 1,4-dioxane (4 days) and ethanol (3 days). The extracts obtained were concentrated using the BUCHI Rotavapor and stored separately at -20 °C until needed.

4.1.1.4 Accelerated Solvent Extraction (ASE)

ASE was developed by Dionex to address several shortcomings of traditional extraction techniques (e.g. Soxhlet extraction), including the use of large quantities of solvent and long duration of extraction. It is an automated extraction method which uses high temperatures and pressures to accelerate extraction (Dionex, 2008).

The high temperatures promote analyte extraction using less solvent and time by (Dionex, 2008):

- Decreasing solvent viscosities (allowing the solvent to penetrate the plant material pores more easily)
- Promoting the disruption of analyte-plant material interactions (e.g. van der Waals forces and hydrogen bonding) and removal of the analytes from the plant material
- Increasing the diffusion rates allowing the analytes to move more quickly from the boundary layer near the plant material surface to the solvent.
- Increasing the capacity of solvents to solubilise the analytes

The highest temperature at which extraction can take place is the boiling point of the solvent. Many of the organic solvents used have low boiling points, limiting the benefits of high temperatures on accelerating extraction. High pressures exerted on the solvents during ASE

allow the use of temperatures above the boiling points of the chosen solvents allowing all of the advantages of using high temperatures during extraction. The use of high pressures also accelerate extraction by assisting the movement of solvent through a packed bed of plant material, forcing the solvent into the pores of the material (Dionex, 2008).

The ASE 350 was compared to Soxhlet extraction by developing an optimal extraction protocol for *C. orbiculata* leaf pulp and comparing the extraction parameters of ASE and Soxhlet extraction to determine which method was more efficient in the extraction of the orbicusides. 1,4-Dioxane could not be used during ASE due to its low auto-ignition point (Dionex, 2008). Instead cyclohexane, toluene and dichloromethane were used. Leaf pulp was mixed with diatomaceous earth (DE), a non-reactive material used as a drying agent to dry the pulp and prevent blockage of the ASE extraction cells. The amount of DE needed for optimal drying was 25 % of the weight of leaf pulp used. Half of the amount of DE used was ground to a fine powder to optimize drying and the other half was kept in its pellet form to avoid the compaction of the plant pulp in the extraction cells which could lead to the blockage of solvent flow and a dangerous increase in cell pressure during extraction. The pulp-DE mixture was placed in stainless steel extraction cells and slightly compacted. Extraction was performed at 1500 psi using the three solvents separately (consecutive extraction of the plant material, as in the case of the Soxhlet extraction method, was not performed during ASE due to the successful extraction of possible cardiac glycoside compounds in the first solvent, cyclohexane (see 4.1.2.1.3)) and three different temperatures (80, 140 and 170 °C) on separate cells to determine the most effective extraction method. The extracts obtained were concentrated using the BUCHI Rotavapor and stored separately at -20 °C until needed.

4.1.2 Evaluation of extraction methods

4.1.2.1 Thin Layer Chromatography (TLC)

TLC was performed using silica gel plates (TLC silica gel 60 G_{254}). The amount of components present and the presence of possible cardiac glycosides in each of the extracts prepared by the extraction methods were determined using various detection reagents. Antimony(III)chloride ($SbCl_3$) and Chloramine T are detection agents that can be used to detect cardiac glycosides. However, these detection agents can also detect steroid and other compounds. It is therefore possible that the bands identified as cardiac glycosides on the TLC plates prepared during the study are plant steroids or other compounds. For the purpose of this study the detection agents were used to aid in the identification of the possible positions of the cardiac glycosides in the various extracts prepared.

4.1.2.1.1 TLC of microwave extraction extracts

TLC of the microwave extraction extracts was performed using methanol, ethyl acetate and dichloromethane (DCM): ethyl acetate: ethanol (1:1:1) as mobile phases (figure 4.1).
The compound bands were visualized using the following spray reagents:

- Anise-aldehyde
- H_2SO_4 (5 % in ethanol)
  Prepared by placing 10 ml H_2SO_4 on ice and very slowly adding ethanol until a final volume of 200 ml was reached.

The plant material was not properly fractionated, because the three extracts contained similar compounds (visual bands that formed were on similar positions). The extracts also contain small amounts of different compounds, possibly indicating the loss of compounds during extraction due to the high temperatures that form during the extraction process.

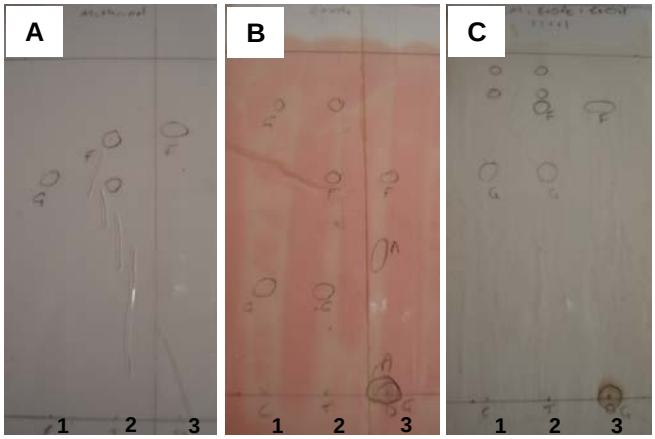


Figure 4.1: TLC of the microwave extraction's (1) cyclohexane, (2) toluene and (3) 1,4-dioxane extracts using (A) methanol as the mobile phase with no detection reagent, (B) ethyl acetate as the mobile phase and anise-aldehyde as the detection reagent and (C) DCM: ethyl acetate: ethanol (1:1:1) as the mobile phase and H_2SO_4 (10 % in ethanol) as the detection reagent.

4.1.2.1.2 TLC of the microwave, maceration and Soxhlet extraction 1,4-dioxane and ethanol extracts

TLC of the 1,4-dioxane and ethanol extracts of the maceration and Soxhlet extraction (figure 4.2 and 4.3) was performed using chloroform: acetone: methanol (70:30:1) and ethyl acetate: methanol: dd H_2O (81:11:8) as mobile phases and the following detection agents:

- $SbCl_3$ solution (25 % (w/v) in chloroform)
- Chloramine T spray reagent:
  A 3 % (w/v) solution of chloramine T in distilled water and a 25 %(w/v) solution of tricarboxylic acid in ethanol was prepared separately. 20ml of the chloramine T solution

was mixed with 80ml of the tricarboxylic acid solution to prepare the chloramine T spray reagent.

Figure 4.2 and 4.3 indicated that the 1,4-dioxane and ethanol extracts of the microwave, maceration and Soxhlet extraction methods contained multiple possible cardiac glycosides. The 1,4-dioxane and ethanol extracts of the Soxhlet extraction method contained the most possible cardiac glycosides, motivating the use of this extraction method and 1,4-dioxane and ethanol as the optimal extraction method.

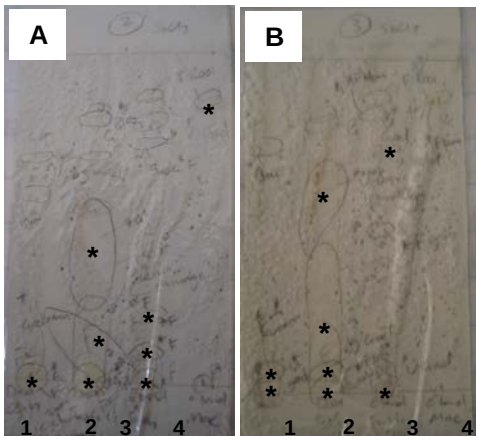


Figure 4.2: TLC of (1) 1,4-dioxane microwave extraction extract, (2) 1,4-dioxane Soxhlet extract, (3) ethanol Soxhlet extract and (4) ethanol maceration extract using $SbCl_3$ as detection reagent and (A) chloroform: acetone: methanol (70:30:1) and (B) ethyl acetate: methanol: dd H_2O (81:11:8) as mobile phases. Bands marked with * indicate possible cardiac glycosides. The flaking of the silica off of the aluminium plate is due to the $SbCl_3$ detection reagent.

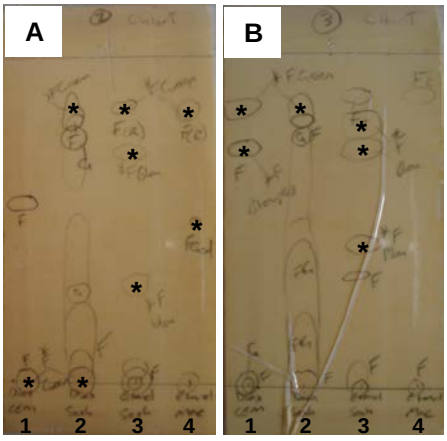


Figure 4.3: TLC of (1) 1,4-dioxane microwave extraction extract, (2) 1,4-dioxane Soxhlet extract, (3) ethanol Soxhlet extract and (4) ethanol maceration extract using Chloramine T solution as detection reagent and (A) chloroform: acetone: methanol (70:30:1) and (B) ethyl acetate: methanol: dd H_2O (81:11:8) as mobile phases. Bands marked with * indicate possible cardiac glycosides.

4.1.2.1.3 TLC of the ASE extracts

TLC of the ASE cyclohexane and toluene extracts using chloroform: acetone: methanol (70:30:1) as mobile phase and $SbCl_3$ and Chloramine T solution as detection reagents showed that both solvents and the high temperature extracts contained multiple possible cardiac glycosides (figure 4.4).

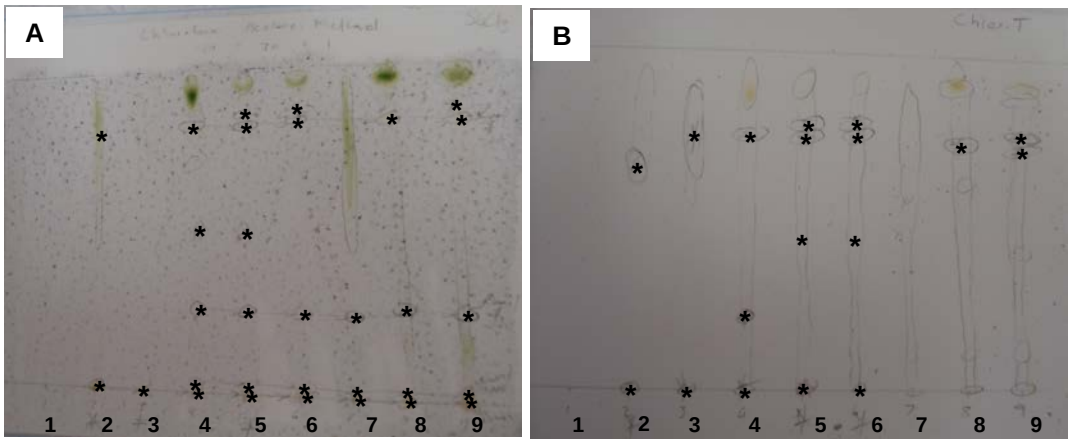


Figure 4.4: TLC of the ASE (1) DE blank sample, (2) cyclohexane extract 70 °C, (3) cyclohexane extract 100 °C, (4) cyclohexane extract 140 °C, (5 + 6) cyclohexane extract 170 °C, (7) toluene extract 100 °C, (8) toluene extract 140 °C and (9) toluene extract 170 °C using (A) $SbCl_3$ and (B) Chloramine T solution as detection reagents and chloroform: acetone: methanol (70:30:1) as mobile phase. Bands marked with * indicate possible cardiac glycosides.

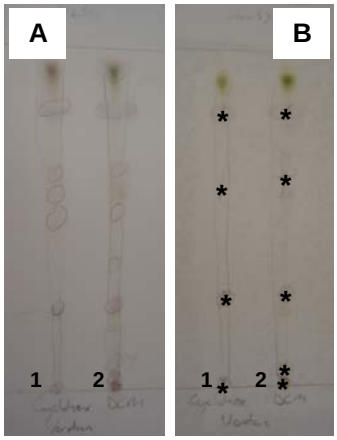


Figure 4.5: TLC of the ASE (1) cyclohexane extract 140 °C and (2) DCM extract 140 °C using (A) H_2SO_4 (5 %) and (B) $SbCl_3$ (25 %) as detection reagents and chloroform: acetone: methanol (70:30:1) as mobile phase. Bands marked with * indicate possible cardiac glycosides.

TLC of the ASE cyclohexane extract 140 °C and DCM extract 140 °C using chloroform: acetone: methanol (70:30:1) as mobile phase and H_2SO_4 and $SbCl_3$ as detection reagents showed that both solvents produced similar extracts (figure 4.5).

4.1.2.1.4 Conclusion

The maceration, microwave and Soxhlet extraction 1,4-dioxane extracts and ASE extracts showed the most prominent indication of cardiac glycosides. The maceration, microwave, Soxhlet and ASE extraction method can therefore be used to extract cardiac glycosides and 1,4-dioxane extracts of the maceration, microwave and Soxhlet extract showed to contain the cardiac glycosides. The ASE method's cyclohexane, toluene and DCM extracts contained the most potential cardiac glycosides. Soxhlet extraction and ASE could therefore be recommended as the ideal methods for the bulk extraction of the orbicusides of *C. orbiculata* when considering the amount of compounds, particularly cardiac glycosides, extracted.

4.1.2.2 HPLC screening of plant extracts

For the HPLC screening of plant extracts an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.10.01 data acquisition and analysis software was used. A Synergi Fusion® RP column 4 µm, 250 x 4.6 mm (Phenomenex, Torrance, CA) and acetonitrile (A) / 0.1 % acetic acid (B) mobile phase at the gradients listed in table 4.1 was used.

Table 4.1: Mobile phase gradients

TIME (MIN)	% A	% B
0	5	95
1	5	95
10	95	5
15	95	5
15.2	5	95
20	5	95

The flow rate was 1.0 ml/min, the injection volume was 25 µl and UV detection was performed at 210 nm and 8 nm bandwidth. Samples of the extracts were dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm with G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter, Pall Life Sciences).

The resulting chromatograms of all the maceration, microwave and Soxhlet extraction extracts showed altered baselines and the presence of recurring peaks at 0 to 6.338 min and 18.315 to 19.739 min, possibly due to artefact formation or contamination in the mobile and/or stationary phase during chromatography. During the identification of potential compound peaks in the extracts, the chromatograms were compared with one another and the recurring peaks identified at 0 o 6.338 min and 18.315 and 19.739 min ignored.

4.1.2.2.1 Maceration

HPLC screening of the maceration cyclohexane extract showed five possible compound peaks at 1.820, 2.601, 5.068, 14.823 and 17.002 min (figure 4.6).

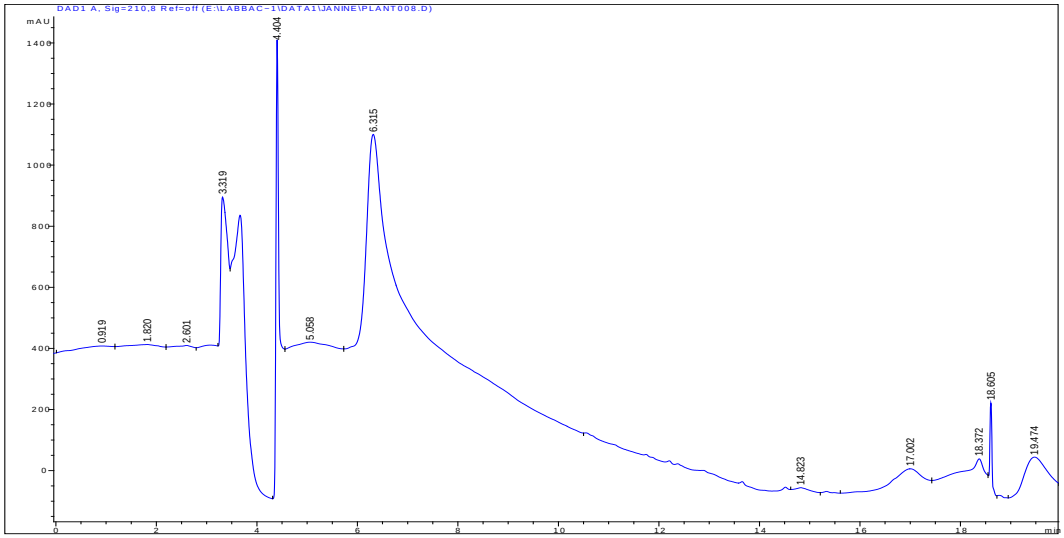


Figure 4.6: HPLC screening of the maceration cyclohexane extract

The toluene extract showed six possible compound peaks at 2.035, 2.592, 3.084, 5.003, 5.448 and 17.164 min (figure 4.7).

The 1,4-dioxane extract contained twelve possible compound peaks at 1.900, 4.806, 5.677, 7.461, 7.690, 8.083, 8.339, 9.629, 13.620, 14.500, 15.320 and 16.527 min (figure 4.8). The ethanol extract showed eleven possible compound peaks at 0.591, 2.894, 4.132, 5.384, 5.778, 7.690, 8.081, 8.416, 8.715, 13.631 and 15.924 min (figure 4.9). However, the peaks of the ethanol extract were positioned similarly to that of 1,4-dioxane, which indicated that the two extracts were similar.

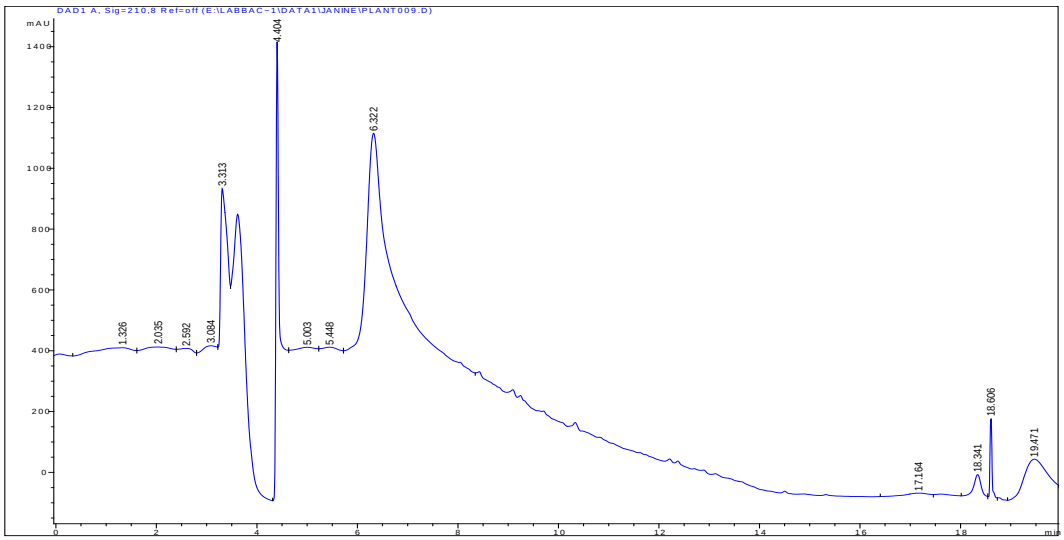


Figure 4.7: HPLC screening of the maceration toluene extract

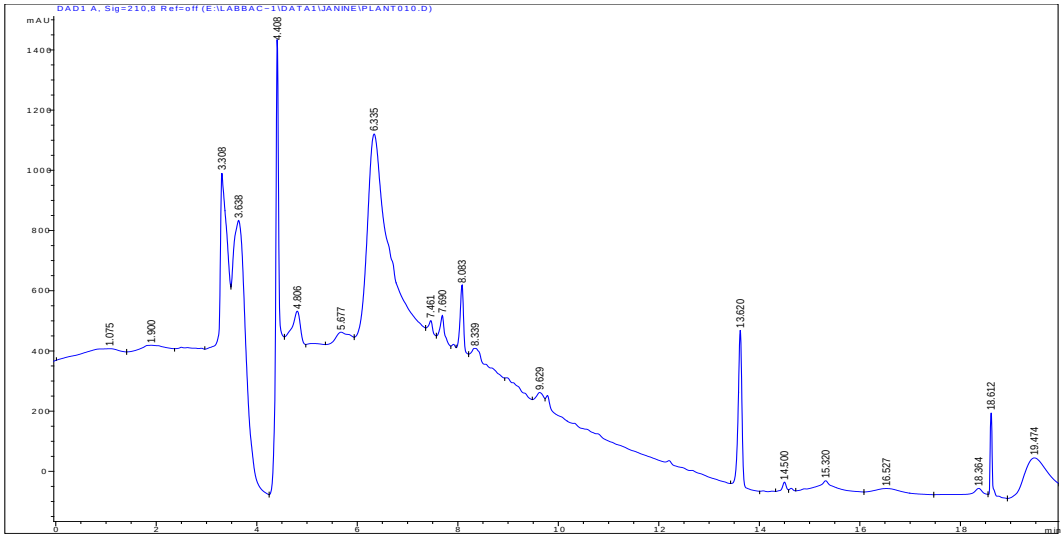


Figure 4.8: HPLC screening of the maceration 1,4-dioxane extract

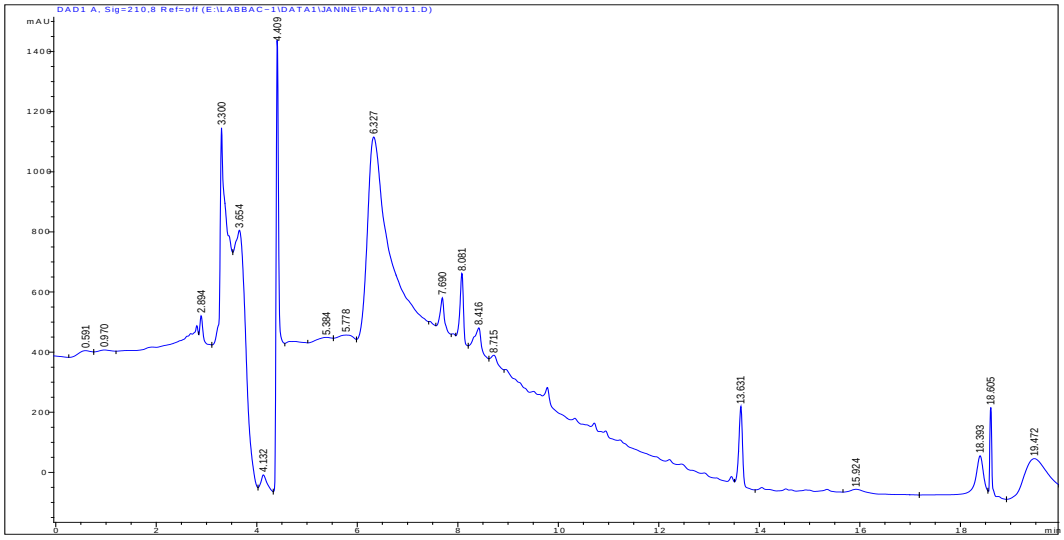


Figure 4.9: HPLC screening of the maceration ethanol extract

The concentrations of the peaks of the cyclohexane and toluene extracts were not significant enough to motivate further extraction or fractionation, whereas the concentrations of certain peaks of the 1,4-dioxane extract (at 8.083 and 13.620 min) and ethanol extract (8.081 and 13.631 min), possibly the same compounds as that of the 1,4-dioxane compounds at 8.083 and 13.620 min, were considered significant enough to target for further fractionation and isolation.

4.1.2.2.2 Microwave extraction

HPLC screening of the microwave extraction extracts indicated that the cyclohexane and toluene extracts (figures 4.10 and 4.11) had no possible compound peaks, whereas the 1,4-dioxane extract (figure 4.12) contained at least nine possible compound peaks (at 0.202, 2.150, 7.680, 8.067, 8.379, 8.670, 9.616, 13.619 and 15.914 min). However, the small peaks indicated that the concentrations were not significant enough to facilitate further fractionation. The extraction of the orbicusides from *C. orbiculata* leaves via microwave extraction was therefore not recommended.

