# Investigation of the Toxicological and Pharmacological Activity of a Hydroethanolic extract of *Boophone disticha* bulb

By

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# DECLARATION

The experimental work described in this thesis was conducted in the Department of Clinical Pharmacology, College of Health Sciences at the University of Zimbabwe under the supervision of Dr D. Tagwireyi (School of Pharmacy, College of Health Sciences, and University of Zimbabwe) and Professor C.F.B. Nhachi (Department of Clinical pharmacology, College of Health Sciences, University of Zimbabwe).

I hereby declare that this thesis, submitted for the degree of Master of Philosophy represents original work composed by myself with the assistance of my supervisors, except where the work of others is duly acknowledged in the text. This work was supported by IFS grant number F/4187-1.

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LOUIS L. GADAGA

*November, 2010*

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Dr DEXTER TAGWIREYI

*Supervisor*

…….....……

Professor CHARLES F.B. NHACHI

*Co-supervisor*

# *To the lost years*

*When you fell how depressingly and slowly you climb,*
*It is well to remember that:*
*Things Take Time*

***Piet Hein;*** *1905-1996*
*Danish Poet & Scientist*

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# ABSTRACT

*Boophone disticha* is a highly poisonous bulb. It has been used systemically in traditional medical practice in Zimbabwe and neighbouring countries for the management of various central nervous system conditions including hysteria. Abuse of the plant by teenagers in Zimbabwe for its claimed hallucinogenic effects has also been reported, with the advent of serious toxicity in some cases.

The aim of the present work was to describe the symptomatology, neurotoxicological effects and lethality of acute and subacute ingestion and of a hydro-ethanolic plant extract of the bulb of *Boophone disticha* in rats. Initially, we set out to estimate the $LD_{50}$ of this extract using a modified Up-and-down procedure for acute toxicity determination. We then used a Functional Observational Battery (FOB) to identify the neurotoxicological effects of the extract after both acute and repeated oral intake. Additionally we studied the genotoxic potential of the *Boophone* extract using the Ames test. Finally we sought to investigate the putative neuropharmacological effects i.e. anxiolytic-like and antidepressant-like activity in a murine model.

Findings in the acute toxicity and neurotoxicological assessment, showedan estimated oral LD50 of between 120 and 240 mg/kg. For doses of 240 mg/kg and less, signs of toxicity began approximately 10 minutes after gavage, and the most prominent initial signs were head tremors (at 50 mg/kg) and body tremors, severe body tremors(>360 mg/kg) followed by convulsions. Generally, symptoms of toxicity lasted approximately 2 hours for doses of 240 mg/kg and less; and 3 hours for doses over 240 mg/kg for animals that survived. These results point to a rapid gastrointestinal absorption of the active principles in the plant extract. The most prominent neurotoxicological effects were increased flaccid limb paralysis and spastic hind-limb paralysis. Tachypnoea was noted at low doses and higher doses produced laboured breathing. The retropulsion observed with higher doses could indicate the reported hallucinogenic effects of the plant extract.

Subacute assessment showed the similar profile symptoms as with acute toxicity.The main subacute toxic effects of *Boophone disticha* like the acute effects seem to be mediated via interference with the neuronal pathways especially the central dopaminergic and motor neurons. Target organs, as observed by changes in organ weight appear to be liver, small and large intestines, stomach, central nervous system and peripheral nervous system. *Boophone disticha* extract was genotoxic from concentrations 1000µg/plate and above with both TA98 and TA100 *Salmonella typhimurium* species irrespective of the metabolic status of the system. The 500µg/plate concentrations of the *Boophone* extract were not associated with any genotoxicity and 2500µg/plate concentration is potential cytotoxic which could have masked the genotoxic effects at this concentration.

*Boophone disticha* extract showed Anxiolytic-like activity in the elevated plus maze test by significantly changing the percentage time spent in open arms, number of open arm entries, rearing, unprotected head dips and stretch attend postures. The 10m/kg dose had the most prominent effects compared to the higher dose which were comparable to the positive control. Repeated dosing enhanced the antidepressant-like activity of the *Boophone* extract.
Therefore from the pharmacological findings of our studies, despite being very toxic *Boophone disticha* proved to be an important source of lead compounds for future drug development. Therefore further investigations are necessary to determine the target site and efficacy of *Boophone* alkaloids with *in vivo* models.

# PUBLICATIONS FROM THIS RESEARCH

1. **Louis L. Gadaga,** Dexter Tagwireyi, Janet Dzangare and Charles F B Nhachi. Acute oral toxicity and neurobehavioural toxicological effects of hydroethanolic extract of *Boophone disticha* in rats. Human and Experimental Toxicology. ***In press.***
2. **LL. Gadaga**, T. Ganga, R. Gwaze, D. Tagwireyi & C.F.B. Nhachi. Sub-acute Toxicity and Genoxicity studies of Hydroethanolic extract of *Boophone Disticha* In Rats. ***Manuscript.***
3. **Louis L. Gadaga**, Donald F. Chuma, Josephine Mudzengi, Aplonia Tanyanyiwa, Dexter Tagwireyi & Charles F.B. Nhachi. Neuropharmacological activity of a hydroethanolic extract of *Boophone disticha* in Balb/c mice. ***Manuscript.***

# TABLE OF CONTENTS

## CHAPTER 4



## CHAPTER 5

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

| | | |
|---|---|---|
| 5-HT | - | 5- Hydroxtryptamine (Serotonin) |
| AC | - | Acetic Acid |
| ANOVA | - | Analysis of Variance |
| BD | - | *Boophone disticha* |
| ATC | - | Acute Toxic Class |
| ATP | - | Adenosine Triphosphate |
| CNS | - | Central Nervous System |
| COX-1 | - | Cyclooxygenase – 1 enzyme |
| COX-2 | - | Cyclooxygenase – 2 enzyme |
| EEG | - | Electroencephalogram |
| EPM | - | Elevated Plus Maze |
| EU | - | European Union |
| FDA | - | Food and Drug Agency |
| FDP | - | Fixed Dose Procedure |
| FOB | - | Functional Observation Battery |
| FST | - | Forced Swim Test |
| GABA | - | Gamma Amino Butyric Acid |
| HE | - | Hydroethanolic |
| $LD_{50}$ | - | Lethal dose where 50% of a population would die after dose administration |
| NA | - | Sodium Azide |

| | | |
|---|---|---|
| NAT | - | Noradrenaline Transporter |
| NF | - | 2-nitrofluorene |
| NMR | - | Nuclear Magnetic resonance |
| NOAEL | - | No Observable Adverse Effects Level |
| OECD | - | Organisation for Economic Cooperation and Development |
| OFT | - | Open Field Test |
| SIRDC | - | Scientific and Industrial Research and Development Centre |
| SEM | - | Standard Error of the Mean |
| SERT | - | Serotonin Transporter |
| SSRI | - | Selective Serotonin Reuptake inhibitor |
| TLC | - | Thin Layer Chromatography |
| TST | - | Tail Suspension Test |
| UDP | - | Up and Down Procedure |
| USEPA | - | United States Environmental Protection Agency |
| WHO | - | World Health Organisation |
| ZINATHA | - | Zimbabwe National Traditional Healers Association |

# CHAPTER 1

# 1. INTRODUCTION

## 1.1. GENERAL INTRODUCTION AND OVERVIEW

The use of plants or plant extracts to treat diseases is a therapeutic modality, which has stood the test of time (Gilani & Rahman, 2005; Saric-Kundalic *et al.,* 2010). In many developing countries, a large proportion of the populace relies heavily on traditional practitioners and herbal plants to meet primary health care needs (WHO, 1999). Recently amongst developed countries, there has been a cultural renaissance, towards more natural methods of healing (Barnes*,* 2003; WHO,1999), and many people in western settings are turning to alternative and complimentary medicines (Makunga *et al.,* 2008). Similarly in Zimbabwe, due to the increase in cost of medicines, and concurrent shortage of drugs, many people are reverting to the use of traditional herbal concoctions and decoctions for their ailments (Tagwireyi *et al.,* 2002).

The increased use of traditional medicines or phytomedicines has led to increasing emphasis on research and development focused toward these preparations (Makunga *et al.,* 2008). Assurance of safety, efficacy, and quality of medicinal plants and herbal products has thus now become a key issue in many countries, with the WHO publishing a series of monographs on selected medicinal plants (Makunga *et al.,* 2008; WHO, 1999).

Africa, and especially Southern Africa, boasts of exceptionally rich plant diversity with an estimated 30,000 species of flowering plants that is almost one tenth of the world's higher plants. There are 10 endemic families, while 80% of the species and 29% of the genera are endemic (Klopper *et al.*, 2006; Stafford *et al.,* 2008; van Wyk, 2008). In South Africa, research on traditional plants has led to the development of several marketed phytomedicinal products. This is evidenced by an increasing body of scientific literature on the use and properties of medicinally important southern African plants (Fennell *et al.*, 2004; Light *et al.*, 2005; Makunga *et al.,* 2008; van Wyk, 2002; van Wyk *et al.,* 1997; van Wyk &Gericke, 2000).

Although there has been a huge increase in ethnopharmacological publications on Southern African medicinal plants, their healing values and bioactivity (van Wyk *et al.*, 1997; Watt & Breyer-Brandwijk, 1962), most of the traditional medicinal plants have never been the subject of exhaustive toxicological tests such as is required for modern pharmaceutical compounds (Fennell &van Staden, 2001; McGaw & Eloff, 2005; Botha & Penrith, 2008). Based on their traditional use for long periods of time, South African medicinal plants are often assumed to be safe (Elgorashi *et al.*, 2003; McGaw and Eloff, 2005; Verschaeve &van Staden, 2008). Phytochemical research has shown that a lot of the purported safe plants have *in vitro* mutagenic (Cardoso *et al.,* 2006; Déciga-Campos *et al.,* 2007; Mohd-Fuat *et al.*, 2007) or toxic and carcinogenic properties (De Sá Ferreira &Ferrão Vargas, 1999; Verschaeve &van Staden, 2008).

In Zimbabwe, whilst the Zimbabwe National Traditional Healers Association (ZINATHA) has suggested efficacy and safety of these preparations, recent reports have shown that traditional medicines are a significant cause of hospital admissions to Zimbabwean hospitals (Nhachi & Kasilo, 1992; Tagwireyi *et al.,* 2002). This has led to advocation for future research to elucidate the toxic components of these traditional medicines (Tagwireyi *et al.,* 2002). Moreover as mentioned above, there is also need for assurance of the safety and efficacy of these preparations. Thus, there is an obvious and immediate need for research focused on elucidating the toxicological profiles of commonly used traditional medicines in the country for both clinical as well as drug development purposes.

*Boophone disticha* (tumbleweed/sore-eye flower; *munzepeti* in the Shona language; *ingcotho* in Isindebele) is a highly poisonous bulb. It is an indigenous psychoactive bulb that is widely found in Southern Africa and its toxic effects are well known by various ethnic people. However, it is commonly used in traditional medicine in Zimbabwe and elsewhere in the region, to treat a variety of ailments including boils, burns and hysteria (Gelfand *et al.,* 1985). Moistened scales are applied to boils, septic wounds and abscesses to alleviate pain and to draw out pus (Watt & Breyer-Brandwijk, 1962). Weak decoctions of the bulb are administered by mouth or as enema for various complaints such as headaches, abdominal pains, weakness and eye conditions, and to 'drive out spirits' (van Wyk *et al.*, 2002). The bulb is also known among traditional healers to possess psychoactive properties and concoctions of the bulb are used traditionally to initiate possession in divination rituals (Gelfand *et al.,* 1985; Mitchell & Breyer-Brandwijk, 1983). Very weak decoctions of the bulb

scales are used as effective sedatives and higher doses are used for divination, and can also induce hallucinations (van Wyk *et al.*, 2002).

Unfortunately, because of it psychoactive properties *Boophone disticha* has been reported as increasingly being used for recreational purposes particularly by youth in Zimbabwe and elsewhere in the region (Acuda & Eide, 1994; Gelfand *et al.,* 1985). This activity has been reported as causing toxicity with some fatal cases reported in the literature (Laing, 1979; du Plooy *et al.,* 2001; Gelfand *et al.,* 1985, Gelfand & Mitchell, 1952). Whilst the characteristic clinical presentation of non-fatal poisoning from *Boophone disticha* have been reported and include rapid development of ataxia, hallucinations, impaired vision, depression, stupor and coma (du Plooy, 2001; Gelfand &Mitchell, 1952), and some of the alkaloids in the plant have been extracted. Still very little is known about the toxicological effects of this plant extract on the major organs and systems, which would result from its acute and chronic abuse. Moreover, there is a paucity of data on the psycho-pharmacological and toxicological effects of *Boophone disticha* plant extract when used in the crude decoction form as it is for recreational purposes. Again there is lack of experimental and clinical evidence for the widely reputed therapeutic effects of the plant. The latter is the more pertinent given the fact that abuse of this plant has been identified in Zimbabwe and is likely to be on the increase. It is against this background that this work is proposed, in order to evaluate the pharmacological & toxicological effects of *Boophone disticha* (crude aqueous extract) after acute and chronic use.

## 1.2. THESIS OUTLINE

A review of the literature on the *Boophone disticha* bulb is presented in section 1.3 of this thesis. The literature review is divided into subsections discussing the botanical features of the bulb, traditional medicinal uses, and the toxicology of the bulb. An overview of the experimental design; selection criteria, neuropharmacological assessments, validity and reliability of the tests are also presented in the literature review. Chapter 2 contains the objectives of the study. Chapter 3 gives specific details and procedures on the materials and methods, and data processing used in the study. The results obtained are presented in Chapter 4. The acute toxicity, neuropharmacology and chronic toxicity of the *Boophone disticha* extract are discussed in Chapter 5. Conclusions and recommendation for further studies are presented in Chapter 6.

## 1.3. REVIEW OF THE LITERATURE

### 1.3.1. Ethnopharmacology in Southern Africa

Southern Africa has a rich and diversified flora that includes a wide variety of plants that have been used for ethnomedical purposes for ages among different cultures of the region (Balandrin *et al.*, 1993; Shale *et al.*, 1999). Today, the use of traditional medicine is still widespread across Southern Africa, and in South Africa most of the indigenous population is still reliant on traditional medicines (Shale *et al.*, 1999; Louw & Korsten, 2002; McGaw *et al.*, 2008; Srivastava *et al.*, 1996; Stafford *et al.*, 2008). This is because most of the people are often subject to shortages of funds, medical facilities and state of the art medicine (Mammem and Cloete, 1996; Shale *et al.*, 1999).The rural population of a country is more

disposed to traditional ways of treatment because of its easy availability and affordability (Banquar, 1993; Fennell *et al.*, 2004). Recently, there has been an increasing trend, worldwide, to integrate traditional medicine with primary health care, particularly in the resource-limited countries (Fennel *et al.*, 2004; WHO, 1999).

In developed countries, historical development of pharmaceuticals and other novel drugs has proceeded primarily through the extraction of efficacious compounds from plants, identified through a variety of bioactivity screening programs (Hunter, 2001). Recently, there has been increasing interest in the drug industry in not only determining the rationale of plant usage in traditional medicine, but in the usage of these plants as leads in drug development (Cox & Balick, 1994; Fabricant and Farnsworth, 2001; Fennell *et al.*, 2004)
Although there has been a huge increase in ethnopharmacological publications on Southern African medicinal plants, their healing values and bioactivity (van Wyk *et al.*, 1997; Watt and Breyer-Brandwijk, 1962), most of the traditional medicinal plants have never been the subject of exhaustive toxicological tests such as is required for modern pharmaceutical compounds (Fennell &van Staden, 2001; McGaw & Eloff, 2005; Botha &Penrith, 2008).Based on their traditional use for long periods, of time they are often assumed to be safe(Elgorashi *et al.*, 2003; McGaw and Eloff, 2005;Verschaeve &van Staden, 2008). Phytochemical research has shown that a lot of the purported safe plants have *in vitro* mutagenic (Cardoso *et al.*, 2006; Déciga-Campos *et al.*, 2007; Mohd-Fuat *et al.*, 2007) or toxic and carcinogenic properties (De Sá Ferreira and Ferrão Vargas, 1999; Verschaeve &van Staden, 2008).

