

**PHARMACOLOGICAL EVALUATION OF SOME CENTRAL NERVOUS
SYSTEM EFFECTS OF *COTYLEDON ORBICULATA*.**

BY

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENT FOR THE DEGREE OF MAGISTER
PHARMACEUTICAE IN THE SCHOOL OF PHARMACY, DEPARTMENT
OF PHARMACOLOGY, AT THE UNIVERSITY OF THE WESTERN CAPE.**



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MAY 2005

DEDICATION:

THIS MASTER'S THESIS IS DEDICATED

TO MY

LATE FATHER

BICAKUMUYANGE JOHN

FOR

HIS HUMILITY, LOVE, CARE AND VALUE OF EDUCATION

MAY GOD REST HIS SOUL AND GIVE HIM ETERNAL PEACE

**PHARMACOLOGICAL EVALUATION OF SOME CENTRAL NERVOUS
SYSTEM EFFECTS OF *COTYLEDON ORBICULATA*.**

KEYWORDS.

Cotyledon orbiculata

Medicinal plants

Extraction

Methanolic extract

Phytochemical and HPLC analysis

Chromatographic techniques

Mice

Pharmacological evaluation

GABAergic and Glutaminergic mechanisms

Anticonvulsant properties

Analgesic properties

ABSTRACT.

PHARMACOLOGICAL EVALUATION OF SOME CENTRAL NERVOUS SYSTEM EFFECTS OF *COTYLEDON ORBICULATA*.

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The use of traditional medicine through the use of medicinal plants in Africa and especially in South Africa has long been considered an important characteristic of people's daily lives and socio-cultural heritage. *Cotyledon orbiculata* Linn. (Family: *Crassulaceae*), is among the medicinal plants that are used by South African traditional practitioners for the treatment of epilepsy and painful conditions such as corns, warts, toothache, earache, boils and various other ailments. However, the claims of therapeutic successes of medicinal plants by traditional medicine practitioners are hardly subjected to scientific scrutiny. This study, therefore, investigated the anti-epileptic property of *C. orbiculata* by studying the effects of the methanol extract of the plant against chemically induced seizures by pentylenetetrazole (PTZ), picrotoxin, bicuculline and N-methyl-DL-aspartic acid (NMDLA) in mice. The study also investigated the analgesic effects of *C. orbiculata* by studying the effect of the plant extract on pain induced by acetic acid and hot plate thermal stimulation.

Methanolic extract of *C. orbiculata* (100 and 200 mg/kg, i.p.) protected 50% of the animals against PTZ-induced seizures and significantly ($P < 0.02$, and 0.05) delayed

the onset of tonic seizures in mice. A dose of 400 mg/kg, i.p. of *C. orbiculata* protected 62.5% of the animals against PTZ-induced seizures. The dose (400 mg/kg, i.p.) significantly ($P < 0.001$) reduced the number of animals convulsing and significantly delayed the onset of PTZ-induced seizures in mice. The standard anti-epileptic drugs, phenobarbitone and diazepam (12 mg/kg and 0.5 mg/kg, i.p. respectively) significantly antagonised the seizures produced by PTZ. Similarly, the same dose of plant extract (100, 200 and 400 mg/kg, i.p.) protected the animals against seizures elicited by picrotoxin and significantly ($P < 0.02-0.001$) delayed the onset of picrotoxin-induced seizures. *C. orbiculata* (100-200 mg/kg, i.p.) protected animals against bicuculline-induced seizures. However, a dose of 100 mg/kg i.p. did not significantly alter ($P > 0.05$) the onset of bicuculline-induced seizures. *C. orbiculata* (400 mg/kg, i.p.) did not protect the mice against seizures-induced by bicuculline but significantly delayed the onset of the seizures. All the animals used were protected against bicuculline seizures either by phenobarbitone or diazepam. N-Methyl-DL-aspartic acid (400 mg/kg, i.p.) elicited seizures in all the animals used. Methanol extract of *C. orbiculata* (100 mg/kg, i.p.) did not affect the incidence of NMDLA-induced seizures, but significantly ($P < 0.005$) delayed the onset of the seizures. *C. orbiculata* (200 and 400 mg/kg, i.p.) neither affected the incidence, nor the onset of seizures induced by NMDLA. Similarly, phenobarbitone, diazepam and DMSO (0.25ml) did not protect any of the animals against NMDLA-induced seizures or affect the onset of the seizures. Methanol extract of *C. orbiculata* (100-400 mg/kg, i.p.) in a dose dependent manner, significantly ($P < 0.02 - 0.001$) reduced the number of acetic acid-induced writhes respectively. Similarly, paracetamol (300 mg/kg i.p.) profoundly reduced the number of writhes elicited by 3% acetic acid. *C. orbiculata* (100 and 200 mg/kg, i.p.) significantly ($P < 0.025-0.005$) delayed the

reaction time of the animals to hot-plate thermal stimulation 30 minutes after the treatment. At a dose of 400 mg/kg, i.p., of *C. orbiculata* significantly prolonged the pain reaction time of the mice to hot-plate thermal stimulation at 15, 30, 45 and 60 minutes after treatment, with significant values of $P < 0.05$, 0.001, 0.005 and 0.001 respectively. Similarly, morphine (10 mg/kg, i.p.) significantly ($P < 0.001$) delayed the reaction time of the animals to hot-plate thermal stimulation throughout the 1 hour period of the experiment. The results obtained in this study shows that the purified methanolic extract of *C. orbiculata* produced both anticonvulsant and analgesic activities at doses as low as 25mg/kg, i.p., unlike the crude extract of *C. orbiculata* which produced its anticonvulsant and analgesic activities at a minimum dose of 100 mg/kg, i.p.

The results obtained in this study indicate that *C. orbiculata* possesses both anticonvulsant and analgesic activities. The results also indicate that *C. orbiculata* may be exerting its anticonvulsant activity by affecting both gabaergic and glutaminergic systems. The data on analgesic effect show that *C. orbiculata* may be exerting its analgesic effect both peripherally and centrally.

The data obtained from the phytochemical analysis of the leaves of *C. orbiculata* indicated the presence of tannin, saponin, cardiac glycosides, reducing sugars and triterpene steroids. The chromatographic spectrum of *C. orbiculata* obtained reveals characteristic peaks at the following retention times (minutes): 6.983, 10.521, 12.088, 12.838 and 13.342.

May 2005 .

DECLARATION

I declare that “*PHARMACOLOGICAL EVALUATION OF SOME CENTRAL NERVOUS SYSTEM EFFECTS OF COTYLEDON ORBICULATA*” is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

JOSEPH KABATENDE

MAY 2005

Signed.....

ACKNOWLEDGEMENTS

Behavioural scientists do things in groups, as indeed, no man is an island. Aware that I have transversed through a great deal of people, benefiting from them in different ways, I wish to express my profound gratitude to quite a great number of them, but by no means all. As always, to God be the glory for his providence in seeing me through against all odds.

My special thanks go to my indefatigable supervisor, George Jimboyeka Amabeoku, Professor of Pharmacology at the School of Pharmacy in the University of the Western Cape. I consider myself exceptionally fortunate in having enjoyed the intellectual stimulation and support from him in the short space of time, and I am glad to have the opportunity to thank him. He is such a supervisor whose attitudinal dispositions transcend the ordinary level of lecturer-student relationship to cordiality and familiarity. I would have been tempted to refer to him as a friend in both need and indeed, but for his scientific status and his intellectual intimidation, I hesitate to do so. I am indeed indebted to him and his amazing hospitable family whose care and love every time I visit make me feel an overwhelming sense of nostalgia.

I wish to equally thank Professor Ivan Robert Green for co-supervising this current work and his valuable input as far as the chemistry part of this work was concerned. I appreciate and wish to give thanks to the government of the Republic of Rwanda for granting me the financial support for the accomplishment of this study.

I wish to thank Professor James Abraham Syce for his valuable help during my admission processes when I was still at home and his continued comments and encouragements throughout the course.

The University of the Western Cape for granting me a place to further my studies.

To Dr. Lyantagaye Sylvester for introducing me to the medicinal plant extraction techniques and his valuable constant inputs and advice during my study period.

To Brian Minnis, Yusuf Alexander, and Vinesh Jeaven for their technical assistance. Thanks go to Mr Franz Weitz for identification of the plant material used in the current pharmacological evaluation.

To my late father Bicakumuyange John, he will continue to receive my posthumous appreciation and thanks for his vision in laying the formation of my academic height. It is impossible to convey the debt of gratitude to my mother, brother, sisters and my uncle and his family for their extraordinary and unassuming care and encouragement to me when abroad for my study.

To a number of individuals who contributed in one way or the other to the success of this work. I am indeed grateful to you all especially my classmates and friends: Claude Bayingana, Denis Muhire, Gaspard Musonera, Egide Kayonga, Bayingana Blaise, Niyonkuru Richard, Kagaba James, Rwigema Jean Bosco, Kabagema David, and Silver Richards Karumba.

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CHAPTER 1. INTRODUCTION

Traditional medicine (TM) occupies an important place in the healthcare systems of developing countries. The World Health Organization (WHO) estimates that more than 80% of healthcare needs in these countries are met through traditional health care practices. The people in developing countries depend on TM, because it is cheaper and more accessible than orthodox medicine (Sofowora, 1982; WHO, 2002; Tabuti *et al.*, 2003). Traditional medicine is also more acceptable because it blends readily into peoples' socio-cultural life. Traditional medicine is the total knowledge, skills and practices based on the theories, beliefs and indigenous cultural experiences, whether explicable or not, used in the maintenance of health, diagnosing, preventing or eliminating physical, mental or social diseases. Such knowledge may rely exclusively on past experience and observations handed down from generation to generation, verbally or in writing (Sofowora, 1982; Van Wyk *et al.*, 2000; WHO, 2002; Tabuti *et al.*, 2003). The traditional medicine system is holistic in that its application usually covers the mind, body and soul (WHO, 2002). The concept includes mystical and magical rituals, herbal therapy, psychiatry and other treatments, which may not be explained by modern medicine. Studies suggest that this therapy is applied to conditions such as cancer, arthritis, chronic back pain, gastrointestinal problems, chronic renal failure, eating disorder, physical, mental or social disease and so on (Sofowora, 1982). Medicinal plants continue to draw wide attention for their roles in case of mild/chronic diseases, and herbal medicines have received an increasing interest as documented by the numerous and rigorous published studies (Mauri *et al.*, 2000).

South Africa has a huge diversity of tribes, which is reflected in the systems of medicine practised. Traditional healers are most commonly known by Zulu people

as, “inyangas”, or herbalists and, “isangomas”, or diviners. However, the distinction between the two has become blurred, with both using herbal medication. Practitioners in other groups are known as, “ixwele”, and, “amaqira”, (Xhosa), “ngaka”, (Sotho) and, “nanga”, “mungome” or “Maine”, (Vhavenda) (Steenkamp, 2003). Most elderly folks in rural areas have knowledge of herbal lore, which they apply. Moreover, there are also faith healers who treat central nervous system disorders and other related problems. Plants have for generations, been a source of various kinds of remedies and have been used for medical purposes to cure all kinds of ailments and will continue to provide remedies for these ailments (Van Wyk *et al.*, 2000).

In South Africa, as in many other countries, traditional medicine is part of the culture of the people that use it. As a result, it is closely linked to their belief and has remained informal. Its successes have been based on oral traditions (Sofowora, 1982). A number of medicinal plants have been shown to offer an alternative to synthetic drug substances in preventing and treating some chronic and mild diseases, provided that they are of adequate quality and properly used. Many factors influence the quality of herbs and these include species variation, environmental conditions, and time of harvesting, storage and processing. For these reasons, the quality control of herbal extracts is an essential part of any research involving safety, efficacy and therapeutic reproducibility. Quality control is never easy because medicinal plant extracts are complex mixtures of different compounds and often their identity is only partially known. Among the active principles present in medicinal plants, flavonoids, terpenes and caffeic acid derivatives have attracted a great interest in scientific research (Mauri *et al.*, 2000).