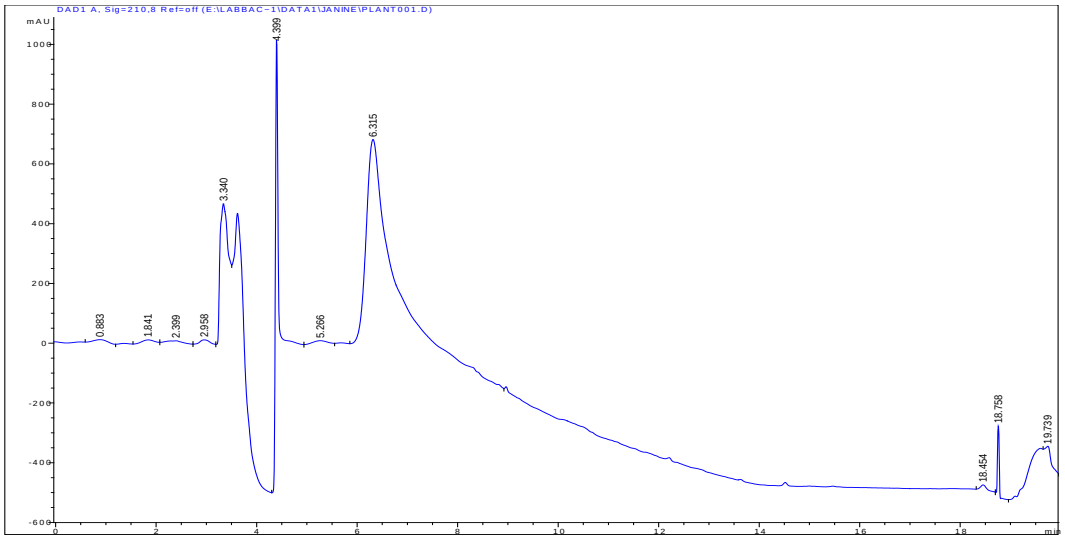


Figure 4.10: HPLC screening of the microwave extraction cyclohexane extract

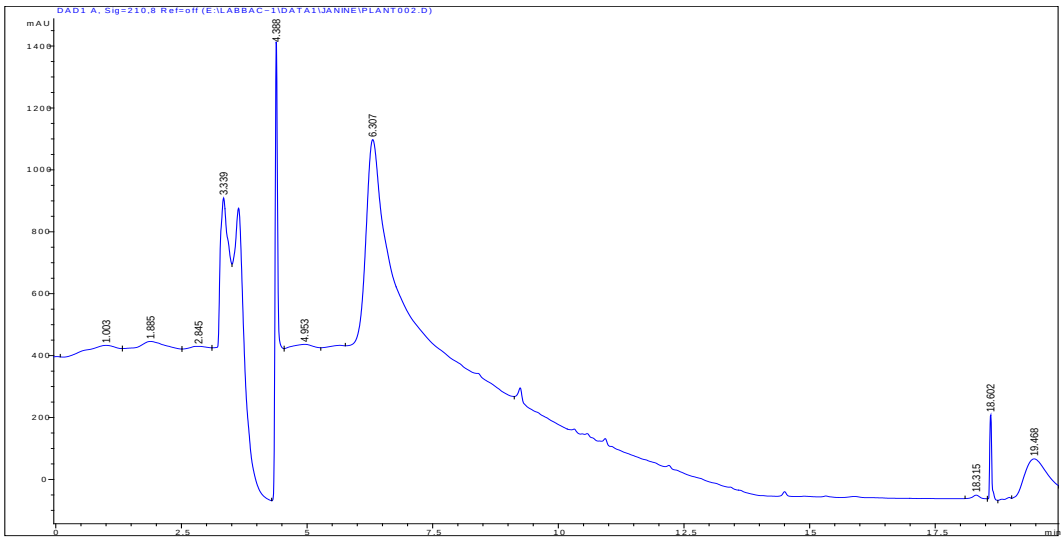


Figure 4.11: HPLC screening of the microwave extraction toluene extract

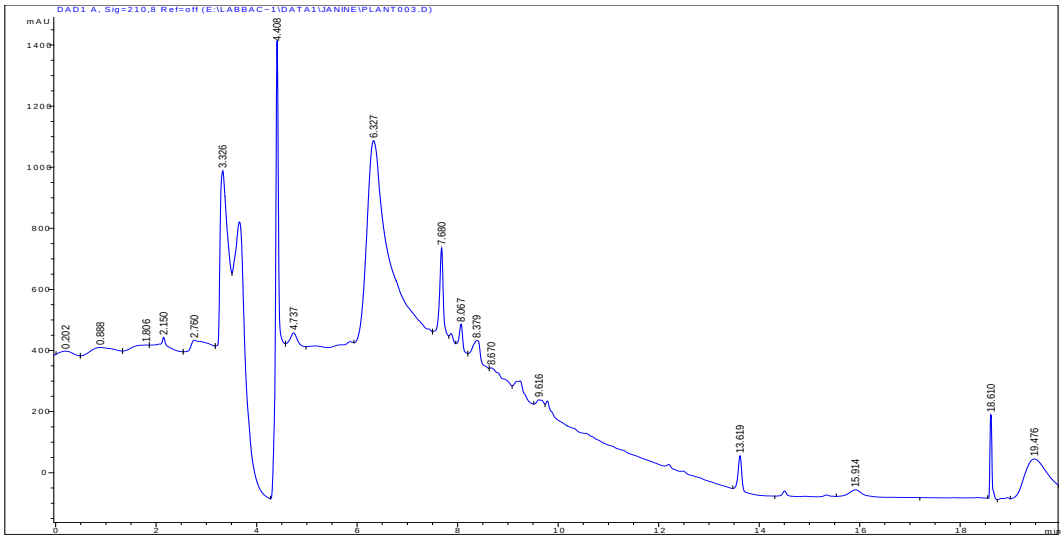


Figure 4.12: HPLC screening of the microwave extraction 1,4-dioxane extract

4.1.2.2.3 Soxhlet extraction

HPLC screening of the Soxhlet extraction's cyclohexane extract showed ten possible compound peaks at 0.969, 1.461, 2.467, 3.122, 5.361, 13.628, 14.506, 15.326, 15.917 and 18.353 min (figure 4.13). The extract did not contain compounds with significant concentrations to motivate further fractionation.

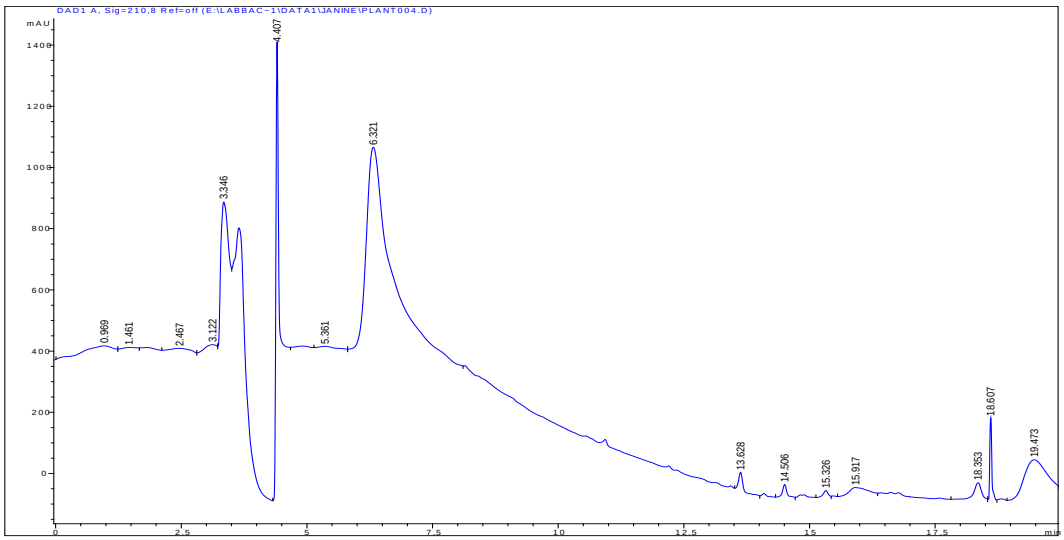


Figure 4.13: HPLC screening of the Soxhlet extraction cyclohexane extract

The toluene extract showed six possible compound peaks at 0.436, 1.245, 1.705, 4.754, 8.050, 8.432, 9.006, 9.247, 9.778, 10.322, 10,478, 10.828, 12.461, 13.128, 13.336, 13.632, 14.083, 14.223, 14.498, 14.885, 15.317, 16.434, 17.026 and 18.312 min (figure 4.14). The extract contained compounds with significant concentrations that could motivate further fractionation (including the peaks at 14.498, 15.317 and 18.312 min).

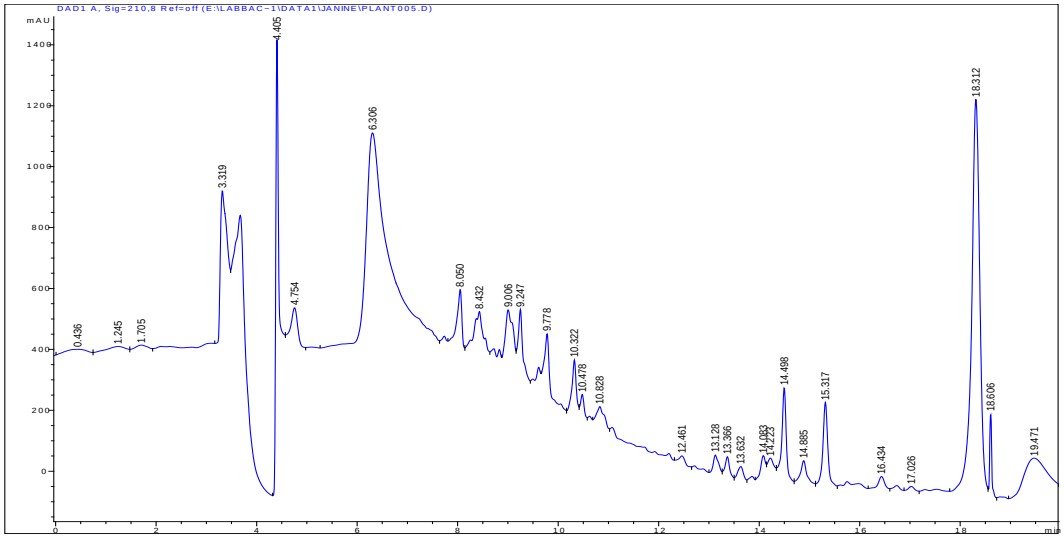


Figure 4.14: HPLC screening of the Soxhlet extraction toluene extract

The 1,4-dioxane extract contained twelve possible compound peaks at 0.286, 1.031, 1.914, 3.506, 4.784, 7.681, 7.904, 9.092, 9.829, 12.668, 13.623, 14.508, 15.007, 15.325, 15.876 and 18.325 min (figure 4.15). The extract contained compounds with significant concentrations that could motivate further fractionation (including the peaks at 4.784, 7.681 and 13.623 min).

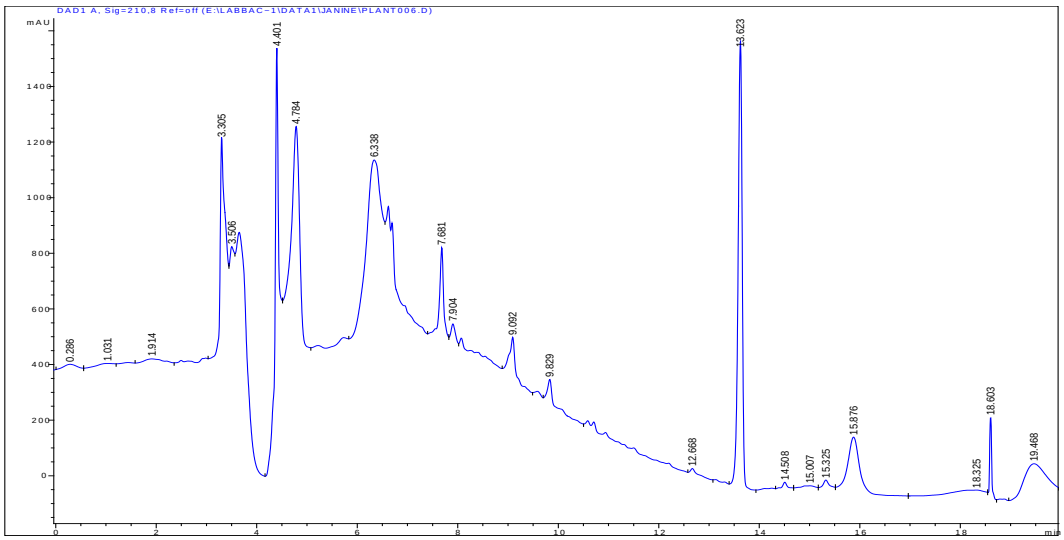


Figure 4.15: HPLC screening of the Soxhlet extraction 1,4-dioxane extract

The ethanol extract showed eleven possible compound peaks at 0.932, 1.824, 2.591, 2.892, 5.720, 7.677, 8.080, 10.587, 10.706, 10.935, 12.222, 13.616, 14.039, 16.453 and 17.248 min (figure 4.16). The extract contained compounds with significant concentrations that could motivate further fractionation (including the peaks at 10.587, 10.706 and 10.935 min).

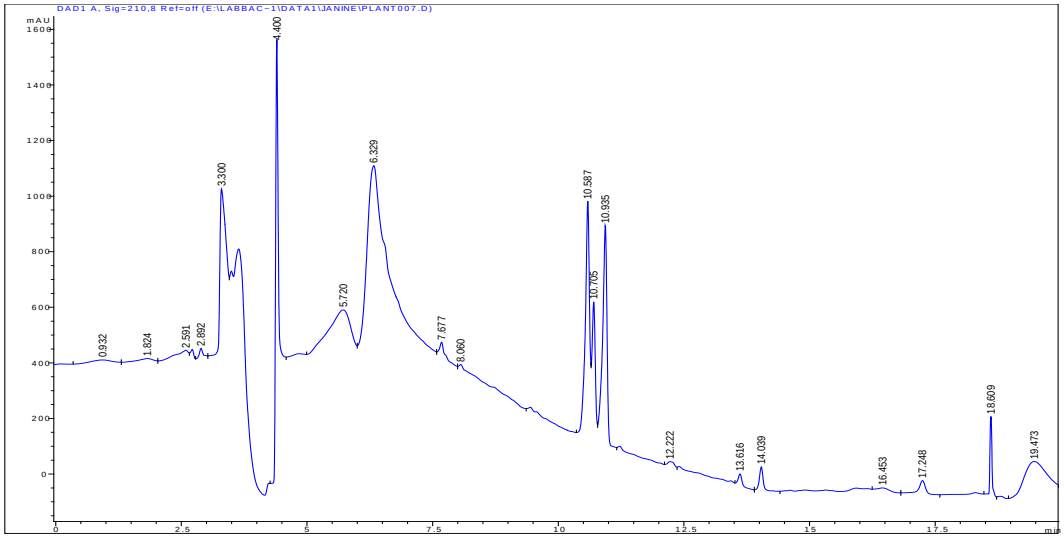


Figure 4.16: HPLC screening of the Soxhlet extraction ethanol extract

4.1.2.2.4 Conclusion

The Soxhlet extraction method's extracts contained the most compounds when compared to maceration and microwave extraction extracts, which was not necessarily ideal when wanting to optimally extract a target compound (the ideal would be to produce an extract containing the target compound and little to no other compounds or impurities). However the 1,4-dioxane and ethanol extracts contained compounds of significant concentrations to use

for further fractionation and Soxhlet extraction could be recommended as the bulk extraction method for the extraction of the orbicusides of *C.orbiculata*.

4.1.2.3 Comparison of extraction parameters

4.1.2.3.1 Amount of extract obtained

Maceration produced the least amounts for all four extracts. 30 ml leaf pulp in 125 ml of solvent produced 5 mg cyclohexane, 4 mg toluene, 168 mg 1,4-dioxane and 6 mg ethanol extract. Microwave extraction of 30 ml leaf pulp in 25 ml solvent also produced a low extract yield (9 mg cyclohexane, 9 mg toluene and 55 mg 1,4-dioxane extract). Soxhlet extraction produced higher amounts of extract (25 mg cyclohexane, 14 mg toluene, 512 mg 1,4-dioxane and 7 mg ethanol extract). ASE extracts produced similar amounts of extract to that of the Soxhlet extracts.

4.1.2.3.2 Time

Microwave extraction produced the extracts in 5-10 minutes per solvent, whereas maceration produced extracts within 24 hours per solvent. These two methods were the fastest but produced the least amount of extract. Producing more sufficient amounts of extract will require repeated extractions and would therefore take longer. Soxhlet extraction took the longest to complete (3-4 days per solvent) but produced the most extract. ASE was the most efficient extraction method in terms of time, producing similar amounts of extract to that of Soxhlet extraction in half the time taken to complete Soxhlet extraction. One extraction cell of the ASE method is similar to that of the sample size used during the extraction method evaluation. Extraction of one extraction cell's plant material took 1 – 2 days.

4.1.2.3.3 Solvent volumes used

Microwave extraction can only use 25 ml of solvent per extraction, the lowest volume of solvent used when the extraction methods were compared. Maceration also used relatively low volumes of solvent (125 ml per solvent), but produced low amounts of extract. Soxhlet extraction used the most solvent. During Soxhlet extraction the solvent is replaced repeatedly until the solvent is clear with no more components being extracted from the plant material. 1 – 2 liters of each solvent was used during the extraction of 30 ml leaf pulp. ASE used larger amounts of solvent than maceration and microwave extraction, but less than Soxhlet extraction, using 1 – 2 liters of solvent during the extraction of 500 g of leaf pulp.

4.1.2.3.4 Reproducibility

Microwave extraction and ASE uses fixed parameters during the extraction process, benefitting reproducibility. Microwave extraction uses 150 Watt at fixed temperatures and

time frames. ASE uses controlled temperatures, pressures and solvent volumes, which is saved as a protocol on the ASE 350's programming, making ASE reproducible. If the maceration's solvent volume and temperature remains constant, the extraction should be reproducible. Soxhlet extraction uses hot plates to heat solvents to reach boiling point. The temperatures used are not controlled or monitored. The temperatures of the atmosphere and possible loss of solvent through evaporation are not monitored during extraction, lowering the reproducibility of this extraction method.

4.1.3 Conclusion

Through comparing maceration, Soxhlet extraction and microwave extraction it was determined that Soxhlet extraction was the most efficient extraction method, producing the most extract containing potential cardiac glycosides. Soxhlet extraction was therefore used for the bulk extraction of the orbicusides. However, Soxhlet extraction used the most solvent and took the longest to complete. Through comparing Soxhlet extraction with ASE it was determined that ASE produced similar amounts of extract, using significantly less solvent and time than Soxhlet extraction. ASE is also a more reproducible extraction method than Soxhlet extraction, by using constant pressures, temperatures and solvent volumes. ASE was therefore identified as the optimal method for the final bulk extraction of the orbicusides of *C. orbiculata* leaves.

4.1.4 Evaluation of the *C. orbiculata* juice not used during extraction

Because there was a possibility that the cardiac glycosides could be in the *C. orbiculata* juice instead of the pulp, the contents of the juice was investigated. A small amount of frozen and fresh juice was filtered to remove pulp and concentrated using the BUCHI Rotavapor at 80 – 100 °C and dotted on a TLC plate (figure 4.17).

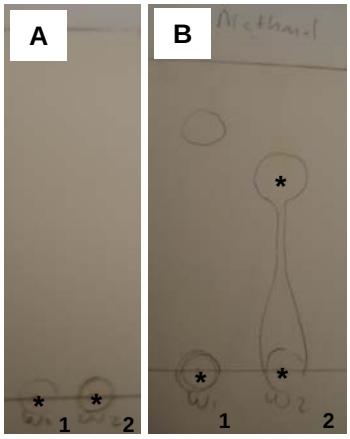


Figure 4.17: TLC of small samples of (1) frozen and (2) fresh *C. orbiculata* juice using $SbCl_3$ as detection reagent and (A) chloroform: acetone: methanol (70:30:1) and (B) methanol as mobile phases. Bands marked with * indicate possible cardiac glycosides.

Chloroform: acetone: methanol (70:30:1) and methanol was used as mobile phases and $SbCl_3$ (25 %) was used as detection agent for cardiac glycosides.

The two juice samples contained very small amounts of cardiac glycosides, forming very light bands not visible in normal light, whereas the cardiac glycoside bands of the various extraction methods' extracts (figure 4.2 to 4.5) were prominent without UV light, indicating that though the juice can contain cardiac glycosides, the concentrations are very low. Cardiac glycosides are therefore not significantly lost when juicing the plant leaves and only using the pulp.

4.1.5 Soxhlet extraction of frozen *C.orbiculata* leaves

To confirm that Soxhlet extraction could be used as the bulk extraction method for frozen plant material, frozen leaf pulp was placed in a 29 x 100 mm extraction thimble and extracted using cyclohexane until the extraction was complete (3 days). The process was repeated using the same plant material with toluene (3 days), 1,4-dioxane (4 days) and ethanol (3 days). The extracts obtained were concentrated using the BUCHI Rotavapor and stored separately at -20 °C until needed. The volume of concentrated 1,4-dioxane extract could not be stored in one polytop and was therefore divided into two polytops (D_1 and D_2 in figure 4.18).

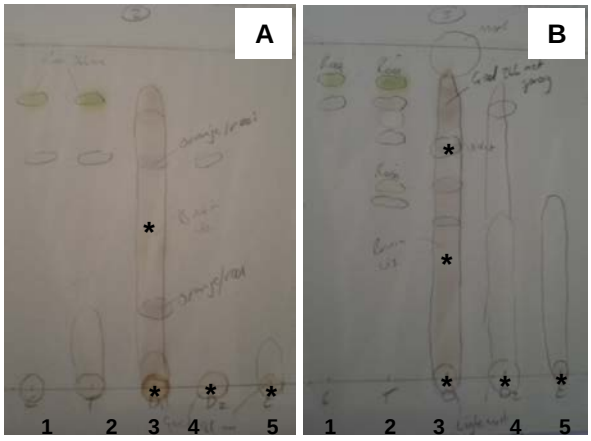


Figure 4.18: TLC of the Soxhlet (1) cyclohexane, (2) toluene, (3 + 4) 1,4-dioxane and (5) ethanol extracts using $SbCl_3$ as detection reagent and (A) chloroform: acetone: methanol (70:30:1) and (B) ethyl acetate: methanol: dd H_2O (81:11:8) as mobile phases. Bands marked with * indicate possible cardiac glycosides.

TLC of the Soxhlet extracts (figure 4.18) using $SbCl_3$ (25 %) as detection agent and chloroform: acetone: methanol (70:30:1) and ethyl acetate: methanol: dd H_2O (81:11:8) as mobile phases indicated multiple possible cardiac glycosides in the 1,4-dioxane extracts and a very light band indicating possible cardiac glycosides in the ethanol extract.

The contents of the extracts were screened via HPLC using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.10.01 data acquisition and analysis software. A Synergi Fusion®-RP column 4 µm, 250 x 4.6 mm (Phenomenex, Torrance, CA) and acetonitrile (A) / 0.1 % acetic acid (B) mobile phase at the gradients listed in table 4.1 was used. The flow rate was 1.0 ml/min, the injection volume was 25 µl and UV detection was performed at 210 nm and 8 nm bandwidth. Samples of the extracts were dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm eith G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter).

According to the HPLC screening the cyclohexane and toluene extracts (figure 4.19 and 4.20) contained similar peaks. Only the highest peaks were considered as potential compounds of considerable concentrations that can be further extracted. However, the recurring peaks at 1.037 and 18.646 min of the cyclohexane extract and the peaks at 1.037 and 18.625 min of the toluene extract could be due to possible artefact formation or contamination of the mobile and/or stationary phase during chromatography and were ignored. The cyclohexane extract, therefore, contained two possible compounds and the toluene extract contained one possible compound of significant concentrations that could be targeted for further fractionation. Due to the cyclohexane and toluene extracts not containing possible cardiac glycosides (figure 4.18) further fractionation of these extracts was not considered.