Therefore, although current research emphasis is on the pharmacological validation of plant traditional medicines, it is important that *in vitro* studies be followed by *in vivo* paradigm studies. In addition, it is imperative that these efficacy studies be combined with toxicological evaluations to ascertain safety. Therefore, the current study seeks to determine the potential value of the bulbous herb *Boophone disticha* and to evaluate the toxicological effects of the herbal extracts and possibly determine safe dosage for therapeutic purposes.

### 1.3.2. *Boophone* the Herb

#### *1.3.2.1. Botanical description and distribution*

*Boophone disticha* belongs to the family Amaryllidaceae, *Boophone* species. The *Boophone* species are found widely distributed throughout Southern Africa extending into Tropical Africa (van Wyk *et al.*, 1997; 2002).Plants of this species are well known for a variety of reasons. Many of these plants have found use in the traditional practices of the indigenous people, while others are reaped for their economic use (Elgorashi *et al.,* 2003). All plants of this species, particularly *Boophone disticha* are known to be highly toxic (Botha *et al.*, 2005).

There are four forms of spelling for William Herbert's Amaryllidaceous genera *Boophone*, namely*Boophone*, *Buphane*, *Boophane* and *Buphone,* but most taxonomists now agree that *Boophone* is correct (Archer *et al.*, 2001).*Boophone disticha* was also known at times as *Buphane toxicaria*, *Haemanthus toxicarius*, *Amaryllis disticha, Brunsvigia toxicaria* and *Boophone toxicarius*(Huttleston, 1960; Archer *et al.*, 2001). Several common names exist for *Boophone disticha*, such as bushman poison bulb, gifbol (Afrikaans for poison bulb), fan

leaved *Boophone*, Poison bulb, Sore eye flower (pollen is irritates the eyes), Tumbleweed, and a host of names in the various indigenous languages (Watt and Breyer-Brandwijk, 1962). In Zimbabwe it is known as Munzepete or Muwandwe in Shona and Ingcotho in Ndebele, these names refer to the medicinal and other uses of the bulb (van Wyk *et al.*, 2002; Gelfand *et al.,* 1985).

*Boophone disticha* is usually found in open grasslands, but it can grow in most places where there is well drained soils and adequate sunlight (Steenkamp, 2005; van Wyk *et al.*, 1997). This bulb has, large strap-like leaves symmetrically arranged in a very distinctive fan-shaped manner (Figure 1.1). Because of the arrangement of the leaves the bulb has also known as the candelabra bulb (Watt and Breyer-Brandwijk, 1962). The bulb produces flowers in spring, and leaves about 45 cm (18 in) long and 5 cm (2 in) wide, in spring and summer. The mature bulbs are 10–15 cm in diameter and are partly exposed above the ground, and have numerous papery scales around the fleshy part. The rounded inflorescence has numerous attractive pink flowers all at an equal distance from the main flowering stalk (Figure 1.1). When dry, the inflorescence breaks off and rolls about in the wind, distributing the seeds (van Wyk *et al.*, 1997; 2002). The plant does not flower every year and when relocated, it will not flower for 2 years (van Wyk *et al.*, 1997, Steenkamp, 2005). Plants that grow in shade are said to be more potent than those growing in the full sun (Watt and Breyer-Brandwijk, 1962).

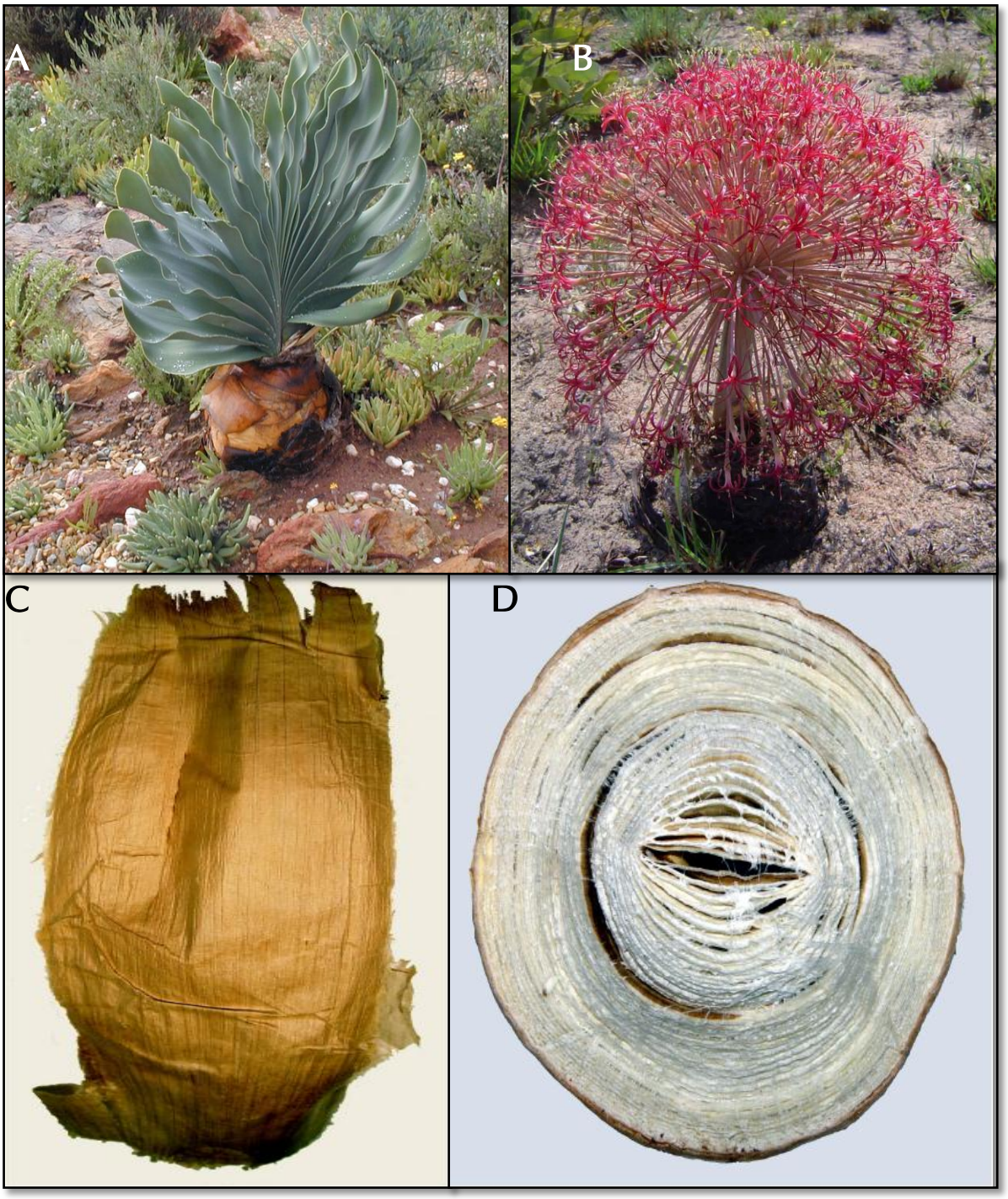


**Figure 1.1.** *BoophoneDisticha*photos showing **A.** the fan-shaped arrangement of the leaves; **B.** the flowering stage; **C.** Papery bulb scales of *Boophone disticha* and **D.** Cross-section of a *Boophone disticha* bulb (adapted from Steenkamp, 2005).

### 1.3.3. Ethno-Medical Uses of *Boophone disticha*

Despite being extremely toxic, *Boophone disticha* is one of the most important medicinal bulbs of Southern Africa. Its high toxicity and claimed antibacterial activity might be the reason it is used Khoi-San mummification, as witnessed by the discovery of a 2000 year old human mummified with *Boophone* scales (Binneman, 1999; van Wyk, 2008). Traditionally, the dried scales of the bulb are used as an outer dressing for circumcision (Watt and Breyer-Brandwijk, 1962). Moistened scales are applied to boils, septic wounds and abscesses to alleviate pain and to draw out pus. Fresh scales are applied to burns and used to treat rashes and skin disorders including eczema. It is also used to relieve rheumatic pains, arthritic swelling, sprains, muscular strains, the pain of abrasions and inflammatory conditions.

The inner bulb is boiled and used as a hot compress in the treatment of oedema. Bulb decoctions are administered by mouth or as enemas to adults suffering from headaches, abdominal pain, weakness, sharp chest pains, persistent bladder pains and eye conditions (Table 1.1). The bulb is also used in the treatment of varicose ulcers and for the relief of urticaria, as well as a treatment for cancer (Watt and Breyer-Brandwijk, 1932; Dyer, 1953; Watt and Breyer-Brandwijk, 1962; Munday, 1988; van Wyk *et al.*, 1997). Laydevant (1932) described its use in initiation ceremonies of the South African Basuto. At the commencement of the initiation period, Basuto boys were given food mixed with the bulb and other ingredients. They were taught that such a remedy would imbue them with the qualities of their ancestors and that it would tend to make men of them. The signs of intoxication were regarded as a token that the spirit of manhood had entered their bodies. Bulbs are reported

to have caused acute and fatal poisoning in humans, following medicinal administration (du Plooy *et al.*, 2001).

**Table 1.1.** Ethno-medical uses of Boophone *disticha*

| Plant Part | Ethno-medical use | Reference |
|---|---|---|
| Bulb scales | Dry outer scale used as outer dressing for wounds, boils and after circumcision | van Wyk *et al.*, 1997; 2002; Watt and Breyer-Brandwijk, 1962; Grierson & Afolayan 1999 |
| Bulb | Weak decoctions administered orally or rectally for headaches, abdominal pains, weakness & eye conditions | Hutchings & van Staden, 1994, van Wyk *et al.*, 1997; Watt and Breyer-Brandwijk, 1962 |
| Bulb | Fresh bulb scales applied to burns, and to treat rashes and skin disorders e.g. eczema | Botha *et al.*, 2005, van Wyk *et al.*, 1997; Steenkamp 2005 |
| Bulb scales | Very weak decoctions used to sedate violent, psychotic patients; higher doses used by traditional healers and diviners to induce hallucinations for divinatory purposes | van Wyk *et al.*, 1997; van Wyk & Gericke, 2000; Sobiecki, 2002 |
| Bulb scales | Used to treat rheumatic pains, arthritic swelling, sprains, muscular pains and inflammatory conditions, chest pains | Botha *et al.*, 2005; Hutchings *et al.*, 1996; van Wyk *et al.*, 1997 |
| Flowers | Ingested in gruel to alleviate the sensation of dizziness | Neuwinger, 2000; Steenkamp, 2005 |
| Bulb | Inner bulb used as hot compress in treating oedema | Botha *et al.*, 2005; Dyer, 1953; Watt and Breyer-Brandwijk, 1962; Munday, 1988; |
| Bulb | Used in treatment of varicose ulcers and for the relief of urticaria and treatment of cancer | Botha *et al.*, 2005; Watt and Breyer-Brandwijk, 1932; Dyer, 1953; Watt and Breyer-Brandwijk, 1962; Munday, 1988; van Wyk *et al.*, 1997 |

## 1.3.4. Pharmacology and Toxicology

### *1.3.4.1. Boophone Alkaloids: Phytochemistry*

*Boophone disticha* and other Amaryllidaceae contain extremely toxic alkaloids. Hence the plant extracts have been used for centuries by the San as an arrow poison (van Wyk *et al.*, 2002; Watt & Breyer-Brandwijk, 1962). The Amaryllidaceae alkaloids represent a still expanding group of isoquinoline alkaloids, which are found exclusively in plants belonging to this family (Viladomat *et al.*, 1997). Early investigations on the plant extract demonstrated that the bulb of *Boophone disticha* contains alkaloids with similar bioactivity as *Datura* alkaloids (Lewin 1912; Watt & Breyer-Brandwijk, 1962). They also show narcotic, hypotensive, vasodilatory and analgesic properties (van Wyk *et al.*, 1997; 2002).

Alkaloids that have been isolated from the bulb include buphanamine, buphanidrine, buphanine, buphanisine, haemanthamine, nerbowdine, undulatine, lycorine (narcissine), crinamidine, crinine, 3-*O*-acetylnerbowdine, ambelline, buphacetine and *distich*amine (see Figure 1.2) (Hautch and Stauffacher, 1961; Raffauf, 1970; Viladomat *et al.*, 1997). Buphanidrine, buphanamine, buphanine, undulatine, buphanisine and nerbowdine emerged as major alkaloids (Hautch and Stauffacher, 1961). Other compounds that have also been isolated are furfuraldehyde, acetovanillone, chelidonic acid, copper, laevulose, pentatriacontane, ipuranol and a mixture of free and combined fatty acids, which are contained in the volatile oil extracted from the bulb (Botha *et al.*, 2005; Watt& Breyer-Brandwijk, 1962).