The use of plant medicines in the treatment of various ailments, including central nervous system disorders, is an age long practice. It is important to note that plant medicines are also gaining popularity in developed countries. Herbal medicine is currently enjoying a revival in popularity in the west and in fact it is the primary form of medicine in many parts of the world (Williamson *et al.*, 1996). With the great reliance on this type of medicine, it becomes pertinent to search for potent, effective and relatively safe plant medicines as well as scientific validation of the success claims about plants already in use by traditional medicine practitioners in order to enhance their safety and efficacy. These are some of the problems making this alternative healthcare system less acceptable, especially by orthodox medicine practitioners.

Although many traditional medicines or complementary, alternative medicine (TM/CAM) therapies have promising potential, and are increasingly used, many of them are untested and their use is not monitored. As a result, knowledge of their potential side effects is limited. This makes identification of the safest and most effective therapies and promotion of their rational use more difficult (WHO, 2002).

According to some authors in the field of plant medicines, the interpretation of data in terms of potential therapeutic application of plant extracts, including pure extracts, must depend on the total pharmacological profile of the extracts (Atta and Alkofani, 1998; De Sarro *et al.*, 1999; Rabbani *et al.*, 2003).

Furthermore, there is very little scientific data on traditional medicinal plants used for central nervous system disorders in this country. Hence, the present study aims to investigate some of the central nervous system effects of *Cotyledon orbiculata* such as antiepileptic and analgesic properties.

CHAPTER 2. LITERATURE REVIEW

2.1. INTRODUCTION.

2.1.1. Overview on traditional medicine.

The interest in traditional knowledge is more and more widely recognised in the development of policies, the media and scientific literature. In Africa, traditional healers and remedies made from plants play an important role in the health of millions of people. The relative ratios of traditional practitioners and orthodox doctors in relation to the whole population in African countries are revealing. In South Africa, for example, in Venda for every traditional medical practitioner, there are 700-200 people, against one university trained doctor for nearly 1639 people (Cunningham, 1997).

Traditional medicine has been described by the World Health Organisation (WHO) as one of the surest means to achieve total healthcare coverage of the world's population. In spite of marginalisation of traditional medicine in the past, the attention currently given by various governments to widespread healthcare application has given a new drive to research, investments and design of programmes in this field in several developing countries (Cunningham, 1993).

Although the main consumers of medicinal plants in Africa have been, until recently, the local population, the field has started to attract a number of local and foreign researchers who have discovered the value of traditional healing. The first undertakings done in this field in Africa were, undoubtedly, of ethno-botanical nature, but since then, the fields of study have expanded to include pharmacology, phytochemistry and chemistry of natural products, organic synthesis and the usefulness of medicinal and aromatic plants.

The pharmaceutical industry has come to consider traditional medicine as a source for identification of bio-active agents that can be used in the preparation of synthetic medicine.

Many of the more pharmacologically (commercially) interesting medicinal plant species in use around the world, are employed in more than one community, and often in more than one country, for multiple uses (Bodeker, 1994). It is important to note that even in contemporary rural Africa, there is no doubt about the efficacy of herbal medicine. Many Africans, especially rural people and the urban poor, rely on the use of herbal medicine when they are ill. In fact, many rural communities in Africa still have areas where traditional herbal medicine is the major source of healthcare available. Thus, there can be no doubt about the acceptability and efficacy of herbal remedy within African society (Sofowora, 1982).

South Africa is blessed with a rich cultural diversity, which is reflected in the formal and informal systems of medicine that are presently practiced in different parts of the country. Traditional medicine is a comprehensive term used to refer to both traditional medicine systems such as traditional Chinese medicine, Indian ayurveda and Arabic unani medicine, and to various forms of indigenous medicine. It is a system of medicine that is based on past experience and cultural beliefs and practices handed down from generation to generation, verbally or in writing. The concept includes mystical and magical rituals, herbal therapy, psychiatry and other treatments, which may not be explained by modern medicine. Traditional medicine practitioners (TMPs) are people recognised by their communities as competent to provide health care by using biotic and a biotic substance and certain other methods

(Sofowora, 1982; Van Wyk *et al.*, 2000; WHO, 2001; Bienvenu *et al.*, 2002; Tabuti *et al.*, 2003).

A traditional healer is commonly described as a person who is recognised by the community to which he or she belongs as competent to provide healthcare. The person can use vegetable, animal and mineral substances and/or certain other methods which take inspiration from social, cultural and religious backgrounds (Sofowora, 1982; Bienvenu *et al.*, 2002).

2.1.2. Advantages and Disadvantages of Traditional Medicine

The Western Cape Province of South Africa is endowed with numerous plants species, which are used for the treatment of various ailments because of their medicinal properties. These include plants like *C. orbiculata* that is used for the treatment of epilepsy and painful conditions (Van Wyk *et al.*, 2000).

However, although many TM/CAM therapies have promising potential and are increasingly used, many of them are untested, and their use not monitored. As a result, knowledge of their potential side effects is limited. This makes identification of the safest and most effective therapies and promotion of their rational use more difficult (WHO, 2001). Plant medicines have, therefore, several disadvantages among which are:

- a) The lack of scientific proof of their efficacy and toxicology profile (Sofowora, 1982).
- b) Toxic chemicals, such a selenium and arsenic are naturally present in some soil and contaminate the medicinal plants growing in such areas (Muhizi, 2002). These contaminated plant medicines may pause serious health hazards.
- c) Due to the fact that the traditional medicine practitioners do not know the pathology of certain

diseases, they end up treating the symptoms rather than the disease because of insufficiency and often the imprecision of diagnosis done by the practitioner before giving the plant medicine (Sofowora, 1982; Muhizi, 2002). According to Williamson *et al.*, (1996), plant medicines are, however, advantageous as being safer and less damaging to the human body than synthetic drugs. Furthermore, underlying this upsurge of interest in plants is the fact that many important drugs in use today were derived from plants or starting molecules of plant origin: digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples. They have also yielded molecules that are extremely valuable tools in the characterisation of enzymes and classification of receptor systems: physostigmine, morphine, muscarine, atropine, nicotine and tubocurarine are important examples.

The efficacy of medicinal plants has thus encouraged chemists and pharmacists to carry out rigorous analysis on the plants in order to establish a relationship between the chemical composition and therapeutic activities (Vickery *et al.*, 1979; Williamson *et al.*, 1996; Bienvenu *et al.*, 2002).

2.1.3. Basic Comparison of Traditional and Modern Medicine.

Plants were once the primary source of medicine in the world, and they still continue to provide mankind with new remedies. Natural products and their derivatives represent more than 50% of drugs in clinical use today, higher plants contribute no less than 25% to the total. South Africa is blessed with a rich cultural diversity, which is reflected in the formal and informal oral-tradition medical systems of the Khoi-San peoples, the Nguni and Sotho-speaking peoples. These systems have not yet been systematised, and are passed on by word of mouth from one generation to the next. These medical systems and their herbal, animal and mineral *materia*

medica have ancient origins, which date back to palaeolithic times. The formal system of medicine, which is well documented and systematised, was introduced to the country over the last three hundred years by the Europeans and other settlers and is exemplified by today's modern western medicine (Van Wyk *et al.*, 2000).

Each system of medicine is the art and science of diagnosing the cause of disease, treating disease and maintaining health in the broadest sense of physical, spiritual, social and psychological well-being. Each culture has found solutions to the preventive, promotive and curative aspects of health that resonate in harmony with the worldview of that culture. The western medicine may diagnose a disease in terms of bacterial infection, for example, and treat that infection effectively with antibiotics. An African traditional healer will seek to understand why the patient became ill in the first place, and the treatment administered will address the perceived cause, usually in addition to specific therapies to alleviate the signs and symptoms of the condition (Van Wyk *et al.*, 2000).

Western biomedicine uses the results of experiments, and disease is regarded as a cause by physiopathological agents, whereas traditional medicine still accepts the fact that disease can be due to supernatural causes or the intrusion of an object into the body (Sofowora, 1982). This system of medicine emphasizes psychological causes of disease and the link between traditional medicine, culture and beliefs (Bienvenu *et al.*, 2002). Plant analysis, therefore, should take account of cultural norms and beliefs of the people and the way they use them.

2.2. PATHOPHYSIOLOGY OF EPILEPSY

2.2.1. Background and definitions.

Epilepsy is a progressive disorder comprising of many seizure types and syndromes. Despite the introduction of eight new anti-epileptic drugs (AEDs) since 1993, a significant percentage of patients with epilepsy continues to experience seizures despite aggressive treatment with one or more AEDs. As a result, there continues to be an unmet clinical need for more effective and less toxic anti-epileptic drugs (Feldman *et al.*, 1997; Barton *et al.*, 2003; Leonard, 2003; Rang *et al.*, 2003). Glutamate is the major excitatory neurotransmitter in the CNS and on release from presynaptic terminals, it binds to G-protein linked receptors either positively coupled to inositol phosphate metabolism or negatively coupled to adenylate cyclase (Webster and Jordan, 1989). Ionotropic receptors such as N-methyl-D-aspartate (NMDA), kainate, and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) are based on their activation by specific agonists. Therefore, the activation of these metabotropic and ionotropic receptors is important for normal CNS functions such as synaptogenesis, synaptic plasticity and the development of functional neural circuits. However, excessive levels of glutamate may be responsible for such pathological CNS processes as seizure generation or neurodegeneration following stroke and ischaemia. Therefore, the development of glutamate receptor agonists and glutamate antagonists has been viewed as a potentially important therapeutic strategy for the treatment of many neurological disorders (Barton *et al.*, 2003).

Anticonvulsants act to prevent the spread of the neuronal excitation by mechanisms that are not fully understood, but which can be roughly divided into those which involve stabilizing effect on excitable cell membranes, and those which involve

enhanced functional activity of neurotransmitters, such as gamma-amino butyric acid (GABA), which then act to inhibit spread of seizure activity by blocking synaptic transmission at some point. *Status epilepticus* is potentially fatal, and is a medical emergency requiring swift and effective treatment to minimize the risk of brain damage (Grammes-Smith and Aronson, 1984).

2.2.2. Types of Epilepsy

The agreed clinical classification of epilepsy recognises two categories, namely; partial seizures and generalised seizures, although there are some overlaps and many varieties of each. A seizure is said to be partial if it is restricted to a regional disturbance. Partial seizures are those in which the discharge begins locally and often remains localised. These may produce relatively simple symptoms without loss of consciousness, such as involuntary muscle contractions, abnormal sensory experiences or autonomic discharge, or they may cause more complex effects on consciousness, mood and behaviour, often termed psychomotor epilepsy (Rang *et al.*, 2003). Psychomotor epilepsy is often associated with a focus in the temporal lobe, the attack may consist of stereotyped purposeless movements such as rubbing or patting movements, or much more complex behaviour such as dressing, walking or hair combing. The seizure usually lasts for a few minutes, after which the patient recovers with no recollection of the event (Webster and Jordan, 1989). Partial seizures are subdivided further into simple partial and complex partial seizures. A simple partial seizure includes focal motor attacks and seizures with somato-sensory signs while complex partial seizures include those forms of temporal lobe or psychomotor seizures where consciousness is impaired (Jarrott, 1999; Porter and Meldrum, 2001; Rang *et al.*, 2003).

Generalised seizures involve the whole brain, including the reticular system, thus producing abnormal electrical activity throughout both hemispheres. Immediate loss of consciousness is characteristic of generalized seizures (Webster 1989; Sudarsky, 1990; Bienvenu *et al.*, 2002; Rang *et al.*, 2003).