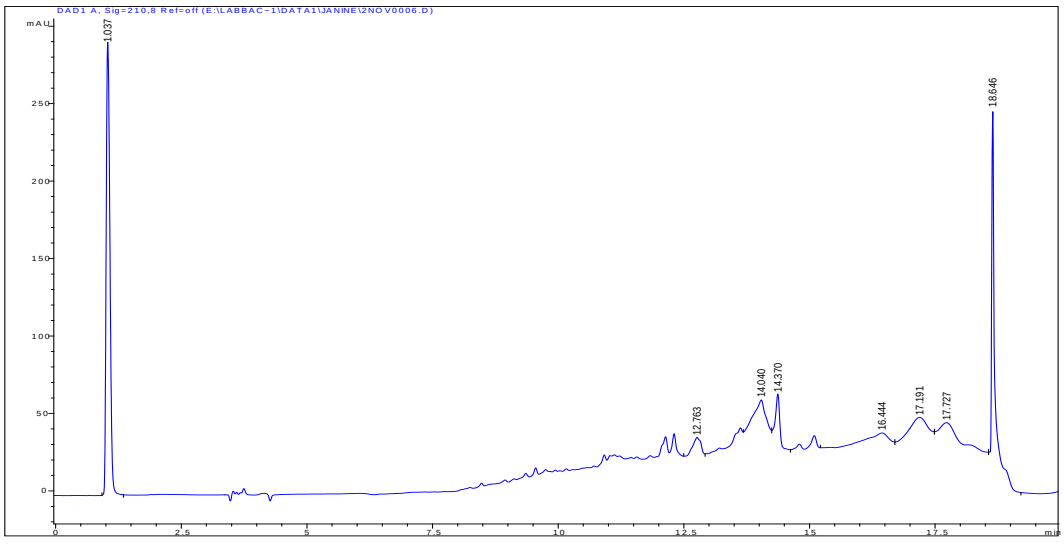


Figure 4.19: HPLC screening of the Soxhlet cyclohexane extract of frozen leaf pulp

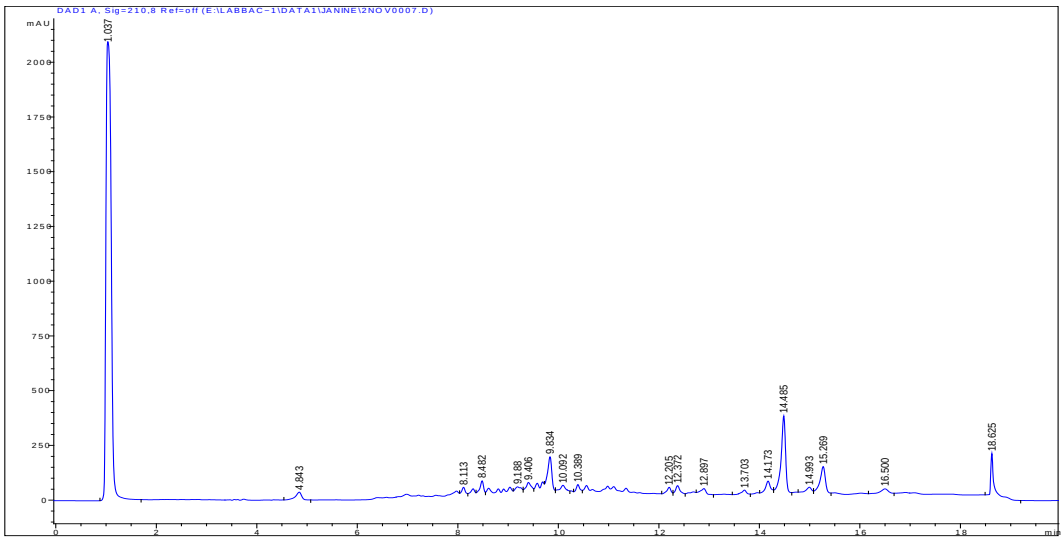


Figure 4.20: HPLC screening of the Soxhlet toluene extract of frozen leaf pulp

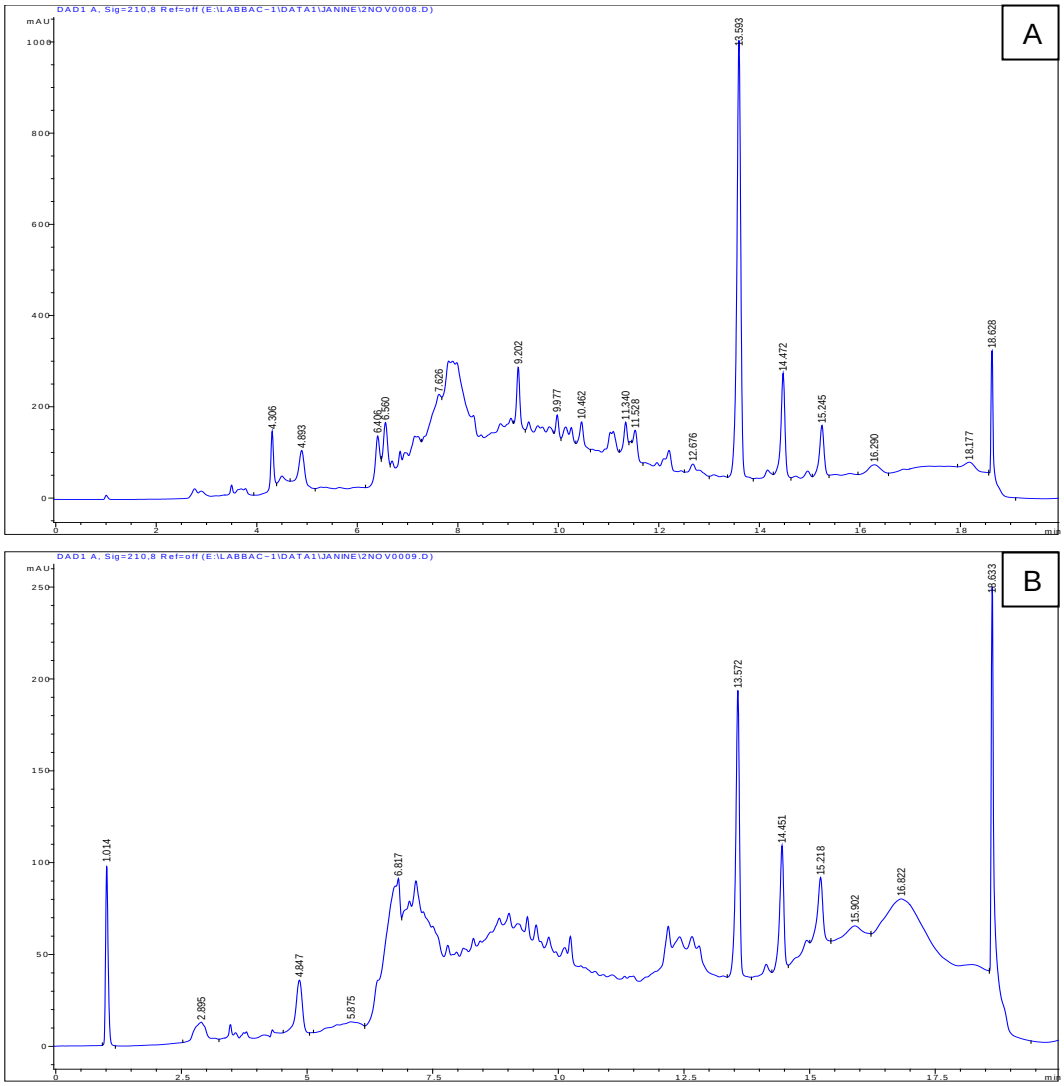


Figure 4.21: HPLC screening of (A) Soxhlet 1,4-dioxane extract D_1 and (B) Soxhlet 1,4-dioxane extract D_2 of frozen leaf pulp

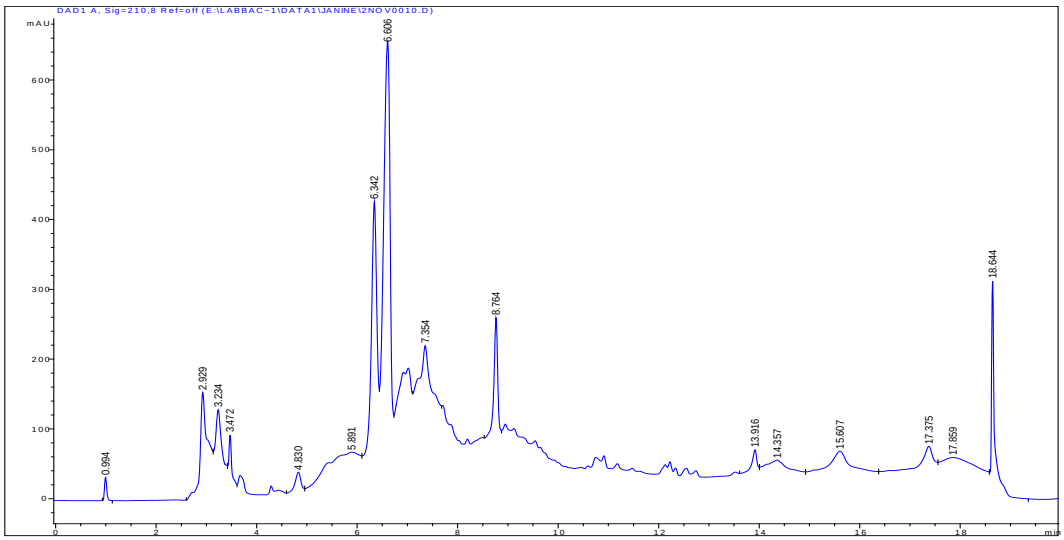


Figure 4.22: HPLC screening of the Soxhlet ethanol extract of frozen leaf pulp

The 1,4-dioxane and ethanol extracts (figure 4.21 and 4.22) contained multiple compounds of significant concentrations that could be targeted for further fractionation. However due to the risk of possible artefact formation, observed by Roux (2012), when using ethanol during extraction, further extraction using ethanol was not considered. The bulk extraction of frozen leaf pulp via Soxhlet extraction and using the 1,4-dioxane extracts for further fractionation to isolate the potential cardiac glycosides was motivated.

4.2 Bulk extraction of orbicusides

4.2.1 Extraction via Soxhlet extraction

Soxhlet extraction of plant material using more than one solvent consecutively is a long procedure. As mentioned, the 1,4-dioxane extracts tested positive for cardiac glycosides. In other words, to produce 1,4-dioxane extract via the consecutive, fractionating extraction of the plant material with cyclohexane, toluene and 1,4-dioxane would take 9 – 12 days. Leaf pulp was placed in a 29 x 100 mm extraction thimble and extracted using only 1,4-dioxane to see whether there is a significant difference in the amount of compounds extracted between the fractionation of the pulp (via extracting compounds using multiple solvents of increasing polarity, namely cyclohexane, toluene and 1,4-dioxane, consecutively) and direct extraction of the orbicusides using the highest polarity solvent used, 1,4-dioxane (figure 4.23).

Figure 4.23: TLC of (A) 1,4-dioxane extract obtained via fractionation and (2) 1,4-dioxane extract obtained via direct extraction using $SbCl_3$ as detection reagent and methanol as mobile phase. The flaking of the silica off of the aluminium plate is due to the $SbCl_3$ detection reagent.

Direct extraction of the cardiac glycosides using only 1,4-dioxane showed to have similar results than the fractionation extraction using multiple solvents. To quicken the bulk extraction of the orbicusides it was decided to use the direct extraction method. The frozen pulp of 10 kg leaves was defrosted and evenly divided and placed in two Soxhlet thimbles (inner diameter and height 60 x 180 mm) and extraction, using 1,4-dioxane, was performed using two Soxhlet apparatus'. The extracts were collected, concentrated and stored at -20 °C.

4.2.1.1 Precipitation of cardiac glycosides

The precipitation of the cardiac glycosides, via dissolving 1,4-dioxane extract in methanol, followed by the addition of dd H_2O to form a ratio of 4:1 (methanol: dd H_2O), was attempted. Addition of 100 ml methanol to the 1,4-dioxane extract resulted in the formation of white flakes. The mixture was filtrated and the flakes collected and dissolved in 1,4-dioxane. TLC of the dissolved flakes, using $SbCl_3$ as detection reagent and chloroform: acetone: methanol (70:30:1), tested negative for cardiac glycosides. 25 ml dd H_2O was added to the filtrate of the methanol-extract mixture, resulting in a colour change of the mixture from orange to milky orange, but no precipitation. Precipitation of the cardiac glycosides in the 1,4-dioxane was therefore unsuccessful.

4.2.1.2 Column chromatography

Column chromatography of the 1,4-dioxane extract was performed, using chloroform: acetone: methanol (70:30:1) as the mobile phase and MN silica gel 60 (0.063 – 0.2 mm / 70 – 230 mesh ASTM for column chromatography), resulting in seven fractions.

The fractions were concentrated and dried using the BUCHI Rotavapor at 80 °C. TLC of the fractions was performed using ethyl acetate: methanol (1:1) (figure 4.24) and chloroform: acetone: methanol (70:30:1) (figure 6.25) as mobile phases and $SbCl_3$ (25 %) as detection agent for cardiac glycosides. All seven fractions tested positive for cardiac glycosides. Due to the similarities in the content of fractions 4 and 5 and fractions 6 and 7, the fractions were combined.

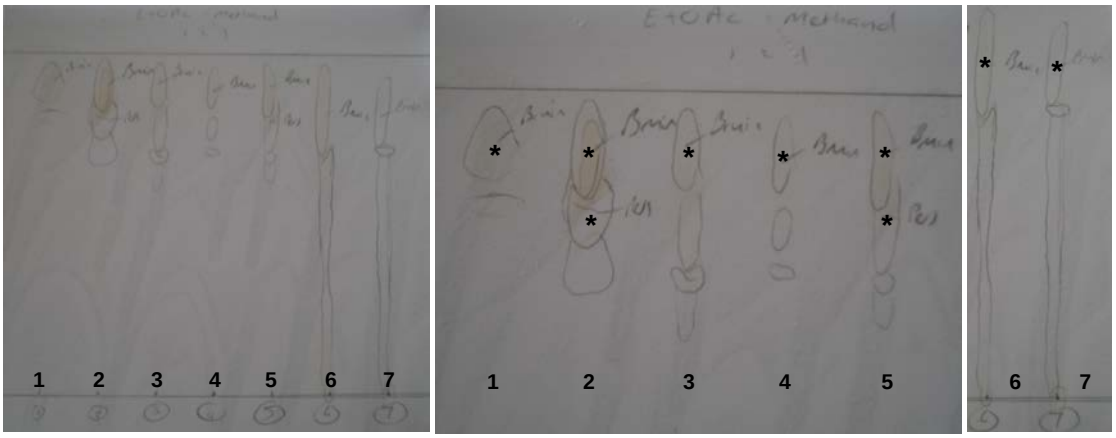


Figure 4.24: TLC of the seven fractions of the Soxhlet 1,4-dioxane extract using ethyl acetate: methanol (1:1) as the mobile phase and $SbCl_3$ as detection reagent. Bands marked with * indicate possible cardiac glycosides.

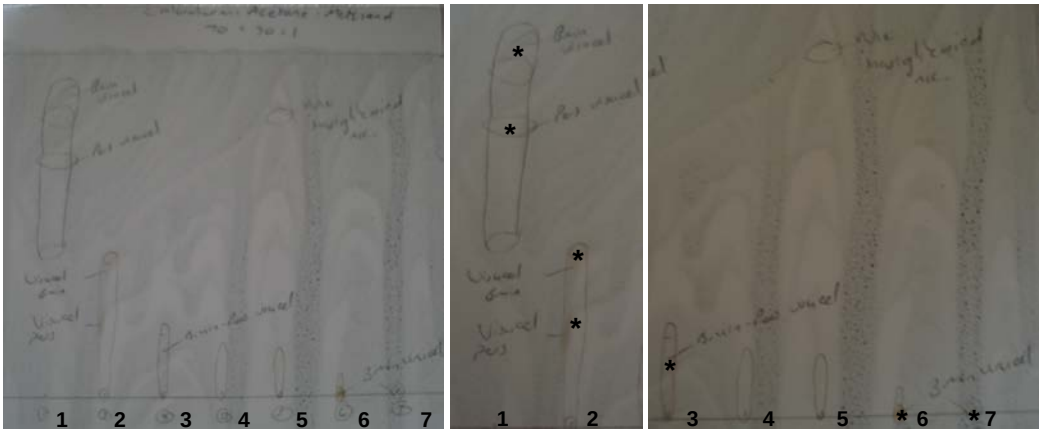


Figure 4.25: TLC of the seven fractions of the Soxhlet 1,4-dioxane extract using chloroform: acetone: methanol (70:30:1) as the mobile phase and $SbCl_3$ as detection reagent. Bands marked with * indicate possible cardiac glycosides.

Additionally, to analyse the contents of the seven fractions, HPLC screening of the fractions (figure 4.26 to 4.30) were performed using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.10.01 data acquisition and analysis software. A Synergi Fusion®-RP column 4 µm, 250 x 4.6 mm

(Phenomenex, Torrance, CA) and acetonitrile (A) / 0.1 % acetic acid (B) mobile phase at the gradients listed in table 4.1 was used. The flow rate was 1.0 ml/min, the injection volume was 25 µl and UV detection was performed at 210 nm and 8 nm bandwidth. Samples of the fractions were dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm eith G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter).

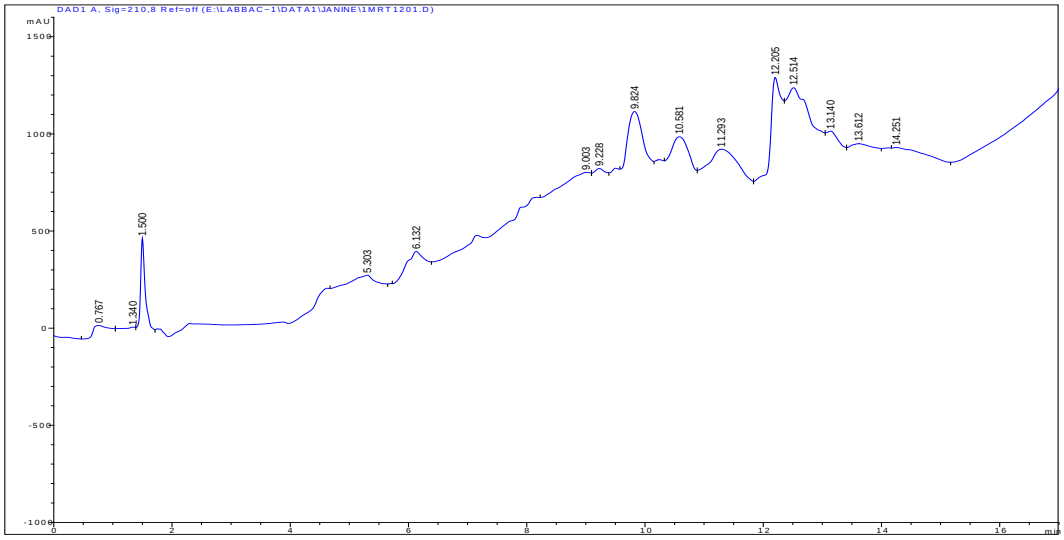


Figure 4.26: HPLC screening of the 1,4-Dioxane Soxhlet extract's first fraction

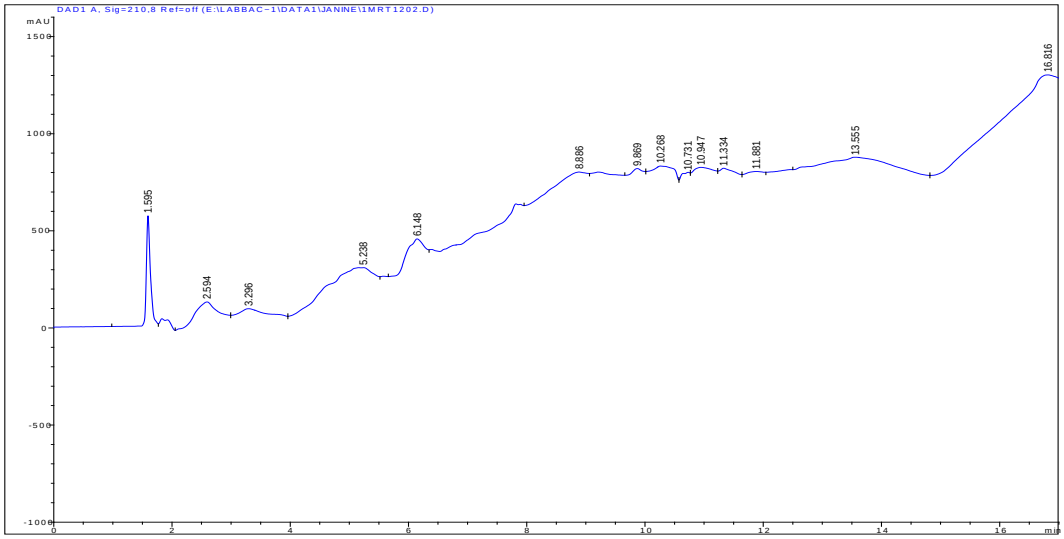


Figure 4.27: HPLC screening of the 1,4-Dioxane Soxhlet extract's second fraction

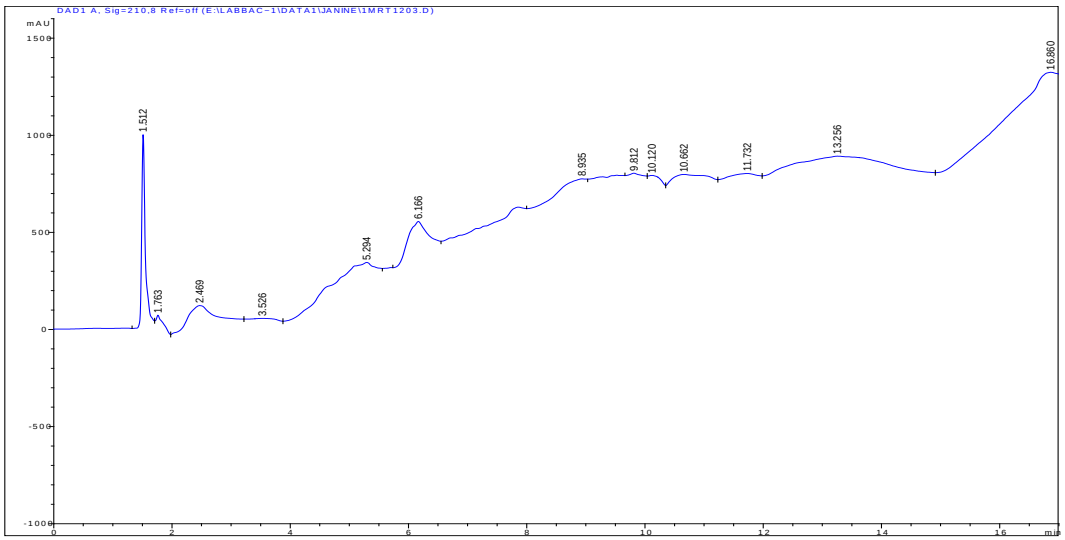


Figure 4.28: HPLC screening of the 1,4-Dioxane Soxhlet extract's third fraction

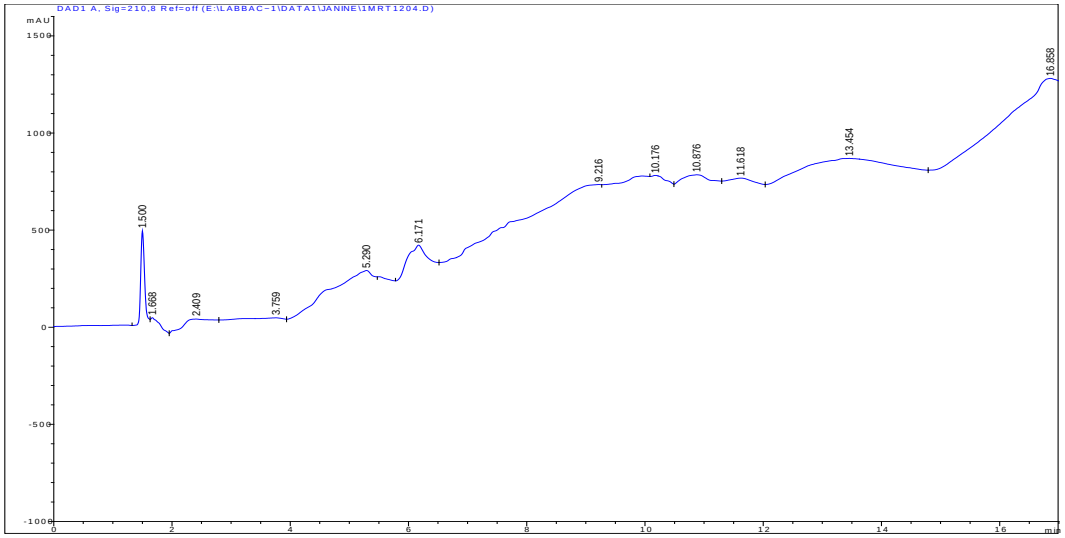


Figure 4.29: HPLC screening of the 1,4-Dioxane Soxhlet extract's fourth and fifth fraction

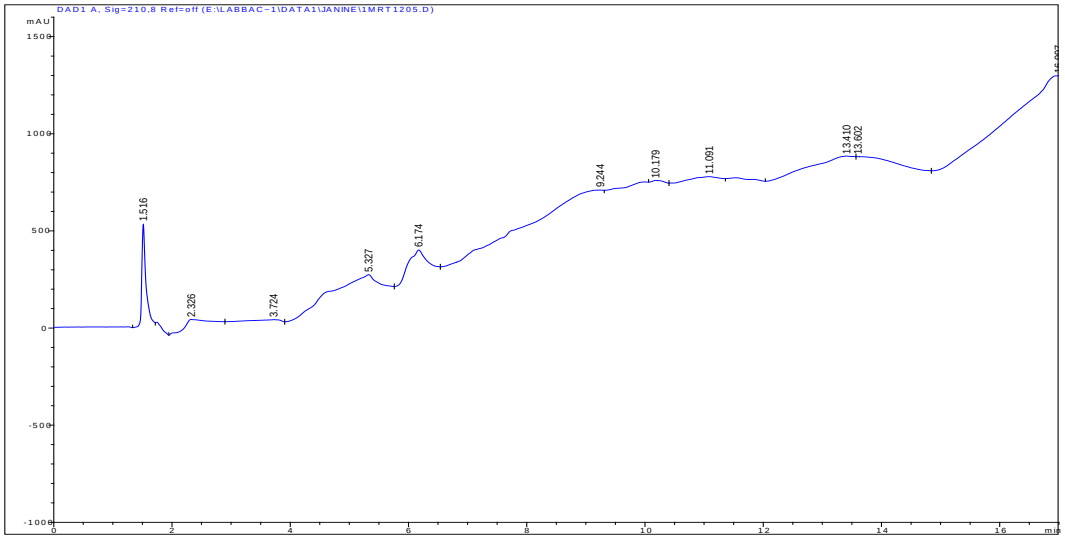


Figure 4.30: HPLC screening of the 1,4-Dioxane Soxhlet extract's sixth and seventh fraction

The results indicated possible retention of the compounds on the column or column contamination, as the positions of the possible compound peaks and cardiac glycosides in the seven fractions are similar. The amounts of fractions were too low to continue further fractionation for the isolation of the cardiac glycosides. Final bulk extraction of the orbicusides via ASE followed.