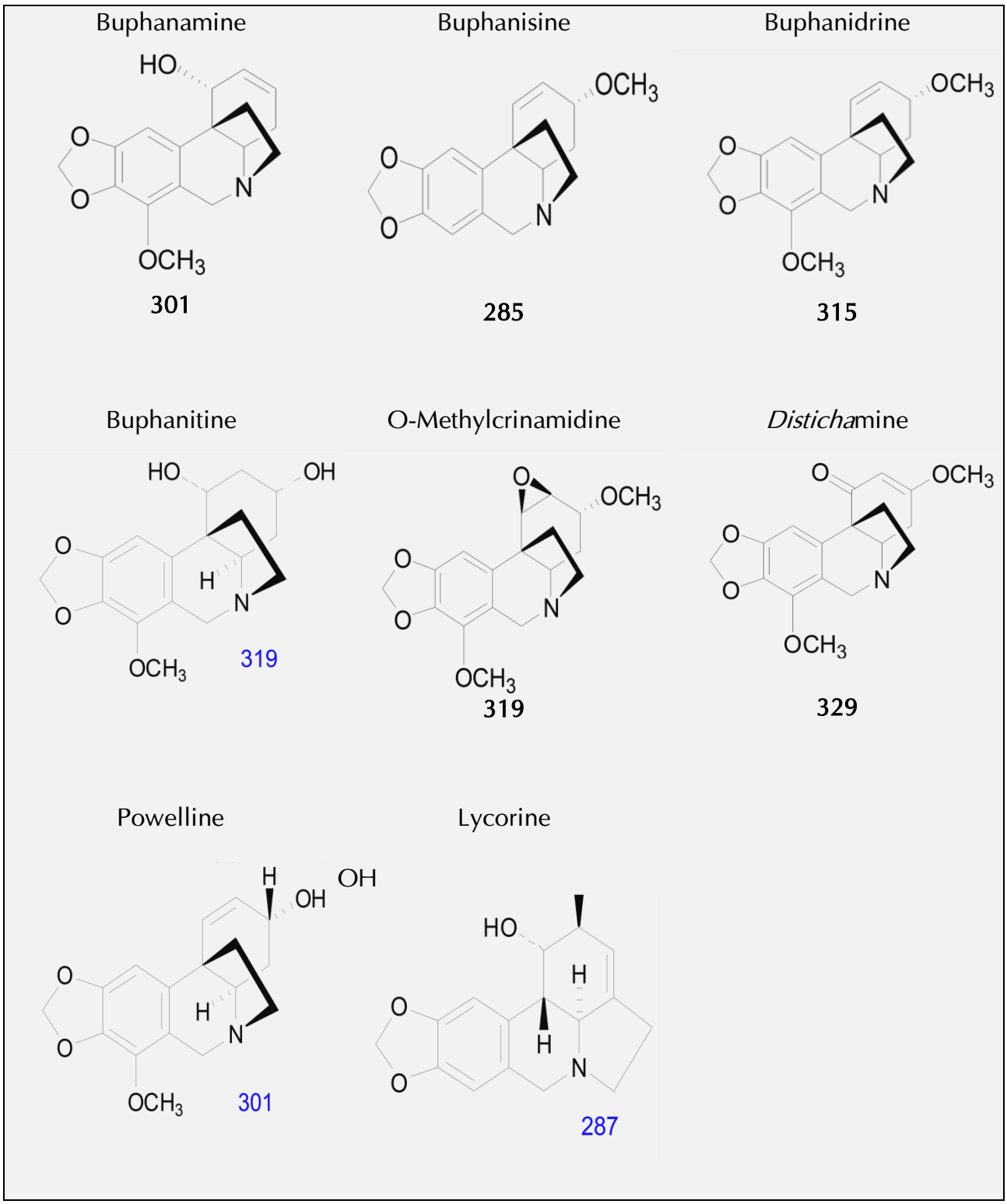


**Figure 1.2.**Structures of some of the major alkaloids extracted from the bulb of *Boophone disticha*. The nominal mass of each structure is shown beneath each structure (adapted from Steenkamp, 2005)

### *1.3.4.2.* Pharmacological Activity

#### *1.3.4.2.1. Anticholinergic and Hallucinogenic Effects*

The purported hallucinogenic activity of *Boophone disticha* has been exploited by the different cultures of Southern Africa. It has been used by the San in trance dance and in Zimbabwe to initiate possession (Gelfand *et al.*, 1985, Watt & Breyer-Brandwijk, 1962). However, although there have been case reports of hallucinogenic effects of *Boophone,* there are no detailed reports about the intentional ingestion of Boophone *disticha for* hallucinatory purposes (De Smet, 1996).

Preliminary investigations on *Boophone disticha* showed that the bulb contained alkaloids with similar activity to Datura alkaloids (Lewin, 1912). Buphanine is mydriatic, inhibits salivary secretion and paralyse the vagus terminations in the heart and leads to death due to respiratory failure. It is postulated to have a similar mode of action to scopolamine, however the effects are weaker and less lasting (Botha *et al.*, 2005; Watt & Breyer-Brandwijk, 1962). The clinical effects of scopolamine overdosage are hallucinations, delirium, coma tachycardia, hypertension, hyperthermia and mydriasis (Goodman & Gilman, 2004).

Haemanthamine has also been extracted and is believed to have some narcotic and emetic properties (Watt and Breyer-Brandwijk, 1962). Nyazema and Ndiweni (1986) did some preliminary work on the effects of a crude extract of *Boophone disticha* on rats. The results showed that the extract induced some psychotic effects that were well managed by chlorpromazine. However, the extract did not show any anticholinergic effects as reported

earlier. This could suggest that the psychotic effect of the *Boophone* alkaloids might be due to a direct effect on the dopaminergic system.

*1.3.4.2.2.Anticholinesterase Activity*

Mental health problems such as schizophrenia, depressive disorders and age-related cognitive disorders are a serious problem in Southern Africa (Stafford *et al.*, 2008). A large number of plants, over 300 species, have been used by traditional healers in the treatment of these ailments. The Amaryllidaceae families comprise the majority of the plants that are used for dementia and age-related mental disorders (Sobiecki, 2002; Stafford *et al.*, 2008). *Boophone disticha* is one of the few plants that have been used in traditional medicine to treat Alzheimer's and as a memory-enhancer (Risa *et al.,* 2004). In a recent investigation of the plants used to treat memory loss in southern Africa using an assay on Thin Layer Chromatography (TLC), extracts of the bulb had significant inhibitory activity on the acetycholinesterase enzyme, which could give an explanation for its use in Alzheimer's disease (Risa *et al.,* 2004).

*1.3.4.2.3.Antidepressant activity*

A number of plants have been used as mood altering substances from prehistoric times, which include *Agapanthus campanulatus, Boophone disticha, Datura ferox, Hypericum perforatum, Mondia whitei, Sceletium tortuosum* and *Xysmalobium undulatum*(Gericke &van Wyk, 2001; Nielsen *et al.*, 2004; Stafford *et al.*, 2008). Two of the above plants, i.e. *Hypericum perforatum* and *Sceletium tortuosum* have been developed into commercial products for the treatment of mild depression (Müller, 2003; Gericke & van Wyk, 2001).

Several antidepressant drugs exert their antidepressant activity by binding to the specific monoamine transporter proteins resulting in inhibition of reuptake of the monoamines back into the neurons (Rang, 2007; Stahl, 1998). In recent studies screening for plants used for anxiety and depression for affinity to the serotonin transporter (SERT), extracts of *Boophone disticha*, *Agapanthus campanulatus, Datura ferox, Mondia whitei* and *Xysmalobium undulatum*had high affinity for the Selective Serotonin Reuptake inhibitor (SSRI) site (Nielsen *et al.*, 2004; Sandager *et al.,* 2005).In a follow-up study these plants were screened for inhibition of SERT, Noradrenaline transporter (NAT) and Dopamine transporter (DAT). *Boophone disticha*, *Agapanthus campanulatus and Mondia whitei* extracts showed high affinity for the SERT, and only *Boophone disticha* and *Agapanthus campanulatus* had some effect on NAT and DAT (Pederson *et al.*, 2008).

*In vivo* investigations were also carried out on the extracts in animal models of depression and all four extracts had some antidepressant effects in various degrees (Pederson *et al.*, 2008). However it is important to note that in the *in vivo* models *Boophone disticha* extract exhibited antidepressive effects at the lowest concentration (125mg/kg), and higher doses caused a loss of the effect. This might possibly indicate that lower doses might have more antidepressive activity and are worth investigating (Pederson *et al.*, 2008).

The alkaloids Buphanamine and buphanidrine were isolated and tested for SERT affinity but only showed slight affinity (Sandager *et al.,* 2005). Structurally buphanamine and buphanidrine have the benzo-1,3-dioxole moiety in common with the clinically used SSRI paroxetine, which could explain their affinity for the SERT (Stafford *et al.*, 2008). Acute

toxicity and analgesic studies have been done on buphanidrine. Its analgesic effect was at a level of 6.2mg/kg in mice and the lethal dose was 8.9mg/kg (van Wyk *et al.*, 2002). However the narrow therapeutic index might make it too toxic and unsuitable for therapeutic use in humans.

*1.3.4.2.4.Anti-inflammatory and Antibacterial Effects*

As already mentioned *Boophone disticha* has been used for the management of inflammatory conditions, bacterial infections, cancer treatment and mummification (Botha *et al.,* 2005; van Wyk, 2008; Watt & Breyer-Brandwijk, 1962). The reputed effects of *Boophone disticha* on components of the immune system and inflammatory response, led to *in vitro* investigations of extracts of *Boophone disticha* on Adenosine triphosphate (ATP) production in isolated human neutrophils and inhibition of superoxide release from neutrophils (Botha *et al.,* 2005). The results of the study showed no significant increase on ATP production but a significant increase in the superoxide production by the neutrophils. This could provide a possible explanation for its traditionally use to relieve rheumatic pains, muscular sprains and other inflammatory conditions.

The antibacterial and antifungal activities of the Amaryllidaceae plants are well known (Elgorashi *et al.*, 2003; Ghosal 1985). In a recent study of Amaryllidaceae alkaloids from crinum species for anti-inflammatory and antibacterial activities, the alkaloids exhibited very low activity against Cyclooxygenase-1(COX-1) and almost no activity against Cyclooxygenase-2 (COX-2) in the cyclooxygenase assay, and very low antibacterial activity with the microtitre plate method (Elgorashi *et al.*, 2003). However, in a preliminary study

*Boophone* extracts were shown to have appreciable anti-inflammatory activity, but little antibacterial activity (Shale *et al.*, 1999). Therefore, from these findings, the antibacterial and anti-inflammatory activity of *Boophone* and other Amaryllidaceae plants cannot be attributed to their alkaloidal content only, and it has been suggested that these effects in the *Boophone disticha* are due to the volatile oil, eugenol (van Wyk, 1997; Watt &Breyer-Brandwijk, 1932).

Therefore, although a lot of work has been done to isolate and characterise *Boophone* alkaloids, and to some extent determine their effects on animal models, very little is still known about their *in vivo* pharmacological and toxicological effects apart from what has been provided by traditional experience, case reports and anecdotal reports.

### *1.3.4.3.* Clinical Toxicology of *Boophone disticha*

#### *1.3.4.3.1. Circumstances & Signs and Symptoms of Intoxication*

A number of *Boophone distich* poisoning cases have been reported around Southern Africa (Du Plooy *et al.*, 2001; Gelfand *et al.* 1985; Laing, 1979; van Wyk, 1997, 2002; Watt and Breyer-Brandwijk, 1962). The majority of cases have been due to accidental poisoning probably due to underestimation of the high toxicity of the bulb or due to overdosage of the bulb decoctions (Steenkamp, 2005). Deliberate poisoning with *Boophone disticha* have also been reported in cases of both homicidal and suicide attempts. Decoctions of the herb have been used as rectal or vaginal enemas in suicidal attempts (Laing, 1979; Steenkamp, 2005).

The toxic effects of *Boophone disticha* are well recognized and numerous cases of poisoning have been reported (Laing, 1979; du Plooy *et al.*, 2001). Symptoms of poisoning include dizziness, restlessness, impaired vision, unsteady gait and intense visual hallucinations. Mild intoxication produces temporary symptoms that diminish within 24hrs, but acute poisoning with large doses of the bulb extract will also induce diarrhoea, vomiting and a general weakness, which will rapidly lead to death (Laing, 1979; du Plooy *et al.*, 2001).

#### *1.3.4.3.2. Case Reports of Boophone disticha Poisoning*

*Case 1(Laing, 1979)*

The hallucinogenic efficacy of *Boophone disticha* was documented by Laing (1979), who reported on three young men presenting at a Zimbabwean hospital the morning after they had taken the bulb. One of them was deeply unconscious, and had dilated pupils, tachycardia, raised blood pressure, a slightly raised temperature and labored respiration. He remained in this state for 24 h and was discharged after 72 h with a normal pulse, blood pressure and temperature. The second young men appeared to suffer from an acute psychotic episode with violent hallucinations. His physical signs were similar to those in the first victim but less marked. He was treated with intravenous chlorpromazine to sedate him and after 36 h he had recovered. The third young man, who was not admitted, claimed that he had taken the decoction with the other two. He had spent the night feeling drunk and seeing visions and felt perfectly well the following morning. On examination, the only abnormal sign was slightly dilated pupils. After recovery, all three men claimed that the hallucinatory effect of the bulb was well known in their area (Gutu district). Botanical

analysis of stomach contents and remnants of the actual bulbs confirmed that they had ingested *Boophone disticha.*

*Case 2 (du Plooy, 2001)*

A number of unpublished cases have been reported at South African hospitals (Botha *et al.*, 2005). The symptoms in these cases where similar to those observed in the above cases. More recently du Plooy and colleagues (2001) reported a fatal case of a man who ingested 150 ml of a concoction to see who placed a spell on him. He started to hallucinate, thinking that somebody was attacking him. He pulled his gun and fired shots randomly, killing one person and injuring others. Gas chromatography and mass spectrometric analysis of a sample of the concoction showed that it contained buphanadrine, buphanine and crinamidine (alkaloids) and eugenol. Although it could not be proved that the concoction was only from *Boophone disticha*, the components were similar to those found in Amaryllidaceae to which *Boophone* belongs. The man's behavioural symptoms can be ascribed to the ingestion of compounds found in B. *disticha*.

*Case 3(Steenkamp, 2005)*

Recently, Steenkamp (2005) reported three cases of accidental intoxication with *Boophone disticha* due to administration of large doses of the bulb decoctions. Analysis of the traditional medicine used indicated the presence of *Boophone* alkaloids. In the first case, two black males went to a traditional healer to obtain a 'muti' (traditional medicine) to "clean their systems", and were given a plant bulb to boil in water. They administered the

'muti' as an enema by using a rubber bulb, and drank the rest of the decoction. They both collapsed – one died and the other person was admitted to a hospital. The empty 'muti' bottle and a small piece of the plant bulb was sent for analysis and five of the alkaloids common to the bulb of *Boophone disticha* were detected in the piece of plant bulb (Steenkamp, 2005).

In another case, a black female visited a traditional healer and was given a powder to mix with water and administer as an enema. This she did and died a few hours later. The last remains of the powdered 'muti' sample and the rubber bulb used to administer the enema were sent for analysis and displayed the characteristic alkaloid fingerprint of the extract of the bulb of *Boophone disticha*. Identical results were obtained when the rubber bulb, used to administer the enema, was analysed (Steenkamp, 2005).

In the last case, a black female consulted a traditional healer to obtain a 'muti' (traditional medicine) to purge her system. She was handed a 750 ml bottle of 'muti' and told to drink half of the bottle and to administer the other half as an enema. She administered the enema and drank the bottle of 'muti', but became violent and sick, vomited repeatedly and died shortly thereafter. Analysis of stomach, liver, kidney samples and the empty 'muti' bottle revealed the presence of *Boophone* (Steenkamp, 2005).

*Case 4 (Gelfand et al., 1985)*

In yet another case, a patient had ingested the milky juice from the *Boophone disticha* bulb (1952) in Zimbabwe. The patient had been told that this could cure abdominal pains. The

patient later told of the burning pain in the epigastrium and immediate dizziness that he felt. He then lost consciousness. In hospital, the patient was found to be irrational, restless and talkative. He displayed intense photophobia. The skin was dry with an elevated temperature, a pulse of 76 and a respiration rate of 36 per minute. There was a right extensor plantar response, the left being equivocal. After twelve hours, the patient was still irrational with dilated pupils that reacted to light. Both plantar responses were flexor, but the tendon jerks were brisk with abdominal distension. The blood pressure was 140/104 mmHg. Gelfand *et al.* (1985) summarised the sign of *Boophone disticha* poisoning as, ataxia and giddiness, impaired vision, talkativeness or quiet and depression, stupor and coma (finally).

*Case 5(Gelfand et al., 1985)*

A patient (18 years old) was deeply unconscious with widely dilated pupils that reacted sluggishly; a tachycardia (110-120 beats per minute), raised blood pressure (150/110 mmHg), a slight temperature (37.4 – 37.6$^{o}$C) and laboured respiration. He remained in this state for 24 hours and then gradually recovered and was discharged after 72 hours with a normal pulse, BP and temperature.

### *1.3.4.4. Necessity of Experimental assessment for Boophone disticha*

To reiterate, the lack of knowledge on the toxicological and pharmacological effects of *Boophone disticha* and its implication in poisoning cases makes it imperative for experimental characterisation of the symptomatology and the lethality of intoxication. Furthermore Boophone has been extensively used in traditional medical practices to treat a variety of ailments (Botha *et al.*, 2005). Thus, its importance in ethnopharmacology in

Southern Africa renders it a worthwhile candidate for preclinical efficacy studies and further drug development. Thus, the following section gives detailed information on the necessary experimental methods, both toxicological and pharmacological which can be employed to achieve the aforementioned goal.