The main categories are generalized tonic-clonic seizures (grand mal) and absences seizures (petit mal). A generalized tonic-clonic seizure consists of an initial strong contraction of the whole musculature, causing a rigid extensor spasm. Respiration stops and defaecation, micturition and salivation often occur. This tonic phase lasts for about 1 minute and is followed by a series of violent, synchronous jerks, which gradually dies out in 2-4 minutes (Sudarsky, 1990). The patient stays unconsciousness for a few more minutes, and then gradually recovers, feeling ill and confused. Injury may occur during the convulsive episode. With optional drug therapy, therefore, epilepsy is controlled completely in about 75% of patients, but about 10% (about 50 000 in Britain) continues to have seizures at intervals of one month or less, which severely disrupt their life and work (Rang *et al.*, 2003). There is, therefore, a need to improve the efficacy of therapy.

2.2.3 Causes of Convulsions

Approximately 1% of the world's population has epilepsy, the second most common neurological disorder after stroke (Porter and Meldrum, 2001). The cause of convulsions must be clearly understood through some precise observations. The type of seizure depends on the site of the focus in the brain. Epileptic attack can be caused by biochemical insults to the brain, such as during hypoglycaemia, anoxia, hypocalcaemia, hyperventilation, water intoxication and sudden withdrawal of certain drugs such as barbiturates or alcohol (Bienvenu *et al.*, 2002). Epilepsy can

also be caused by previous active pathology, such as birth trauma to the brain, during or following meningitis, trauma to the skull and brain later in life, cerebral abscesses, cerebral infarction, cerebral haemorrhage or subarachnoid haemorrhage (Biller, 1997; Bienvenu *et al.*, 2002).

Further analysis shows that the blockade of post-synaptic gamma-amino butyric acid (GABA) receptors or an inhibition of GABA synthesis is the principal origin of brain discharge (Delgado *et al.*, 1970; Muhizi, 2002). According to Bienvenu and co-workers (2002), an epileptic attack can be triggered by a sensory stimulus, which is specific for individuals. To date, there is no single unifying explanation as to how these diverse factors cause seizures. Hence, it is difficult to determine the exact cause of epilepsy, even though it has been possible to investigate the physiological events which participate in the genesis of epilepsy (Sudarsky, 1990)

2.2.4. Neuropathology of Epilepsy.

The pathology found in the brains of patients with epilepsy is of three types:

- a) Focal lesions that are themselves the primary causes of secondary epilepsy. These include congenital malformations (vascular), neoplasm, traumatic lesions, infarcts abscesses, cysts and parasitic infestations (Webster and Jordan, 1989; Porter and Meldrum, 2001; Rang *et al.*, 2003). In general, there is no clear pathological difference between such lesions, whether they occur in patients with or without epilepsy.
- b) Degenerative disease (diffuse infective), that is primary, and has no specific relationship to epilepsy but may be associated with epilepsy (including focal seizures and myoclonic syndrome). This group includes pathologies as diverse as the

leucodystrophies, cerebral malaria, Huntington's chorea, and Alzheimer's disease (Webster and Jordan, 1989).

c) Epileptic brain damage, a pathological syndrome which is found to varying degrees both in patients with primary or idiopathic epilepsy and those in whom epilepsy is clearly secondary to some focal or generalised disorder. It is characterised by highly selective neuronal loss and by glial proliferation, which is partly selective and partly diffuse (Webster and Jordan, 1989). The lesions are either a consequence of seizure activity or in the perinatal period or early childhood.

In the hippocampus, the neuronal loss involves selectively the pyramidal neurons in the endfolium and the sommer sector. In this case, the cerebellum, the purkinje and the basket cells are selectively involved, while in the neocortex, smaller pyramidal neurons, particularly in the third cortical lamina, are most vulnerable. The evidence that such pathological changes are secondary to epilepsy comes from both clinical observations and animal experiments. Animal studies of drug induced *status epilepticus* (either generalised, due to bicuculline or allylglycine or focal limbic due to kainic acid) also show neuronal loss or its prodromal manifestation, ischaemic cell change occurring with similar selectivity in the hippocampus, neocortex and cerebellum (Webster and Jordan, 1989; Rang *et al.*, 2003).

2.2.5. GABA and Epileptogenesis

The major amino acid neurotransmitters in the brain are GABA, an inhibitory transmitter and glutamic acid, an excitatory transmitter. GABA is widely distributed in the mammalian brain and has been shown to contribute to over 40% of the synapses in the cortex alone. While it is evident that a reduction in GABAergic activity is associated with seizures, and most anticonvulsant drugs either directly or indirectly facilitate GABAergic transmission, GABA also has a fundamental role in the brain by shaping, integrating and refining information transfer generated by the excitatory transmitters (Leonard, 2003).

The GABAergic system has long been implicated in epilepsy with defects in GABA neurotransmission being linked to epilepsy in both experimental animal models and human syndromes. However, to date, no human epileptic syndromes may be directly attributed to an altered GABAergic system (DeLorey, 1999). The observed defects in GABA neurotransmission in human epileptic syndromes may be the indirect result of a brain besieged by seizures (DeLorey, 1999). There is now evidence that many epilepsy-associated genes participate in secondary cellular plasticity during brain development, which is likely to influence downstream events. These influences may account for the delayed temporal onset of seizures observed in some epileptic syndromes (DeLorey, 1999). It is becoming increasingly clear that pathophysiological features associated with seizures and their treatments in mature brain do not necessarily apply to the developing brain. Despite this, relatively little attention has been focused on how pathophysiology, during the development, may render the brain epileptic later in life (DeLorey, 1999).

Drugs have been developed to modulate GABA function; the inhibitors of GABA transaminases, which metabolize GABA, have been shown to be effective

anticonvulsants. These are derivatives of valproic acid that do not only inhibit the metabolism of GABA, but also act as antagonist of GABA autoreceptor and thereby enhance the release of the neurotransmitter (Leonard, 2003).

GABA_A receptor is directly linked to chloride ion channels, activation of which results in an increase in the membrane permeability to an influx of chloride ion, and thereby the hyperpolarization of cell bodies. GABA_A receptors are also present extrasynaptically where, following activation, can depolarize neurons. The convulsant drug, bicuculline, acts as a specific antagonist of GABA on its GABA_A receptor site, while the convulsant drug, picrotoxin, binds and blocks the chloride ions channels linked to GABA_A receptors and directly decrease chloride ion influx. Barbiturates, on the other hand, have the opposite effect on the chloride ion channel, which remains open to allow influx of chloride ions into the brain cells (Leonard, 2003; Burt, 2003).

2.2.5.1. Clinical Implications.

GABA mediates its action in the mammalian brain via at least two classes of receptors, GABA_A (ionotropic) and GABA_B (metabotropic) receptors, which differ in terms of their pharmacological profile, mechanisms of transduction and regional distribution (De Sarro *et al.*, 2000). GABA released from pre-synaptic terminals, acts on both GABA_A and GABA_B receptors. GABA_A receptors are post-synaptic while GABA_B receptors are both pre- and post-synaptic. Previous studies have suggested that, the functional role of the pre-synaptic GABA_B receptors is to regulate the release of both excitatory and inhibitory neurotransmitters (in particular, baclofen, a selective GABA_B receptor agonist, is to elicit a down-regulation of GABA_B function and to decrease transmitter release whilst GABA_B receptor

antagonists increase the release of both excitatory amino acids and GABA (De Sarro *et al.*, 2000). Both GABA_A and GABA_B receptors are involved in the control of neuronal excitability and epileptogenesis but, whereas much is known about the involvement of GABA_A receptors in the control of generalised convulsive seizures, the role of the GABA_B receptors in epileptic process is not fully understood (De Sarro *et al.*, 2000).

Effects on synaptic transmission have been sought for many anti-epileptic drugs. Enhancement of GABA-mediated inhibition can be produced in many different ways, involving either direct action on the GABA_A - receptor-chloride channel ionophore complex, or action on reuptake or metabolism of GABA (Porter and Meldrum, 2001). This mechanism provides protection against generalised tonic - clonic and focal seizures.

2.2.6. Glutamic Acid and Epilepsy.

Glutamate is the most important excitatory neurotransmitter in all rapidly conducting relay pathways of the motor and sensory systems of the outer tube of the central nervous system. It produces fast or prolonged synaptic excitation, and triggers various calcium dependent processes in the target cells, including production of nitric oxide (Bienvenu, *et al.*, 2002). Glutamate also plays a major role in synaptic plasticity and in the processes of learning and memory (Rang *et al.*, 2003).

According to Westmoreland *et al.*, (1994), glutamate is a transmitter in the corticospinal, corticostriatal pathways, intrahemispheric and interhemispheric association pathways, hippocampal circuits, primary afferents, and somatosensory and special sensory pathways, cerebellar afferents and excitatory inter-neurons.

Epilepsy may arise as a consequence of a dramatic release of glutamate from central nerve terminals. Sustained seizures of the limbic system in experimental animals result in brain damage that resembles that due to glutamate toxicity. Similar changes are seen at autopsy in patients with intractable epilepsy. In animals, such seizure related brain damage may be reduced by the administration of non-competitive NMDA receptor antagonists, but it would appear that not all seizure activity is suppressed by such drugs (Leonard, 2003). The precise mechanism whereby persistent seizure activity results in neuronal degeneration is not completely understood. It seems possible that repetitive depolarization and repolarization of the nerve membrane eventually leads to an energy-deprived state within the cell, thereby preventing the restoration of the cell membrane potential. Each depolarization will also lead to an influx of calcium ions and efflux of potassium ions, which if prolonged, can result in cell death. The reduced efficiency of glial cells to remove potassium ions, and the ability of high extracellular concentration of potassium ions to depolarize neurons and cause neurodegenerative changes also play a critical role in causing the degenerative changes that are a feature of *status epilepticus* and intractable epilepsy (Leonard, 2003). Recent advances have indicated that gamma (γ)-amino butyric acid (GABA_A) receptors work synergistically with N-methyl-D-aspartic acid (NMDA) receptors to increase the flux of Ca²⁺ ions into neuroblasts and immature neurons. This is essential for the modulation of early CNS development (DeLorey, 1999). It is evident that, GABA is a critical inhibitory transmitter, and seizures can readily be elicited by pharmacological disruption of GABAergic mechanism (Feldman *et al.*, 1997). Drugs have also been developed to modulate glutamic acid function. Reduction of excitatory glutamatergic neurotransmission is potentially important; AMPA receptor blockade probably contributes to the

antiepileptic effect. Topiramate and NMDA receptor blockade contribute to the antiepileptic effect of drugs such as lamotrigine (Porter and Meldrum, 2001).

2.2.7. Animal Models of Epilepsy.

Animal models of epilepsy also show a differential effectiveness of anticonvulsant drugs. Additionally, they permit the investigation of underlying biochemical changes. Several animal syndromes of epilepsy appear to be genetically determined. In mice there are at least 12 single locus mutations that produce neurological syndromes with spontaneous seizures. One syndrome known as tottering has aroused much interest because the spontaneously occurring seizures resemble absence attacks both behaviourally and electroencephalographically (EEG). Among the syndromes showing seizures precipitated by sensory stimulation, sound-induced and posturally induced seizures in mice have been extensively studied biochemically (Webster and Jordan, 1989; Leonard, 2003). Genetic and kindling models have been widely used to investigate possible neurotransmitter defects that cause different types of epilepsy. Rodent models in which seizures are induced by electroshock or by convulsant drugs such as pentylenetetrazole, picrotoxin or bicuculline, are frequently used in screening procedures to identify potential anticonvulsants (Leonard, 2003; Rang *et al.*, 2003).

Studies of the biochemical or molecular mode of action of anticonvulsant drugs have also indicated many ways in which disturbed neurotransmitter function can facilitate or provoke seizures (Webster and Jordan, 1989).

2.3. ANALGESIA.

2.3.1. Introduction

Pain is considered to be a sensation resulting from any tissue-damaging stimulus and is essential for survival. Pain receptors, unlike the more specialised receptors of the other senses, can be activated by a variety of stimuli, including heat, cold, electrical impulses, pressure of stretching, cuts or tears and chemical irritants (Feldman *et al.*, 1997; Rang *et al.*, 2003). The quality of pain also varies and may be described as pricking, stabbing, burning, throbbing, aching and so on.