4.2.2 Final bulk extraction using accelerated solvent extraction

During the development of an optimal ASE method for the orbicusides of *C. orbiculata* it was determined that the solvents cyclohexane, toluene and DCM produced similar extracts when extraction was performed at 140 °C (figure 4.4 and 4.5). Bulk extraction of the pulp of 5 kg leaves was performed with cyclohexane as extraction solvent. Cyclohexane was later replaced with DCM for the extraction of the pulp of 10 kg leaves, because it is readily available in the department's bulk storage, whereas cyclohexane had to be ordered regularly at great expense. The cyclohexane extract prepared before changing to DCM was analised via column chromatography.

4.2.2.1 Column chromatography of the ASE cyclohexane extract

The initial extract of the pulp of 5 kg leaves using cyclohexane and 140 °C was fractionated via column chromatography (using chloroform: acetone: methanol (70:30:1) as the mobile phase and MN silica gel 60 (0.063 – 0.2 mm / 70 – 230 mesh ASTM for column chromatography), producing 10 fractions that test positive for cardiac glycosides (figure 4.31). The fractions were concentrated and dried using the BUCHI Rotavapor at 70 °C.

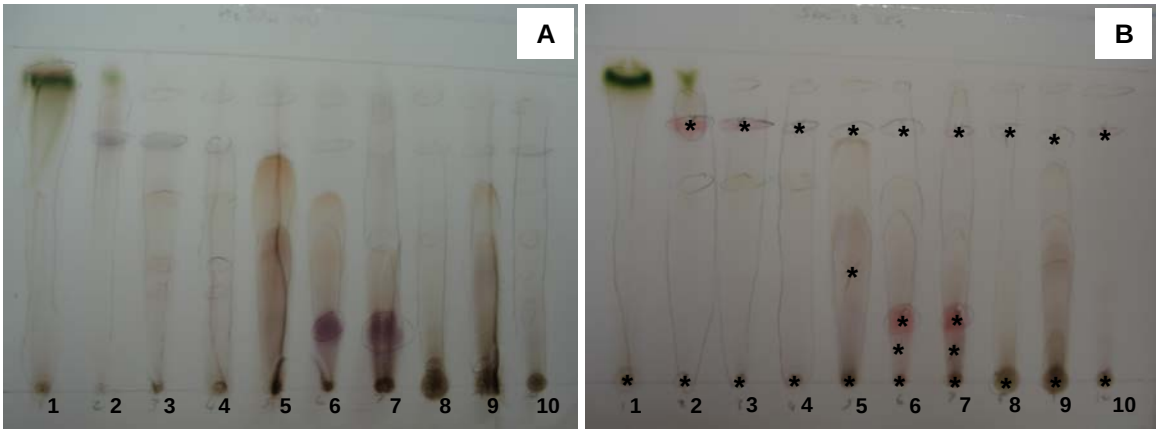


Figure 4.31: TLC of the ten fractions of the ASE cyclohexane extract 140 °C using chloroform: acetone: methanol (70:30:1) as the mobile phase and (A) H_2SO_4 and (B) $SbCl_3$ as detection reagents. Bands marked with * indicate possible cardiac glycosides.

Fraction 3, 4 and 9 contained two possible cardiac glycosides and were further fractionated via column chromatography (using chloroform: acetone: methanol (70:30:1) as the mobile phase and MN silica gel 60 (0.063 – 0.2 mm / 70 – 230 mesh ASTM for column chromatography). Fractionation of Fraction 3 produced four fractions. Two of the fractions contained the two possible cardiac glycoside compounds, but the samples were too small for NMR or analytical screening. The other two fractions still contained impurities and needed further fractionation. Fractionation of Fraction 4 produced four fractions. Two of these fractions contained cardiac glycosides similar to those of Fraction 3 and were added to those fractions. The other two fractions were similar to the two fractions of Fraction 3 that contained impurities and were added to those fractions. Fractionation of Fraction 9 produced four fractions. One of the fractions contained a third possible cardiac glycoside, but the sample was too small for NMR and analytical screening. The second fraction contained a cardiac glycoside similar to that found in Fraction 3 and was added to the corresponding fraction. The other two fractions contained impurities and required further fractionation.

The final fraction mixtures containing the three isolated cardiac glycosides were not sufficient for further analytical testing. The 5 kg of plant material used produced approximately 1 mg of each cardiac glycoside, meaning that further extraction of at least 75 kg of plant material using ASE and cyclohexane would be required for the production of sufficient amounts of sample for characterisation and analytical screening.

4.2.2.2 HPLC Fractionation of the ASE DCM extract

HPLC screening of the ASE DCM extract was performed (figure 4.32) using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software. A Synergi Fusion®-RP column 4 µm, 250 x 4.6 mm (Phenomenex, Torrance, CA) and acetonitrile (A) / 0.1 % acetic acid (B) mobile phase at the gradients listed in table 4.1 was used. The flow rate was 1.0 ml/min, the injection volume was 25 µl and UV detection was performed at 210 nm and 8 nm bandwidth. A sample of the ASE DCM extract was dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm eith G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter).

Fractionation of the ASE DCM extract was performed via HPLC fractionation. The ASE DCM extract was dissolved in methanol and the solution filtered through an Acrodisc PSF syringe filter (a Pall Life Sciences product, 25 mm with G x F / 0.45 µm GHP membrane with a glass fiber pre-filter). HPLC fractionation was performed on an Agilent 1200 series HPLC connected to an Agilent 1200 Series fraction collector using a Phenomenex Synergi® Max RP C18 column (150 x 4.6 mm). The mobile phase was a gradient of 50 % acetonitrile (ACN)

and 50 % water. An injection volume of 20 µl and detection via a diode array detector at 210 nm was used. After 10 minutes a gradient system was increased to 95 % ACN until 30 minutes was reached. HPLC fractionation of the DCM extract of the pulp of 10 kg plant leaves produced four fractions (figure 6.32) which were concentrated and dried using the BUCHI Rotavapor at 80 – 100 °C.

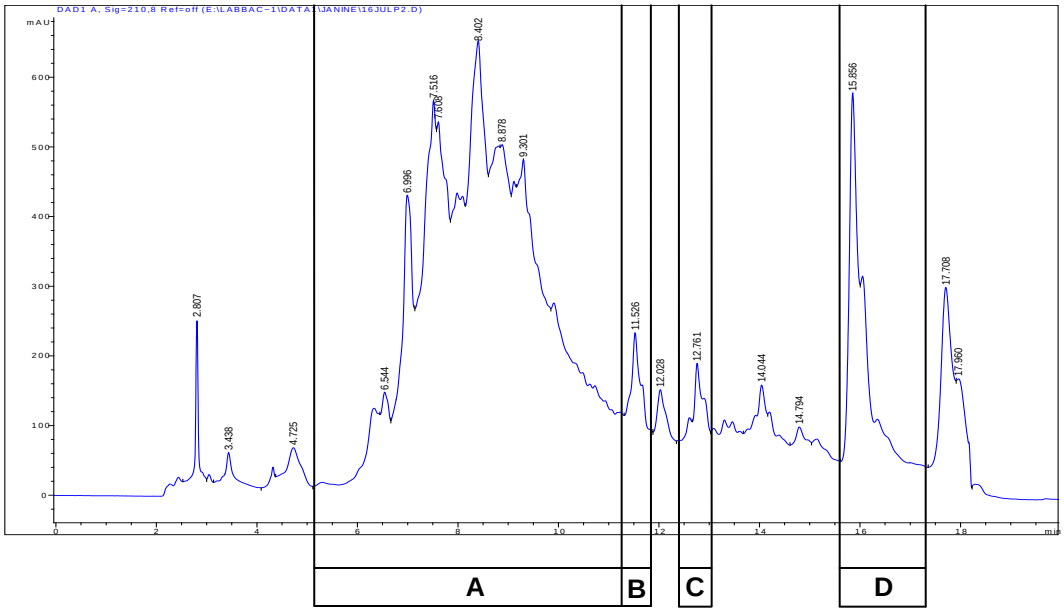


Figure 4.32: HPLC screening of the ASE DCM extract and the peaks targeted for HPLC fractionation ((A) Fraction 1, (B) Fraction 2, (C) Fraction 3 and (D) Fraction 4)

HPLC screening of the four ASE DCM extract fractions was performed (figure 4.33 to 4.36) to visualise the resulting fractions using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software. A Synergi Fusion®-RP column 4 µm, 250 x 4.6 mm (Phenomenex, Torrance, CA) and acetonitrile (A) / 0.1 % acetic acid (B) mobile phase at the gradients listed in table 4.1 was used. The flow rate was 1.0 ml/min, the injection volume was 25 µl and UV detection was performed at 210 nm and 8 nm bandwidth. Small samples of the fractions were dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm eith G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter).

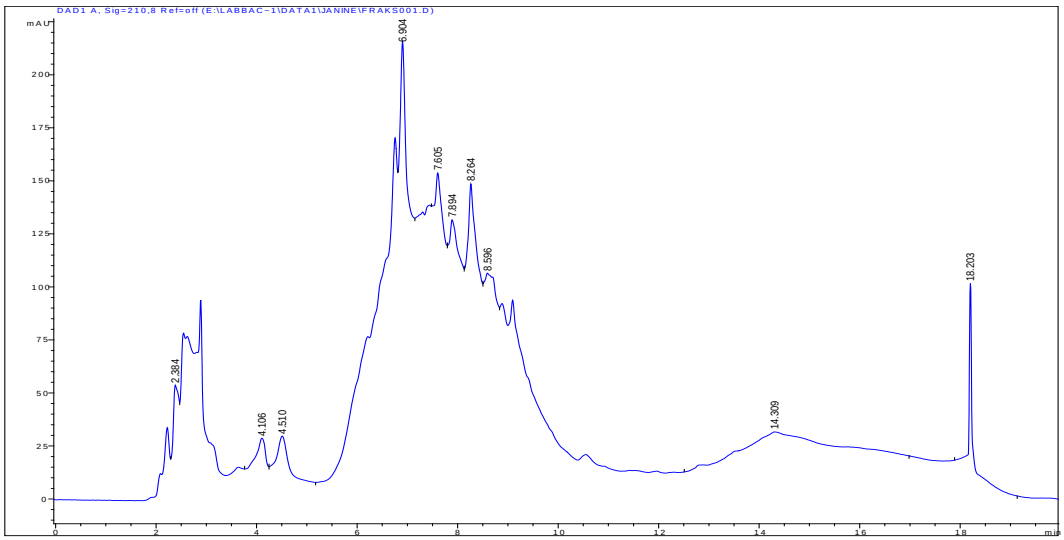


Figure 4.33: HPLC screening of Fraction 1 of the ASE DCM extract

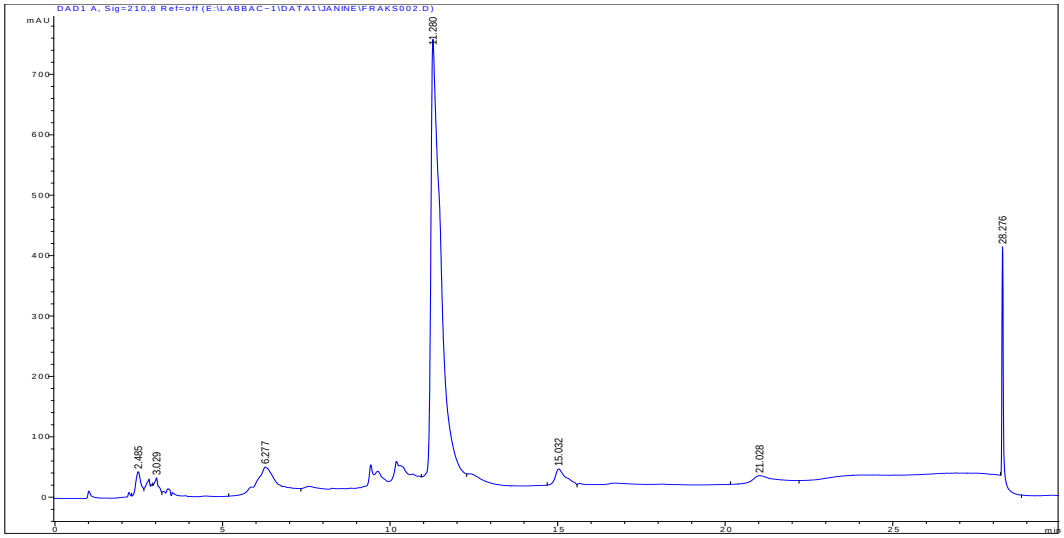


Figure 4.34: HPLC screening of Fraction 2 of the ASE DCM extract

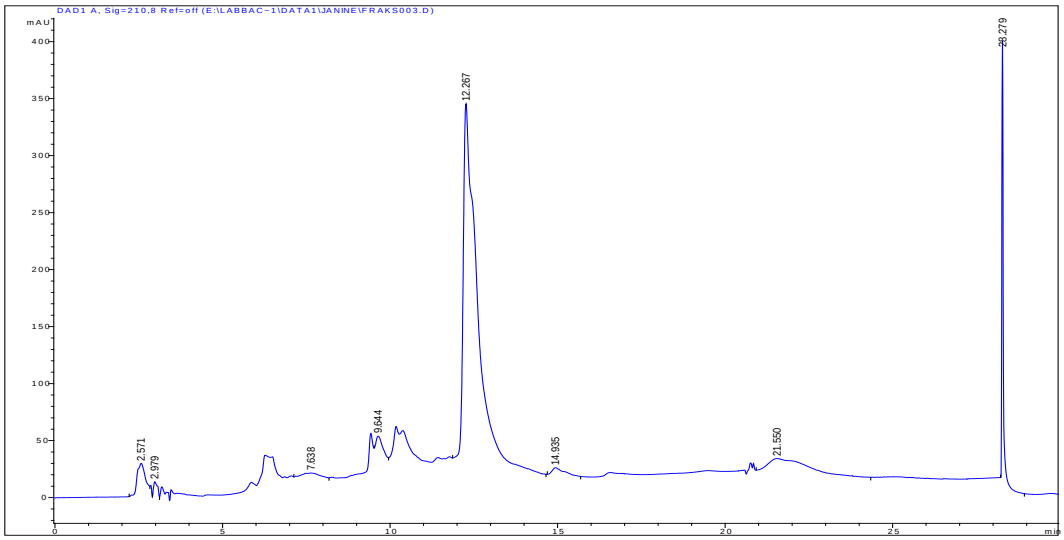


Figure 4.35: HPLC screening of Fraction 3 of the ASE DCM extract

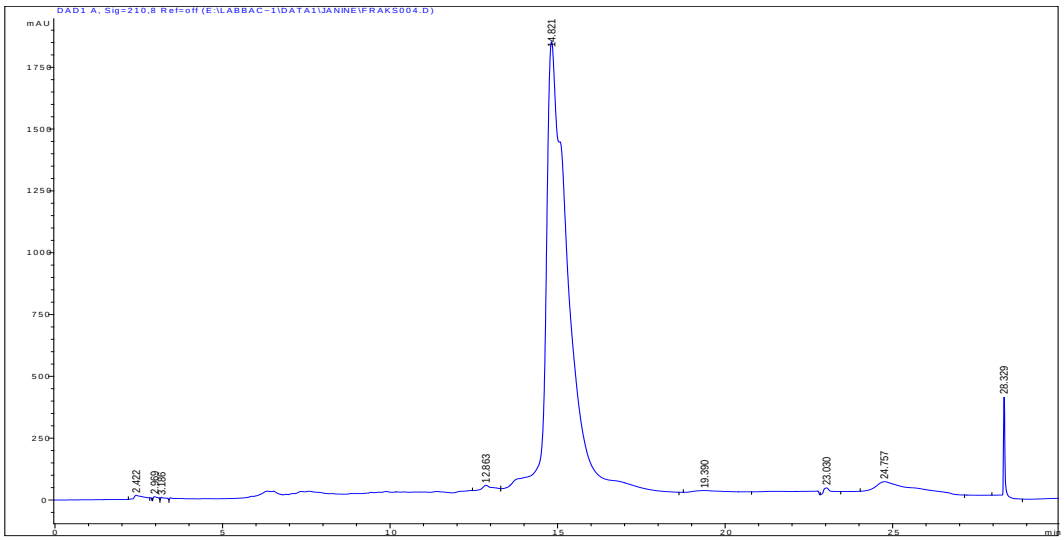


Figure 4.36: HPLC screening of Fraction 4 of the ASE DCM extract

TLC of the four fractions was performed with chloroform: acetone: methanol (70:30:1) and methanol as mobile phases and $SbCl_3$, Chloramine T solution, Lieberman Buchard and H_2SO_4 as detection reagents (figure 4.37). Lieberman Buchard solution was prepared by placing ethanol (25 ml) on ice and slowly adding acetic acid anhydride (2.5 ml) and concentrated H_2SO_4 (2.5 ml). TLC of the fractions showed that each fraction contained multiple possible cardiac glycosides.

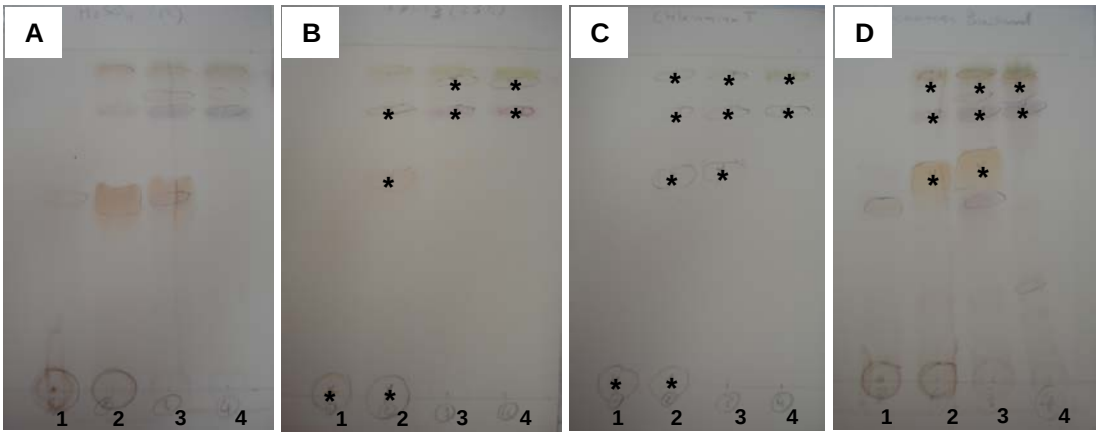


Figure 4.37: TLC of the four HPLC fractions of the ASE DCM extract 140 °C using chloroform: acetone: methanol (70:30:1) as the mobile phase and (A) H_2SO_4 (5 %), (B) $SbCl_3$ (25 %), (C) Chloramine T solution and (D) Lieberman Buchard solution as detection reagents. Bands marked with * indicate possible cardiac glycosides.

To analyse the four fractions HPLC purity determinations were performed using an Agilent 1100 series HPLC equipped with a gradient pump, autosampler, Diode array UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software. A Venusil® XBP C18 column (150 x 4.6 mm) was used. The mobile phase was a gradient of 30 % ACN and 70 %

water. An injection volume of 20 µl and detection via a diode array detector at 210 nm was used. A gradient system was increased to 85 % ACN and 15 % water after 5 minutes, held to 10 minutes and re-equilibrated to 15 minutes. Samples of the fractions were dissolved in methanol and filtered with an Acrodisc PSF syringe filter (Premium 25 mm eith G x F / 0.45 µm GHP membrane, HPLC certified with a glass fiber prefilter).

The purity determination of HPLC Fractions 1 and 2 (figure 4.38 and 4.39) showed that the fractions did not contain any compounds of significant concentrations. The purity determination of HPLC Fractions 3 and 4 (figure 4.40 and 4.41) showed that the fractions still contained multiple compounds or impurities.

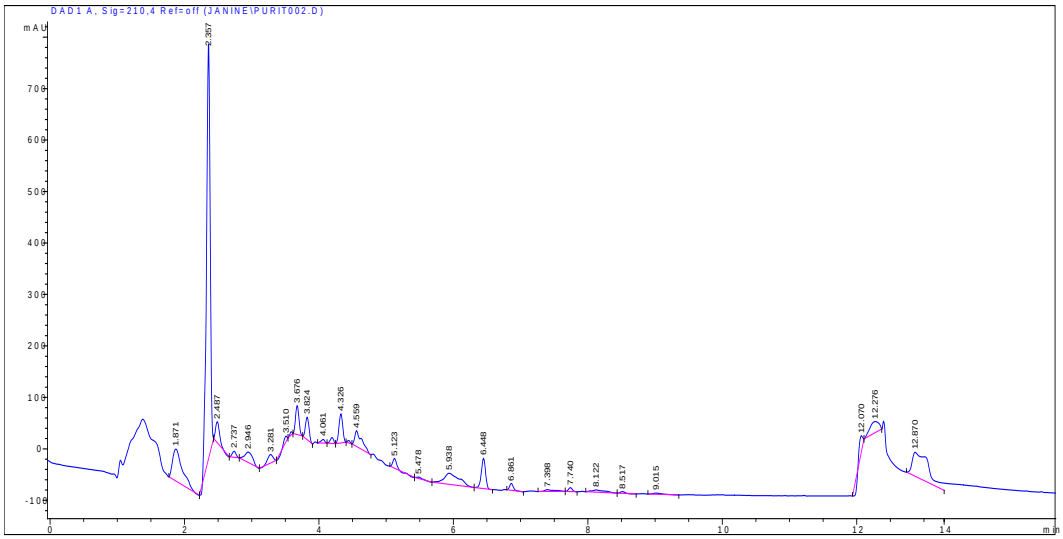


Figure 4.38: HPLC chromatogram of the purity determination of Fraction 1

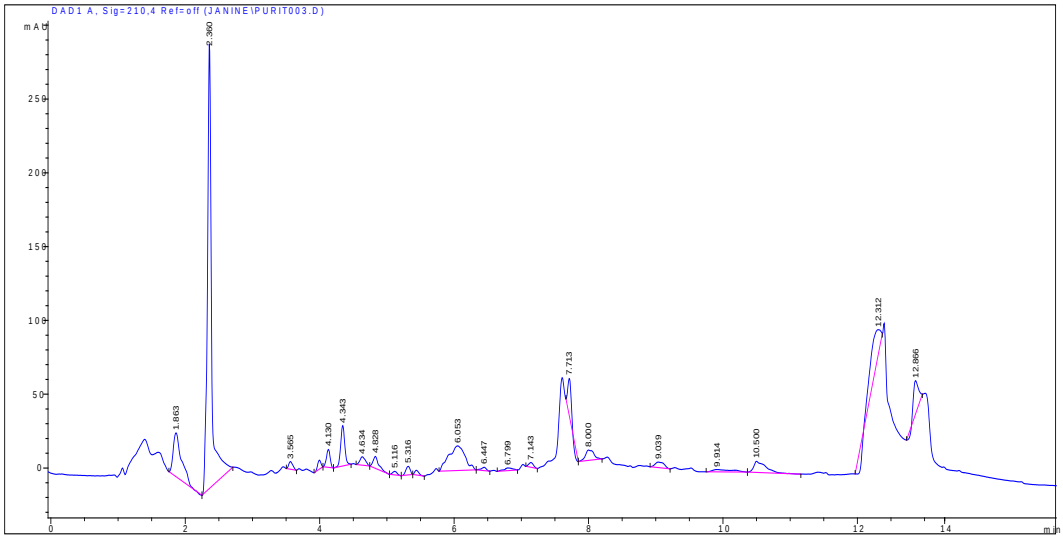


Figure 4.39: HPLC chromatogram of the purity determination of Fraction 2

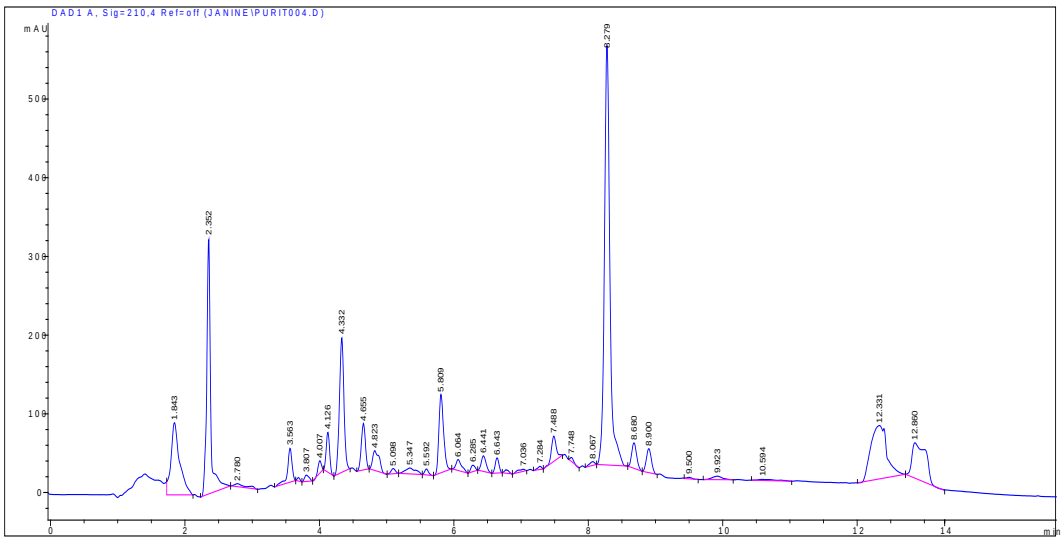


Figure 4.40: HPLC chromatogram of the purity determination of Fraction 3

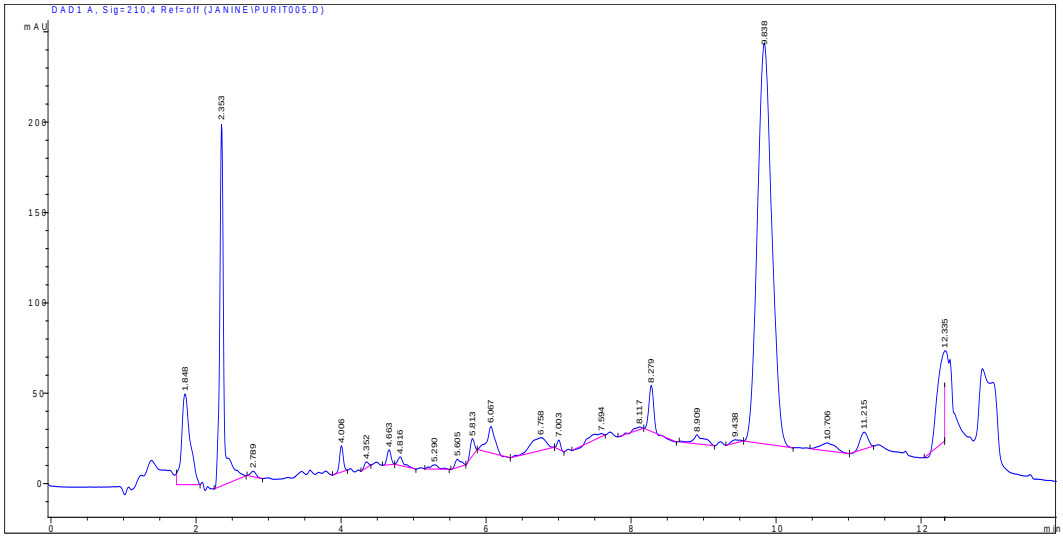


Figure 4.41: HPLC chromatogram of the purity determination of Fraction 4

4.3 Conclusion

Steyn *et al.*, (1986) successfully extracted sufficient amounts of the orbicusides of *C. orbiculata* leaves using maceration. However, 212 kg leaves were required to extract 390 mg of orbicuside A, 180 mg of orbicuside B and 145 mg of orbicuside C. Comparison of the maceration, microwave extraction, Soxhlet extraction and ASE during this study indicated that maceration produced the least and Soxhlet extraction and ASE produced the most amounts of extract, with Soxhlet extraction and ASE producing three to four times the amount extracted by maceration. It was therefore expected that Soxhlet extraction and ASE would produce more cardiac glycosides during extraction in less plant material than maceration.