### 1.3.5. Experimental Pharmacology and Toxicology Approaches

#### *1.3.5.1. General Principles*

The best way of predicting the clinical and adverse effects on human health is to test potentially toxic substances directly on human subjects. However, this approach is often difficult and in many situations is unethical (DiPasquale& Hayes, 2001). Some pharmacological and toxicological data can be derived directly from humans, through case reports of accidental exposures to industrial chemicals, cases of food-related poisoning, epidemiological studies and clinical investigations. However, often the nature and extent of available human toxicological data are too incomplete to serve as the basis for an adequate assessment of potential health hazards (EHC223; WHO, 2001). Consequently, in preliminary toxicity and safety investigations of various drugs, herbs and chemicals, animals are commonly used to predict risk in humans. The suitability of experimental animal data is an important contemporary issue in toxicology (Wilson *et al.*, 2001; Brown, 1988). The ultimate goal of toxicology assessments is to characterize toxicity in animal models and extrapolate to the human situation. There are basically two main guiding principles to experimental toxicology which are; that animal assays, when properly qualified, serve as useful predictors of potential human effects, and that exposure of test animals to high doses is a valid

determinant of human hazard (Wilson, 1990; Wilson *et al.*, 2001). Therefore the use of adequate test systems is critical to the predictive ability of animal toxicity studies.

*In vitro* studies have been used to complement whole-animal experiments. They are valuable in providing information on basic mechanistic processes in order to refine specific experimental questions to be addressed in the whole animal (Goldberg & Frazier, 1989; Harry *et al.,* 1998, WHO, 2001; Wilson *et al.*, 2001). However, *in vitro* studies have limitations. Neurobehavioral effects such as loss of memory or sensory dysfunction, organ system interactions and organ system effects cannot be modelled with the current *in vitro* strategies (Harry *et al.,* 1998).

The effects on human health of many plants and herbs used in traditional medicine systems are from experience or claims from ethnomedicinal usage. But little is known about both the exact pharmacological effects and the toxicological effects of these medicinal plants and herbs. The World Health Organisation (WHO) has been recently advocating for the integration with traditional medicine systems with conventional medical practices in resource poor countries, and recent surveys in southern Africa have shown that the majority of the population rely on traditional medicines (Fennell *et al.*, 2004; WHO, 1999). This unfortunately, has exposed many people to potentially toxic plants with little or no documented evidence of efficacy. Therefore the WHO and many research institutes in tropical countries are placing emphasis on research on plants used in ethnomedical practices particularly in the developing world. In line with this, the major aim of this study is

to validate the traditional claims and determine the toxic effects of the *Boophone disticha* in an *in vivo* model.

### *1.3.5.2.* Experimental Design of Animal Experiments

#### *1.3.5.2.1.Model Species Selection*

The use of experimental animal data to extrapolate human hazards is not without controversy. Relative sensitivity across species as well as between sexes is a constant concern. Overly conservative risk assessments, based on the assumption that humans are always more sensitive than a tested animal species, can result in poor risk management decisions. Conversely, an assumption of equivalent sensitivity in a case where humans actually are more sensitive to a given agent can result in under-regulation, which might have a negative impact on human health. Therefore, proper and informed selection of an animal species is vital in the experimental design of toxicity studies (Wilson, 1990). No strain or species is likely to be ideal, and selection is based on practical considerations and the species that most closely mimics man in its response. The routine procedure is for two species to be used and these are generally a rodent and a non-rodent species.

The rat is the most frequently used species for toxicology studies (Brown, 1988). Its responses to chemical exposure are more likely to be sensibly interpreted than those of other species. Their short lifespan, ease of handling and breeding allow them to be used extensively in routine acute, subacute and Subchronic, and lifespan studies (Brown, 1988; Wilson, 1990; Wilson *et al.*, 2001). Furthermore, carcinogenicity and mechanistic studies require large numbers of animals to be more sensitive, and this is only practical with rats and mice

(Traystman, 2003). However their small size limits the amount of blood that can be drawn for use in clinical chemistry studies. Another considerable advantage of using rodents is that neurosensory and motor behaviour measurements can be made relatively easy (Traystman, 2003).

Although, using large animal species such as guinea pigs, rabbits, primates and dogs is very relatively costly compared with small animals, it has got its own advantages. Firstly, imaging techniques such as Nuclear Magnetic Resonance (NMR) and spectroscopy imaging can be easily done with large animals, and the brain of large animals is closer in structure to the human brain (Klein and Nelson, 2002; Landi, 2001; Traystman, 2003). Again, sophisticated physiological monitoring such as evoked potential monitoring, Electroencephalography (EEG), arterial blood gases, blood pressure, blood glucose etc. can be done simultaneously on the same animal (Traystman, 2003).

### 1.3.6. Risk Assessment and Toxicological Evaluation Studies

Risk assessments of safety pharmacology studies were originally developed to test new chemical entities and novel pharmaceuticals. However, they can also be applied and have been recommended for testing the safety of herbal products, animal and plant toxins. The purpose of the studies is to assess the adverse effects of a compound on the physiological function of one or more organs or organ systems (e.g. neurotoxicity studies); thus are carried out on whole animal models (Hite, 1997). Toxicity testing can be considered to be composed of several major types of studies, which include acute, subacute, subchronic and chronic tests, and these are discussed below.

### *1.3.6.1. Acute Toxicity studies*

Acute toxicity is defined as that toxicity which arises soon after administration and unless death occurs recovery is complete (Wilson, 1990; Brown, 1988). The median lethal dose($LD_{50}$) of a substance, or the amount required to kill 50% of a given test population. The LD50 value for a substance will vary according to the species involved. The substance may be administered any number of ways, including orally, topically, intravenously, or through inhalation. The most commonly used species for these tests are rats, mice, rabbits, and guinea pigs(Wilson, 1990; Brown, 1988).

Over the years acute toxicity testing has been taken to be synonymous with the lethal dose, yet the $LD_{50}$ is only one of many indices used in defining acute toxicity. This misconception rose due to the relative ease of determination and frequent use of the $LD_{50}$ (Brown, 1988). However there are two aspects of that are not addressed by the $LD_{50}$ value that is symptomatology and pathological changes. Proper experimental design of an acute toxicity study should allow determination of non-lethal parameters together with the $LD_{50}$ (DiPasquale & Hayes, 2001).

The significance of the $LD_{50}$ value has been questioned by many toxicologists (DiPasquale & Hayes, 2001; Kennedy *et al.,* 1986). The determination of $LD_{50}$ with 95% confidence limit has been described as wasteful in terms of number of animals sacrificed (Rowan, 1983; Kennedy *et al.,* 1986). Aninter-laboratory comparison study of $LD_{50}$ by Hunter *et al.,* (1979), showed wide variations on the $LD_{50}$ values. The $LD_{50}$ has been shown to vary according to species, age, weight, sex, strain, nutrition and environment factors (Rowan, 1983). Another

weakness is that it does not take into account toxic effects that do not result in death but are nonetheless serious. However, estimation of the $LD_{50}$ value could still provide valuable information about the toxicity of a compound. The United States Environmental Protection Agency (EPA) and other international bodies now recommend the use of a limit test to estimate the $LD_{50}$ (OECD, 2000).

*1.3.6.1.1.Alternatives methods for the oral $LD_{50}$ Test*

A number of methods (Table 1.2) based on the concept of a Limit Test that use fewer animals as alternatives to estimate the acute lethal dose (Walum, 1998; Rispin *et al.,* 2002) have been developed over the years, and include the fixed dose procedure (FDP), the acute toxic class (ATC) method and the Revised Up-and-Down procedure (UDP) (OECD; 1992, 1996, 1998). All three methods emphasize humane treatment of animals undergoing testing and each assay utilizes a major endpoint other than lethality as its determining value (Huggins, 2003).

**Table 1.2.** The principles of the three alternative methods (adapted from Botham, 2004)

| | FIXED DOSE PROCEDURE | ACUTE TOXIC CLASS | UP AND DOWN PROCEDURE |
|---|---|---|---|
| *Methodology* | Single bolus dose. Young adult rats (one sex). Oral gavage with constant volume or concentration, clinical observations, bodyweight, mortality over 14 days. Necropsy at termination. | | |
| *Sighting study* | Yes | No | No |
| *Dose levels* | Fixed doses 5,50,300,2000 (5000)mg/kg | Fixed doses 5,50,300,2000 (5000)mg/kg | Starting at best estimate of $LD_{50}$ (or 175mg/kg) and using dose progression factor of 3.2, single animals dosed until one of three stopping criteria |
| *Aim* | Identify lowest fixed dose causing evident toxicity | Identify lowest fixed dose causing mortality | Estimate $LD_{50}$ |
| *Output* | Range estimate of $LD_{50}$<br>Signs of acute toxicity.<br>Target organ(s) | Range estimate of $LD_{50}$<br>Signs of acute toxicity.<br>Target organ(s) | Point estimate of $LD_{50}$ with confidence intervals<br>Signs of acute toxicity.<br>Target organ(s) |

*Acute Toxic Class Method*

The ATC method (OECD TG 423; OECD 1996) consists of a stepwise procedure with the use of three animals of a single sex (usually female rats) per step. Mortality at one step determines progression to the next stage i.e. either stopping test, dosing of 3 additional animals with the same dose or dosing 3 additional animals at the next higher or lower dose. The starting dose is selected from one of four fixed levels i.e. 5, 50, 300 and 2000mg/kg and should be the one postulated to most likely produce mortality. When there is no information on a substance it is recommended to use a starting dose of 300mg/kg (OECD, 1996). This does not allow calculation of a specific $LD_{50}$ value but allows determination of a dosage

range of where lethality is expected. Its main advantage over the traditional $LD_{50}$ is that it uses substantially fewer animals and produces sufficient information about the signs of toxicity (Huggins, 2003).

*Fixed Dose Procedure*

The FDP (OECD TG 420; OECD 1992) consists of dosing groups of animals of a single sex (usually females) in a stepwise procedure using fixed doses (5, 50, 300 and 200mg/kg). The starting dose is selected as the one expected to produce signs of toxicity without causing severe toxic effects or mortality. Further testing with higher or lower doses is dependent on the occurrence and severity of the signs of toxicity. The procedure continues until the dose causing evident toxicity is identified (OECD, 1992).

The method has been validated international and it has been shown to produce consistent results (van den Heuvel, 1990). The FDP enables substances to be ranked according to the EU classification system as it provides the necessary information on the nature, time of onset, duration and outcome of intoxication required for risk assessment (Botham, 2004). It also causes less pain, distress and mortality than the other methods.

*Up-and-Down Procedure*

The UDP aims to estimate the $LD_{50}$ value by testing individual animals sequentially, with the dose for the next animal being adjusted up and down (by a progression factor of 1.3 times the initial dose) depending on the outcome for the previous animal. The first dose is estimated from the best estimate of the $LD_{50}$ (OECD, 2001). This procedure allowed

convergence on the region of the $LD_{50,}$ which is the inflection point corresponding to the median response of the log dose-response curve. The UDP works best if there is some knowledge of the approximate $LD_{50}$ and slope, however poor choice of starting dose could lead to a delayed converge (Rispin *et al.,* 2002). This drawback led to the Revision of UDP to incorporate a sighting study before the main test and allows use with substances with a wide range of slopes (Rispin, 2002; OECD, 2001). Other changes that were incorporated into the revised UDP included use of single sex (female) only to reduce variability, increase of the dose progression factor from 1.3 to 3.2, and increase of the time between dosing (OECD, 2001).

In the present study, acute toxicity testing involved behavioural changes, histopathological evaluations and estimation of the $LD_{50}$ using a modification of the revised up and down procedure (OECD, 2001).

### *1.3.6.2. Repeated Dose Toxicity Studies*

The objective of repeated dose toxicity studies is to screen for potential adverse effects of substances using animal models as surrogates (Wilson*et al.,* 2001). These studies are of varying duration, generally 1 to 4 weeks for short-term (subacute), 3 months for sub-chronic and 6-12 months for chronic studies (Wilson*et al.,* 2001; USEPA, 1998).

#### *1.3.6.2.1.Subacute Studies*

Short-term tests are performed to obtain information on the toxicity of a substance after repeated administration and are generally required for the successful design of subchronic

studies (Eaton & Klaasen, 2001; Wilson*et al.,* 2001). The major objective of short-term studies is to determine adverse effects at low doses, dose response and sometimes to identify target organs (Brown, 1988).

*1.3.6.2.2. Subchronic Studies*

The principal goals of subchronic toxicity studies are to identify adverse effects not detected in subacute studies, to establish a non-observable adverse effects level (NOAEL) and to further identify specific target organs or sites of action (Brown, 1988; Eaton & Klaasen, 2001; Wilson*et al.,* 2001). Subchronic studies data alone may be sufficient to predict the hazard of long-term exposure (Wilson et *al.,* 2001).

*1.3.6.2.3. Chronic Studies*

Chronic studies are performed in a similar manner to subchronic studies, except that the duration of exposure is prolonged. They are performed to assess the cumulative toxicity of substances and the majority of chronic effects. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects, histopathological effects and often include carcinogenic evaluation (Eaton & Klaasen, 2001; USEPA, 1998). These studies are usually performed with rodent species, rats and mice, so as to extend over the lifetime of the species (Eaton & Klaasen, 2001; Nelson *et al.,* 2001).

### *1.3.6.3. Genotoxicity Studies*

Genetic toxicology testing is a basic component in preclinical assessment of drug candidates, industrial and environmental chemicals (Brambilla & Martelli, 2004; Witte *et al.*, 2007). Various guidelines have been established to identify potential risk for carcinogenicity and heritable mutations (Kirkland *et al.*, 1994; Muller *et al.*, 1999). The currently recommended approach is to carry out the following battery of tests i.e. an *in vitro* bacterial gene mutation test, an *in vitro* mammalian cell test and an *in vivo* test for chromosomal damage using rodent haematopoietic cells (Brambilla & Martelli, 2004; Witte *et al.*, 2007). However, a reduced battery one or two *in vitro* tests, can be acceptable in cases where physicochemical and metabolic properties indicate lack of genotoxicity activity (Eisenbrand *et al.,* 2002).

Generally the *in vitro* tests are used as screening tests before progressing to the hazard assessment *in vivo tests.* The most commonly used screening *in vitro* bacterial test is the Ames *salmonella* test due to its simplicity and relative cheap cost (Eisenbrand *et al.,* 2002). The comet assay is used for both *in vitro* and *in vivo* studies of assessment of chromosomal damage. Other tests that can be used to evaluate genotoxicity potential include the chromosome aberration assay micronucleus assay, saccharomyces cerevisiae mutation assay etc. (Brambilla & Martelli, 2004; Witte *et al.*, 2007).

#### *1.3.6.3.1. The Ames Test*

The Ames test developed by Ames *et al.* in1973, uses *Salmonella typhimurium* strains that have lost their ability to grow in the absence of histidine (Mortelmans & Zeiger, 2000).

Reverse mutations caused by exposure to mutagenic compounds can reactivate their ability to synthesize histidine and thus grow in the absence of histidine. The number of colonies at different concentrations of the test compound is compared with that of the negative control and indicates the degree of mutagenicity (Ames *et al.,* 1973; Maron & Ames, 1983). The most commonly used *Salmonella* strains are TA98 and TA100 as they detect the great majority of mutagens (De Sa Ferreira &Ferrao Vegas, 1999). Strain TA98 gives an indication of frame-shift mutations, while a positive response from strain TA100 indicates base-pair substitution.