2.3.2. Pain and Nociception

The perception of noxious stimuli (known as nociception) is not the same thing as pain, which is a subjective experience, and includes a strong emotional component. The amount of pain that a particular stimulus produces depends on many factors other than the stimulus itself (Rang *et al.*, 2003). A stabbing sensation in the chest will cause much more pain if it occurs spontaneously in a middle-aged man than if it occurs in a 2-year old poking him in the ribs with a sharp stick. The nociceptive component may be much the same, but the effective component is quite different (Rang *et al.*, 2003). Animal tests of analgesic drugs commonly measure nociception, and involve testing the reaction of an animal to a mildly painful stimulus, often mechanical or thermal. Such measurements include the hot plate and the tail-flick tests (Williamson *et al.*, 1996). Clinically, spontaneous pain of neuropathic origin is coming to be recognised as particularly important, but this is difficult to model in animal studies because of technical and ethical reasons. It is recognised clinically that many analgesics, particularly those of morphine-type can greatly reduce the distress associated with pain even though the patients reports no great change in the

intensity of the actual sensation (Grammes-Smith and Aronson, 1984; Rang *et al.*, 2003).

Polymodal nociceptors (PMN) are the main type of peripheral sensory neurons that respond to noxious stimuli. The majority are non-myelinated c-fibres whose endings respond to thermal, mechanical and chemical stimuli. Chemical stimuli acting on PMN to cause pain include bradykinin, 5-HT and capsaicin. PMN are sensitized by prostaglandins, which explains the analgesic effect of aspirin-like drugs, particularly in the presence of inflammation. Nociceptive fibres terminate in the superficial layers of the dorsal horn, forming synaptic connections with transmission neuron running to the thalamus. PMN neurons release glutamate and various peptides, which act as slow transmitters. Peptides are also released peripherally and contribute to neurogenic inflammation. Neuropathic pain, associated with damage to neurons of the nociceptive pathway rather than an excessive peripheral stimulus, is frequently a component of chronic pain states, and may respond poorly to opioid analgesics (Rang *et al.*, 2003).

2.3.3. Analgesic Drugs

Pain may be modified by psychological factors and attention to these is essential in pain management. Drug treatment aims to modify the peripheral and central mechanisms involved in the development of pain (WHO, 2002). Neurogenic pain generally responds poorly to conventional analgesics.

The two main groups of analgesic drugs are the non-opioid and the opioid analgesics such as morphine. Non-opioid analgesics are particularly suitable for pain in musculoskeletal conditions, whereas opioid analgesics are more suitable for

moderate to severe visceral pain. The non-opioid analgesics, non-steroidal anti-inflammatory drugs (e.g. NSAIDs) have predominantly peripheral effects, although some poorly characterised central effects have been described. By contrast, the opiates produce analgesia by central actions although their side effects arise from central and peripheral actions (Webster and Jordan, 1989; WHO, 2002; Rang *et al.*, 2003).

2.3.4. Pharmacology of non-opioid analgesics

The analgesic effect of non-steroidal anti-inflammatory drugs (NSAIDs) may result from interference with the prostaglandins involved in pain. Prostaglandins appear to sensitize pain receptors to mechanical stimulation or to other chemical mediators. NSAIDs inhibit synthesis of prostaglandins peripherally and possibly centrally. Their anti-inflammatory action may also contribute indirectly to their analgesic effect. NSAIDs are used principally for symptomatic relief of mild to moderate pain and inflammation. Non-opioid analgesics include diclofenac sodium, diflunisal, etodolac, fenoprofen calcium, flurbiprofen sodium, ibuprofen, acetylsalicylic acid and paracetamol, to mention but a few (Webster and Jordan, 1989; Fawcett, 1999; WHO, 2002; Rang *et al.*, 2003).

2.3.5. Pharmacology of opioid analgesics.

The term 'opioid' applies to any substance, whether endogenous or synthetic, that produces morphine-like effects that are blocked by antagonists such as naloxone (Rang *et al.*, 2003). Opioids act as agonists at specific opiate receptor binding sites in the central nervous system (CNS) and other tissues. These are the same receptors occupied by endogenous opioid peptides to alter CNS response to painful stimuli.

The opiate agonists do not alter the cause of pain, but only the patients' perception of the pain. They relieve pain without affecting other sensory functions. Opiate receptors are present in highest concentration in the limbic system, thalamus, striatum, hypothalamus, midbrain and spinal cord (Stahl, 2000). Opioids produce varying degrees of analgesia and have antitussive, anti-diarrhoeal and sedative effects. They may be used in the symptomatic management of moderate to severe pain associated with acute and chronic disorders, including renal or biliary colic, myocardial infarction, acute trauma, post-operative pain or terminal cancer. Opioid analgesic drugs include: morphine sulphate, pethidine, codeine phosphate, nalorphine, levallorphan, alfentanil hydrochloride, codeine sulphate to mention but a few. Morphine remains the most valuable analgesic for the treatment of severe pain. In addition to pain relief, it confers a state of euphoria and mental tolerance, but this should not be a deterrent for its use in the control of pain in terminal illness (Fawcett, 1999; WHO, 2002; Rang *et al.*, 2003).

2.3.6. Pain Transmissions.

Pain receptors, unlike other sensory receptors such as pacinian corpuscles that respond to skin indentation, have not been identified. It is, therefore, presumed that nociceptors, pain sensory receptors, must be the free bared nerve endings found profusely in the dermal and epidermal layers. Pain is transmitted from these free nerve endings by an unknown transduction process, but endings connect to the spinal cord, where the first synapses occur, by the Ad, fine myelinated or c-fibres which remain unmyelinated. Since these fibres have little or no myelin, they conduct slowly, Ad fibres in the range of 430m/s and c-fibres at less than 2.5m/s. These primary afferent fibres, accessible to electrophysiological techniques in man and animals, are responsive to noxious stimuli. In addition to the innervations of

cutaneous tissue, Ad and c-fibres innervate cardiac muscle and the tooth pulp and analogous fibres carry painful information from the skeletal muscles (Webster and Jordan, 1989; Rang *et al.*, 2003).

The non-opioid analgesics block cyclooxygenase, which converts arachidonic acid to prostaglandins, and so are able to prevent the sensitisation of receptors to allogeic substances, hence their peripheral analgesic effects. Local anaesthetics, can block transmission of the sensory signal either by a direct action on the fibres or on the dorsal root ganglion, and the opiates can reduce pain messages at the spinal level either by direct actions within the cord or through control descending from the brain (Webster and Jordan, 1989; Rang *et al.*, 2003).

2.4. DESCRIPTION OF THE PLANT, *COTYLEDON ORBICULATA*

2.4.1. Introduction

Cotyledon orbiculata Linn, belongs to the family Crassulaceae. *C. orbiculata* is among the medicinal plants that are used by South African traditional practitioners for the treatment of various ailments (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk *et al.*, 1997; Van Wyk *et al.*, 2000).

This common succulent is a small shrub with woody branches and thick, fleshy leaves. The leaves are bright green to grey, often with a reddish margin and usually covered with a waxy layer on the surface. Orange or red tubular flowers are borne on a long, slender stalk (Fig.1). *C. orbiculata* is a very variable species and several different varieties have been described. (Van Wyk *et al.*, 2000).

Cotyledon orbiculata is widely distributed practically over the whole of Southern Africa. But it is usually confined to rocky outcrops in grassland fynbos and karoo regions. Black frost will damage the flowers if planted in an unprotected spot, but the plant itself will tolerate moderate frosts. *C. orbiculata* is known locally as “Seredile” in Sotho and Tswana, “Plakkie” in Afrikaans, “Imphewula” in Xhosa, “intelezi” or “ipewula” in Zulu, and “pig’s ears” in English (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk *et al.*, 2000; Harris, 2004).

The fresh part of the leaf is applied to corns and warts to soften and remove them. Van Wyk *et al.*, (2000) and Hutchings (1996) reported that a single leaf is eaten as a vermifuge juice and that the warmed juice can be used as drops for toothache or earache. They also reported that the fresh leaf juice has been used to treat epilepsy in the Cape. Warmed leaf poultices are applied to boils and other inflammations by Xhosa people (Watt and Breyer-Brandwijk, 1962).



Fig.1. *Cotyledon orbiculata* plant photographed by Kabatende, J. 2004, at Kristenbosh National Botanical Gardens.

2.4.2. Objectives

The use of traditional medicine through the use of medicinal plants in Africa and especially in South Africa has long been considered an important characteristic of people's daily lives and socio-cultural heritage (Van Wyk *et al.*, 2000). Plants were once a primary source of medicines in the world and they still continue to provide mankind with new remedies. However, the claims of therapeutic successes by traditional medicine practitioners have not been exhaustively subjected to scientific scrutiny. Therefore, plants with medicinal properties are selected based on their perceived ability to restore harmony and frequently used in a manner which doesn't allow pharmacological interpretation of their efficacy (Sofowora, 1982; Williamson *et al.*, 1996; Van Wyk *et al.*, 2000; Bienvenu *et al.*, 2002). *C. orbiculata* is used in this way for its potential anti-epileptic and analgesic activities. The danger with this type of practice is that, little is known about the mechanisms through which its actions are manifested and also its potential level of toxicity.

These arguments are supported by the fact that little or no scientific data exists about *C. orbiculata* particularly as it pertains to its anti-epileptic and analgesic properties.

This study, therefore, investigated the anti-epileptic property of *C. orbiculata* by studying the effects of the methanol extract of the plant against chemically induced seizures by pentylenetetrazole (PTZ), picrotoxin, bicuculline, N-methyl-DL-aspartic acid (NMDLA) in mice. The study also investigated the analgesic effects of *C. orbiculata* by studying the effect of the plant extract on pain induced by acetic acid and thermal stimulation.

CHAPTER 3. MATERIALS AND METHODS:

3.1. PLANT MATERIALS

3.1.1. Selection, collection and identification of plant material

Cotyledon orbiculata was selected based on the reported claims by traditional healers for its usefulness in epilepsy and painful conditions. The plant material was collected from Kirstenbosch National Botanical Garden, Cape Town, Republic of South Africa. The botanical identity of the plant was confirmed by Mr. Franz Weitz, a taxonomist in the Department of Biodiversity and Conservation Biology. A voucher specimen of *C. orbiculata* (GEORGE 01) was deposited in the Herbarium, University of the Western Cape.

3.1.2. Preparation of crude methanol extract of *C. orbiculata*.

The leaves (16,766.4g) of the plant species collected were washed with distilled water, cut into small pieces and dried in a ventilated oven at 40°C for 120 hrs. The dried plant material (817.2g) was milled to a fine powder using the Waring Commercial laboratory blender and passed through a 850µm sieve. The dried powder (800.6g) was extracted in a Soxhlet extractor with methanol for 72 hrs. The methanol extract was evaporated to dryness using a Büchi RE11 rotavapor and Büchi 461 water bath and a yield of 583.3g of crude methanol extract was obtained. The resultant methanol extract was then stored in a desicator for further investigations.

Fresh solutions of *C. orbiculata* were prepared on each day of the experiment by reconstituting a weighed quantity of the crude methanol extract in a minimum amount of dimethylsulfoxide (DMSO) and then made up to the appropriate volume

with physiological saline. The plant extract was injected intraperitoneally (i.p.) into mice in a volume of 1ml/100g of body weight.

3.1.3. Phytochemical analysis of *C. orbiculata*.

Phytochemical analysis was performed using the methods of Harborne (1984) and Ikhiri *et al.* (1992) to screen for chemical compounds present in the leaves of *C. orbiculata*.

3.1.3.1. Alkaloids

0.5g of the powdered *C. orbiculata* was boiled with 10 ml of dilute hydrochloric acid (alcoholic) in a test tube for 5 minutes. The mixture was cooled and the debris was allowed to settle. The supernatant liquid was filtered into another test tube and 1ml of the filtrate was taken into which three drops of Dragendorff's reagent (potassium bismuth iodide solution) was added, shaken and observed for the appearance of an orange-red spot and a precipitate formation.