The pulp of 5 kg of plant leaves produced approximately 1 mg of possible cardiac glycoside compounds when using ASE with cyclohexane. HPLC fractionation with DCM of the pulp of 10 kg plant leaves over a period of two months produced 7 mg of Fraction 1, 17 mg of Fraction 2, 6 mg of Fraction 3 and 7 mg of Fraction 4, of which none are useable without further fractionation (Fractions 1 and 2 did not contain detectable amounts of compounds and Fractions 3 and 4 still contained more than one compound (impurities)). This meant that further extraction of much larger amounts of plant material (at least 75 kg) using ASE would be required for the production of sufficient amounts of sample for characterisation and analytical screening. The extraction of the orbicusides from *C. orbiculata* leaves was therefore unsuccessful due to low concentrations in the plant.

Due to the insufficient amounts of samples obtained, the large amounts of both plant material and solvents (extraction solvents and HPLC grade solvents) used and the amount of time spent on these extraction and fractionation processes to produce the insufficient amounts of samples, it was decided to cease further extraction and isolation processes as it was not justifiable to continue.

The evaluation of the antioxidant activity and toxicity of two commercial bufadienolides analogous to the orbicusides of *C. orbiculata* followed. The commercial bufadienolides were used to determine whether the orbicusides have potential as antioxidant compounds because the orbicusides could not be extracted to analise directly.

5 EVALUATION OF THE ANTIOXIDANT ACTIVITY AND TOXICITY OF COMMERCIAL BUFADIENOLIDES, BUFALIN AND CINOBUFOTALIN

The production of analogues of compounds with known activity can potentially increase a desired activity and/or reduce the toxicity of a compound. The orbicusides have potential as antioxidants due to their aglycone structures having both a steroid and 2-pyrone moiety (paragraph 2.2.1 and 2.6).

C. orbiculata leaves contained orbicuside levels too low for sufficient extraction. The orbicusides could therefore not be screened for antioxidant activity and toxicity. Two commercial bufadienolides analogous to the orbicusides, namely bufalin and cinobufotalin, were used to determine whether or not the orbicusides had potential of having antioxidant activity. Additionally, the antioxidant activity and toxicity of the commercial bufadienolides was compared with that of synthesised bufadienolide analogues to determine whether changes in the aglycone structure of a bufadienolide (orbicuside) could improve antioxidant activity and reduce toxicity.

The lipid peroxidation and NBT assay (paragraph 2.7) were used to evaluate the antioxidant activity of the commercial bufadienolides.

5.1 Lipid peroxidation assay

The same method and reagents used for the TBA assay of the juice and fractions of *C. orbiculata* (paragraph 3.3.1.1) was used here. Bufalin and cinobufotalin was purchased at Sigma Aldrich. The pre-prepared and frozen aliquots of rat brain homogenate (paragraph 3.3.1.1.3) were used during the assay.

5.1.1 Preparation of samples

A 10 mM stock solution of bufalin was prepared by dissolving bufalin (3.9 mg) in ethanol (1ml). The necessary precautions were taken when handling the bufalin.

0.08 ml of the bufalin stock solution was placed in an Eppendorf tube and 1.92 ml ethanol added to produce a 0.4 mM sample (2 ml).

1 ml of the 0.4 mM bufalin solution was placed in an Eppendorf tube and 1 ml ethanol added to produce a 0.2 mM sample (2 ml).

0.5 ml of the 0.2 mM bufalin solution was placed in an Eppendorf tube and 0.5 ml ethanol added to produce a 0.1 mM sample (1 ml).

A 10 mM stock solution of cinobufotalin was prepared by injecting ethanol (2.181 ml) into a 10 mg cinobufotalin injection vial. The necessary precautions were taken when handling the cinobufotalin.

0.08 ml of the cinobufotalin stock solution was withdrawn from the vial and placed in an Eppendorf tube and 1.92 ml ethanol added to produce a 0.4 mM sample (2 ml).

1 ml of the 0.4 mM cinobufotalin solution was placed in an Eppendorf tube and 1 ml ethanol added to produce a 0.2 mM sample (2 ml).

0.5 ml of the 0.2 mM cinobufotalin solution was placed in an Eppendorf tube and 0.5 ml ethanol added to produce a 0.1 mM sample (1 ml).

5.1.2 Standard curve and assay

The standard curve prepared in paragraph 3.3.1.1.5 was used during this assay. The samples were prepared and the assay performed as according to the method in paragraph 3.3.1.1.4.

5.1.3 Results

The levels of MDA formed (in nmol/mg tissue) in the tested samples were determined and summarised in tables 5.1 and 5.2 and figures 5.1 and 5.2. Statistical analysis of the results was performed with the Student-Newman-Keuls multiple comparisons test and one-way analysis of variance (ANOVA) via GraphPad software. The results of the samples were statistically compared with that of the Toxin.

Table 5.1: The effect of bufalin on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	± Standard error of the mean (SEM) n = 5
Negative Control	0.390	0.0156
***Toxin**	0.501	0.02235
Trolox (Positive Control)	0.120	0.008424
Bufalin 0.4 mM	0.590	0.01443
Bufalin 0.2 mM	0.585	0.01502
Bufalin 0.1 mM	0.527	0.02701

***Toxin: A combination of $FeCl_3$, H_2O_2 and vitamin C was used to induce lipid peroxidation in the rat brain homogenate**

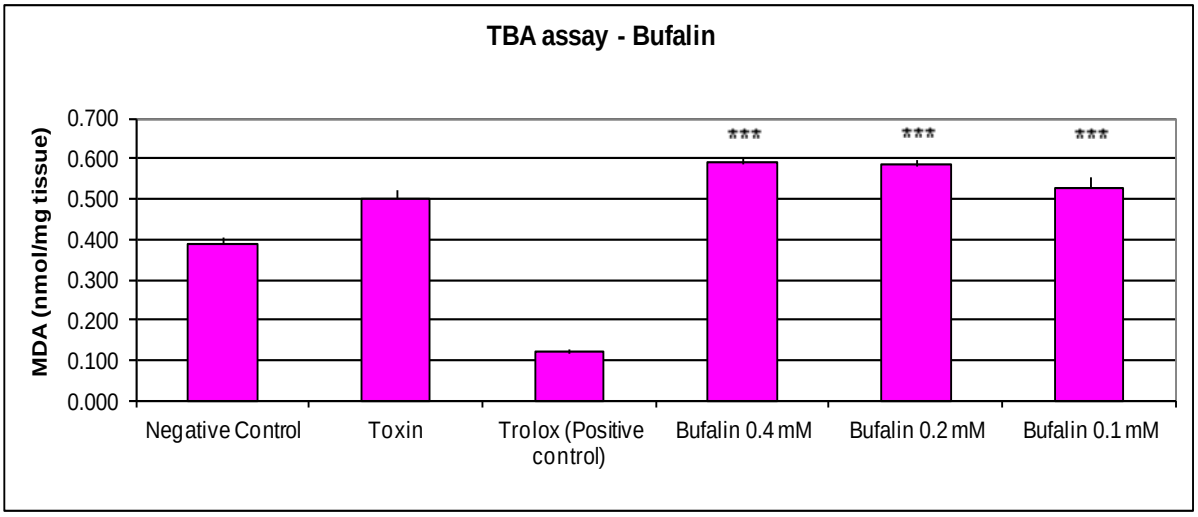


Figure 5.1: The effects of bufalin on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). *** $p < 0.001$ vs Toxin.

Bufalin showed significant pro-oxidant activity (figure 5.1).

Table 5.2: The effect of cinobufotalin on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	± Standard error of the mean (SEM) n = 5
Negative Control	0.390	0.0156
Toxin	0.501	0.02235
Trolox (Positive Control)	0.120	0.008424
Cinobufotalin 0.4 mM	0.494	0.01161
Cinobufotalin 0.2 mM	0.493	0.01928
Cinobufotalin 0.1 mM	0.491	0.01697

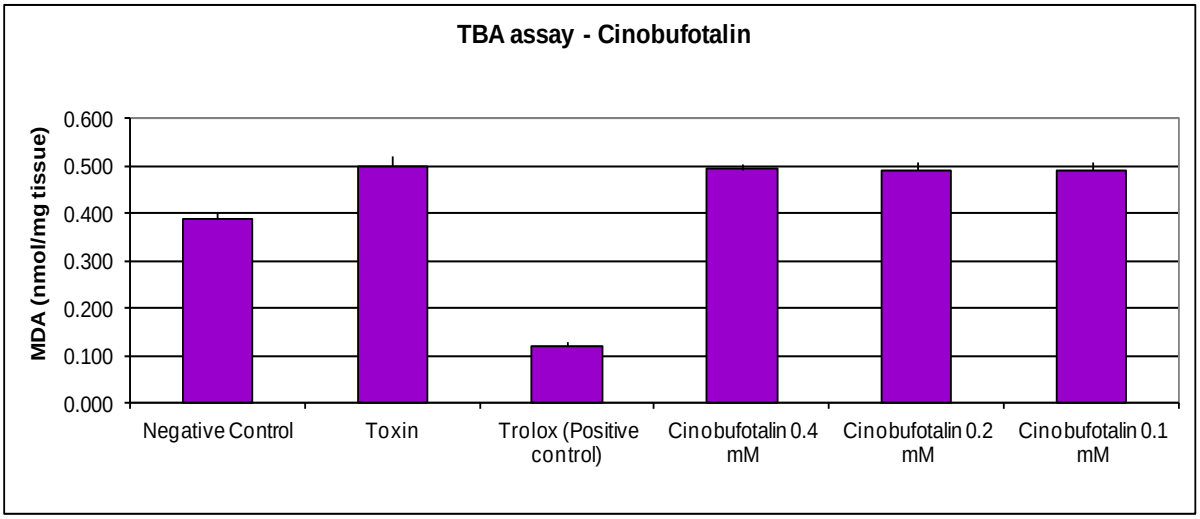


Figure 5.2: The effects of cinobufotalin on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). The sample results were statistically compared with that of the toxin.

Cinobufotalin showed no significant activity (figure 5.2).

5.1.4 Discussion

The pro-oxidant activity of bufalin and non-significant activity of cinobufotalin indicated that there is a possibility that the orbicusides may not have potential antioxidant effects against lipid peroxidation.

5.2 NBT assay

The same method and reagents used for the NBT assay of the juice and fractions of *C. orbiculata* (paragraph 3.3.1.2) were used here. The pre-prepared and frozen aliquots of rat brain homogenate (paragraph 3.3.1.1.3) were used during the assay.

5.2.1 Preparation of samples

The bufalin and cinobufotalin samples prepared in paragrapgh 5.1.1 were used during this assay.

5.2.2 Standard curve and assay

The standard curve and Bradford protein assay prepared in paragraph 3.3.2.1.3 and 3.3.2.1.5 was used during this assay. The assay was performed according to the method in paragraph 3.3.2.1.4.

5.2.3 Results

The levels of NBD formed (in µmol/mg protein) in the tested samples were determined and summarised in tables 5.3 and 5.4 and figures 5.3 and 5.4. Statistical analysis of the results was performed with the Student-Newman-Keuls multiple comparisons test and one-way analysis of variance (ANOVA) via GraphPad software. The results of the samples were statistically compared with that of the Toxin.

Table 5.3: The effect of bufalin on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	73.928	8.453
***Toxin**	169.875	4.258
Trolox (Positive Control)	55.924	4.118
Bufalin 0.4 mM	182.970	5.920
Bufalin 0.2 mM	168.784	2.509
Bufalin 0.1 mM	167.069	2.253

***Toxin: KCN was used to induce $O_2^{\bullet-}$ production in the rat brain homogenate**

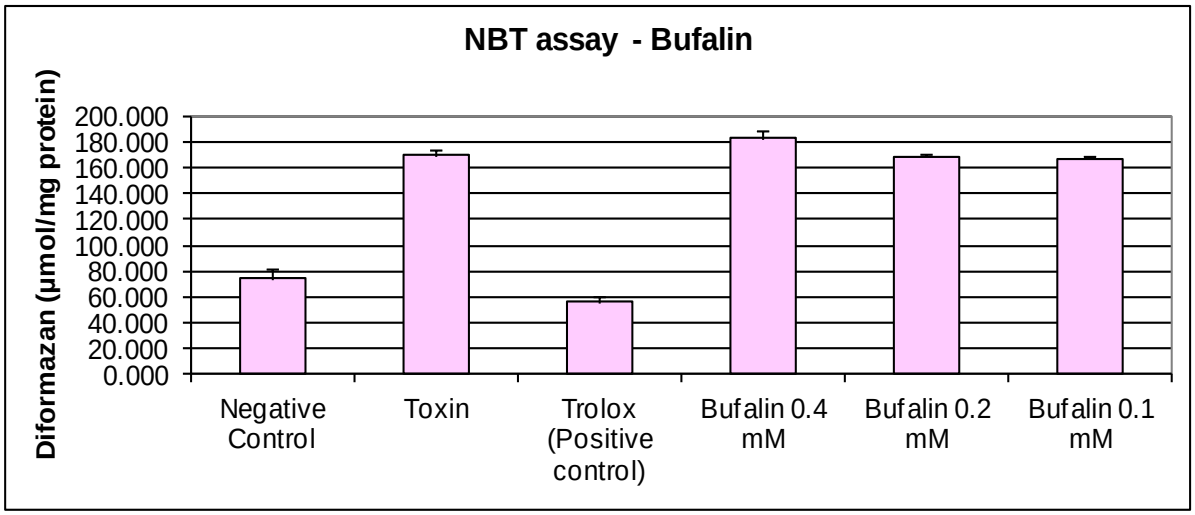


Figure 5.3: The effects of bufalin on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). The sample results were statistically compared with that of the toxin.

Bufalin showed no significant activity (figure 5.3).

Table 5.4: The effect of cinobufotalin on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	31.372	1.469
Toxin	109.102	3.518
Trolox (Positive Control)	41.664	1.300
Cinobufotalin 0.4 mM	110.520	1.907
Cinobufotalin 0.2 mM	110.513	3.384
Cinobufotalin 0.1 mM	108.210	4.349

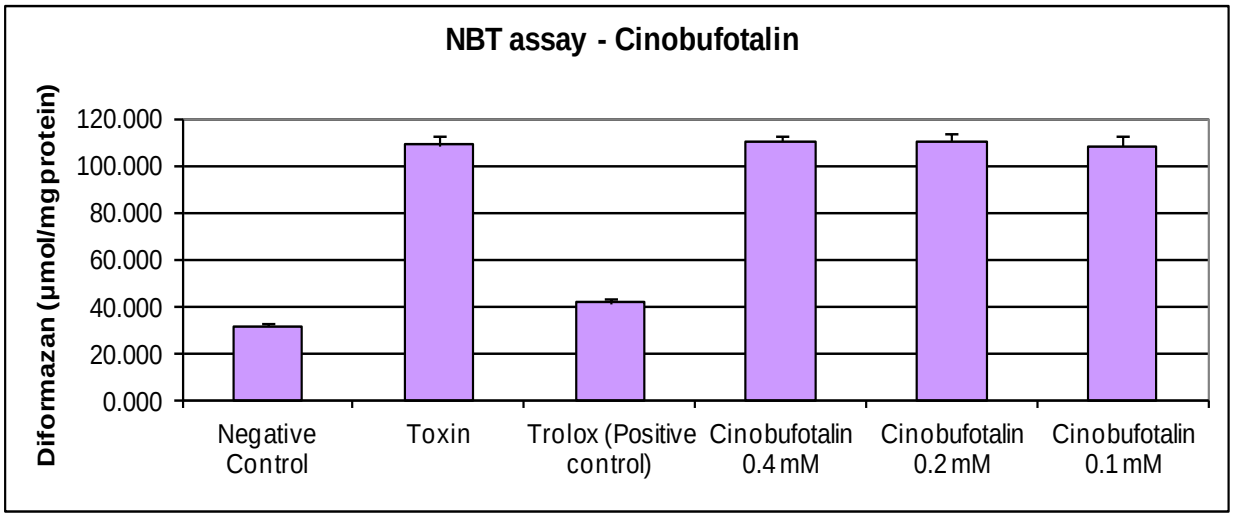


Figure 5.4: The effects of cinobufotalin on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). The sample results were statistically compared with that of the toxin.

Cinobufotalin showed no significant activity (figure 5.4).

5.2.4 Discussion

The lack of significant activity of bufalin and cinobufotalin indicated that there is a possibility that the orbicusides may not have potential antioxidant effects against $O_2^{\bullet-}$.

5.3 Toxicity evaluation

To evaluate the toxicity of the commercial bufadienolides, the MTT assay (refer to paragraph 2.8) was performed.

5.3.1 Method

5.3.1.1 Chemicals and reagents

The chemicals used are of the highest chemical purity and purchased at Sigma Aldrich and Separations.

The PBS buffer prepared for the TBA assay (paragraph 3.3.1.1.2) was used.

15 ml Tryptan EDTA aliquots were prepared to prevent repetitive freezing and thawing of the original product packaging.

5 % stock solution of MTT was prepared by diluting MTT (500 µl) with PBS buffer (10 ml).

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin and sopropanol was purchased at Separations.

Penicillin and streptomycin (PenStrep) aliquots, to prevent the infection of the cells, were prepared to prevent repetitive freezing and thawing of the original product packaging.

Fungizone aliquots, to prevent the infection of the cells, were prepared to prevent repetitive freezing and thawing of the original product packaging.

Cell growth medium was prepared by placing DMEM (40 ml) in 60 ml Falcon tubes and adding FBS (10 ml), PenStrep (500 µl) and Fungizone (500 µl). The Falcon tubes were stored at 2 – 5 °C.

5.3.1.2 Cell line

Neuroblastoma cells (SH-SY-SY) were used during the MTT assay, to evaluate the toxicity of the samples in a neurological environment. The neuroblastoma cells were kept in a chest freezer at -150 °C.

5.3.1.2.1 Preparation of the cell suspension

Neuroblastoma cells were thawed, 15 ml placed in a Falcon tube containing cell growth medium (10 ml), centrifuged for 10 minutes at 1000 x g. The supernatant was collected and discarded, cell growth medium (10 ml) added and the Falcon tube shaken for the cells to become suspended in the medium. Cell growth medium (20 ml) was added to a 150 ml cell culture flask with a vented cap, followed by the addition of the cell suspension. The cells were incubated at 37 °C in an incubator with CO_2 (5 %) and relative humidity (100 %).

5.3.1.2.2 Maintaining the cells

Sterile or aseptic conditions were maintained when the culture flask, growth medium and cells were handled throughout the assay.

To ensure that the cells have an optimal growth environment, the cells were investigated daily and cell growth medium replaced (by removing the old cell growth medium and adding new cell growth medium (30 ml) to the culture flask) two to three times a week or as needed.

When the cell suspension reached a total confluence of approximately 80 %, the cell line was split. The growth medium was removed and tryptan EDTA (2 ml) added to rinse any remaining medium from the culture flask. The tryptan EDTA was then discarded and new tryptan EDTA (3 ml) added and the culture flask placed in the incubator for 5 minutes. The culture flask was then shaken and placed back into the incubator for 5 minutes. The contents of the culture flask was split into two by adding growth medium (7 ml) to the tryptan EDTA cell suspension, mixing the solution to evenly spread the cells throughout the suspension,

dividing the suspension into two portions of 5 ml each and placed into two new cell culture flasks. Cell growth medium (25 ml) was added to each of the culture flasks, forming two 30 ml suspensions. The two suspensions were placed back into the incubator. This splitting process was gradually repeated, forming ten culture suspensions. Six of these suspensions were used to perform the MTT assay. The remaining four suspensions were maintained by splitting them to form eight culture suspensions and were maintained to repeat the MTT assay if necessary.

5.3.1.3 Preparation of samples

The samples were prepared under aseptic or sterile conditions in a laminar flow cabinet and filtered before use.

A 10 mM bufalin stock solution was prepared by dissolving bufalin (3.9 mg) in ethanol (1 ml). The necessary precautions were taken when handling the bufalin.

A 10 mM stock solution of cinobufotalin was prepared by injecting ethanol (2.181 ml) into a 10 mg cinobufotalin injection vial. The necessary precautions were taken when handling the cinobufotalin.

Three concentrations (250 µM, 25 µM and 2.5 µMl) of bufalin and cinobufotalin, respectively, were prepared in a fume hood. The stock solutions and ethanol (1 % in dd H_2O) were filtered. 10 mM stock solution (50 µl) was diluted with the 1 % ethanol (1.950 µl) to form a 250 µM solution (2 ml). 250 µM solution (200 µl) was diluted with 1 % ethanol (1.8 ml) to form a 25 µM solution (2 ml). 250 µM solution (20 µl) was also diluted with 1 % ethanol (1.98 ml) to form a 2.5 µM solution.

5.3.1.4 Assay

5.3.1.4.1 Seeding of the cells

On the first day of the MTT assay six culture flasks containing cell suspension were incubated until confluent. A cell stock suspension was then prepared by gently rinsing each culture flask with tryptan EDTA (2 ml), to remove the cell growth medium, followed by adding tryptan EDTA (3 ml) to each flask and incubating them for 10 minutes at 37 °C. After 5 minutes the flasks were shaken to loosen the cells and placed back into the incubator. After 10 minutes the flasks were inspected under a microscope to ensure that the cells were in suspension. Cell growth medium (7 ml) was added to each suspension and the suspensions removed and placed into a Falcon tube.

To determine the cell count of the cell stock suspension, the cell suspension had to be diluted. 50 µl of the cell stock suspension was collected and added to cell growth medium (450 µl). The remaining cell stock suspension was placed on a rotating stand to prevent the cells from adhering to the walls of the Falcon tube. 20 µl of the diluted cell stock suspension was placed on a haemocytometer to microscopically count the cells. The cells on each of the nine squares of the haemocytometer were counted and an average calculated from the total. The dilution factor was adjusted and the amount of cells needed per well calculated using the following equation:

$$\text{Volume of cells required} = \left[\frac{\text{Total number of cells per well}}{\text{Number of counted cells}}\right] \times \text{Final volume of cells and DMEM required to seed}$$

The suspension containing the volume of cells required for seeding was prepared and 1 ml of the suspension was added to the wells of six 24 well cell culture plates. The culture plate was then placed into the incubator for 24 hours.

5.3.1.4.2 Pre-treatment of the sample wells

After the 24 hour incubation period, the cell growth medium in the wells was removed and the wells prepared according to table 5.5.

5.3.1.4.3 Spectrophotometric analysis

After the 24 hour incubation period, the 5 % MTT stock solution was prepared and 200 µl added to the wells to terminate cell growth and provide the colour reaction to measure cell viability. The plates were then incubated for 2 hours at 37 °C. The supernatant in the wells were removed and isopropanol (250 µl) added and the plates slightly shaken to dissolve the formazan crystals that formed in the viable cells. 100 µl of the resulting formazan solution of each well was collected and placed in a 96 well cell culture plate and analysed spectrophotometrically at 560 nm and 650 nm (a reference wavelength).