For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds must be more than twice the number of colonies produced on the solvent control plates (i.e., a ratio above 2.0). In addition, a dose–response should be evident for the various concentrations of the mutagen tested. Toxicity can be checked by investigation of the background layer of bacteria (Elgorashi *et al.,* 2003; Reid *et al.*, 2006; Verschaeve *et al.,* 2004).

Most carcinogens require biotransformation to reactive species that can exert a genotoxic effect, but in bacteria cells xenobiotic metabolizing enzyme systems are lacking. Therefore to circumvent this limitation exogenous metabolic system (rat liver S9-mix) enzymes are usually added to approximate the whole animal scenario (Brambilla & Martelli, 2004; Eisenbrand *et al.,* 2002).

### 1.3.7. Neurobehavioural Assessment in Toxicity Studies

There is a growing concern on the potential neurotoxicity of many new drug entities and environmental chemicals. Considerable emphasis is now being placed on the need of neurotoxicity testing of chemicals to be done at the screening or first-tier level (Moser, 1991). Several international expert panels such as the WHO, OECD and Food and Drug Agency (FDA) recommend neurotoxicity assessments at screening level of new chemicals, drug entities and herbal products (Hayes, 2001; USEPA, 1998, WHO, 1986). The United States Environmental Protection Agency (USEPA) recommends a neurotoxicity screening, battery which consists of neurobehavioural tests i.e. a functional observational battery (FOB and motor activity) and neuropathological evaluations for testing pesticides and natural toxins (Moser, 1994; McDaniel and Moser, 1993; USEPA, 1991).

Neurobehavioural screening batteries are intended to detect profiles of neurological effects, define the dose-range and time-course of effectiveness and provide direction for second-tier testing (Moser, 1994). Because both the pharmacological and toxicological effects of *Boophone disticha* from both the case reports and traditional use are indicative of a neurotoxicological mechanism, the present study has concentrated on assessing the potential neurotoxicity of the herb using both the FOB and motor activity. The FOB and motor activity tests are described below. Other potential effects on the central nervous system like antidepressant effects, anxiolytic and antiepileptic effects need to be assessed and methods to assess them are also discussed below.

### *1.3.7.1. Functional Observational Battery*

The functional observational battery (FOB) consists of a series of tests to assess sensory, neuromuscular and autonomic function. The component tests involved in the FOB are listed in Table 1.3 below. The components of a FOB can also be categorised according to the functional domain (Table 1.4). Procedural descriptions of the tests can be found in appendix 1. The FOB has been shown to be sensitive in detecting neurological activity at doses of chemicals, which are not detectable with other neurobehavioural tests. In addition, the tests in the FOB are relatively specific, non-invasive and non-neurotoxin agents have very few, if any, effects on the test (Moser, 1990).

**Table 1.3.** Components of the Functional Observational Battery (adapted from Moser, 1990)

| Home cage & Handling Open Field | Manipulative | Physiologic |
|---|---|---|
| Posture (D) | Ease of removal (R) | Body temperature (I) |
| Convulsions, tremors (D) | Handling reactivity (R) | Body weight (I) |
| Palpebral closure (R) | Palpebral closure (R) | |
| Lacrimation (R) | Approach response (R) | |
| Piloerection(R) | Click response (R) | |
| Salivation (R) | Touch response (R) | |
| Vocalisations (Q) | Tail pinch response (R) | |
| Rearing (C) | Righting reflex (R) | |
| Urination (C) | Landing foot splay (I) | |
| Defecation (C) | Forelimb grip strength (I) | |
| Gait (D,R) | Hindlimb grip strength | |
| Arousal (R) | (I) | |
| Mobility (R) | Pupil response (Q) | |
| Stereotypy (D) | | |
| Bizarre behaviour(D) | | |

D-descriptive data; R-rank order data; Q-quantal data; I-interval data; C-count data.

**Table 1.4.** Measures of the Functional Observational Battery by Functional Domain (adapted from Moser, 1990)

| *Autonomic* | *CNS Excitability* | *CNS Activity* |
|---|---|---|
| ➢ Lacrimation | ➢ Ease of removal | ➢ Home cage posture |
| ➢ Salivation | ➢ Handling reactivity | ➢ Palpebral closure |
| ➢ Pupil response | ➢ Clonic movements | ➢ Rearing |
| ➢ Palpebral closure | ➢ Tonic movements | ➢ Motor activity |
| ➢ Defecation | ➢ Arousal | |
| ➢ Defecation | ➢ Vocalisations | |
| ➢ Urination | | |
| *Neuromuscular* | *Sensorimotor* | *Physiological* |
| ➢ Gait score | ➢ Tail pinch response | ➢ Body temperature |
| ➢ Mobility | ➢ Click response | ➢ Body weight |
| ➢ Landing foot splay | ➢ Touch response | ➢ Piloerection |
| ➢ Forelimb Grip strength | ➢ Approach response | |
| ➢ Hindlimb Grip strength | | |
| ➢ Righting reflex | | |

### *1.3.7.2. Motor Activity*

Motor activity is included in the neurobehavioural battery both as a measure of motor function and as an apical test (Tilson *et al.*, 1987). Assessment of motor activity is non-invasive and has been used to evaluate the effects of acute and repeated exposure to neurotoxicants (MacPhail *et al.*, 1989). Numerous devices are used in the measurement of motor activity and these include the figure 8 maze, video-image analysis, telemetry, beam walking and the animal activity meter (photoactometer) (Reiter and MacPhail, 1979; Porsolt *et al.,* 2002). These tests involve placing the test animal, which is usually a rodent, onto the apparatus for a fixed observation time after administration of the test substance and

measuring any changes in locomotion. Since most of the tests are automated, a large number of animals can be tested simultaneously and different aspects of locomotion such as rearing, small or large displacements can also be measured at the same time. These tests can be used to measure both exploration and habituation behaviour.

Habituation is known to be affected by changes to the cholinergic system and the muscarinic antagonist scopolamine is known to impair habituation (Bà & Seri, 1995; Ehman & Moser, 2006). However antimuscarinic agents like atropine do not affect habituation and thus it has been suggested that habituation is not exclusively mediated through the cholinergic system alone, but is also is affected also through the GABAergic system (Giovannini *et al.,* 2001; Ehman and Moser, 2006).

*Boophone* alkaloids have been postulated to have some anticholinergic effects (Risa *et al.,* 2004), and therefore investigation of the bulb extract's effects on explorative and habituation behaviour in the open field could give some insight on the effects on the cholinergic system.

*1.3.7.2.1.The Rota-rod Test*

Motor coordination is commonly assessed using a rota rod, rope climb and beam walking (Roegge *et al.,* 2004). In the rota rod test, the animal is placed on a rotating rod (Figure 1.3) and the time to fall off the rod is measured. The animals are given prior habituation before administration of the substance, and this is done to decrease test variability (Porsolt *et al.,* 2002). The rota rod can only detect the capacity of a substance to decrease neuromuscular coordination, however in conjunction with other locomotor test in the FOB, it can provide

some quantification of the safety margin between doses which alter spontaneous motor activity, and those which impair motor function (Porsolt *et al.,* 2002).

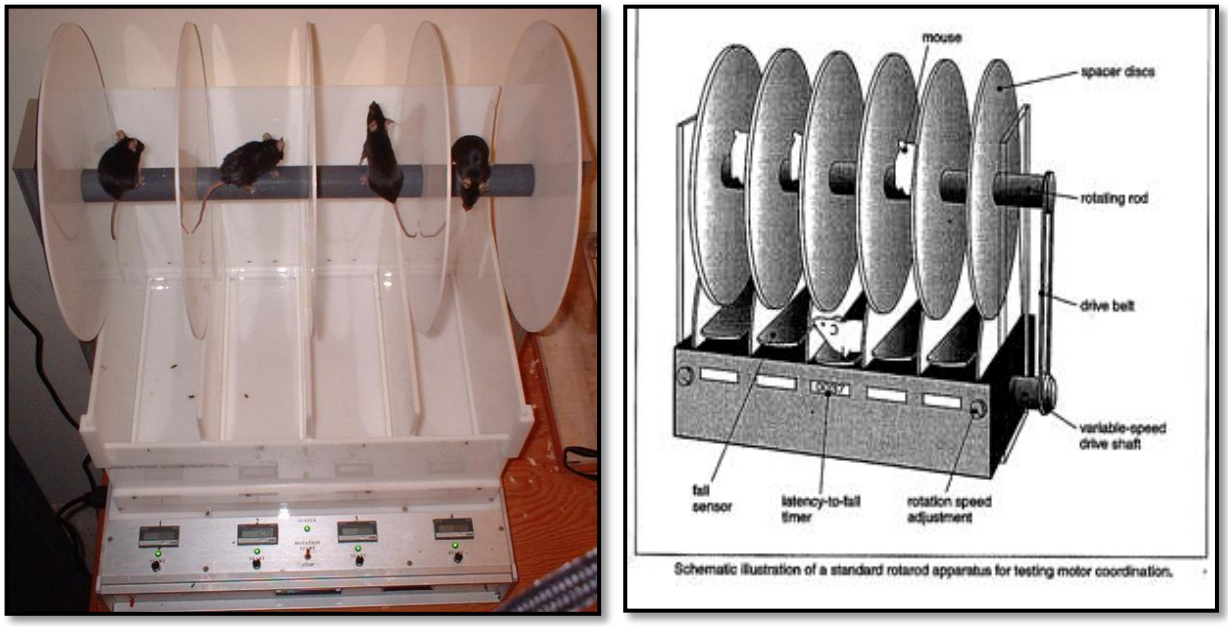


**Figure 1.3.** Rota-rod Apparatus

The rota-rod test provides a simple automated way of assessing motor coordination and balance in rodents (Carter *et al.*, 1999, 2001; Dunnett *et al.*, 2003; Stanley *et al.*, 2005). The rats or mice have to keep their balance on a rotating rod, which can either be at different speeds or continuously accelerating (Carter *et al.*, 1999, 2001; Dunnett *et al.*, 2003). In all the versions, the latency to fall provides the single measure of performance (Dunnett *et al.*, 2003). The rota-rod test is basically divided into two models, a stepped version and a continuously accelerating version. In the stepped version the animal is tested at constant speed for a number of trials, with the speed being increased progressively until the final trial (Carter *et al.*, 2001; Dunnett *et al.*, 2003). In the accelerating version the animal is tested on

a single trial with the rod accelerating gradually until it reaches a specific speed (Carter *et al.*, 1999; Dunnett *et al.*, 2003; Fox *et al.*, 2001). The accelerating version is simpler and more efficient compared to the stepped version, however it is less sensitive (Dunnett *et al.*, 2003; Fox *et al.*, 2001).

The rota-rod test has been widely used to predict clinical sedation (Stanley *et al.*, 2005), using ataxia as predictor of human sedative effects. However, the rota-rod test is not able to separate sedation from motor incoordination and Stanley *et al.*, (2005) showed it to be a less sensitive test in determining benzodiazepine-induced motor incoordination than the beam walking assay. Despite its shortcomings, the rota-rod test has been adopted as the most reliable test for screening relevant transgenic mouse models of Huntington's disease (Dunnett *et al.*, 2003).

*1.3.7.2.2.Open Field Test*

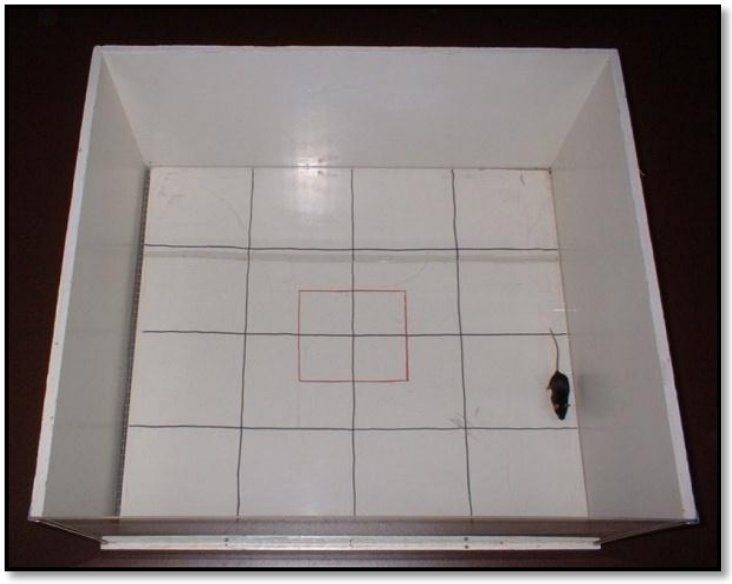

**Figure 1.4.** Open Field Apparatus

The Open Field Test (OFT) is used to assess simultaneously locomotor activity, exploratory drive and anxiety (Prut &Belzung, 2002; Walsh & Cummins, 1976). The test was originally described by Hall (1934) for the study of emotionality (Prut & Belzung, 2002). It consists of placing a rodent in the centre of an inescapable novel arena (circular square or rectangular), surrounded by walls for a period ranging from 2 to 20 minutes (usually 5min.). Its behaviour is then measured. Like most behavioural experiments, it is based on the natural approach-avoidance behaviour of animals in novel environment.

In a novel situation rodents spontaneously prefer the periphery of the arena to activity in the centre. When anxious rats and mice have a strong tendency to walk or stay close to walls, the behaviour is called thigmotaxis (Belzung, 2001; Prut & Belzung, 2002). Therefore the ratio of time spent in the periphery to time in the centre of the open field gives an estimate of anxiety-like behaviour (Belzung, 1999; Walsh & Cummins, 1976; Prut & Belzung, 2002). Rodent behaviour in the open field depends mainly on tactile factors, and thigmotaxic behaviour is depended on the use of vibrissae and mechanoreceptors to sense walls. Mice without vibrissae do not exhibit thigmotaxic behaviour (Prut & Belzung, 2002). Behaviour in the open field is also dependant on lighting conditions and the light-dark cycle, therefore it is important to control lighting conditions, and ensure that treatment doses not alter circadian behaviours (Prut & Belzung, 2003).

In the OFT, the frequency of line crossings and rearings are used as measures of locomotor activity. However they are also indicative of both explorative and anxiety-like behaviour (Prut & Belzung, 2002). Defecation and urination in the open field are used as indices of

emotionality and anxiety-like behaviour (Hall, 1934), but their value as measures of anxiety-like behaviour have been questioned (Bindra & Thompson, 1953; Lister, 1990). Risk assessment behaviour, such as stretch attend posture are also one of the valuable indices that can be assessed in this test. They indicate hesitancy to explore, to move from present to a new position (Blanchard *et al.*, 2001). Anxiolytic substances decrease risk assessment behaviours and increase open area activity (Dawson & Tricklebank, 1995). Rearing is another important parameter noted, and it is a component of vertical exploratory behaviour (Võikar, 2006).

Grooming behaviour is a displacement response that can be elicited by novelty, social contact and stressors (Espejo, 1997; Võikar, 2006). Novelty-induced grooming behaviour decreases with repeated exposure to the testing apparatus (Espejo, 1997). Extended time of repeated exposure to the OFT apparatus can result in habituation to the test system, that is decreased responsiveness due to repeated stimulation (Bolivar *et al.*, 2000; Choleris *et al.*, 2001; Hughes, 2007; Walsh & Cummins, 1976). Initially, when the animals are presented to the novel OFT apparatus, they exhibit more fear-related behaviours (e.g. stretch attends and thigmotaxis).