3.1.3.2. Saponins

A little (0.2 g) of the powdered *C. orbiculata* was shaken with water and the mixture was observed for a persistent froth.

3.1.3.3. Tannins

0.2 g of the powdered *C. orbiculata* was boiled in 5ml of water. The mixture was cooled and filtered. A few drops (3 drops) of 5% ferric chloride solution were added to the filtrate and observed for a blue-black precipitate formation.

3.1.3.4. Reducing sugars

0.2 g of the powdered *C. orbiculata* was boiled in 5ml of water. The mixture was cooled and filtered. An equal quantity (5ml) of Fehlings A and B solutions were

added to the filtrate, heated in a water-bath, and then observed for a red-brown precipitate formation.

3.1.3.5. Anthraquinones

0.1 g of the powdered *C. orbiculata* was shaken in 10 ml of 15% ferric chloride solution and 5 ml of hydrochloric acid, and immersed in a water bath for 10 minutes. The mixture was filtered immediately. The filtrate was cooled and extracted with 10 ml of carbon tetrachloride. The carbon tetrachloride layer was separated, washed with 5 ml of water and shaken with 5 ml of dilute ammonia solution. The resultant mixture was observed for a rose pink to cherry red colour in the ammoniacal layer.

3.1.3.6. Cardiac glycosides

0.5 g of powdered *C. orbiculata* was boiled in 5 ml of 70% ethyl alcohol for 2 minutes. The mixture was filtered and 10 ml of water and 5 ml of chloroform were added to the filtrate. It was then shaken. The lower chloroform layer was separated off and evaporated to dryness in a water-bath. The cooled chloroform residue was dissolved in 3 ml of glacial acetic acid containing 0.1 ml of ferric chloride. The solution was carefully transferred to the surface of 2 ml of sulphuric acid and observed for a reddish-brown layer that was formed at the interface and whether the upper layer gradually acquired a bluish-green colour.

3.1.3.7. Flavonoids.

10 g of powdered *C. orbiculata* was boiled for 2 to 3 minutes in 100 ml of water in a water-bath. To 3 ml of the filtrate, 3 ml of acid-alcohol (Ethanol: Water: concentrated hydrochloric acid in a ratio of 1:1:1), solid magnesium (1 cm) and 1 ml of t-amyl-alcohol were added. The mixture was then observed for a rose-orange or violet colour change.

3.1.3.7. Triterpene Steroids.

1g of powdered *C. orbiculata* was extracted for 24 hours in ether. 1 ml of the filtrate was evaporated to dryness and the residue redissolved in several drops of acetic anhydride and then several drops of sulphuric acid were added to solution. The mixture was then observed for a green colour change.

3.2. PURIFICATION PROCEDURES

3.2.1. Purification of the Methanol Extract.

The isolation of active compounds from the methanol extract was done using thin layer chromatography and column chromatography. Hexane and ethyl acetate used in the isolation experiment were previously distilled.

3.2.2. Choice of solvents.

A small amount of methanol extract of *C. orbiculata* was dissolved in 1 ml of acetone. By means of Pasteur pipette, a small amount of the methanol extract was spotted at 0.8 cm from the bottom of the plate (TLC, aluminium sheets 20x20 cm, silica gel 60 F254, Merck Germany). The spotted plate was left to dry and then placed in a tank containing a small quantity (3 ml) of the solvent system to be studied (a mixture of ethyl acetate and hexane). The solvent mixture was allowed to rise up the thin layer chromatography plate until it was 1.5 cm from the top of the plate. The plate was then removed and the level of the solvent front was marked for the determination of the retardation factor (R_f) for each compound detected. The plates were allowed to dry before the various bands were identified. Four different strengths of ethyl acetate/hexane mixture: 20%, 30%, 40% and 50% by volume were tested and 20% was identified to be the most suitable solvent for the column. The spots were identified by visible light and UV light at 366 nm.

3.2.3. Isolation of active compounds.

In the present investigation, thin layer chromatography and column chromatography were used. During column chromatography, the stationary phase was silica gel (70-230) and the mobile phase was a mixture of ethylacetate and hexane in proportion of 2:8 by volume. LC-200 fraction collector was used to collect the small fractions. The time/drop volume was set at 5 minutes on the column. The thin layer chromatography was needed to pool together similar fractions which contain the same compounds.

3.2.4 Sample to be separated.

4.83 g of methanol extract of *C. orbiculata* was dissolved in a small volume of acetone. The resultant solution was mixed with a small quantity of coarse silica gel in a round bottom flask and the acetone evaporated with a rotary evaporator at 50°C. The solute obtained was pre-adsorbed onto the silica gel. The pre-adsorbed mixture was then fractionated over silica gel (70-230) column chromatography and eluted with a mixture of ethylacetate: hexane (2:8). The collector was used to collect small fractions and switched on when the first drop from the column started flowing. The resultant fractions were analysed by thin layer chromatography and viewed under UV light. Fractions 21-42, which were shown to contain active ingredients by TLC spotting, were combined to afford an enriched sample of the active agents referred to as “S” for further investigation for anticonvulsant and analgesic properties.

Fresh solutions of “S” were prepared on each day of the experiment by reconstituting a weighed quantity of the “S” in a minimum amount of dimethylsulfoxide (DMSO) and then made up to the appropriate volume with

physiological saline. The solution was injected intraperitoneally (i.p.) into mice in a volume of 1 ml/100 g body weight.

3.3. HPLC ANALYSIS.

With the application of standard techniques of chromatography, the spectral profile of *C. orbiculata* (plant extract) was analyzed.

The chromatographic system used includes a Beckman HPLC system consisting of a double pump programmable Solvent Module 126, Diode Array detector Module 168, with 32 Karat Gold software supplied by Beckman; Column C18 Bondapak 5 µm and dimensions of 250x4.6 mm.

The chromatographic conditions were as follows: Mobile phase, solvent A: methanol (MeOH); solvent B: 5% acetic acid (CH₃COOH); Model: gradient, increasing the organic phase (MeOH) from 20% to 90% over 18 minutes; flow rate: 1ml/min; reference standard. Rutin (2.5g dissolved in 10 ml MeOH). The run time was 25 minutes.

3.4. EXPERIMENTAL ANIMALS

Male albino mice bred in the Animal House of the Department of Pharmacology, School of Pharmacy, University of the Western Cape, Bellville, South Africa, weighing between 18 and 30g were used in this study. The mice were normally housed in groups of eight per cage and were maintained on tap water and food *ad libitum*. Each mouse was used for one experiment only. The animals were used for all the pharmacological screening experiments.

3.5 DRUGS AND CHEMICALS

Pentylentetrazole (PTZ, Sigma Co.), picrotoxin (Sigma Co.), N-methyl-DL-aspartic acid (Sigma Co.), phenobarbitone (Gardenyl[®], Rhone-poulenc Rorer, South Africa) and morphine (Bodene) were all dissolved in physiological saline to appropriate volumes. Bicuculline (Sigma Co.) was suspended in 0.5ml of Tween 80 and diazepam (Valium[®], Roche, South Africa) was suspended in polyethylene glycol 400 (Fluka AG, Buchs). Both suspensions were separately adjusted to appropriate volumes with physiological saline. Paracetamol (Sigma Co) was dissolved in a minimum volume of propylene glycol 400 (BDH, UK) and adjusted to the appropriate volume with physiological saline. Acetic acid (Merck) was dissolved in physiological saline to an appropriate strength. Preliminary studies were carried out to establish the doses of plant extract and drugs as well as the pretreatment times to be used. All drugs were injected intraperitoneally (i.p.) in a volume of 1 ml/100 g body weight. Control animals received equal volume injections of the appropriate vehicle. Fresh drug solutions were prepared on each day of the experiment.

3.6. PHARMACOLOGICAL SCREENING

3.6.1. Assessment of Anticonvulsant Activity.

The method of Vellucci and Webster (1984), modified by Amabeoku and Chikuni (1993) was used to assess the anticonvulsant effect of the plant extract. The mice were housed singly in a transparent Perspex cage for 30 minutes before commencement of the experiment in order to acclimatize them to their new environment. Standard convulsant agents such as pentylentetrazole (PTZ, 95 mg/kg), bicuculline (40 mg/kg), picrotoxin (12 mg/kg) and N-methyl-DL-aspartic acid (NMDLA, 400 mg/kg) given intraperitoneally, were used to induce convulsions

in the animals. The animals were observed for 30 minutes for tonic convulsions. Seizures were manifested as tonic hind-limb extensions. The ability of the plant extract to prevent this feature or prolong the latency or onset of the tonic hind limb extensions was taken as an indication of anticonvulsant activity (Amabeoku, 1993; Amabeoku *et al.*, 1998; Erasmus, 2004). The time of the onset of seizures and proportion of animals convulsing or not convulsing were obtained during this period of observation. Animals that did not convulse during the period of observation were considered as not having convulsed (Amabeoku *et al.*, 1998). Experiments were repeated with other groups of animals pre-treated 15 minutes prior to the administration of the plant extract, standard anticonvulsant drug (phenobarbitone, 12 mg/kg, i.p or diazepam, 0.5 mg/kg, i.p), or control vehicle (DMSO) prior to the administration of either of the convulsant agents.

3.6.2 Assessment of analgesic activity.

Two assay models, acetic acid- induced writhing and hot plate reaction time method were employed to detect analgesic activity. In both models, control group of the animals received normal saline intraperitoneally, while the test animals received *C. orbiculata* at doses of 100, 200 and 400 mg/kg body weight.

3.6.2.1. Acetic acid writhing test.

The methods of Koster *et al.*, (1959); Williamson *et al.*, (1996) and García *et al.*, (2004) were used. Mice were used in groups of eight per dose of plant extract or drug. The animals were kept singly in transparent perspex cages (25cmx15cmx15cm) for 30 minutes to acclimatize to their new environment before the commencement of the experiment. Control mice were pre-treated with normal

saline in a volume of 1 ml/100 g of body weight and after 15 minutes each mouse was injected with 0.2 ml of 3% acetic acid. 5 minutes after the administration of acetic acid, the writhes were counted for 20 minutes. Other groups of animals were pre-treated with plant extract or paracetamol 15 minutes prior to injecting them intraperitoneally with 0.2 ml of 3% acetic acid. All experiments were performed between 08:00 and 16:00h in a quiet laboratory with an ambient temperature of 22 ± 1 °C. The ability of the plant extract to significantly reduce the number of acetic acid-induced writhes was taken as an analgesic activity.

3.6.2.2. Hot-plate test.

The methods of Eddy and Leimback (1953) and Williamson *et al.*, (1996) were employed. Control animals were pre-treated with normal saline and after 15 minutes were then placed individually in a 2l glass beaker placed on a thermostatically controlled hot plate, model HC500 (Bibby Sterilin Ltd, England), maintained at 50-55°C. The pain threshold is considered to be reached when the animals lift and lick their paws or attempt to jump out of the beaker. The time taken for the mice to exhibit these characteristics (time reaction) was noted by means of a stopwatch. The animals were tested before and 15 min, 30 min, 45 min and 60 min after intraperitoneal administration of normal saline. The experiments were repeated using other groups of animals, which were tested before, and 15 min, 30 min, 45 min and 60 min after the administration of either plant extract or morphine. All experiments were carried out between 08:00 and 16:00 h in a quiet laboratory with an ambient temperature of 22 ± 1 °C. A cut off time of 60s was used to avoid harm to the mice. The ability of the plant extract to delay the reaction time was taken as an analgesic activity.

3.7. STATISTICAL ANALYSES.

All the data obtained from the pharmacological tests, with the exception of the proportion of animals convulsing, were analyzed using the “Paired Student’s t-test”. The proportion of animals convulsing was analyzed using the Chi-squared test (Tallarida and Murray, 1981; Amabeoku *et al.*, 2001; Bienvenu *et al.*, 2002; Erasmus, 2004). Data obtained were expressed as mean (\pm S.E.M).