Table 5.5: Preparation of the 24 well cell culture plates for the MTT assay of bufalin and cinobufotalin

Wells prepared (three of each)	Content
No cells	No cells, 500 µl medium
Negative control	Cells, 400 µl cell growth medium and 100 µl ethanol (1 % in dd H_2O)
Positive control	Cells, 400 µl growth medium and 100 µl formic acid (1.65 % in ethanol)
Bufalin 250 µM	Cells, 400 µl growth medium and 100 µl of the 250 µM bufalin solution (to produce 50 µM bufalin in the well)
Bufalin 25 µM	Cells, 400 µl growth medium and 100 µl of the 25 µM bufalin solution (to produce 5 µM in the well)
Bufalin 2.5 µM	Cells, 400 µl growth medium and 100 µl of the 2.5 µM bufalin solution (to produce 0.5 µM in the well)
Cinobufotalin 250 µM	Cells, 400 µl growth medium and 100 µl of the 250 µM cinobufotalin solution (to produce 50 µM bufalin in the well)
Cinobufotalin 25 µM	Cells, 400 µl growth medium and 100 µl of the 25 µM cinobufotalin solution (to produce 5 µM in the well)
Cinobufotalin 2.5 µM	Cells, 400 µl growth medium and 100 µl of the 2.5 µM cinobufotalin solution (to produce 0.5 µM in the well)

5.3.2 Results

The results were expressed as the percentage of viable cells. The following calculations were used:

- The difference between the absorbance at 650 nm and the absorbance at 560 nm of each sample was calculated (Δ Absorbance)
- The difference between the mean absorbance of the blank at 650 nm and the mean absorbance of the blank at 560 nm (Δ Blank)
- The difference between the absorbance of the cell control at 650 nm and the absorbance of the cell control at 560 nm (Δ Control)
- The percentage of viable cells was then calculated with the following equation:
  Percentage of viable cells = (Δ Absorbance – Δ Blank) / (Δ Control – Δ Blank) x 100

Table 5.6 and figure 5.5 summarises the cell viability of bufalin and cinobufalin.

Table 5.6: Cell viability of bufalin and cinobufotalin

	% Cell viability
No cells (Blank)	0.000
Negative Control	100.000
Positive Control	-0.367
Bufalin 250 µM	19.186
Bufalin 25 µM	12.150
Bufalin 2.5 µM	34.323
Cinobufotalin 250 µM	20.891
Cinobufotalin 25 µM	25.838
Cinobufotalin 2.5 µM	49.918

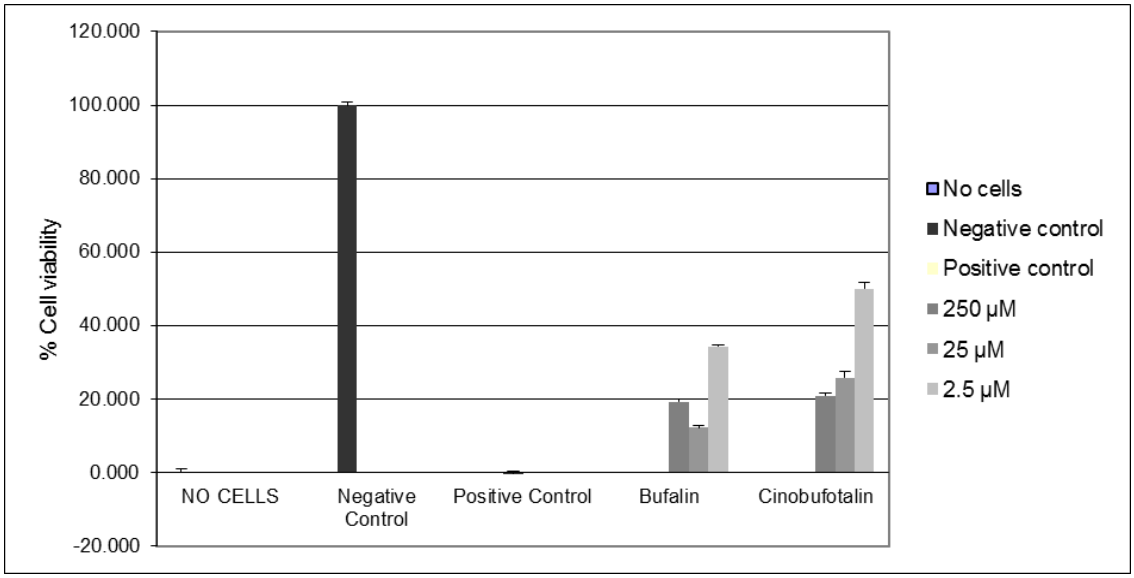


Figure 5.5: Cell viability of bufalin and cinobufotalin. Each bar represents the mean ± SEM (n = 3).

5.3.3 Discussion

Both bufalin and cinobufotalin showed significant toxicity, which was expected as bufadienolides are neurotoxic compounds (Joubert, 1981, Kellerman *et al.*, 2005, Sun *et al.*, 2011, Xie *et al.*, 2011).

5.4 Conclusion

The toxicity of bufalin and cinobufotalin, pro-oxidant activity of bufalin in the TBA assay and lack of any significant activity of cinobufotalin in the TBA assay and both bufalin and cinobufotalin in the NBT assay indicate that the orbicusides may not have potential as antioxidants. However, it does not necessarily mean that bufadienolides cannot be used to produce therapeutic antioxidants. There is still a possibility that the aglycone structure of bufadienolides can be chemically altered to improve antioxidant activity and reduce toxicity.

6 SYNTHESIS OF COMPOUNDS ANALOGOUS TO THE ORBICUSIDES OF *C. ORBICULATA* AND THE EVALUATION OF THEIR ANTIOXIDANT ACTIVITY AND TOXICITY

6.1 Synthesis of the bufadienolide analogues

Alterations in the aglycone structure of a bufadienolide could potentially improve the compound's antioxidant activity and reduce the compound's toxicity. Pyrones, particularly, have been shown to have antioxidant properties (paragraph 2.6) and were chosen as the target moieties to undergo structural alterations during analogue synthesis. Two compounds (with 2-pyrone esters) analogous to the orbicusides of *C. orbiculata* were synthesised to determine whether changes to the bufadienolide's 2-pyrone moiety and its position on the bufadienolide aglycone could improve its antioxidant activity.

6.1.1 Method

The analogues were synthesised using the method of Tomoda *et al.* (2010) by the esterification of transandrosterone and androstanolone, respectively, using coumalic acid, producing Compound **1** and Compound **2** (figure 6.1 and 6.2).

6.1.2 Chemicals and reagents

Nitrogen (g) was purchased from Afrox, Potchefstroom.

Trans-androsterone, androstanolone, DCM, ethyl acetate, anhydrous sodium sulfate (Na_2SO_4), coumalic acid, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCl) and 4-(Dimethylamino)pyridine (DMAP) was purchased from Sigma Aldrich.

A 1 N hydrochloric acid (HCl) solution was prepared by diluting a 32 % HCl solution (49.11 ml) with dd H_2O to produce 500 ml.

6.1.3 Synthesis of the two bufadienolide analogues

6.1.3.1 Analogue with 2-pyrone ester on position C-3

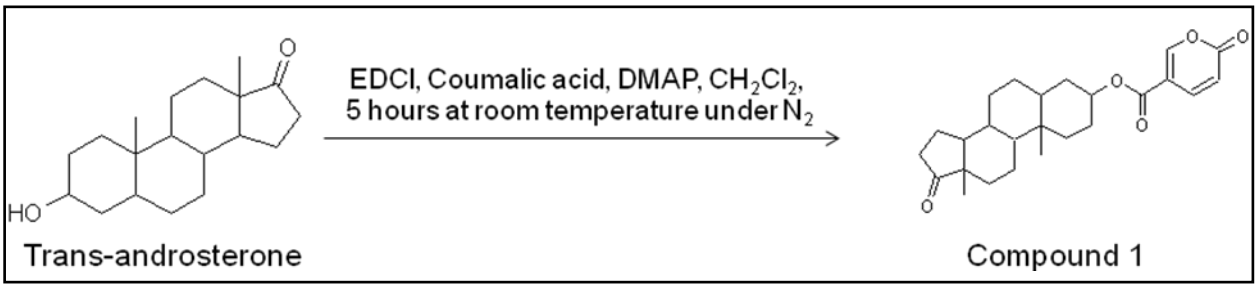


Figure 6.1: The synthesis of Compound **1**

Figure 6.1 illustrates the synthesis of Compound **1**. In a nitrogen atmosphere, trans-androsterone (501 mg, 27,7 µmol) was dissolved in DCM (31.25 ml). EDCl (500 mg, 41.6 µmol), coumalic acid (267,047 mg, 30.5 µmol) and DMAP (107 mg, 13,9 µmol) was added to the solution and the mixture stirred for five hours at room temperature. The reaction mixture was diluted with ethyl acetate and the organic layer washed with the 1 N HCl solution and dd H_2O. The organic layer was dried over Na_2SO_4. The solution was filtered and concentrated using the BUCHI Rotavapor at 40 °C.

Column chromatography of the dried residue with ethyl acetate: petroleum ether (1:1) as mobile phase and MN silica gel 60 (0.063 – 0.2 mm / 70 – 230 mesh ASTM for column chromatography) was used to purify the reaction product, a 2,15-dimethyl-5-oxotetracycloheptadecan-14-yl 2-oxo-2H-pyran-5-carboxylate (Compound **1**).

6.1.3.2 Analogue with 2-pyrone ester on position C-17

Figure 6.2 illustrates the synthesis of Compound **2**. In a nitrogen atmosphere, androstanolone (500 mg, 27,7 µmol) was dissolved in DCM (31.25 ml). EDCl (500 mg, 41.6 µmol), coumalic acid (267,047 mg, 30.5 µmol) and DMAP (107 mg, 13,9 µmol) was added to the solution. The reaction mixture was stirred for 5 hours at room temperature. A small sample was collected and a TLC plate prepared to determine the progress of the reaction. TLC of the reaction sample and androstanolone indicated that the reaction was complete, with no androstanolone present in the reaction sample (figure 6.3).

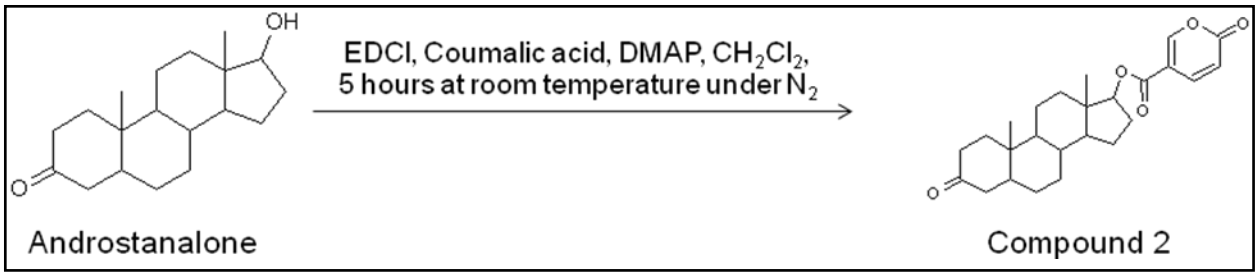


Figure 6.2: Synthesis of Compound **2**

The reaction mixture was diluted with ethyl acetate and the organic layer washed with the 1 N HCl solution and dd H_2O. The organic layer was dried over Na_2SO_4. The solution was filtered and concentrated using the BUCHI Rotavapor at 40 °C. Column chromatography of the dried residue with ethyl acetate: petroleum ether (1:1) as mobile phase and MN silica gel 60 (0.063 – 0.2 mm / 70 – 230 mesh ASTM for column chromatography) was used to purify the reaction product, a 2,15-dimethyl-14-oxotetracycloheptadecan-5-yl 2-oxo-2H-pyran-5-carboxylate (Compound **2**).

Figure 6.3: TLC of a small sample of (1) androstanolone and (2) the reaction mixture after 5 hours of stirring at room temperature using H_2SO_4 (5 % in ethanol) as detection reagent and ethyl acetate: petroleum ether (1:1) as mobile phase.

6.1.4 Validation of the analogue structures

The structures of Compound **1** and Compound **2** were validated by ^{1}H and ^{13}C NMR, high resolution mass spectrometry and Fourier transform infrared spectroscopy (refer to appendix C to J).

(10,13-dimethyl-17-oxo-1,2,3,4,5,6,7,8,9,11,12,14,15,16-tetradecahydrocyclopenta[a]-phenanthren-3-yl) 6-oxopyran-3-carboxylate (Compound **1**): cream coloured powder; m.p. 258.2 – 266.3 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.24 (dd, *J* = 2.6, 1.1 Hz, 1H), 7.75 (dd, *J* = 9.8, 2.6 Hz, 1H), 6.29 (dd, *J* = 9.8, 1.1 Hz, 1H), 4.90 – 4.81 (m, 1H), 2.40 (dd, *J* = 19.3, 8.5 Hz, 1H), 2.03 (dt, *J* = 19.3, 9.1 Hz, 1H), 1.95 – 1.83 (m, 2H), 1.82 – 1.52 (m, 7H), 1.52 – 1.38 (m, 2H), 1.37 – 1.17 (m, 6H), 1.09 – 0.91 (m, 2H), 0.84 (s, 3H), 0.82 (s, 3H), 0.70 (ddd, *J* = 12.2, 10.4, 4.0 Hz, 1H); ^{13}C NMR(151 MHz, $CDCl_3$) δ 162.4, 159.9, 157.9, 141.8, 115.1, 112.4, 75.0, 54.2, 51.3, 47.7, 44.6, 36.6, 35.8, 35.6, 34.9, 33.8, 31.4, 30.7, 28.2, 27.3, 21.7, 20.4, 13.7, 12.2; APCI-HRMS: m/z: calculated for $C_{25}H_{33}O_5$, 413.2323, found 413.2297 $[M+H]^+$; v_{max} cm^{-1}: 2931.21, 2855.78, 1748.80, 1731.08, 1701.44, 1291.65, 1235.58, 1058.00, 995.98, 776.94, 762.11.

(10,13-dimethyl-3-oxo-1,2,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydrocyclopenta[a]-phenanthren-17-yl) 6-oxopyran-3-carboxylate (Compound **2**): white powder; m.p. 216.3 – 217.9 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.24 (dd, *J* = 2.5, 1.2 Hz, 1H), 7.74 (dd, *J* = 9.8, 2.6 Hz, 1H), 6.30 (dd, *J* = 9.8, 1.2 Hz, 1H), 4.75 (t, *J* = 8.5 Hz, 1H), 2.35 (td, *J* = 14.6, 6.5 Hz, 1H), 2.30 – 2.16 (m, 3H), 2.05 (ddd, *J* = 15.1, 4.2, 2.2 Hz, 1H), 1.98 (ddd, *J* = 13.2, 6.6, 2.2 Hz, 1H), 1.76 – 1.63 (m, 1H), 1.61 – 1.41 (m, 4H), 1.39 – 1.23 (m, 5H), 1.19 (td, *J* = 12.9, 4.0 Hz, 1H), 1.08 (td, *J* = 11.7, 7.2 Hz, 1H), 0.99 (s, 3H), 0.90 (qd, *J* = 12.6, 4.9 Hz, 4H), 0.84 (s,

3H), 0.74 (td, *J* = 12.1, 3.9 Hz, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 211.7, 162.8, 159.8, 157.8, 141.7, 115.2, 112.3, 83.9, 53.6, 50.4, 46.5, 44.6, 42.9, 38.4, 38.0, 36.8, 35.6, 35.1, 31.1, 28.6, 27.5, 23.5, 20.8, 12.3, 11.4; APCI-HRMS m/z: calculated for $C_{25}H_{33}O_5$, 413.2323, found 413.2315 $[M+H]^+$; v_{max} cm^{-1}: 2930.16, 2851.62, 1760.30, 1708.69, 1307.16, 1232.49, 1094.78, 771.80.

6.1.4.1 NMR analysis

The structures of the Compound **1** and Compound **2** (figure 6.4) were confirmed with ^{1}H and ^{13}C NMR spectra (Refer to Appendix C, D, G and H for the NMR spectra).

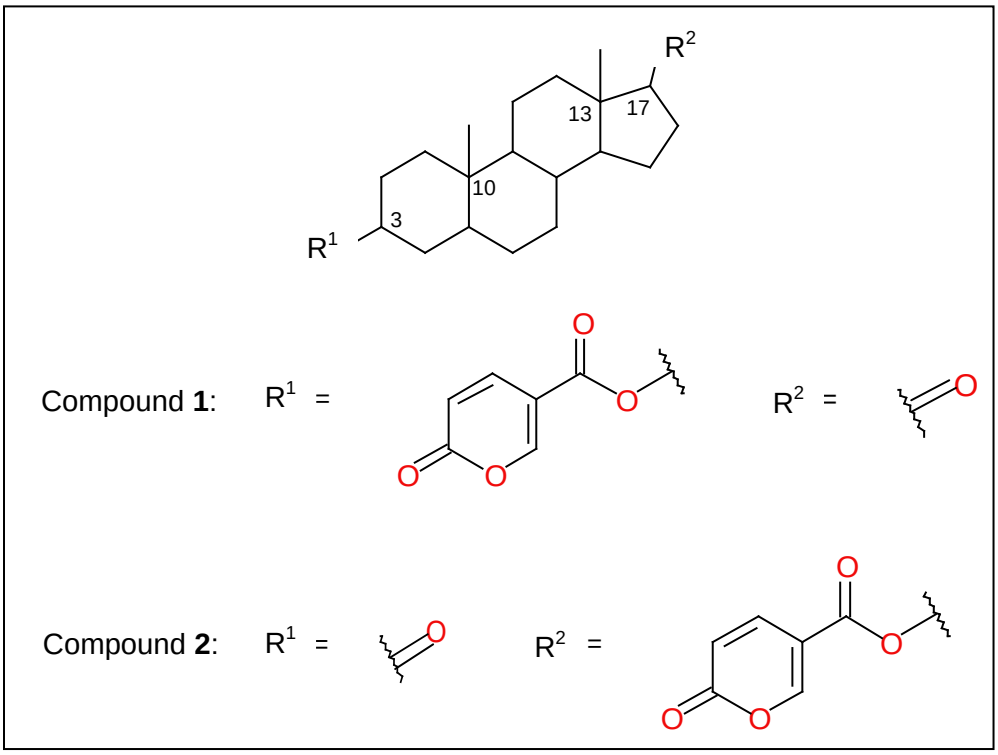


Figure 6.4: Structures of Compound **1** and Compound **2**

35 mg of Compound **1** and Compound **2** was dissolved in Chloroform-D1 (from Merck Chemicals), filtered and placed in separate NMR tubes. A Bruker Advance III 500; 5 mm BBO-Z probe was used to obtain ^{1}H and ^{13}C NMR spectra of the samples. The NMR spectra were recorded at 30 °C. Chemical shifts are in units of ppm, referenced to the residual protonated solvent (chloroform) at 6.24 ppm for ^{1}H NMR spectra and at 77.0 ppm for ^{13}C NMR spectra. Topspin version 2.1, patch level 6 was used to record the NMR data.

In the ^{1}H NMR spectrum of Compound **1** the three deshielded protons of the 2-pyrone ring were positioned at 8.24, 7.75 and 6.29 ppm. The proton on position C-3 occurred as part of the multiplet at 4.90 – 4.81 ppm. The protons of the two methyl groups on position C-10 and C-13 were present as two singlets at 0.84 and 0.82 ppm. In the ^{13}C NMR spectrum the

carbonyl group of the aglycone on C-17 was absent, possibly due to the long relaxation time of this quaternary carbon. The signals at 162.4 and 159.9 ppm represented the ester and the carbonyl carbons of the 2-pyrone moiety. The peaks at 157.9, 141.8, 115.1 and 112.4 represented the remaining carbons of the 2-pyrone ring. The carbon signals of C-1 to C-16 of the aglycone structure were represented by the 16 signals situated at 75.0 to 20.4 ppm. The methyl groups on position C-10 and C-13 were represented by the signals at 13.8 and 12.2 ppm.

In the ^{1}H NMR spectrum of Compound **2** the three deshielded protons of the 2-pyrone ring were positioned at 8.24, 7.74 and 6.30 ppm. H-3 was positioned at 4.75 ppm. The protons of the two methyl groups, C-18 and C-19, were present as a singlet at 0.99 ppm and as part of a multiplet at 0.96 – 0.82 ppm. In the ^{13}C NMR spectrum the carbonyl group of the aglycone on C-3 was visible at 211.7 ppm. The signals at 162.8 and 159.9 ppm represented the ester and the carbonyl of the 2-pyrone ring.The signals at 157.8, 141.7, 115.2 and 112.3 ppm represented the remaining carbons of the 2-pyrone ring. C-1, C-2 and C-4 – C-17 of the aglycone structure were represented by the 16 signals situated between 83.9 and 20.8 ppm. The methyl groups, C-18 and C-19, were represented by the signals at 12.3 and 11.42 ppm.

6.1.4.2 Mass spectrometry

The structures of the Compound **1** and Compound **2** were validated with APCI – high resolution mass spectrometry (refer to Appendix E and I for the mass spectrometry spectra) using an Accurate-Mass Time-of-Flight spectrometer (micrOTOF-Q II) and Bruker Compass DataAnalysis 4.0 software. The mass spectrometer parameters were:

- Source type APCI
- Scanning range from 50 to 1500 m/z
- Ion polarity in positive mode
- Set capillary 4500 V
- Set end plate offset -500 V
- Set collision cell RF of 100 Vpp
- Nebulizer pressure 1.6 Bar
- Set dry heater temperature 200 °C
- Set dry gas flow 8 l/min

Mass spectrometry of Compound **1** and Compound **2**, with the same molecular formulas ($C_{25}H_{32}O_5$) confirmed the presence of the synthesised compounds. For Compound **1** in particular, the mass data provided confirmation of the structure as the signal of the C-17 carbonyl group was not visible in the ^{13}C NMR spectrum.

6.1.4.3 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy was performed with a Bruker Alpha Platinum-ATR (attenuated total reflection) and Opus 7.0 software. For Compound **1** the peaks at 2931 and 2856 cm^{-1} most likely represent the C-H bonds of the 2-pyrone moiety and/or the lactone ring's O=C-O bond. The peaks of the C-17 ketone carbonyl group and the two carbonyls of the ester groups were situated at 1749, 1731 and 1701 cm^{-1}. These data provided further confirmation of the presence of the carbonyl on position C-17, which was not detetectable in the ^{13}C NMR spectrum. The infrared data of Compound **2** was similar, with the peaks at 2930 and 2852 cm^{-1} likely to represent the C-H bonds of the 2-pyrone moiety and/or the lactone ring's O=C-O bond. Only two carbonyl peaks were visible on the infrared spectrum (situated at 1760 and 1709 cm^{-1}). However, the ^{13}C NMR spectrum confirmed the presence of three carbonyl groups.

6.1.4.4 Melting points

A Buchi Melting Point B-545 (Labotec) was used to determine the melting points. The melting point of Compound **1** was determined as 258.2 – 266.3 °C. The melting point of Compound **2** was determined as 216.3 – 217.9 °C.

6.2 Lipid peroxidation assay

The same method and reagents used for the TBA assay (paragraph 2.7) of the juice and fractions of *C. orbiculata* (paragraph 3.3.1.1) was used here. The pre-prepared and frozen aliquots of rat brain homogenate (paragraph 3.3.1.1.3) were used during the assay.

6.2.1 Sample preparation

A 10 mM stock solution of Compound **1** was prepared by mixing Compound 1 (4.12 mg) in ethanol (1 ml) at 50 °C until dissolved.

0.08 ml of the Compound **1** stock solution was placed in an Eppendorf tube and 1.92 ml ethanol added to produce a 0.4 mM sample (2 ml).

1 ml of the 0.4 mM Compound **1** solution was placed in an Eppendorf tube and 1 ml ethanol added to produce a 0.2 mM sample (2 ml).

0.5 ml of the 0.2 mM Compound **1** solution was placed in an Eppendorf tube and 0.5 ml ethanol added to produce a 0.1 mM sample (1 ml).

A 10 mM stock solution of Compound **2** was prepared by mixing Compound 1 (4.12 mg) in ethanol (1 ml) at 50 °C until dissolved.

0.08 ml of the Compound **2** stock solution was placed in an Eppendorf tube and 1.92 ml ethanol added to produce a 0.4 mM sample (2 ml).

1 ml of the 0.4 mM Compound **2** solution was placed in an Eppendorf tube and 1 ml ethanol added to produce a 0.2 mM sample (2 ml).

0.5 ml of the 0.2 mM Compound **2** solution was placed in an Eppendorf tube and 0.5 ml ethanol added to produce a 0.1 mM sample (1 ml).

6.2.2 Standard curve and assay

The standard curve prepared in paragraph 3.3.1.1.5 was used during this assay. The assay was performed as according to the method in paragraph 3.3.1.1.4.

6.2.3 Results

The levels of MDA formed (in nmol/mg tissue) in the tested samples were determined and summarised in tables 6.1 and 6.2 and figures 6.5 and 6.6. Statistical analysis of the results was performed with the Student-Newman-Keuls multiple comparisons test and one-way analysis of variance (ANOVA) via GraphPad software. The results of the samples were statistically compared with that of the Toxin.