With repeated trials, more exploration and locomotor activity is observed. However this has been shown to be strain depended in rodents (Bolivar *et al.*, 2000). Finally the OFT has been shown to have face and construct validity. However, it lacks predictive validity as it is lack sensitivity with alprazolam and chronic Selective Serotonin Reuptake Inhibitors, which are effective in anxiety disorders (Belzung, 2001; Prut & Belzung, 2002).

### 1.3.8. Neuropharmacological Assessments

The search for new molecules that act on the central nervous system and can be used to treat conditions such as Alzheimer's disease, anxiety and depression has been a challenge for pharmacologists. Recently, plants with molecules that have such activity have become attractive targets for drug development (Gomes *et al.*, 2009; Kumar, 2006).

*Boophone disticha* has been used to treat anxiolytic disorders, depression, and for memory enhancement (Botha *et al.*, 2005; Pedersen *et al.*, 2008; Risa *et al.*, 2004). This has also been supported by preliminary *in vitro* screening tests (Sandager *et al.,* 2005; Stafford *et al.*, 2008). Therefore, this evidence provides a basis for further in *vivo* neuropharmacological efficacy assessments. The following subsection details some of the animal tests that can in neuropharmacological screening.

#### *1.3.8.1. Behavioural Evaluation of Antidepressant Activity*

Various paradigms have been developed to detect the antidepressant-like potential of novel compounds. They were developed based on behavioural consequences of stress, drugs and lesions. These tests include the Forced Swim Test (FST), Tail Suspension Test (TST), chronic mild stress and learned helplessness (Cryan *et al.,* 2002; Deussing, 2006; Holmes *et al.,* 2002). Among the screening tests, the behavioural despair tests are the most commonly used to assess antidepressant-like activity and of these, the Forced Swim Test is the most frequently used paradigm (Deussing, 2006, Holmes, 2003; Holmes *et al.,* 2002). This test offers a number of advantages compared to the other methods which require much more extensive manipulations.

*1.3.8.1.1.Forced Swim Test*

**Figure 1.5.**Forced Swim Test setup

The Porsolt's Forced Swim Test (Porsolt *et al.*, 1977a,b) or the Behavioural despair test has been used extensively to evaluate the antidepressant activity of drugs and antidepressant-like behaviour in transgenic mice (Cryan & Mombereau, 2004; Kurtuncu *et al.*, 2005; Porsolt, 2000). The widespread use of this test is due largely to its ease of use, interlaboratory reliability and ability to detect activity of a broad spectrum of substances with antidepressant activity (Cryan *et al.*, 2002; Deussing, 2006; Lucki, 1997).The test was original originally developed to test rats and it has also been modified for use in mice (Cryan *et al.*, 2002; Kurtuncu *et al.*, 2005; Porsolt, 2000). The test is based on the observation that rodents, when placed in an inescapable cylinder filled with water, after initial escape-oriented movements, rapidly develop a characteristic immobile posture (Castagne *et al.*, 2006; Cryan *et al.*, 2002, Cryan *et al.*, 2005; Deussing, 2006). In the FST, immobility is interpreted as a passive stress-coping strategy or depression-like behaviour (behavioural despair or learned helplessness) (Cryan *et al.*, 2002; Deussing, 2006; Kurtuncu *et al.*, 2005; Lucki, 1997).

Antidepressants have been shown to decrease the duration of immobility (Castagne *et al.*, 2006; Cryan *et al.*, 2002; Lucki, 1997). Rodents also exhibit two other behavioural patterns in the FST, swimming and climbing movements and these are indicative of non-depressive behaviour, and scoring of these behaviours has been noted provide a more sensitive model (Crowley *et al.*,2004; Cryan *et al.*, 2002; Deussing, 2006; Kurtuncu *et al.*, 2005). The major drawback with FST is that it is unreliable in detecting the antidepressive effects of selective serotonin uptake inhibitors(SSRIs) (Cryan *et al.*, 2002; Lucki, 1997).

Rats do not display stable behaviour when tested on a single session, therefore the original FST is performed in two sessions. In the first day session it is tested for a 15minute period and on the second session (24hr later), it is tested for only 5 minutes. Mice are tested in the modified Forced Swim Test, which was developed to enhance sensitivity of the test to SSRIs antidepressive effects. In the modified FST the water depth is increased from 15-18cm in the rat FST to 30cm. The first session was removed and the increased the swimming session was increased to 6minutes. The first 2 minutes of the FST are accepted as a habituation period and immobility behaviour is recorded in 5s intervals (Cryan *et al.*, 2002; Kurtuncu *et al.*, 2005; Porsolt, 2000). The modified FST has an advantage over the traditional FST in that quantification of non-depressive behaviours has predictive value to differentiate catecholaminergic and serotonergic substances. Catecholaminergic agents are noted to decrease immobility with a corresponding increase in climbing behaviour, whereas serotonergic agents such as SSRIs decrease immobility but increase swimming behaviour (Cryan & Lucki, 2000; Cryan *et al.*, 2002; Deussing, 2006).

### *1.3.8.2. Behavioural Evaluation of Anxiolytic Effects*

Several assays of anxiety-like behaviors have been developed. The most common are the so-called exploration-based tests (Holmes, 2003). The premise of these tests is that for some species such as rodents, the innate drive to explore a novel place will be inhibited as aversion to the new space increases. A simple version is the open-field test. High levels of exploration of the open, brightly illuminated area of an enclosure are interpreted as low-anxiety behavior (Holmes, 2003).

Highly anxious mice stay near the wall of the enclosure. Administration and scoring of this test has been automated, and several commercial products for performing the test are available. Defecation constitutes an additional measure of anxiety; high rates of bolus production are correlated with anxiety in wild-type rodents. Treatment with anxiolytics increases the time spent in the "open" portion of the open field and reduces the number of boluses produced (Holmes, 2003).

Obviously, gene manipulations that affect metabolism or food intake could affect bolus production and confound the assay of anxiety. Other exploration-based tests of anxiety include the elevated plus maze, the light–dark exploration test, the emergence test, and the free-exploration test (Belzung & Griebel, 2001).

*1.3.8.2.1. The Elevated Plus Maze Test*

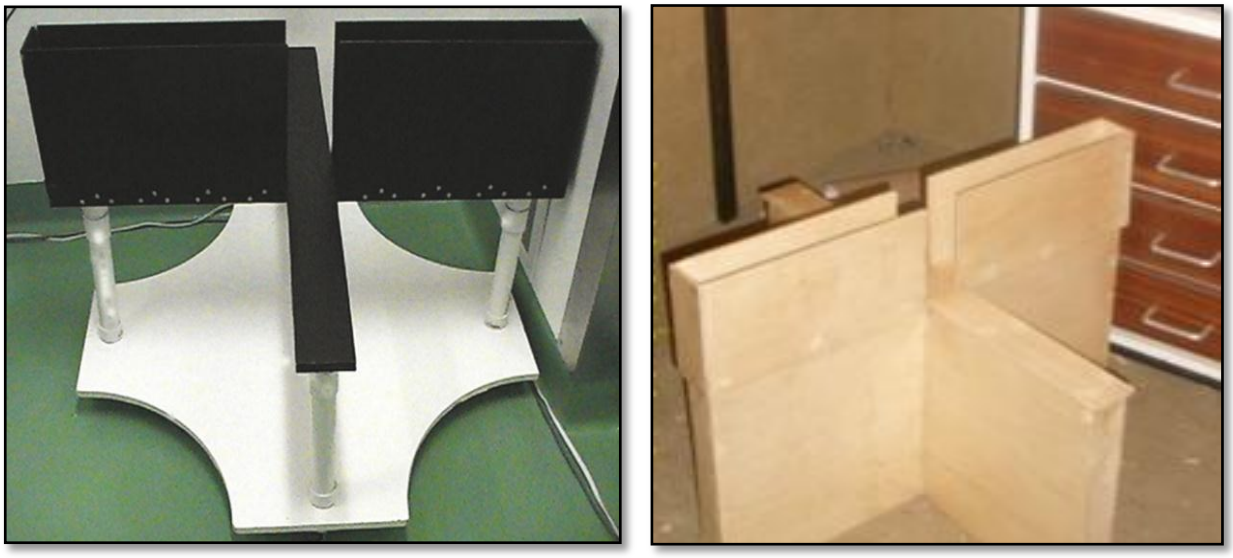

**Figure 1.6.** Elevated Plus maze apparatus (adapted from Walf & Frye, 2007)

The elevated plus maze (EPM) has become the most commonly used screen for novel anxiolytics, as well as a probe for anxiety in transgenic mice (Holmes, 2003). The elevated plus maze is shaped like a plus sign, has two open arms and two enclosed arms, and is usually raised at least 1 meter above the floor (Lister, 1987). The test animal is placed in the open center of the plus maze, and the number of entries into the closed arms is compared with the number of entries into the open arms over some period (commonly 5–15 minutes). High levels of anxiety correlate with more time spent in the enclosed arms.

The EPM test has been widely validated and provides a reliable, sensitive and consistent measure of anxiety in rodents, and its success has led to development of other variants (Carobrez & Bertoglio, 2005; Castagne *et al.*, 2006; Lister, 1987; Ohl, 2003; Pellow *et al.*, 1985; Rodgers *et al.*, 1997; Rodgers & Dalvi 1997). However, due to its success and

adoption by many research laboratories, a significant degree of variation exists in the composition of the apparatus, organisms used and testing protocols (Hogg *et al.*, 1996; Rodgers *et al.*, 1997). The test is based on the natural aversion of rodents for high and open spaces, and faced with a choice they prefer enclosed arms, which have a burrow like ambience and therefore spend greater amount of time in the enclosed arm (Castagne *et al.*, 2006; Rodgers *et al.*, 1997; Ohl, 2003). The EPM apparatus consists of an elevated runway with four arms that are interconnected by a central platform to form a plus-shaped runway. Two opposing walls are closed by side- and end-walls and the other two arms are open i.e. unprotected (Lister, 1987; Pellow *et al.*, 1985; Dawson & Tricklebank, 1995). The animal is usually placed on the central platform and observed for period of 5 to 10 minutes.

The behaviours scored in the EPM test can be categorized into conventional (spatiotemporal) and ethological parameters (Rodgers & Dalvi 1997; Rodgers *et al.*, 1997). The spatiotemporal behaviours that are typically recorded are the time spent and entries made on the open and closed arms. Ethological parameters include explorative activity (head dips, rearings, flatback approach), risk assessment behaviour (sniffing, stretch attend postures), grooming, freezing, defecation and urination (Rodgers *et al.*, 1997; Võikar, 2006). However there are some inter-laboratory variations in some of the ethological parameters recorded (Hogg, 1996; Võikar, 2006). In the test animals do not usually have prior exposure to the EPM, and recent studies suggest that pre-exposure to the EPM can result in test decay and altered response in subsequent trials (Bertoglio & Carobrez, 2000, 2002; Carobrez & Bertoglio, 2005; Fernandes & File, 1996).

### 1.3.9. Validity of Neurobehavioural Tests

Validity refers to how well the test measures what it is supposed to measure in the context of its intended purpose (Vorhees, 1987; Moser, 1990). Generally, screening battery tests should be reliable, sensitive, specific and efficient (MacPhail *et al.,* 1997). Validation of neurobehavioural tests involves the assessment of their ability to measure neurological function and how well the test identifies potential neurotoxicants (Moser, 1990). Neurobehavioural tests should be able to differentiate neurotoxic from non-neurotoxic agents as well as the neurological functions affected (Moser, 1991; MacPhail *et al.,* 1997). The validity in toxicology is addressed as criterion, predictive, concurrent and construct validity (Moser, 1990).

#### *1.3.9.1.* Criterion Validity

Criterion validity is the ability of a test to estimate or predict standard external to the test itself (Moser, 1990). In neurotoxicity tests criterion validity can be determined by assessing the degree to which the test battery detects chemicals known to be neurotoxic (Moser, 1990), for example comparing known human signs and symptoms with effects in animal models.

#### *1.3.9.2.* Face Validity

Face validity describes the degree of analogy of an animal model to the effects observed in humans for example, the behavioural dysfunction seen in an animal model and in humans affected by a particular neurobehavioural disorder. However it is considered the weakest of

all validation criteria because sometimes animals have their own species-specific behaviours. Again it is not possible to model many major symptoms of neurodegenerative disorders that are subjective in animals e.g., hallucinations (Bortolato & Godar, 2010; van der Staay, 2006).

#### *1.3.9.3.* Construct Validity

Construct validity indicates the degree to which one or several endpoints serve as measures of the same broad biological function. Rating scales have been developed to assess this type of validity, for example the rating scale for quantifying behavioural effects of dopaminergic agonists on dopaminergic function. The FOB has been shown to display a high degree of both criterion and concurrent validity, making it a suitable test for neurotoxicity screening (Moser, 1990).

#### *1.3.9.4.* Predictive Validity

Predictive validity refers to extrapolation of effects from one species or another. It is important since in preclinical studies animal models are commonly used to determine both pharmacological and toxicological effects, which are then extrapolated to humans. In drug development it refers to the ability of a screening test or animal model to correctly identify the efficacy of a putative therapeutic agent (van der Staay *et al.,* 2009).

#### *1.3.9.5.* Concurrent Validity

Concurrent validity is the ability of a test battery to predict or indicate other effects occurring at the same time that are measured independently. In neurotoxicology, this could be the

extent to which a test can predict other types of neurological effects e.g. neuropathological changes. It can be assessed by comparing the effects of a toxicant using different tests, or comparing different endpoints such as behaviour and neuropathology (Moser, 1990).

### 1.3.10. Weight of Evidence and Motivation

There are an increasing number of reported cases resulting from *Boophone* intoxication in Zimbabwe (Acuda & Eide, 1994). In South Africa many undocumented cases of poisoning with fatal consequences have been reported (Botha *et al.*, 2005). Although no substantial experimental studies have been done to determine the actual physiological effects of the *Boophone* extracts, the weight of the evidence from clinical poisoning cases reported in the region is indicative of some adverse neurological effects. In a number of the cases hallucinations, aggressiveness, irrational thinking and many other central nervous effects have been recorded (Gelfand *et al.,* 1985; Botha *et al.,* 2005).

However, despite *Boophone* toxicity and its wide spread abuse, its wide use in ethnomedicine for a variety of ailments (Botha *et al.*, 2005; Gelfand *et al.,* 1985) and preliminary evidence of *in vitro* activity of some the extracted components (Botha *et al.*, 2005;Risa*et al.,* 2004; Stafford *et al.*, 2008) renders it a promising lead candidate for future drug development. It is therefore of particular relevance to the region that more detailed experimental studies be done to give a better understanding of the toxicological profile and potential neuropharmacological effects of extracts of this herb.

### 1.3.11. Research question

Therefore given the above discussions and review of literature on *Boophone disticha*, the following research questions where formulated:

- What are the signs and symptoms of *Boophone disticha* intoxication after oral administration?
- What is the lethality of a crude extract of *Boophone disticha* in rodent models?
- Is *Boophone disticha* genotoxic?
- Does *Boophone* crude extract have any anxiety-like and antidepressant-like activity?

# CHAPTER 2

# 2. OBJECTIVES

## 2.1. MAIN OBJECTIVES

The main objective of the study is to determine the toxicological effects of both acute and chronic exposure to a crude extract of the *Boophone disticha* herb and also to investigate the neuropharmacological effects of decoctions of the *Boophone distich* in rodent models. The potential genotoxicity of the extract is also investigated.