3.8. ETHICAL CONSIDERATIONS.

All animals used in this study were treated according to the University of the Western Cape Regulations Act concerning animal experiments.

CHAPTER 4. RESULTS

4.1. PHYTOCHEMICAL ANALYSIS.

Data obtained from the phytochemical analysis of the leaves of *C. orbiculata* indicated the presence of the following chemical components: tannin, saponin, cardiac glycosides, reducing sugars and triterpene steroids. However, the tests performed showed that the leaves of *C. orbiculata* did not contain alkaloids, anthraquinones and/or flavonoids.

Table 1. Phytochemical analysis of *Cotyledon orbiculata*.

Components	Results.
Alkaloids	-
Cardiac glycosides	+
Saponins	+
Tannins	+
Reducing sugars	+
Anthraquinones	-
Triterpene steroids	+
Flavonoids	-

Key

+ (Positive) means present

- (Negative) means absent

4.2. ANTICONVULSANT ASSESSMENT.

4.2.1. Effect of Crude methanol extract of *Cotyledon orbiculata* on pentylene tetrazole -induced seizures.

Pentylene tetrazole (95 mg/kg, i.p.) produced tonic seizures in all the animals used. A dose of 50 mg/kg of *C. orbiculata* did not protect any of the animals since they all convulsed. A dose of 100 mg/kg, i.p. of *C. orbiculata* protected 50% of the animals against pentylene tetrazole-induced seizures and significantly reduced the onset of seizures. *C. orbiculata* (200 mg/kg, i.p.) protected 50% of mice against pentylene tetrazole -induced seizures and significantly delayed the onset of tonic seizures in mice. It was also noted that a dose of 400 mg/kg, i.p. of *C. orbiculata* protected 62.5% of mice against pentylene tetrazole-induced seizures. The dose significantly reduced the number of animals convulsing and significantly delayed the onset of pentylene tetrazole-induced seizures in mice. The standard anti-epileptic drugs, phenobarbitone (12 mg/kg, i.p.) and diazepam (0.5 mg/kg, i.p.) significantly antagonized the seizures produced by Pentylene tetrazole. DMSO used as vehicle, did not alter pentylene tetrazole-induced seizures in the mice (Table2).

Table 2. Effect of crude methanol extract of *Cotyledon orbiculata* (CO) on pentylenetetrazole (PTZ)- induced seizures in mice.

Dose (mg/kg)					Onset of tonic		
PTZ	CO	Pheno- barbitone	Diazepam	DMSO	No convulsed/ No used	convulsion (min) Mean ± SEM	
95	-	-	-	-	8/8	4.75	0.98
95	50	-	-	-	8/8	4.63	1.41
95	100	-	-	-	4/8	13.00**	2.87
95	200	-	-	-	4/8	13.25*	3.30
95	400	-	-	-	3/8 ^F	14.12***	2.10
95	-	12	-	-	1/8 ^{FF}	14.80***	0.85
95	-	-	0.5	-	1/8 ^{FF}	16.41***	0.70
95	-	-	-	0.25ml	8/8	4.70	1.02

*P< 0.05, **P< 0.02, ***P< 0.001 compared with PTZ control, Student's t-test (n=8).

^FP< 0.05, ^{FF}P<0.005 compared with PTZ control, Chi-squared test (n=8). DMSO did not alter PTZ- induced seizures in mice.

4.2.2. Effect of crude methanol extract of *Cotyledon orbiculata* on picrotoxin-induced seizures.

Picrotoxin (12 mg/kg) produced tonic seizures in all animals used. A dose of 100 mg/kg of *C. orbiculata* protected 12.5% of mice and significantly delayed the latency of picrotoxin-induced seizures. A dose of 200 mg/kg of *C. orbiculata* protected 25% of the animals against and also significantly delayed the onset of

picrotoxin-induced seizures. *C. orbiculata* (400 mg/kg) similarly, protected 12.5% of the animals and also significantly prolonged the onset of picrotoxin-induced seizures. The standard anticonvulsant drug, phenobarbitone (12 mg/kg) protected 50% of mice and significantly delayed the onset of tonic convulsion in the mice. Diazepam (0.5 mg/kg) also protected 50% of the animals and also significantly delayed the onset of picrotoxin-induced seizures. DMSO, used as a vehicle, did not alter picrotoxin-induced seizures in the mice (Table 3).

Table 3. Effect of crude methanol extract of *Cotyledon orbiculata* (CO) on picrotoxin-induced seizures in mice.

Dose (mg/kg)					No convulsed/ No used	Onset of tonic convulsion (min)	
Picro- Toxin	CO	Pheno- barbitone	Diazepam	DMSO		Mean	± SEM
12	-	-	-	-	8/8	10.75	2.01
12	100	-	-	-	7/8	20.57 ^{FF}	0.98
12	200	-	-	-	6/8	18.0 ^F	1.72
12	400	-	-	-	7/8	17.29 ^F	1.30
12	-	12	-	-	4/8	24.5 ^{FF}	0.94
12	-	-	0.5	-	4/8	24.25 ^{FF}	0.67
12	-	-	-	0.25ml	8/8	11.75	0.45

^FP<0.02, ^{FF}P< 0.001 compared with picrotoxin control, Student's ttest (n=8).

DMSO did not alter picrotoxin-induced seizures in mice.

4.2.3. Effect of crude methanol extract of *Cotyledon orbiculata* on bicuculline-induced seizures.

Bicuculline (40 mg/kg) induced seizures in all the animals used. *C. orbiculata* (100 mg/kg) protected 25% of mice. However, this dose of the plant extract did not significantly alter the onset of bicuculline-induced seizures. At a dose of 200 mg/kg, i.p. of *C. orbiculata* protected 75% of animals against seizures induced by bicuculline (40 mg/kg) and significantly delayed the onset of the seizures. *C. orbiculata* (400 mg/kg) did not protect mice against seizures induced by bicuculline but significantly delayed the onset of the seizures.

The standard anti-epileptic drugs, phenobarbitone (12 mg/kg) and diazepam (0.5 mg/kg), profoundly antagonised seizures produced by bicuculline. All the animals used were protected against bicuculline seizures, either by phenobarbitone or diazepam. DMSO, which was used as a vehicle, did not alter bicuculline-induced seizures in the mice (Table 4).

Table 4. Effect of crude methanol extract of *Cotyledon orbiculata* (CO) on bicuculline-induced seizures in mice.

Dose (mg/kg)							Onset of tonic convulsion (min)	
Bicuculline	CO	Pheno-barbitone	Dia-zepam	DMSO	No Convulsed/No used	Mean	± SEM	
40	-	-	-	-	8/8	4.5	0.53	
40	100	-	-	-	6/8	8.5	3.40	
40	200	-	-	-	2/8 ⁺	16.5*	1.75	
40	400	-	-	-	8/8	19.38*	1.60	
40	-	12	-	-	0/8 ⁺⁺	0*		
40	-	-	0.5	-	0/8 ⁺⁺	0*		
40	-	-	-	0.25ml	8/8	5.5	0.5	

*P<0.001 compared with bicuculline control, Student's t-test (n=8).

⁺P<0.01, ⁺⁺P<0.001 compared with bicuculline control, Chi-squared test (n=8).

DMSO did not alter bicuculline-induced seizures in mice.

4.2.4. Effect of Crude methanol extract of *Cotyledon orbiculata* on N-methyl-DL-aspartic acid (NMDLA)-induced seizures.

N-Methyl-DL-aspartic acid (400 mg/kg) elicited seizures in all the animals used. Methanol extract of *C. orbiculata* (100 mg/kg) did not affect the incidence of NMDLA-induced seizures but significantly delayed the onset of the seizures. *C. orbiculata* (200 and 400 mg/kg) neither affected the incidence nor the onset of seizures induced by NMDLA (400 mg/kg). Similarly, phenobarbitone (12 mg/kg), Diazepam (0.5 mg/kg) and DMSO (0.25 ml) did not protect any of the animals against NMDLA-induced seizures, or affect the onset of the seizures (Table 5).

Table 5. Effect of Crude methanol extract of *Cotyledon orbiculata* (CO) on N-methyl-DL-aspartic acid (NMDLA)-induced seizures in mice.

Dose (mg/kg)		Pheno barbitone	Diazepam	DMSO	No convulsed/ No used	Onset of tonic convulsion (min)	
NMDLA	CO					Mean ± SEM	
400	-	-	-	-	8/8	2.13	0.30
400	100	-	-	-	8/8	4.38 ^F	0.46
400	200	-	-	-	8/8	3.63	1.24
400	400	-	-	-	8/8	2.88	0.55
400	-	12	-	-	8/8	2.45	0.74
400	-	-	0.5	-	8/8	2.90	1.83
400	-	-	-	0.25ml	8/8	2.37	0.84

^FP<0.005 compared with NMDLA control, Student's t-test (n=8).

4.3. ANALGESIC TESTS.

4.3.1 Acetic acid writhing test.

0.2 ml of 3% acetic acid produced a substantial number of writhes in control mice pre-treated with physiological saline. Methanol extract of *C. orbiculata* (100-400 mg/kg) in a dose dependent manner, significantly reduced the number of acetic acid-induced writhes. 100 mg/kg of *C. orbiculata* protected 51% of animals against the writhes. 200 mg/kg and 400 mg/kg of *C. orbiculata* protected 67% and 76% of the mice against 0.2 ml of 3% acetic acid-induced writhes respectively. Similarly, paracetamol (300 mg/kg) profoundly reduced the number of writhes elicited by 0.2 ml of 3% acetic acid. 93% of the animals were protected against the writhes by paracetamol. DMSO did not significantly alter acetic acid-induced writhes in mice (Table 6).

Table 6. Effect of Crude methanol extract of *Cotyledon orbiculata* on acetic acid-induced writhing in mice.

Treatment groups	Dose (mg/kg)	Writhes Mean \pm SEM		Protection (%)
Normal saline	-	28.13	3.92	-
<i>C. orbiculata</i>	100	13.83*	3.17	51
	200	9.20**	3.04	67
	400	6.75***	1.97	76
Paracetamol	300	2.10***	0.24	93
DMSO	-	29.04	2.73	0

*P<0.02, ** P<0.005, ***P<0.001 compared with 0.2 ml of 3% acetic acid control, Student's ttest (n=8). Writhes are expressed as number of counts per 20 minutes. 0.25 ml of DMSO was injected into animals intraperitoneally.

4.3.2. Hot-plate test

Mice pre-treated with physiological saline reacted to hot-plate thermal stimulation at 50-55°C either by lifting and licking their paws, or attempting to jump out of the beaker within 2.75 - 6.63 seconds and throughout the 1 hour duration of the experiment.

The crude methanolic extracts of *C. orbiculata* (100 mg/kg) significantly delayed the reaction time of the animals to hot-plate thermal stimulation 30 min after treatment. At a dose of 200 mg/kg, i.p., *C. orbiculata* significantly delayed the pain response of the mice to hot-plate thermal stimulation 30 minutes after treatment. *C. orbiculata* (400 mg/kg) significantly prolonged the pain reaction time of the mice to hot-plate thermal stimulation at 15, 30, 45 and 60 minutes with significant values of $P < 0.05$, 0.001, 0.005 and 0.001 respectively. Similarly, morphine (10 mg/kg, i.p.) significantly ($P < 0.001$) delayed the threshold pain response of the animals to hot-plate thermal stimulation throughout the 1-hour duration of the experiment. DMSO, which was used as a vehicle to dissolve the plant extract, did not affect the reaction times of the mice to hot-plate thermal stimulation at the different times of measurement (Table 7).

Table 7. Effect of Crude methanol extract of *Cotyledon orbiculata* on hot-plate induced nociception in mice.