Table 6.1: The effect of Compound **1** on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	0.365	0.006135
***Toxin**	0.527	0.01695
Trolox (Positive Control)	0.074	0.01094
Compound 1 0.4 mM	0.538	0.005896
Compound 1 0.2 mM	0.512	0.01052
Compound 1 0.1 mM	0.620	0.02772

***Toxin: A combination of $FeCl_3$, H_2O_2 and vitamin C was used to induce lipid peroxidation in the rat brain homogenate**

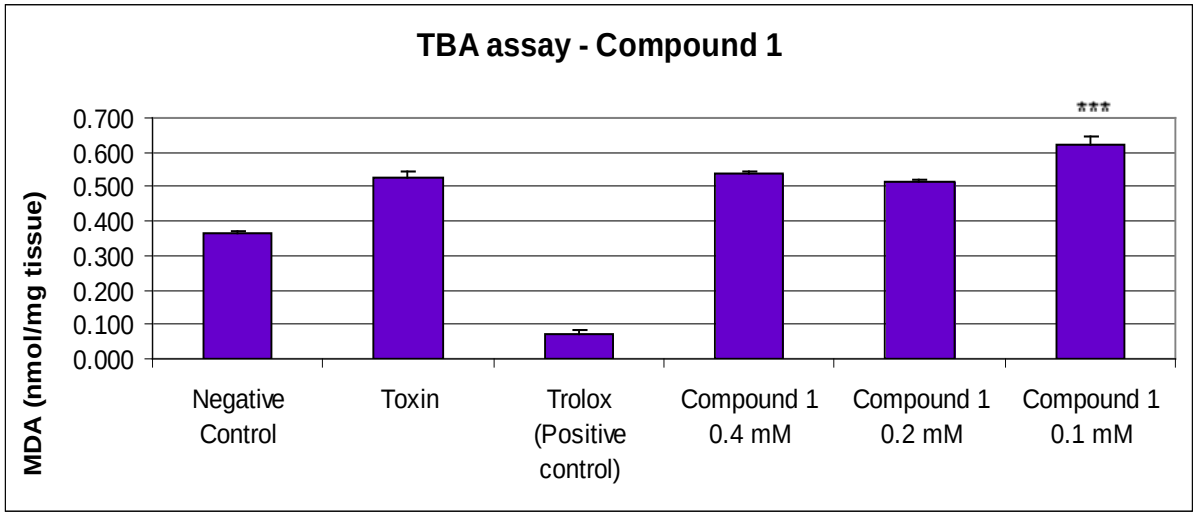


Figure 6.5: The effects of Compound **1** on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). *** $p < 0.001$ vs Toxin.

Compound **1** showed significant pro-oxidant activity (figure 6.5).

Table 6.2: The effect of Compound **2** on lipid peroxidation induced in rat brain homogenate

	MDA (nmol/mg tissue)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	0.365	0.006135
Toxin	0.527	0.01695
Trolox (Positive Control)	0.074	0.01094
Compound 2 0.4 mM	0.511	0.005774
Compound 2 0.2 mM	0.533	0.02149
Compound 2 0.1 mM	0.528	0.004411

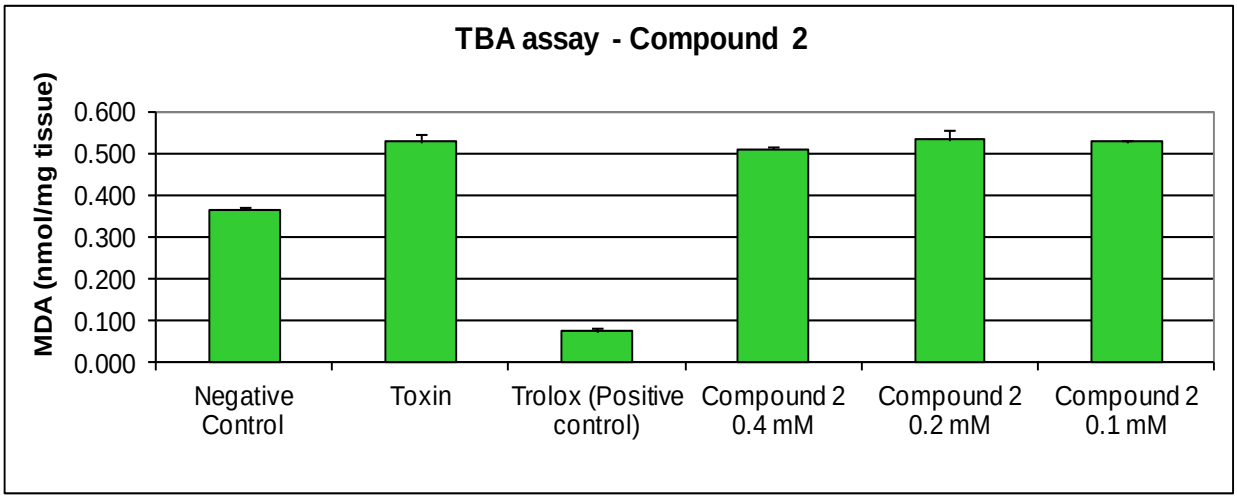


Figure 6.6: The effects of Compound **2** on lipid peroxidation induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). The sample results were statistically compared with that of the toxin.

Compound **2** showed no significant activity (figure 6.6).

6.2.4 **Discussion**

The pro-oxidant activity of Compound **1** indicated that altering the structure of the 2-pyrone moiety and its position on the aglycone of a bufadienolide from C-17 to C-3 did not improve its antioxidant activity against lipid peroxidation. The lack of significant activity of Compound **2** indicated that altering the structure of the 2-pyrone moiety on position C-17 of the aglycone of a bufadienolide did not improve its antioxidant activity against lipid peroxidation.

6.3 NBT assay

The same method and reagents used for the NBT assay of the juice and fractions of *C. orbiculata* (paragraph 3.3.1.2) were used here. The pre-prepared and frozen aliquots of rat brain homogenate (paragraph 3.3.1.1.3) were used during the assay.

6.3.1 Sample preparation

The Compound **1** and **2** samples prepared in paragraph 6.2.1 were used during this assay.

6.3.2 Standard curve and assay

The standard curve and Bradford protein assays prepared in paragraph 3.3.2.1.3 was used during this assay. The assay was performed according to the method in paragraph 3.3.2.1.4.

6.3.3 Results

The levels of NBD formed (in µmol/mg protein) in the tested samples were determined and summarised in tables 6.3 and 6.4 and figures 6.7 and 6.8.

Table 6.3: The effect of Compound **1** on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	37.647	0.5983
***Toxin**	117.329	1.558
Trolox (Positive Control)	49.073	2.155
Compound 1 0.4 mM	111.124	2.456
Compound 1 0.2 mM	117.631	1.599
Compound 1 0.1 mM	124.139	2.74

***Toxin: KCN was used to induce $O_2^{\bullet-}$ production in the rat brain homogenate**

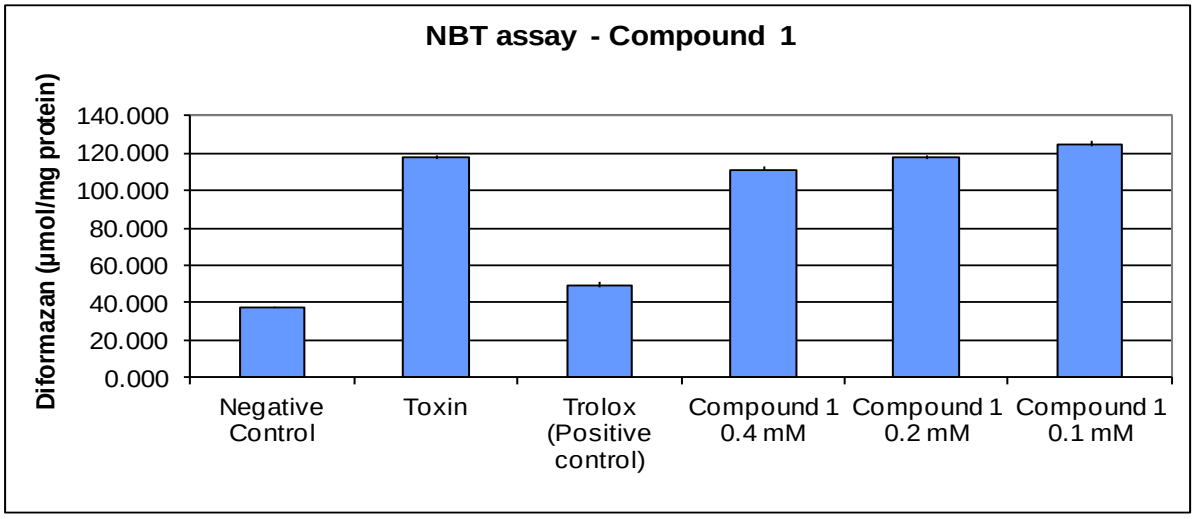


Figure 6.7: The effects of Compound **1** on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). The sample results were statistically compared with that of the toxin.

Compound **1** showed no significant activity (figure 6.7).

Table 6.4: The effect of Compound **2** on $O_2^{\bullet-}$ production induced in rat brain homogenate

	NBD (µmol/mg protein)	**± Standard error of the mean (SEM)** **n = 5**
Negative Control	37.647	0.5983
Toxin	117.329	1.558
Trolox (Positive Control)	49.073	2.155
Compound 2 0.4 mM	106.886	2.248
Compound 2 0.2 mM	110.670	3.42
Compound 2 0.1 mM	119.523	3.597

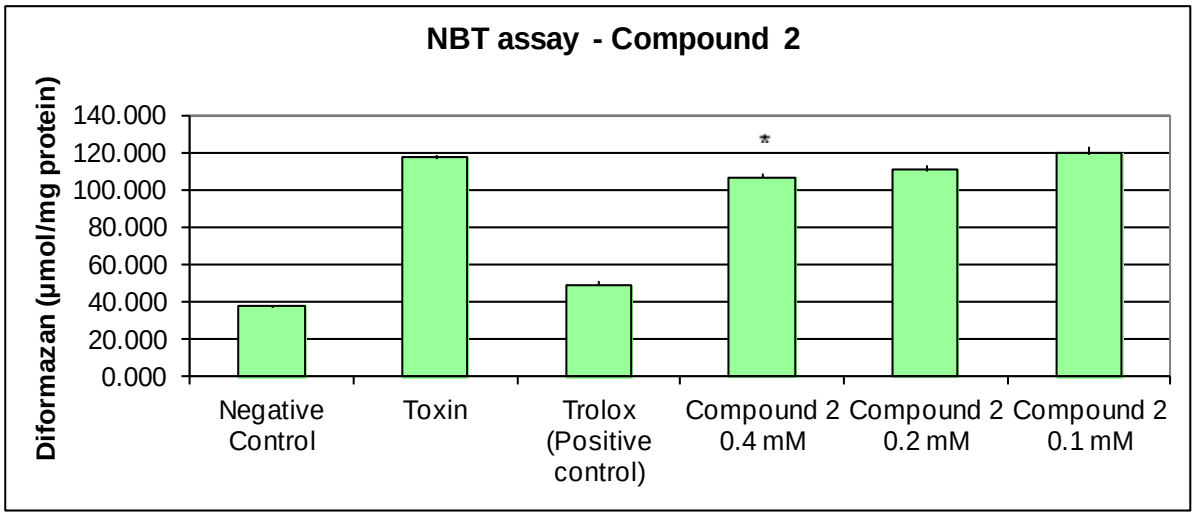


Figure 6.8: The effects of Compound **2** on $O_2^{\bullet-}$ production induced in rat brain homogenate. Each bar represents the mean ± SEM (n = 5). * $p < 0.05$ vs Toxin.

Compound **2** showed slight antioxidant activity (figure 6.8).

6.3.4 Discussion

The lack of significant activity of Compound **1** indicated that altering the structure of the 2-pyrone moiety and its position on the aglycone of a bufadienolide from C-17 to C-3 did not improve its antioxidant activity against $O_2^{\bullet-}$ production. In the NBT assay the diformazan levels of Compound **2** was lower than that of the Toxin (where $O_2^{\bullet-}$ production was induced in rat brain homogenate) but not near or lower than the levels of the negative control (which

contained only rat brain homogenate), indicating that Compound **2** could slightly reduce the induced $O_2^{\bullet-}$ production but was not potent enough to be considered as an effective therapeutic antioxidant (could not reduce the $O_2^{\bullet-}$ levels back to the levels of that of the healthy or uninduced rat brain homogenate or negative control). The slight antioxidant activity of Compound **2** did, however, indicate that altering the structure of the 2-pyrone moiety on position C-17 of the aglycone of a bufadienolide can slightly improve its antioxidant activity ($O_2^{\bullet-}$ scavenging activity).

6.4 Toxicity evaluation

To determine whether altering the 2-pyrone moiety of a bufadienolide and its position on the aglycone structure could reduce the toxicity of the compounds, the MTT assay (refer to paragraph 2.8) method in paragraph 5.3.1 was followed.

6.4.1 Preparation of samples

The samples were prepared under aseptic or sterile conditions in a laminar flow cabinet and filtered before use.

10mM stock solutions of both Compound **1** and Compound **2** were prepared by suspending Compound **1** (4.1 mg) and Compound **2** (4.1 mg) in ethanol (1ml), respectively, and heating the suspensions at 50 °C until dissolved.

Three concentrations (250 µM, 25 µM and 2.5 µMl) of Compound **1** and Compound **2**, respectively, were prepared in a fume hood. The stock solutions and ethanol (1 % in dd H_2O) were filtered. 10 mM stock solution (50 µl) was diluted with the 1 % ethanol (1.950 µl) to form a 250 µM solution (2 ml). 250 µM solution (200 µl) was diluted with 1 % ethanol (1.8 ml) to form a 25 µM solution (2 ml). 250 µM solution (20 µl) was also diluted with 1 % ethanol (1.98 ml) to form a 2.5 µM solution.

6.4.2 Results

The cell viability was calculated according to the method in paragraph 5.3.2. Table 6.5 and figure 6.9 summarises the cell viability of Compound **1** and Compound **2**.

Table 6.5: Cell viability of Compound **1** and Compound **2**

	% Cell viability
No cells (Blank)	0.000
Negative Control	100.000
Positive Control	-0.367
Compound 1 (250 µM)	91.387
Compound 1 (25 µM)	95.914
Compound 1 (2.5 µM)	92.670
Compound 2 (250 µM)	65.091
Compound 2 (25 µM)	99.230
Compound 2 (2.5 µM)	93.861

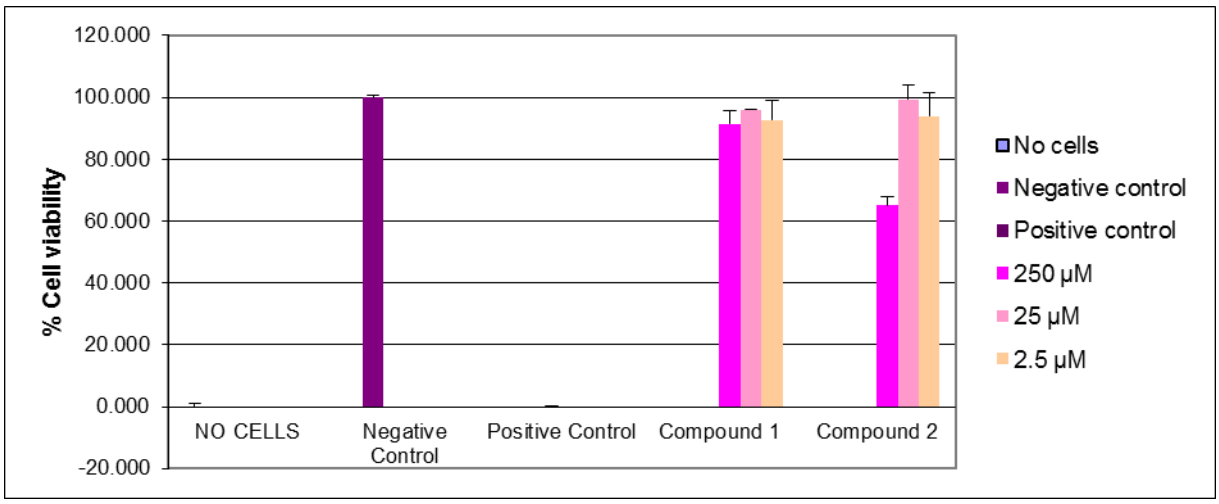


Figure 6.9: Cell viability of Compound **1** and Compound **2**. Each bar represents the mean ± SEM (n = 3).

6.4.3 Discussion

The three concentrations of Compound **1** showed no toxicity due to its high cell viability values (91.387, 95.914 and 92.670 % respectively). The 250 µM concentration of Compound **2** showed moderate toxicity (65.091 %), whilst the 25 and 2.5 µM concentrations showed no toxicity (99.230 and 93.861 % respectively).

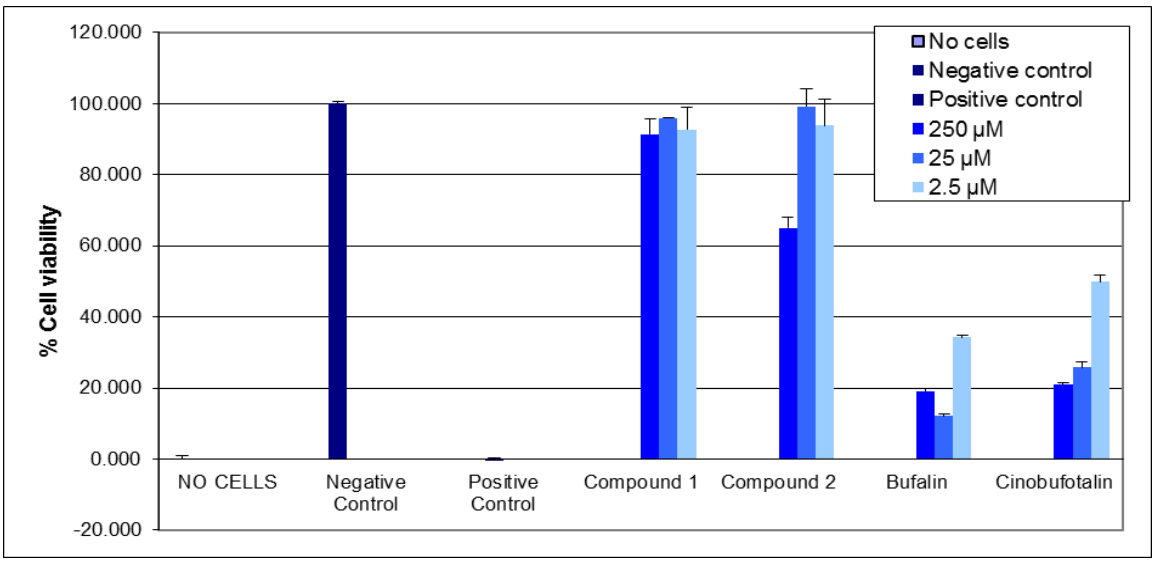


Figure 6.10: Cell viability of the two synthesised bufadienolide analogues (Compound **1** and Compound **2**) and commercial bufadienolides (bufalin and cinobufotalin). Each bar represents the mean ± SEM (n = 3).

Figure 6.10 shows the cell viability of the two synthesised bufadienolide analogues and commercial bufadienolides. By comparing the cell viability of the two synthesised bufadienolide analogues (Compound **1** and Compound **2**) with the cell viability of the two commercial bufadienolides, bufalin and cinobufotalin, it was evident that altering the 2-pyrone moiety of a bufadienolide aglycone to a 2-pyrone ester (as in the case of Compound **2**) could significantly improve the toxicity of the compound. However, these compounds were still moderately toxic and further investigation is required. Altering the position of the 2-pyrone ester on the aglycone structure of a bufadienolide (as in the case of Compound **1**) also significantly improved the toxicity of the compound, as the three concentrations evaluated in the MTT assay showed no toxicity.

6.5 Conclusion

The pro-oxidant activity of Compound **1** in the TBA assay and lack of any significant activity of Compound **2** in the TBA assay and Compound **1** in the NBT assay indicated that bufadienolides may not have potential in the development of antioxidant analogues against lipid peroxidation. However, when comparing the lack of significant activity of the two commercial bufadienolides, bufalin (figure 5.3) and cinobufotalin (figure 5.4), with the slight antioxidant activity of Compound **2** (figure 6.8) observed in the NBT assay, it was concluded that altering the 2-pyrone moiety of a bufadienolide did have the potential to improve the antioxidant activity ($O_2^{\bullet -}$ scavenging activity) of a bufadienolide.

The MTT assay of the synthesised bufadienolide analogues confirmed that alterations to the 2-pyrone moiety and its position on the aglycone structure of a bufadienolide can reduce a

bufadienolide's toxicity. Compound **2**, with slight antioxidant activity showed moderate toxicity. However this measured toxicity is significantly lower than that of the commercial bufadienolides, bufalin and cinobufotalin, indicating that the structural alteration of the 2-pyrone moiety can have potential in both reducing the toxicity and improving the antioxidant activity of bufadienolide compounds.

Further investigation concerning the use of bufadienolides to develop analogues with improved antioxidant activity and reduced toxicity to produce antioxidant compounds treat epilepsy (if oxidative stress pathology is involved) is strongly motivated.

7 RESEARCH CONCLUSION

Kabatende (2005) and Amabeoku *et al.* (2007) determined that *C. orbiculata* extracts had anticonvulsant effects and attributed the effects to GABAergic and glutaminergic activity. Louw (2009) and Roux (2012) determined that *C. orbiculata* extracts had antioxidant activity. The hypothesis was that the antioxidant effects could have contributed to the anticonvulsant effects observed by Kabatende (2005) and Amabeoku *et al.* (2007). In this study the potential of the orbicusides of *C. orbiculata* as antioxidant treatment for epilepsy (in epilepsy pathophysiology where oxidative stress is involved) was evaluated.

Roux (2012) and Louw (2009) confirmed that various extracts of *C. orbiculata* leaves have antioxidant activity. However, the antioxidant activity of the plant as a whole and the dosage form used in traditional medicine to treat epilepsy (leaf juice) have not yet been studied. *C. orbiculata* leaf juice showed significant pro-oxidant activity in both the TBA and NBT assays. Fraction 1 showed slight antioxidant activity in the NBT assay and pro-oxidant activity in the TBA assay. Fraction 2 showed slight antioxidant activity in both assays. The observed antioxidant activity was too low to motivate further investigation.

Steyn *et al.* (1986) observed that the toxic orbicusides of *C. orbiculata* could be isolated using methanol. Roux (2012) determined that methanol *C. orbiculata* extracts have antioxidant activity and Kabatende (2005) and Amabeoku *et al.* (2007) confirmed that methanol *C. orbiculata* extracts have GABAergic and glutaminergic effects, which reduced induced epileptic seizures. However, the exact compound(s) and mechanisms involved in the observed activities were not identified.

The isolation and screening of the orbicusides was not possible. The extraction of the orbicusides from 5 kg to 10 kg of leaves via Soxhlet extraction (with 1,4-dioxane) and accelerated solvent extraction (with dichloromethane) produced concentrations of the orbicusides too low for further analytical analysis. Commercial and synthesised bufadienolide compounds analogous to the orbicusides of *C. orbiculata* were used to determine whether or not the orbicusides had potential as antioxidants. The commercial bufadienolides showed significant toxicity and mainly pro-oxidant activity. These observations were supported by Sun *et al.* (2011) and Xie *et al.* (2011), who determined that bufalin, a chemotherapeutic bufadienolide, causes ROS-mediated apoptosis and autophagy-mediated cell death. These results and the low concentrations of orbicusides in the plant material indicated that the orbicusides may possibly not be involved in the antioxidant effects of *C. orbiculata* extracts as observed by Louw (2009) and Roux (2012).

To date the antioxidant activity of bufadienolides have not yet been determined. However, various steroid and pyrone compounds and analogues have been found to elicit antioxidant activity in *in vitro* studies (Ahmad *et al.*, 2013; Goel & Ram, 2009; Klinger *et al.*, 2002; Lee *et al.*, 2010; Mohamed *et al.*, 2012; Thuong *et al.*, 2010). Bufadienolides, in this case orbicusides, therefore have promising potential as antioxidant compounds and altering the aglycone structures of bufadienolides may improve the antioxidant activity and reduce the toxicity of these compounds (refer to paragraph 2.6). The synthesised bufadienolide analogue, Compound **1**, showed pro-oxidant activity in the TBA assay, whereas Compound **2** showed slight antioxidant activity in the NBT assay. The slight antioxidant activity of Compound **2** indicates that bufadienolides do have potential in the development of antioxidants by altering their pyrone moieties and should be investigated further. Compound **1** and Compound **2** showed significantly reduced toxicity (despite Compound **2** showing moderate toxicity) when compared to the toxicity of bufalin and cinobufotalin, indicating that bufadienolide toxicity can be reduced by analogue synthesis.