## 2.2. SPECIFIC OBJECTIVES

- To determine the acute toxic effects of oral exposure to a hydroethanolic extract of *Boophone disticha* by estimating the $LD_{50}$ of the crude extract in rats
- To determine the acute central nervous system effects of oral exposure to a hydroethanolic extract of *Boophone distich*a in a rat model through functionality changes using a Neurobehavioural Function Observational Battery in rats
- To assess the potential genotoxic effects of a hydroethanolic extract of *Boophone disticha* using *Salmonella typhimurium* bacterial species
- To investigate the anxiolytic-like activity of a hydroethanolic extract of *Boophone disticha* in mice after oral administration
- To investigate the potential antidepressant-like activity of *Boophone disticha* extracts in the Forced Swim test after repeated dosing in a mouse model.

# CHAPTER 3

# 3. MATERIALS AND METHODS

## 3.1. MATERIALS

**Table 3.1.** Laboratory equipment and reagents used in the experimental studies

| MATERIAL | MANUFACTURER/SUPPLIER | TEST |
|---|---|---|
| ***EXTRACTION*** | | |
| Grinding mill | | |
| 70% & 95 % Ethanol | McDonald Scientific, Zimbabwe | All |
| 500ml beakers | Pyrex, England | |
| 2000ml conical flasks | Pyrex, England | |
| Reflux equipment | Pyrex, England | |
| Buchner flask s | | |
| Buchner funnel s | | |
| Mutton cloth | | |
| Whatman No. 1 filter paper | Whatman Limited, England | |
| Whatman No. 99 filter paper | Whatman Limited, England | |
| Heidolph 4000 Rotavapor | Heidolph, Germany | |
| Freeze drier | | |
| Dessicator | | |
| ***TOXICOLOGICAL ASSESSMENTS*** | | |
| Laboratory Trolley | | OFT |
| Rota-rod apparatus | Letica | RRT |
| Gavage tube | | |
| 2ml syringes | Braun Melsungen AG, Germany | |
| Formalin | Merck Laboratories supplies | |

| MATERIAL | MANUFACTURER/SUPPLIER | TEST |
|---|---|---|
| Chloroform | Merck Laboratories supplies | |
| Electronic Balance | Sartorius AG Gottingen, Germany | |
| *Salmonella typhimurium* strains; TA98 and TA100 | Sigma | |
| Sodium azide [Cas no. 26628-22-8] [Lot no.19,993-1] | Sigma | |
| 2-nitrofluorene [Cas no. 607-57-8] [Lot no. N1.675-4] | Sigma | |
| Vogel-Bonner E agar | | |
| Nutrient agar | | |
| Metabolic activation system | Sigma | |
| Autoclave | | |
| Bijow bottles | Pyrex, England | |
| Sterile pipettes(1ml) | Pyrex, England | |
| Colony counters | | |
| ***PHARMACOLOGICAL ASSESSMENTS*** | | |
| Zinc oxide tape | | OFT |
| Stopwatch | | All |
| Thermometers | | FST |
| Webcam | Labtec | All |
| Lamp heater | | FST |
| PC | HP Compaq | All |
| Rota-rod apparatus | Letica | RRT |
| Elevated Plus Maze | In-house built | EPM |
| Laboratory Trolley | | OFT |
| Cylindrical water tanks | | FST |

EPM – **Elevated Plus Maze Test;** FST – **Forced Swim Test;** OFT – **Open-field Test;** RRT – **Rota-rod Test**

## 3.2. EXTRACTION

### 3.2.1. Plant Material

*Boophone Disticha* was collected in December 2005 in Mashonaland West province about 60km from Harare. The plant sample was authenticated by a taxonomist from the Botanical Gardens and National Herbarium, and a voucher specimen was refrigerated in the department of Clinical Pharmacology, College of Health Sciences, University of Zimbabwe.

### 3.2.2. Preparation of the Fermented crude extract

The fresh bulbs scales (5 bulbs were used) were peeled and then sun dried until all the scales were papery dry. The dried bulb scales were ground in a small mill until a coarse powder (1173 g) was obtained. The powder was then mixed (1:5 w/v) with aqueous ethanol (70%v/v; 4 L) and then refluxed for 60 minutes at 100$^{o}$C. After ( 24 hours) the extract was then filtered using a mutton cloth to remove the coarse material. The filtrate obtained was further vacuum filtered to remove the finer particulates. The aqueous-ethanolic extract was volume reduced by rotary evaporation with a Heidolph 4000 Rotavapor (Heidolph, Germany) to a thick paste (100 ml). The extract was then freeze dried from -40 to -20$^{o}$C for 3 days. The extract was then ground to a fine powder, which was kept in tightly sealed container in a cool dark place.

## 3.3. EXPERIMENTAL PROTOCOLS

### 3.3.1. Study 1: Acute Toxicity and Neurobehavioural Effects of a Hydroethanolic extract of *Boophone distich*a in rats

#### *3.3.1.1. Animals and Animal Husbandry*

Thirty-three adult, non-pregnant female Sprague Dawley rats (6-12 weeks old; 180-280g) purchased from the Animal House Faculty of Veterinary Sciences, University of Zimbabwe, were used. The animals were acclimatised to the laboratory conditions for at least 5 days prior to the experiments. The rats were housed in groups of up to five per cage, with wood shavings or shred paper bedding. They were allowed standard rodent food and tap water *ad libitum*. The animal facility was maintained at 19- 21$^{o}$C and had a 12hour dark – light cycle with light on at approximately 0630 to 0700. The relative humidity was less than 70 %.

#### *3.3.1.2.* Administration of the Extracts

The animals were randomly selected and marked to permit individual identification prior to dosing. The rats were fasted overnight and then weighed prior to dosing to avoid dose discrepancies. The test extract was administered in a single dose by oral gavage using an intubation cannula at volume of 1ml/100g body weight. After the sample administration and behavioural tests, food but not water was withheld for a further 3-4 hours.

#### *3.3.1.3. Sighting Study*

A sighting study was carried out to allow selection of an appropriate starting dose for the main study. The test substance was administered to single animals in a sequential manner,

with doses increasing by a log cycle increase, starting at a dose of 5mg/kg. If the animal showed any evidence of toxicity that dose was used in the main study, and dosing was stopped when a dose was reached which showed significant evidence toxicity. A period of at least 24 hours was allowed between the dosing of each animal. All animals were observed for at least 2days. Single rats were given doses of 5, 50 and 500 mg/kg. The 50mg/kg dose produced observable pharmacological effects and the 500mg/kg dose caused death. Therefore doses for the main study were selected within the range 50 to 1000 mg/kg. Doses for LD50 estimation were increased by approximately a quarter log cycles starting at 50mg/kg (50, 120, 240, 360, 500 & 700 mg/kg).

#### *3.3.1.4. Main Study: Estimation of the $LD_{50}$ of the crude extract*

The rats were divided into six treatment groups of 5 female rats and one group with 3 female rats. The animals were fasted overnight and then were weighed prior to the experiment to avoid dose discrepancies. Six concentrations of the test extract were prepared by serial dilution of the crude extract. The concentrations were spaced appropriately to permit an acceptable determination of the LD50. The concentrations were made so as to keep the volume of liquid administered to each animal constant, which is 1ml/100g. The concentrations prepared are shown in Table 3.2 below.

The extract was administered by oral gavage using an intubation cannula at volume of 2ml/100g. After administration of the extracts the animals were routinely observed for 48hrs. Animals that died during the test were necropsied, and some of those that survived and appeared moribund were humanely sacrificed by carbon dioxide asphyxiation and

necropsied. Observations made included changes in the skin fur, eyes and mucous membranes, respiratory rate, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern.

**Table 3.2.** Doses administered in the estimation of the $LD_{50}$

| *Group* | *Dose of the Extract in mg/kg* |
|---|---|
| A | 50 |
| B | 110 |
| C | 240 |
| D | 360 |
| E | 500 |
| F | 700 |

### *3.3.1.5. Functional Observational Battery*

The functional observational battery (FOB) was used to evaluate neurobehavioural and physiological changes. The experimental protocol for the FOB was based on procedural details (Appendix 1). Scoring criteria for FOB where described by McDaniel and Moser (1993). Some of the parameters were scored, and a few others were descriptive.

On the test day, rats were transported to an observation room and allowed at least one hour to acclimate before testing began. The observer then removed the rat, held it, and scored lacrimation, salivation, miosis, piloerection and handling reactivity, according to defined criteria. The rat was then placed on an open field (a laboratory cart 60×90 cm surrounded with a 10 cm perimeter barrier). During 5 minutes of exploration, the observer counted the

number of rears and evaluated and scored any gait abnormalities, ataxia, arousal, activity level, involuntary motor movements, stereotypical behaviour and excretion level (urination, defecation). Aerial righting reflex was also ranked. After the open field observations, sensorimotor responses were assessed according to responses to a variety of stimuli

#### *3.3.1.6. Histopathological Evaluations for Acute Toxicity tests*

All animals that died or were sacrificed were given a complete postmortem examination. The abdominal, thoracic and cranial cavities were observed for any abnormalities, and all the organs were removed and examined for grossly visible lesions, and the following organs weighed; brain, heart, liver, thymus, spleen, kidneys, adrenals, ovaries and/or testes. After weighing the organ, samples were placed in 10%neutral buffered formalin. Tissues were then imbedded in paraffin, sectioned to a thickness of 4-6 µm, transferred to slides, and stained with haematoxylin and eosin for light microscopic examination.

#### *3.3.1.7. Statistical Analysis*

Data analyses were carried out using Graphpad® Prism 5.0 software package. Functional Observational Battery (FOB) end-point data was analysed by non-parametric Kruskal-Wallis followed by Dunnett's multiple comparison test to determine differences between the groups. Differences were considered at $p<0.05$.

### 3.3.2. Study 2: Subacute Toxicity of a Hydroethanolic extract of *Boophone distich*a in rats

#### *3.3.2.1. Animals and Extract administration*

##### *3.3.2.1.1.Animal Husbandry*

Sprague Dawley rats (4-5 weeks old) of both sexes (4 females & 20 males) were used for the experiments. The rats were housed in wire mesh cages with a maximum of 6 animals per cage. Animals were housed, dosed and fed as per Organisation for Economic Co-operation and Development (OECD) guidelines for chemical testing. All rats were allowed free access to food and tap water in the home cages. The animal holding room was allowed natural light during the day and darkness during the night. There was equal distribution of male and female rats across all groups. The experiments were carried out between 08:00 and 16:00 in the light phase of the light-dark cycle. The rats were carried to the test room in their home cage, and they were left undisturbed for about 30 minutes. Rats were allocated to four treatment groups (n=6); control (normal saline), three experimental group (100, 200 & 400mg/kg *Boophone extract*). The rats were dosed daily by oral gavage using an intubation tube for 28days. Animals that died during the test period were necropsied. FOB and motor activity testing were carried out on day 1, 14 and 28 of the study.

#### *3.3.2.2. FOB and Motor Activity*

The functional observational battery (FOB) was used to evaluate neurobehavioural and physiological changes. The experimental protocol for the FOB was based on procedural details and scoring criteria for FOB as described by McDaniel and Moser (1993). The procedures for the FOB are described in the experiment above (Study 1 & Appendix 1). On the test days, rats were transported to an observation room and allowed at least one hour to

acclimate before testing began. Animals were then observed using the FOB, 30 minutes after dosing. Soon after the FOB, the rats were tested on the Rota-rod according to the procedures. Each rat was given three successive trials and the longest period it remained on the rod taken as its score.

#### *3.3.2.3. Gross Necropsy & Histopathology*

On the final day (day 28) of experiments, all the rats that survived were weighed and sacrificed by Chloroform asphyxiation. The rats were immediately dissected and blood was collected from the heart using a syringe and needle. The testes, kidneys, spleen, stomach, small intestines, large intestines, liver, heart, lungs, and brain were harvested in that order. Organs were weighed and immediately preserved in formalin. Blood samples were all centrifuged within 30 minutes at 3000rpm for 5minutes and refrigerated at 0 $^{o}$C overnight before being stored at -80 $^{o}$C.

#### *3.3.2.4. Statistical Analysis*

Data analyses were carried out using Graphpad® Prism 5.0 software package. Functional Observational Battery (FOB) end-point data was analysed by non-parametric Kruskal-Wallis followed by Dunnett's multiple comparison test to determine differences between the groups. Differences were considered at $p<0.05$. Body weights and organ weights were analysed using one-way ANOVA followed by Dunnett's Test.

### 3.3.3. Study 3: Genotoxicity effects of a HE extract of *Boophone disticha*

#### *3.3.3.1. Organisms and Media Preparation*

Two bacterial strains of *Salmonella typhimurium*, TA98 and TA100, were employed in this test. For each strain, fresh bacterial cultures were grown to the late exponential phase of the growth where there is an approximate of about $10^9$ cells per ml. Mammalian liver post mitochondrial fraction (S9) was used as an exogenous metabolic activation system. The S9 fraction prepared from male Sprague–Dawley rat livers induced with Aroclor 1254 was obtained from Sigma Chemical Co. Pvt Ltd, stored frozen at approximately 80°C, thawed just prior to use and supplemented with an NADPH-generating system (S9-mix). Final concentrations of S9 fraction in S9-mix were 10% (v/v) in the Ames test. The minimal agar used is the Vogel agar prepared with the following formula all in g/l

**Table 3.3.** Formula for preparing Vogel agar.

| *Formulae* | *g/l* |
|---|---|
| Tryptone | 10.0 |
| Yeast extract | 5.0 |
| Mannitol | 10.0 |
| Dipotassium phosphate | 5.0 |
| Lithium chloride | 5.0 |
| Glycine | 10.0 |
| Phenol red | 0.025 |
| Agar | 16.0 |

Sixty-one grams of the final mixture was suspended in one litre of distilled water and brought gently to boil to dissolve completely. The resultant paste was sterilised by autoclaving at 121°C for 15 minutes. This was then cooled to 50 °C and added to it was 3.5 % potassium tellurite. The overlay agar used was the basic nutrient agar.

### *3.3.3.2. Ames Test Procedure*

The method was carried out following the recommendations of Maron and Ames (1983) and according to the current OECD guideline (TG471; 1997). Two *Salmonella typhimurium* histidine autotrophic strains (TA98 and TA100) were used. *Salmonella* strains were obtained from Scientific and Industrial Research and Development Centre (SIRDC). A frozen sample of each tester strain was thawed, pure cultures in Nutrient Broth Agar plates were established and late exponential cultures of each tester strain set up from single colonies. Two independent experiments were performed, both without and with metabolic activation, using triplicate plates for each substance and treatment condition. Acetic acid (0.02% solution) was used as a negative control. Sodium azide (NA) and 2-nitrofluorene (NF) were used as positive controls for TA100 and TA98 respectively. Briefly, 0.1 ml of test solution, 0.1 ml of bacterial culture and 0.5 ml of S9-mix (phosphate buffer for treatment without activation) were mixed and poured onto Vogel Bonner-E minimal agar plates after addition of 2ml of top agar at 42$^{o}$C. Triplicate plating was used at each dose level for an adequate estimate of variation. Plates were inverted and incubated for 70 hours at 37$^{o}$C in the dark. Revertant colonies were counted and the background lawn was inspected for signs of toxicity or compound precipitation.

### *3.3.3.3. Statistical Analysis*

Data analyses were carried out using Graphpad® Prism 5.0 software package. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used to test for differences among the groups at $p<0.05$.