Treatment Groups	Dose (mg/kg)	Response time(s)				
		0 min	15 min	30 min	45 min	60 min
Normal saline	-	4.13 ± 0.13	6.63 ± 0.60	4.38 ± 0.78	3.38 ± 0.68	2.75 ± 0.31
<i>C.orbiculata</i>	100	6.63 ± 0.92	10.25 ± 1.46	11.75** ± 1.07	7.13 ± 1.27	5.13 ± .08
	200	6.63 ± 0.85	7.38 ± 1.08	12.38 ⁺ ± 1.43	6.88 ± 1.57	6.25 ± 1.45
	400	5.25 ± 0.47	19.13* ± 5.01	26.63 ⁺⁺ ± 3.35	22.63 ⁺ ± 3.41	24.5 ⁺⁺ ± 2.55
Morphine	10	3.38 ± 0.64	26.63 ⁺⁺ ± 4.83	36.5 ⁺⁺ ± 6.55	22.88 ⁺⁺ ± 2.93	16.63 ⁺⁺ ± 2.07
DMSO	-	5.0 ± 0.76	6.13 ± 0.99	3.88 ± 0.38	4.75 ± 0.86	4.50 ± 0.91

*P<0.05, **P<0.025, ⁺P<0.005, ⁺⁺P<0.001 compared with normal saline control, Student's t-test (n=8). The response time in seconds was expressed as Mean ± SEM. 0.25ml of DMSO was injected into animals intraperitoneally.

4.4.1. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on pentylenetetrazole (PTZ)-induced seizures.

The results obtained with “S” were comparable to those obtained with the crude extract. However, at a lower dose of 50 mg/kg, i.p. “S” significantly reduced the number of PTZ-induced convulsing animals. 50 mg/kg,i.p. of “S” protected 62.5% of the animals against the PTZ-induced seizures. 25 mg/kg,i.p. of “S” protected 50% of the animals against the seizures. “S” (100-200 mg/kg) significantly delayed the onset of PTZ-induced seizures. However, 100 mg/kg, i.p. of “S” protected 50% of

the mice against the seizures, while 200 mg/kg,i.p. of “S” did not alter the incidence of PTZ-induced seizures (Table 8).

Table 8. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on pentylenetetrazole (PTZ)-induced seizures in mice.

Dose (mg/kg)		No convulsed/ No used	Percentage protection (%)	Onset of convulsion (min)	
PTZ	S			Mean	± SEM
95	-	8/8	0	3.13	0.48
95	25	4/8	50	3.75	0.34
95	50	3/8 ⁺	62.5	4.33	0.74
95	100	4/8	50	10.00 ^F	2.31
95	200	8/8	0	12.00 ^F	1.72

^FP<0.001 compared to PTZ control, Student’s t-test (n=8).

⁺P<0.05 compared to PTZ control, Chi-squared t-test (n=8).

4.4.2. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on bicuculline-induced seizures.

“S” (25-100 mg/kg) significantly delayed the onset of seizures elicited by bicuculline (40 mg/kg). 25 mg/kg of “S” protected 12.5% of animals against the seizures while 50 mg/kg of “S” protected 50% of animals and 100 mg/kg protected 25% of animals. 200 mg/kg of “S” protected 12.5% of animals, but did not significantly affect the onset of bicuculline-induced seizures (Table 9).

Table 9. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on bicuculline-induced seizures in mice

Dose (mg/kg)		No convulsing/ No used	Percentage protection (%)	Onset of convulsion (min) Mean ± SEM	
Bicuculline	S				
40	-	8/8	0	4.50	0.33
40	25	7/8	12.5	7.00*	0.65
40	50	4/8	50	6.50*	0.35
40	100	6/8	25	9.67**	1.26
40	200	7/8	12.5	5.57	0.53

*P<0.005, **P<0.001 compared to bicuculline control, Student's t-test (n=8).

4.4.3. Effect of purified methanol extract of *cotyledon orbiculata* (S) on NMDLA-induced seizures.

Low dose (25 mg/kg) of “S” protected 25% of the animals against the seizures elicited by NMDLA (350 mg/kg) but did not significantly delay the NMDLA-induced seizures. 50 mg/kg of “S” significantly delayed NMDLA-induced seizures but did not affect the incidence of the seizures. (100-200 mg/kg) did not alter the seizures induced by NMDLA (350 mg/kg) (Table 10).

Table 10. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on N-methyl-DL-aspartic acid (NMDLA)-induced seizures in mice.

Dose (mg/kg)		No convulsed/ No used	Percentage protection (%)	Onset of convulsion (min) Mean ± SEM	
NMDLA	S				
350	-	8/8	0	2.38	0.38
350	25	6/8	25	2.83	0.52
350	50	8/8	0	3.75*	0.37
350	100	8/8	0	2.71	0.49
350	200	8/8	0	2.46	0.71

*P<0.025 compared to NMDLA control, Student's t-test (n=8).

4.4.4. Effect of purified methanol extract of *Cotyledon orbiculata* (S) on acetic acid-induced writhes.

“S” (25-200 mg/kg) significantly reduced the number of writhes produced by 0.2ml of 3% acetic acid. 25 mg/kg and 100 mg/kg of “S” protected 65% of the animals against the pain elicited by acetic acid, while 50 mg/kg and 200 mg/kg protected 62% of the animals (Table 11).

Table 11. Effect of purified methanol extract of *cotyledon orbiculata* (S) on acetic acid-induced writhes in mice

Dose (mg/kg)		Writhes		Percentage protection
Acetic acid	S	Mean	± SEM	(%)
Normal saline	-	43.5	6.11	0
0.2ml	25	15.38 ^{**}	2.87	65
0.2ml	50	16.63 [*]	4.13	62
0.2ml	100	15.38 [*]	3.59	65
0.2ml	200	16.38 [*]	3.34	62

*P<0.005, **P<0.001 compared to 0.2 ml of 3% acetic acid control, Student's t-test (n=8). The writhes are expressed as counts per 15 minutes.

4.5. HPLC ANALYSIS.

The chromatographic spectrum of the crude methanol extract of *C. orbiculata* obtained revealed major peaks at the following retention times (minutes): 6.983, 10.521, 12.088, 12.838 and 13.342 (Fig. 2). The reference standard, Rutin, showed a major peak at the retention time (minute): 13.767 (Fig. 3). Rutin was used to standardize the retention time.

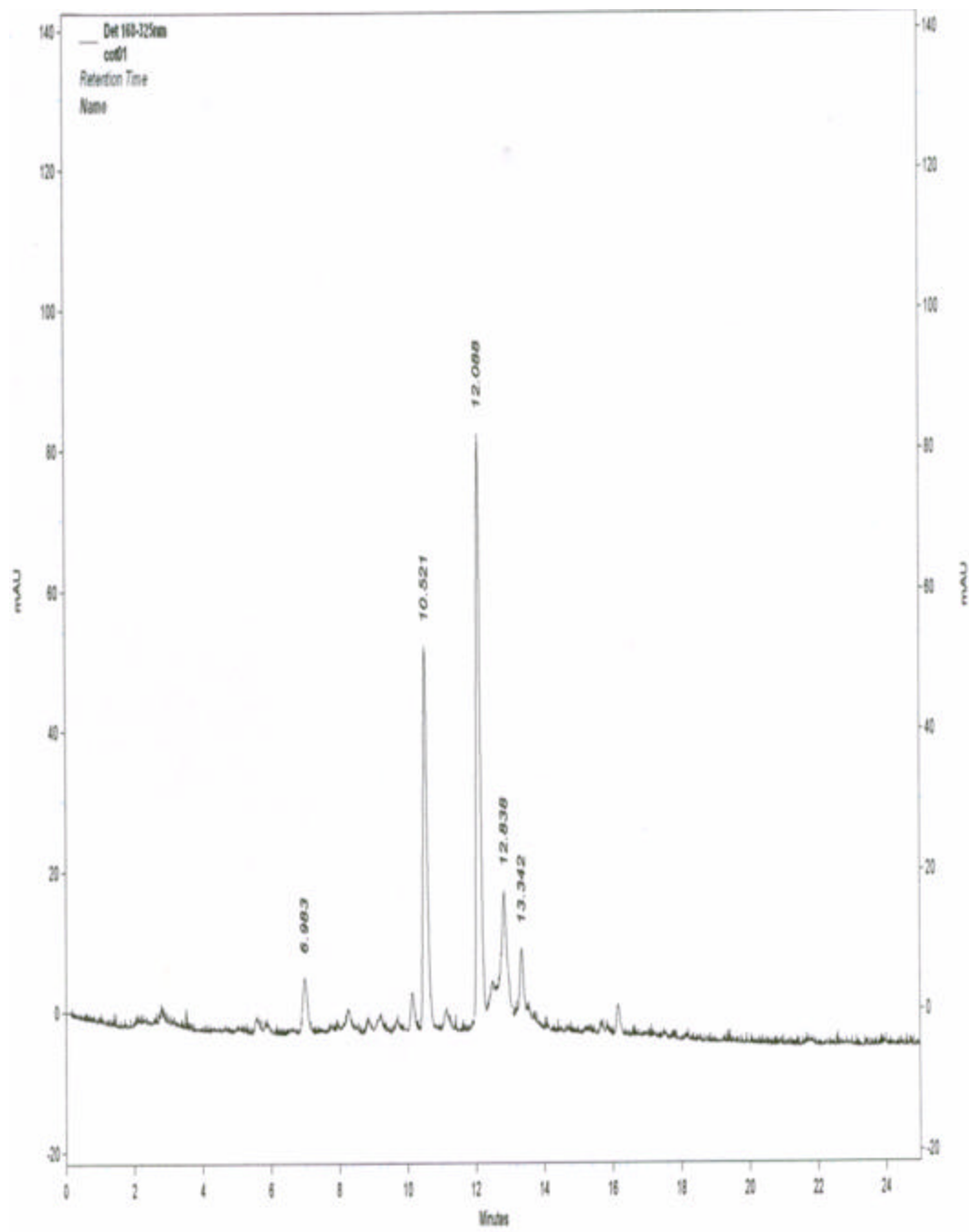


Fig. 2. HPLC chromatogram of crude methanol extract of *Cotyledon orbiculata*.