The pro-oxidant activity of the commercial bufadienolides indicated that the orbicusides may not have antioxidant properties. The pro-oxidant activity and toxicity of the commercial bufadienolides also discourages the use of bufadienolides as potential epilepsy treatment. However, the reduced toxicity and slight antioxidant activity observed from the synthesised bufadienolide analogues indicated that altering the pyrone moiety of a bufadienolide aglycone can improve the antioxidant activity and reduce the neuronal toxicity of the compounds. This motivates further investigation for the discovery and development of novel antioxidants and, possibly, novel neuroprotective epilepsy treatment that can prevent epilepsy from progressing and becoming a chronic disease. Novel antioxidants resulting from further investigation into bufadienolide analogues can also have potential as the treatment of other neurodegenerative diseases, e.g. Parkinson's and Alzheimer's disease, and this also motivates further investigation.

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9 APPENDIX A: LIST OF REFERENCES USED FOR FIGURE 2.3

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10 APPENDIX B: LIST OF REFERENCES USED FOR FIGURE 2.4

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Ono, T. & Galanopoulou, A. S. 2012. Epilepsy and epileptic syndrome. (*In* Ahmad, S. I., *ed.* 2012. Neurodegenerative diseases. New York: Springer Science + Business Media. p. 99-113).

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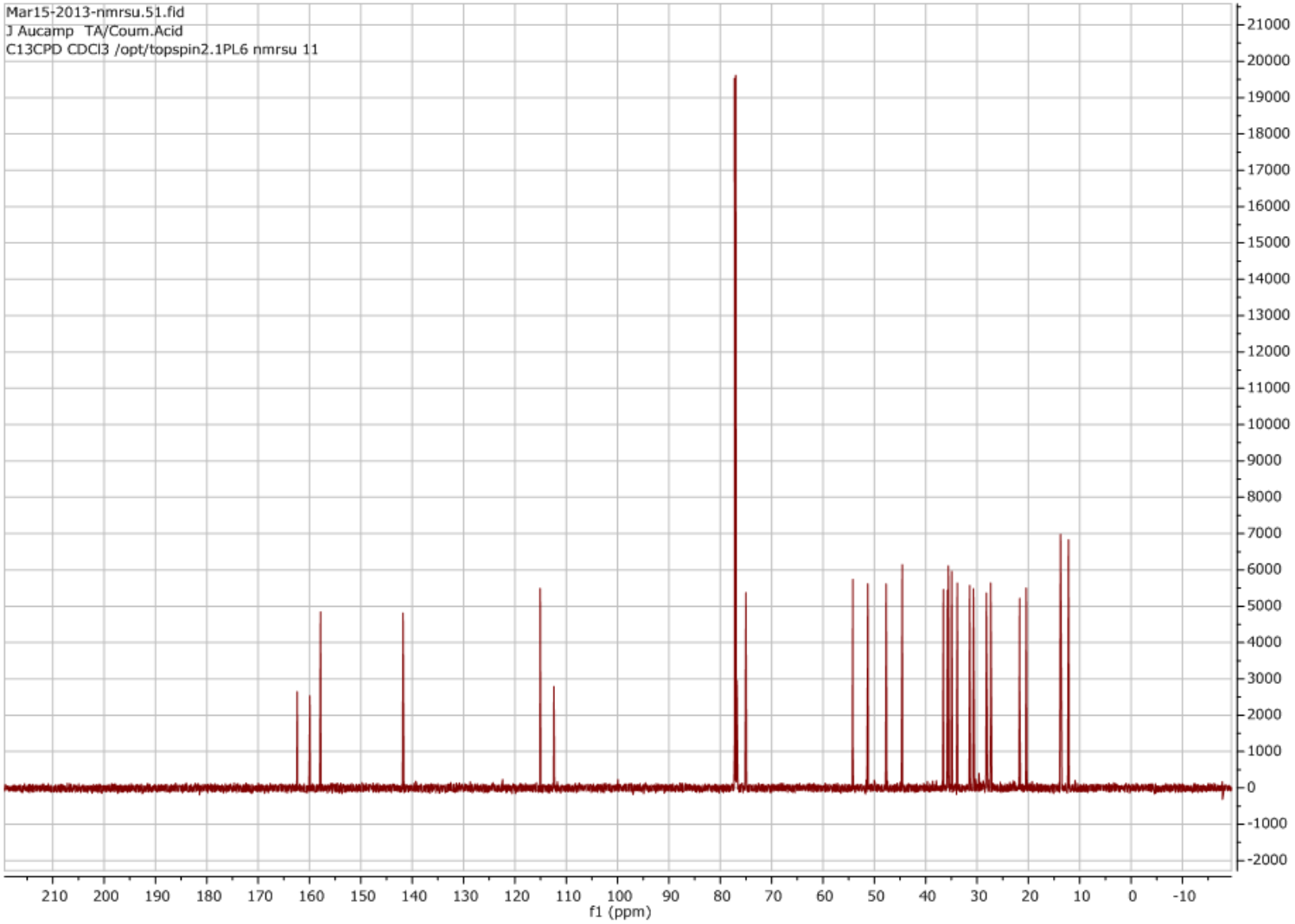
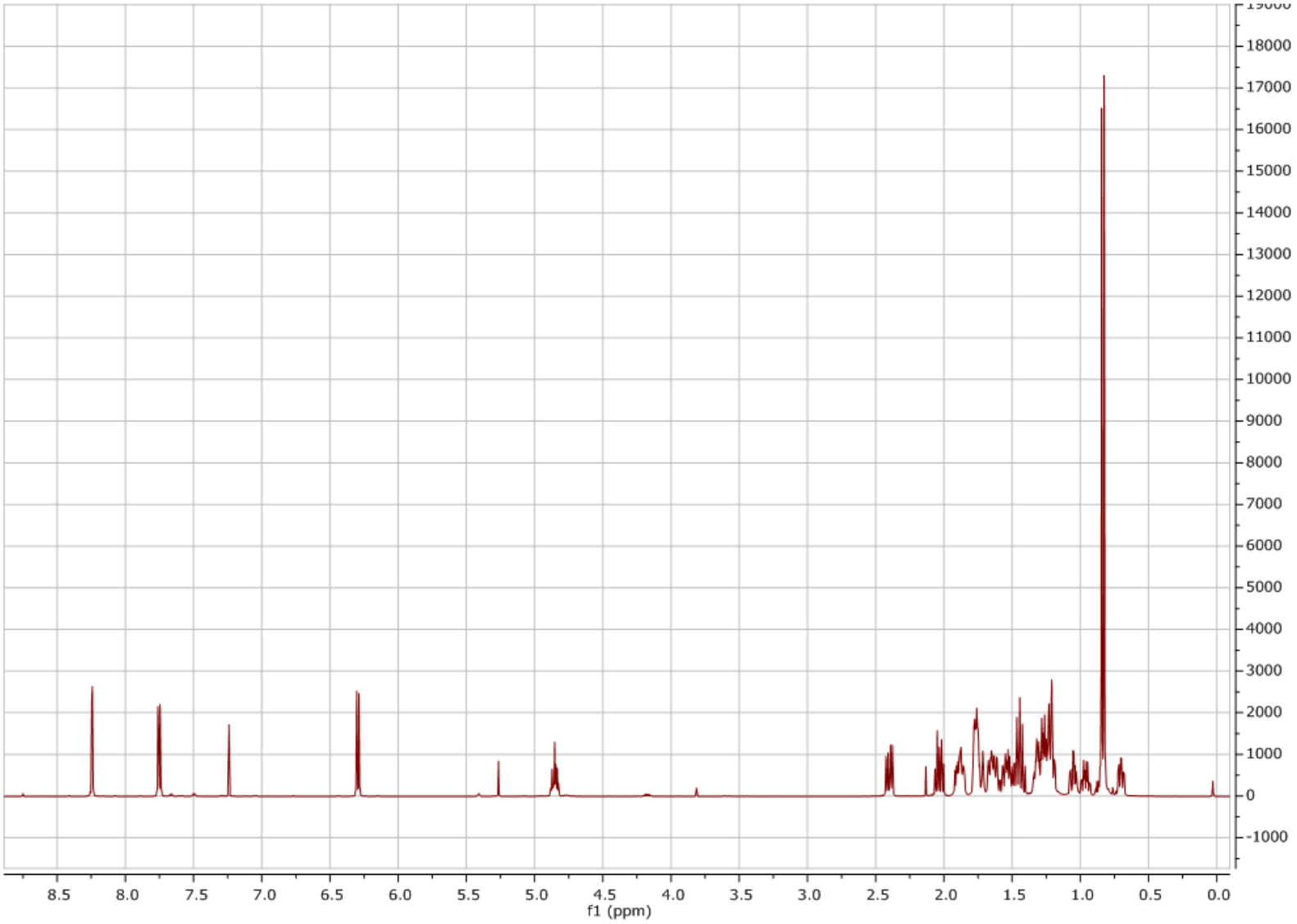
11 APPENDIX C: ^{13}C NMR SPECTRA OF COMPOUND 1



12 APPENDIX D: ^{1}H NMR SPECTRA OF COMPOUND 1



13 APPENDIX E: MASS SPECTROMETRY DATA OF COMPOUND 1

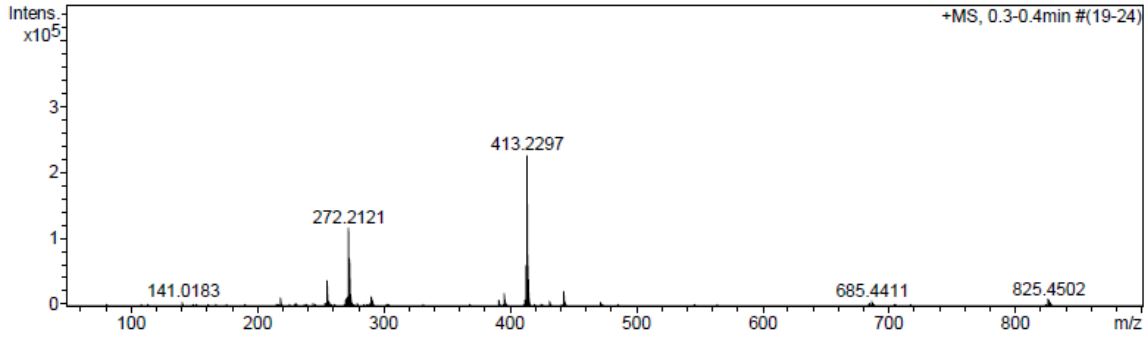
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\14062013\LADMS000005.d	Acquisition Date	6/14/2013 9:45:20 AM
Method	tune_low12062013.m	Operator	JHL Jordaan
Sample Name	TA_COUM_ACID	Instrument / Ser#	micrOTOF-Q II 10390
Comment			

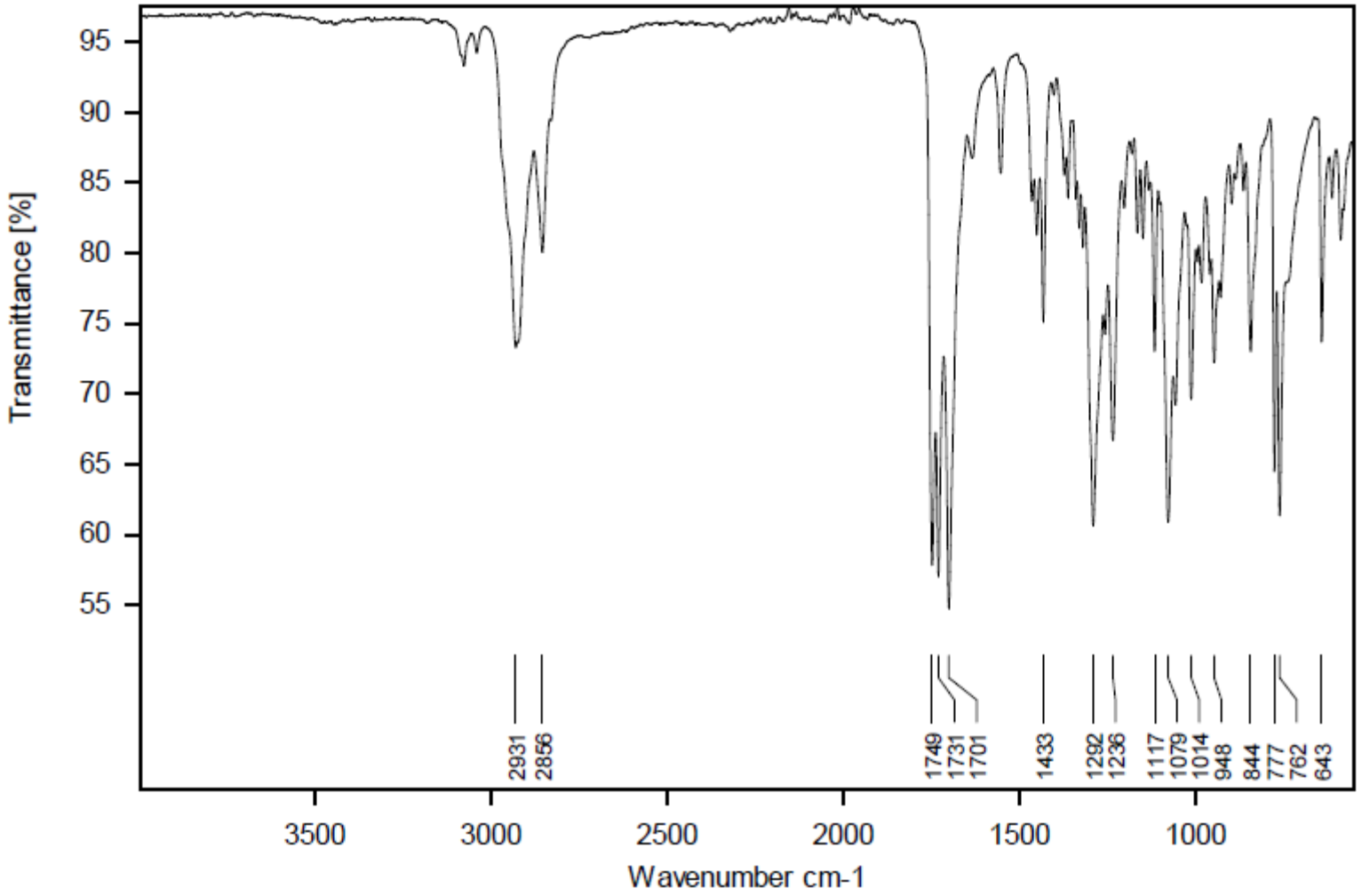
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

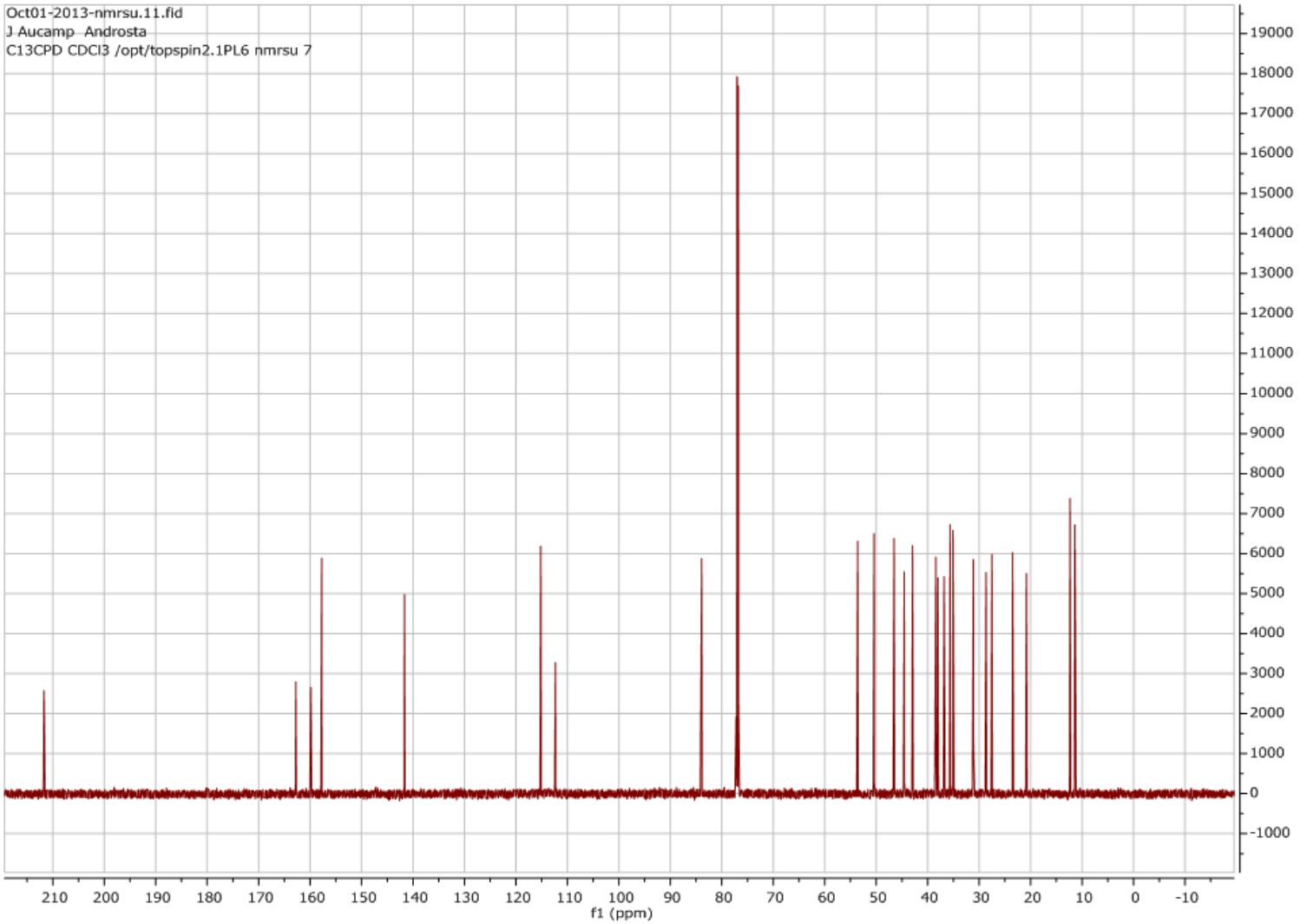


Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
255.2098	1	C 19 H 27	100.00	255.2107	0.9	3.7	63.3	6.5	ok	even
	2	C 14 H 27 N 2 O 2	12.58	255.2067	-3.1	-12.1	74.7	2.5	ok	even
272.2121	1	C 19 H 28 O	100.00	272.2135	1.4	5.2	227.3	6.0	ok	odd
	2	C 14 H 28 N 2 O 3	2.87	272.2094	-2.6	-9.6	253.7	2.0	ok	odd
412.2219	1	C 24 H 26 N 7	100.00	412.2244	2.6	6.2	607.1	15.5	ok	even
	2	C 23 H 30 N 3 O 4	23.23	412.2231	1.2	2.9	617.1	10.5	ok	even
	3	C 19 H 26 N 9 O 2	1.75	412.2204	-1.5	-3.6	626.8	11.5	ok	even
413.2297	1	C 25 H 33 O 5	100.00	413.2323	2.6	6.3	3.7	9.5	ok	even
825.4502	1	C 50 H 65 O 10	100.00	825.4572	7.0	8.5	11.3	18.5	ok	even

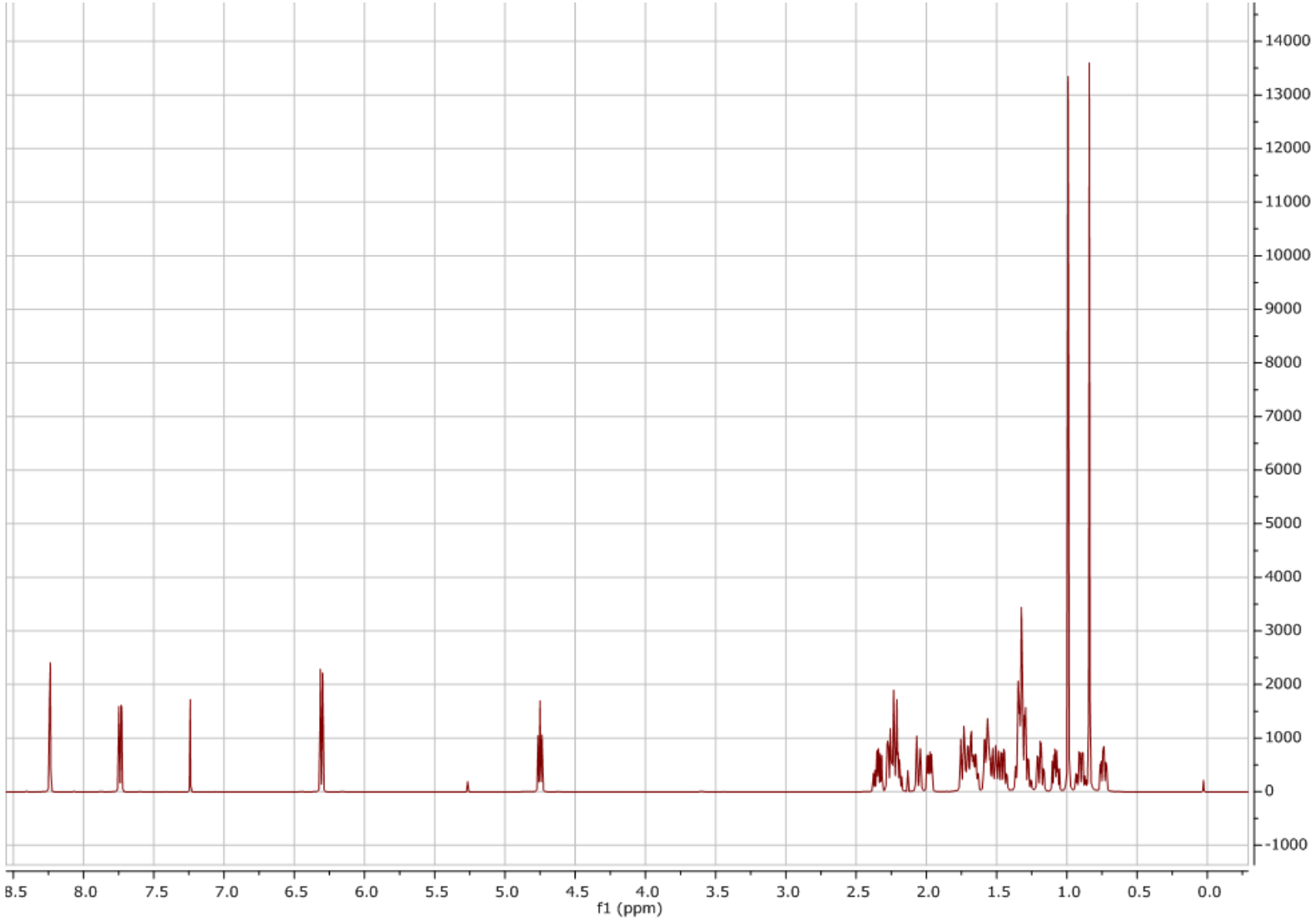
14 APPENDIX F: INFRARED SPECTROSCOPY DATA OF COMPOUND 1



15 APPENDIX G: ^{13}C SPECTRA OF COMPOUND 2



16 APPENDIX H: ^{1}H SPECTRA OF COMPOUND 2



17 APPENDIX I: MASS SPECTROMETRY DATA OF COMPOUND 2

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Mass Spectrum SmartFormula Report

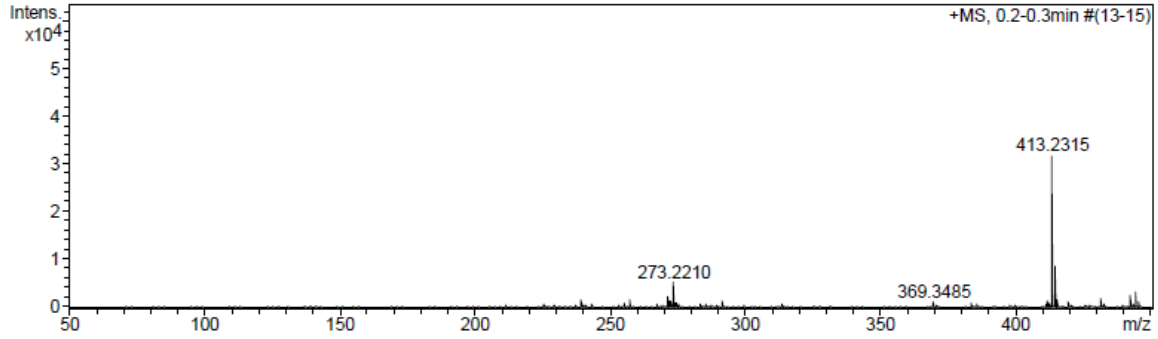
CHEMICAL RESOURCE BENEFICIATION
CHEMIESE HULPBRONVERRYKING

Analysis Info

Analysis Name	D:\Data\03102013\LADMS000003.d	Acquisition Date	10/3/2013 9:04:51 AM
Method	tune_low10092013.m	Laboratory	Laboratory for Analytical Services
Sample Name	AS_COUM_ACID	Operator	JHL Jordaan
Comment		Instrument / Ser#	micrOTOF-Q II 10390

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	N-Rule	e⁻ Conf
413.2315	1	C 25 H 33 O 5	100.00	413.2323	0.8	1.8	1.5	9.5	ok	even

18 APPENDIX J: INFRARED SPECTROSCOPY DATA OF COMPOUND 2