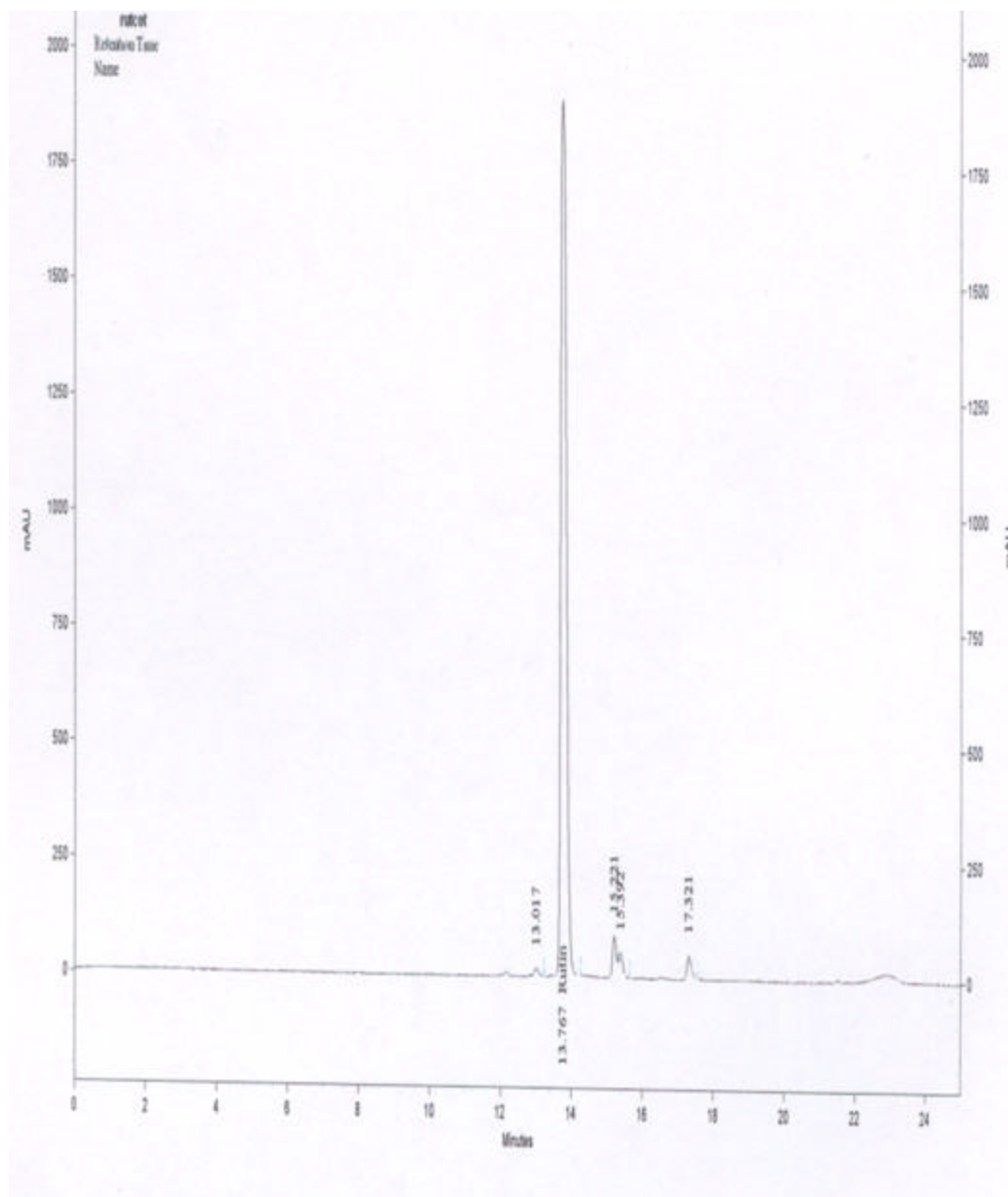


Fig. 3. HPLC chromatogram of Rutin (reference standard for *Cotyledon orbiculata*).

CHAPTER 5. DISCUSSION AND CONCLUSION

5.1. DISCUSSION.

The results of the present study demonstrate that *C. orbiculata* has both anticonvulsant and analgesic activities.

A dose of 100 mg/kg, i.p. of the crude methanol extract of *C. orbiculata* protected 50% of the animals against pentylenetetrazole-induced seizures, and significantly reduced the onset of seizures. *C. orbiculata* (200 mg/kg) protected 50% of the mice against pentylenetetrazole-induced seizures and significantly delayed the onset of tonic seizures in the mice. It was also noted that, a dose of 400 mg/kg of *C. orbiculata* protected 62.5% of the mice against pentylenetetrazole-induced seizures. 400 mg/kg, i.p. of *C. orbiculata* significantly reduced the number of animals convulsing and significantly delayed the onset of pentylenetetrazole-induced seizures in the mice. The standard anti-epileptic drugs, phenobarbitone (12 mg/kg) and diazepam (0.5 mg/kg) significantly antagonized the seizures produced by pentylenetetrazole. Pentylenetetrazole (PTZ) most likely produces seizures by inhibiting gamma aminobutyric acid (GABA) neurotransmission (De Sarro *et al.*, 2000). Gamma aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain, and the inhibition of its neurotransmission has been thought to be the underlying factor in epilepsy (Amabeoku *et al.*, 1998). The enhancement of the GABAergic neurotransmission is reported to antagonise seizures, while the inhibition of the neurotransmission promotes seizures (Gale, 1992; Leonard, 2003; Erasmus, 2004).

The protection of mice against PTZ-induced seizures by the standard anticonvulsant drugs, phenobarbitone and diazepam is expected, since various authors have shown that they exert their anticonvulsant activities by enhancing GABA-mediated

inhibition (Olsen, 1981; Amabeoku *et al.*, 1998; Leonard, 2003; Rang *et al.*, 2003; Leonard, 2003; Shaun, 2004;). The antiseizure effect of the methanol extract of *C. orbiculata* may, therefore, be due to its ability to attenuate PTZ-induced seizures by interfering with GABAergic transmission.

The methanol extract of *C. orbiculata* and standard anticonvulsant drugs, phenobarbitone and diazepam, antagonised seizures induced by picrotoxin in the present study. A dose of 100 mg/kg of *C. orbiculata* protected 12.5% of mice and significantly delayed the latency of picrotoxin-induced seizures. A dose of 200 mg/kg of *C. orbiculata* protected 25% of the animals against picrotoxin-induced seizures, and also significantly delayed the onset of picrotoxin-induced seizures. *C. orbiculata* (400 mg/kg) similarly, protected 12.5% of the animals and also significantly prolonged the onset of picrotoxin-induced seizures. The standard anticonvulsant drug, phenobarbitone (12mg/kg) protected 50% of mice and significantly delayed the onset of tonic convulsion. Diazepam (0.5 mg/kg) also protected 50% of the animals and also significantly delayed the onset of picrotoxin-induced seizures.

GABA_A receptors have been shown to be linked functionally to benzodiazepine receptors, barbiturate receptors and chloride (Cl⁻) ion channels to form the GABA-chloride ionophore complex, which is involved in modulation of GABAergic inhibitory transmission (Olsen, 1981; Seller, 1985; Amabeoku and Chikuni, 1993; Rang *et al.*, 2003; Bennett and Brown, 2003; Shaun, 2004). This hypotheses supports the finding that, *C. orbiculata* has an anti-epileptic effect. Picrotoxin, an antagonist of GABA_A-receptors, produces seizures by blocking the chloride ion

channels, which are linked to GABA_A-receptors to prevent the influx of chloride ions into the brain neuron (Amabeoku and Chikuni, 1993; Rang *et al.*, 2003). Diazepam, a benzodiazepine, and phenobarbitone, a barbiturate, antagonise picrotoxin-elicited seizures because they are thought to produce their anticonvulsant effect by increasing chloride flux through chloride channels at GABA_A receptor sites to enhance GABAergic systems (Amabeoku and Chikuni, 1993; Rang *et al.*, 2003). It is, therefore, possible that *C. orbiculata* may be antagonising seizures induced by picrotoxin by enhancing GABAergic systems.

Bicuculline, a selective antagonist of GABA_A receptors, which have been widely implicated in epilepsy (Gale, 1992; Amabeoku *et al.*, 1998; Rang *et al.*, 2003), produces seizures by blocking the effect of GABA at central GABA_A-receptors. In the present investigation, the standard anticonvulsant drugs, phenobarbitone and diazepam, which are thought to enhance GABA neurotransmission, antagonised bicuculline-induced seizures. Doses of *C. orbiculata* at (200 and 400 mg/kg) significantly antagonised the seizures induced by bicuculline. Based on the results obtained, it is suggested that *C. orbiculata* may be exerting its effects by affecting gabaergic systems.

NDMLA is a specific agonist at NDMLA receptors, which are implicated in the pathogenesis of epilepsy. It produces effects similar to glutamic acid at NDMLA receptors and exerts its convulsant effect by activating the receptors to enhance glutaminergic neurotransmission (Watkins and Evans 1981; Chapman and Meldrum, 1993). The data from the present study show that *C. orbiculata* significantly delayed the onset of NMDLA-induced seizures. However, phenobarbitone and diazepam were shown not to significantly alter the seizures elicited by NMDLA. In view of

the results obtained, it is possible that *C. orbiculata* may also be affecting the glutaminergic system.

Two assay models, viz., acetic acid-induced writhing response and the hot-plate reaction time methods, were employed in evaluating the analgesic activity of the methanolic extract of *C. orbiculata*. It is necessary to employ different testing models with respect to stimulus quality, intensity and duration, to fully obtain a complete picture of the analgesic properties of a substance using behavioural nociceptive tests (Tjolsen *et al.*, 1992; Shinde *et al.*, 1999; Olufunmilayo and Okpaka, 2004). The present study shows that the methanol extract of *C. orbiculata* (100-400 mg/kg), in a dose-dependent manner, significantly reduced the number of acetic acid-induced writhes. 100 mg/kg of *C. orbiculata* protected 51% of animals against acetic acid-induced writhes. 200 mg/kg and 400 mg/kg of *C. orbiculata* methanol extract protected 67% and 76% of the mice against 0.2 ml of 3% acetic acid-induced writhes respectively. Similarly, paracetamol (300 mg/kg), a standard peripherally acting analgesic drug antagonised the acetic acid-induced writhing elicited by 0.2 ml of 3% acetic acid. 93% of the animals were protected against the writhes by paracetamol. Therefore, results obtained in this study probably suggest that *C. orbiculata* possess significant analgesic activity relative to the control group in the pain model (Fawcett 1999; WHO, 2002).

In the hot-plate test model, *C. orbiculata* (100 – 400 mg/kg) significantly prolonged the reaction time of the animals to thermal stimulation over the period of observation. Similarly, morphine, a standard centrally acting analgesic drug (Fawcett 1999; WHO, 2002; SAMF, 2003) delayed the reaction time to the thermal stimulation. According to Eddy and Leimback, 1953; Koster *et al.* 1959; Williamson *et al.*, 1996; and Amabeoku *et al.*, 2001, acetic acid writhing and hot-plate tests are

normally used to study the peripheral and central analgesic effects of drugs respectively. Since, *C. orbiculata* significantly antagonised the writhes induced with by 0.2 ml of 3% acetic acid, and also significantly delayed the reaction time of the animals to thermal stimulation, it is likely, therefore, that the plant extract could be producing its analgesic effects both peripherally and centrally.

The results of the phytochemical studies carried out using the methods of Harborne (1984) and Ikhiri *et al.*, (1992), show that the leaves of *C. orbiculata* contain, cardiac glycosides, saponins, tannins, reducing sugars and triterpene steroids. According to Chauhan *et al.*, (1988), triterpenoids evaluated for anticonvulsant activity against PTZ-induced seizures in mice protected 10-40% of the animals. It is possible, therefore, that saponins which may be of triterpenoid type and the triterpene steroid present in *C. orbiculata* might contribute to the anticonvulsant activity of the plant. Further more, Bruneton (1999) reported that saponins are responsible for the analgesic properties of *Platycodon grandiflorum* or of the various *Dianthus*. It is, therefore, tempting to suggest that saponins present in *C. orbiculata* may also contribute to the analgesic activity of the plant species.

The HPLC fingerprints of the crude methanol extract of *C. orbiculata* show major peaks at the following retention times (min) 6.983, 10.521, 12.088, 12.838 and 13.342 (Fig.1).

In the present study, the purified methanolic extract of *C. orbiculata* (S) produced comparable anticonvulsant and analgesic activities to the crude extract of the plant. However, unlike the crude methanol extract of *C. orbiculata*, which produced anticonvulsant and analgesic activities at a minimum dose of 100mg/kg, the purified

extract produced both anticonvulsant and analgesic activities at doses as low as 25 mg/kg.

5.2. CONCLUSION.

The results obtained in this study indicate that *C. orbiculata* possesses anticonvulsant activity and the methanolic extract of the plant is thought to affect both gabaergic and glutaminergic systems. It is, therefore, possible that the anticonvulsant activity of the plant may be exerted through more than one mechanism. It is also possible that saponin content of the plant's extract may contribute to the anticonvulsant activity of the plant.

In addition, since *C. orbiculata* antagonised the pain produced by both acetic acid and hot-plate analgesic test methods, it is possible that the plant produces its analgesic activity both peripherally and centrally. It is also possible that the saponins in the plant may be responsible for the analgesic activity of the plant.

5.3. RECOMMENDATIONS.

Further studies including neurochemical analysis should be done to elucidate the mechanism of action of *C. orbiculata* in epilepsy and analgesia. Secondly, toxicological studies can be done to establish the safety level of the plant. Thirdly, considering the fact that lower doses of the purified methanol extract of *C. orbiculata* produced anticonvulsant and analgesic effects compared to the higher doses of the crude methanol extract, detailed studies to determine the active compounds will be very vital. Fourthly, structural elucidation of the chemical constituents of the extract should also be carried out.

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