## 3.3.4. Study 4: Anxiolytic effect of a HE extract of *Boophone Distich*a in Balb/c mice

### *3.3.4.1. Animal & Extract Administration*

#### *3.3.4.1.1. Animals*

Forty BALB/c male mice (3months old) were purchased from the Provincial Veterinary Services, Harare. Food and water were made available *ad libitum* and bedding of wood shavings were changed every three days. Routine handling of the animals was done for two weeks before the experiments were started in order to familiarize the animals with such handling. On test days, the animals were carried from the Animal holding to the test room in their cages.

#### *3.3.4.1.2. Drug Administration*

The mice were randomly placed into five treatment groups (n=8) i.e., negative control (normal saline); *Boophone* extracts (10, 25 & 40mg/kg); and Positive Control (Diazepam 1mg/kg). The *Boophone disticha* extracts were administered 1hr before the test trial. The experiments were performed in a sound proof room located at the Old Health Sciences Building at the College of Health Sciences, University of Zimbabwe.

### *3.3.4.2. Elevated Plus Maze (EPM) Test*

#### *3.3.4.2.1.Elevated Plus Maze Procedures*

The experiments were carried out between 0800 and 1200 in the light phase of the light: dark cycle. The mice were carried to the test room in their home cage, and they were left undisturbed for about 30 minutes. Thirty minutes after dose administration (normal saline, negative control; 10, 100, 200 mg/kg p.o. *Boophone* extract; Diazepam 2mg/kg, positive

control), each mouse was placed at the centre of the maze, facing one of the open arm opposite were the experimenter was (sat quietly about 1m away from the apparatus), and observed for a period of 5 minutes (Kulkarni& Verma, 1993; Thakur& Mengi, 2005; Walf& Frye, 2007). The video tracking of and timing of each trial started just before the animal was released in the open field so that the behavior of each animal would be recorded consistently for 5 minutes. At the end of the 5minutes trail period, the mouse was removed from the maze and returned to its home cage. Any faeces left in the apparatus were removed and the arena thoroughly wiped with first water and then 70% ethanol, and allowed to dry before the next trial.

The parameters noted were; percentage preference for open arm as first entry, percentage of number of entries into open arms relative to the total number of entries and duration of stay in the open arm. Entry into an arm was defined as the point when the animal placed all four paws onto the arm. Other ethologically derived measures (grooming, rearing, stretched-attend postures and head-dipping) were also determined (Rodgers and Dalvi, 1997).

#### *3.3.4.3. Statistical Analysis*

Statistical analyses were carried out using Graphpad® Prism 5.0 software package. Data were analysed by a one-way analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons. Statistical significance was set at $p<0.05$.

### 3.3.5. Study 5: Subchronic Antidepressant-like effects of a Hydroethanolic extract of *Boophone disticha* in Balb/c mice

#### *3.3.5.1. Animal Husbandry*

Forty male Balb/c mice (4-6months old) were used for the subchronic antidepressant activity assessment. Food and water were provided *ad libitum* and bedding of wood shavings were changed every three days. Routine handling of the animals was done for two weeks before the experiments were started in order to familiarize the animals with such handling. On test days and for daily dosing, the animals were carried from the Animal holding to the test room in their cages. The Subchronic study was for 21days and the animals were dosed daily for the entire study period

They were divided into four groups (n=6) and treated as follows: negative control (water); *Boophone* extract (10mg/kg, 20mg/kg & 40mg/kg) and the positive control (Fluoxetine 20mg/kg). The animals were dosed by oral gavage using an intubation cannula.

#### *3.3.5.2. The Forced Swim Test*

*3.3.5.2.1.Apparatus and Setup*

The apparatus consisted of four transparent cylinders (12cm diameter 25cm height) divided by cardboard so that the animals were not able to see each other in the different cylinders. Warm water was filled up to approximately 17cm and was replaced after each session. A camera connected to a computer was set up in position to record the footage of the video of the tests.

*3.3.5.2.2.Procedures*

The test was carried out on day one and day twenty-two during the light phase of the light-dark cycle. No dosing was carried out on day twenty-two. The mice were carried to the test room in their home cages, and they were left undisturbed for about 30 minutes. The swimming test was performed for 6 minutes with a recording on the webcam. The behavioural responses were scored by the observer from the video footage afterwards. After each swimming session, each mouse was gently dried with a towel. The water in the swim chamber was changed after each session. Increase in active responses, such as climbing or swimming, and reduction in immobility, were considered as behavioural profiles consistent with an antidepressant-like action (Cryan *et al.,* 2002).

### *3.3.5.3. Statistical Analysis*

Statistical analysis was carried out using Graphpad® Prism 5.0 software and presented as mean ± SEM. The total swimming, climbing and immobility times were analysed using one-way analysis of variance (ANOVA) followed Dunnett's test for multiple comparison. Comparison between day1 and day 21 were analysed using two-way ANOVA followed by the Bonferroni test. Statistical significance was set at $p<0.05$.

# CHAPTER 4

# 4. RESULTS

## 4.1. Acute Oral Toxicity and Neurobehavioural Effects of Hydroethanolic Extract of *Boophone distich*a in Rats

### 4.1.1. Acute Oral Toxicity and Estimation of the $LD_{50}$

The toxicological effects of acute administration of the crude extract of *Boophone disticha* are summarized in Table 4.1. No lethal effects were observed during the 14day observation period for the 50 and 120mg/kg dosage groups. However, deaths were observed with doses equal to or higher than 240mg/kg. In all cases the animals died between 30minutes and 3hours after dose administration. In these cases the most prominent symptoms preceding death were generalized convulsions, respiratory distress, tachypnoea and flaccid paralysis. The estimated oral $LD_{50}$ of the crude plant extract was determined to be between 120 and 240 mg/kg.

**Table 4.1.** Toxicity after single dose administration by oral gavage of the hydroethanolic extract of *Boophone disticha.*

| Dose (mg/kg) | T/M | Latency | Signs of Toxicity observed |
|---|---|---|---|
| Control | 5/0 | - | - |
| 50 | 5/0 | >10minutes, <2hours | Piloerection, mydriasis, Head tremors, hypoactivity, increased respiratory rate, lethargy |
| 120 | 5/0 | >10minutes, <2hours | Piloerection, mydriasis, Body tremors, hypoactivity, increased respiratory rate, lethargy |
| 240 | 5/3 | >10minutes, <2hours | Piloerection, mydriasis, Body tremors, convulsions, hypoactivity, hind paralysis, ataxia, laboured breathing, back arching, retropulsion |
| 360 | 5/4 | > 5minutes, <3hours | Piloerection, mydriasis, Body tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion, excessive sniffing |
| 500 | 5/4 | > 5minutes, <3hours | Piloerection, mydriasis, Tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion |
| 700 | 3/3 | > 5minutes, | Piloerection, mydriasis, Tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion |

T/M, Total initial Number of rats in Group/Number of rats that died due to the treatment

### 4.1.2. Neurotoxicological Assessment with FOB

The data obtained from neurotoxicological evaluations are presented in Tables 4.1 and 4.2. Generally, home-cage and Open-field observations, autonomic and activity endpoints were indicative of CNS depression with severity increasing with increasing dose. For doses of 240mg/kg and less, signs of toxicity began approximately ten minutes after dosing, with the most prominent initial signs according to home cage observations being head tremors (at 50mg/kg) and body tremors. Generally symptoms of toxicity lasted approximately 2 hours for

doses of 240mg/kg and less; and 3 hours for doses over 240mg/kg in animals that survived. Of the parameters assessed in the home-cage only handling reactivity was significantly decreased as the dose was increased in experimental groups.

The most prominent neurotoxicological effects with doses 240mg/kg and higher were increased flaccid limb paralysis, retropulsion (backward movement) and hypoactivity. This is evidenced by significantly increased rearing, reduced mobility and gait scores (Table 4.2.). However, sensorimotor evaluations showed no significant difference between all experimental groups and the control group (Table 4.2).

### 4.1.3. Histopathological Analysis

Gross examination and microscopic histopathological analysis of the extracted organs did not show any extract-related abnormalities. However, microscopic minimal to mild gastrointestinal sequela was noted in a few animals including controls.

**Table 4.2.** Acute effects of the hydroethanolic extract of *Boophone disticha* on behavioural endpoints of the FOB.

| FOB Endpoint | Overall significance $\chi^2$ (p-value) | Dose (mg/kg p.o.) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | 50 | 120 | 240 | 360 | 500 | 700 |
| **Activity/Reactivity** | | | | | | | | |
| Posture | Not significant | 1.00 | 1.00 | 1.00 | 2.00 | 1.00 | 2.00 | 6.00 |
| Removal reactivity | Not significant | 3.00 | 2.80 | 2.00 | 2.00 | 2.00 | 1.80 | 2.00 |
| Handling reactivity | $\chi^2$ = 15.916 (0.0071) | 3.00 | 3.20 | 2.00 | 1.80 | 2.00 | 1.80 | 2.00 |
| Arousal | Not significant | 3.00 | 3.60 | 3.40 | 3.20 | 3.00 | 2.40 | 2.00 |
| Open-field rears | $\chi^2$ = 10.038 (0.0742) | 28 | 14.4 | 9.00 | 3.40 | 4.80 | 4.20 | 0.67 |
| Involuntary movements | $\chi^2$ = 12.563 (0.0278) | 3.00 | 3.4 | 3.40 | 4.40 | 4.00 | 4.20 | 4.33 |
| **Autonomic** | | | | | | | | |
| Lacrimation | $\chi^2$ = 16.221 (0.0062) | 1.00 | 1.00 | 1.00 | 1.00 | 1.60 | 2.00 | 2.00 |
| Salivation | $\chi^2$ = 17.308 (0.0040) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.67 |
| Palpebral closure | $\chi^2$ = 14.775 (0.0140) | 1.00 | 1.80 | 1.40 | 2.00 | 2.00 | 2.20 | 3.00 |
| Defecation/urination | Not significant | 1.00 | 0.80 | 0.80 | 0.40 | 0.80 | 1.00 | 1.67 |
| **Neuromuscular** | | | | | | | | |
| Gait Score | $\chi^2$ = 12.880 (0.0245) | 1.00 | 1.20 | 1.80 | 2.20 | 3.00 | *3.60 | *4.33 |
| Mobility Score | $\chi^2$ = 11.084 (0.0497) | 1.00 | 1.20 | 1.80 | *2.20 | *3.00 | *3.60 | *4.00 |
| Righting reflex | Not significant | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| **Sensorimotor** | | | | | | | | |
| Approach response | Not significant | 1.00 | 1.00 | 1.60 | 1.60 | 1.80 | 1.60 | 1.00 |
| Tail pinch response | Not significant | 3.00 | 3.00 | 2.60 | 2.80 | 3.00 | 2.80 | 2.67 |
| Touch response | Not significant | 2.00 | 1.60 | 1.60 | 2.00 | 2.00 | 1.60 | *1.00 |
| Click response | Not significant | 2.00 | 2.80 | 2.60 | 2.60 | 2.60 | 2.80 | 2.33 |

* Significant difference versus Control Group using Kruskal-Wallis comparison

## 4.2. Subacute Toxicity of a Hydroethanolic extract of *Boophone disticha in* Rats

Initially, there were 28 rats and only 16 rats survived to the final day. Onset of toxic responses were from 5 minutes to 15 minutes post dosing and lasted between 35 and 60 minutes, followed by a rapid recovery. It was also observed that the latency to toxic responses decreased with increasing dose and with repeated dosing. Signs of toxicity included stupor followed by whole body tremor. In the severe cases, the animal would progress from tremors at rest with the head sloping to one side to explosive Tonic-clonic jerks and eventually death in the majority of the cases. This occurred most with the high doses, particularly with the 800 mg/kg dose after single dosing. This led to the abandonment of this dose for the repeated dosing study. Death usually occurred within 10minutes of the onset of severe toxic responses. Lower doses were associated with mild responses, including piloerection, palpebral closure, drunken gait, walking on tiptoe, and hunched body position. Retropulsion was observed in some rats after repeated dosing.

### 4.2.1. Body Weight

At the commencement of the study mean Body weight for all the groups was below 100 g. The mean weight for 100 mg/kg and 400 mg/kg groups increased more rapidly for the first 2 weeks, compared to the other groups (Figure 4.1). However the mean body weight started falling from the second week until the conclusion of the study for both groups particularly for the 400 mg/kg group. The growth rate for 200 mg/kg group was comparable to that of the control group and there was no statistical significant difference (Figure 4.1).

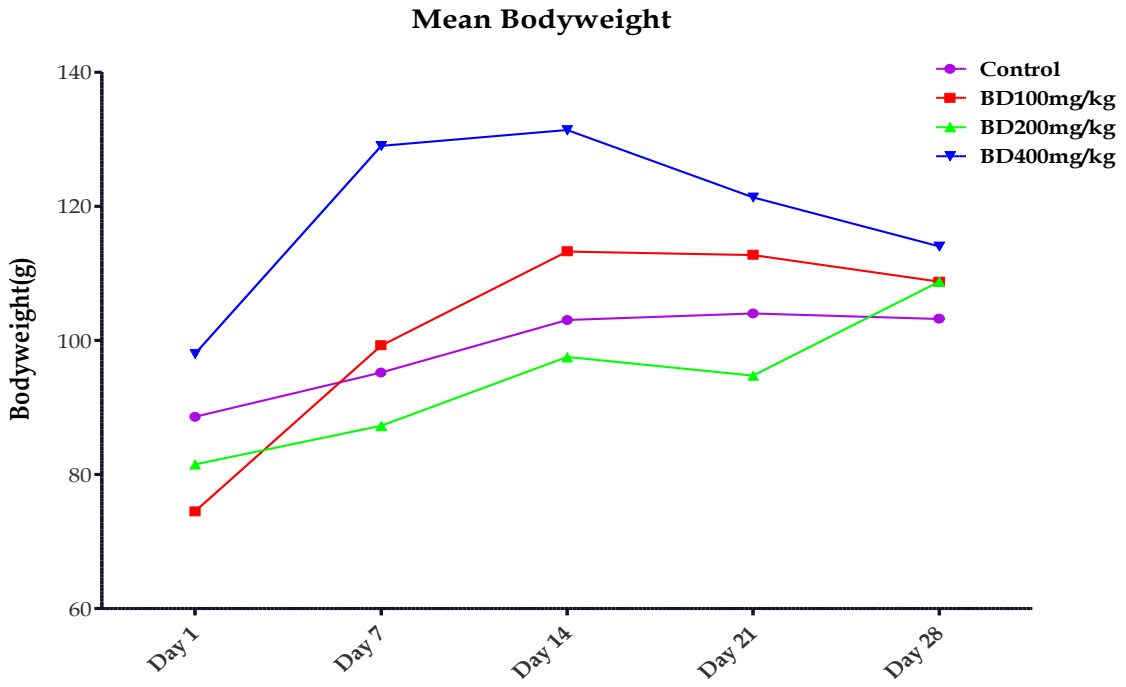


**Figure 4.1.** Effects of *Boophone* oral treatment on Bodyweight

### 4.2.2. Relative Organ Weight

Significant relative organ weight differences were noted for the stomach, large intestines, small intestines, and the liver (Figure 4.2). The weight of these four organs all showed a dose dependent increase in relative organ weight, with the control group and 100 mg/kg dose groups having the smallest mass to body ratio and the 200 mg/kg& 400 mg/kg groups having larger ratios that were statistically significant ($p<0.05$). The testes showed a trend of decreasing mass with increase in dose, however, this differences were not statistically significant ($p>0.05$). There was a general enlargement of the hearts of all dosing groups as compared to the control, but this was not statistically significant ($p>0.05$). Lung size of all test groups was generally less than of the control group with no statistically significant difference ($p>0.05$).

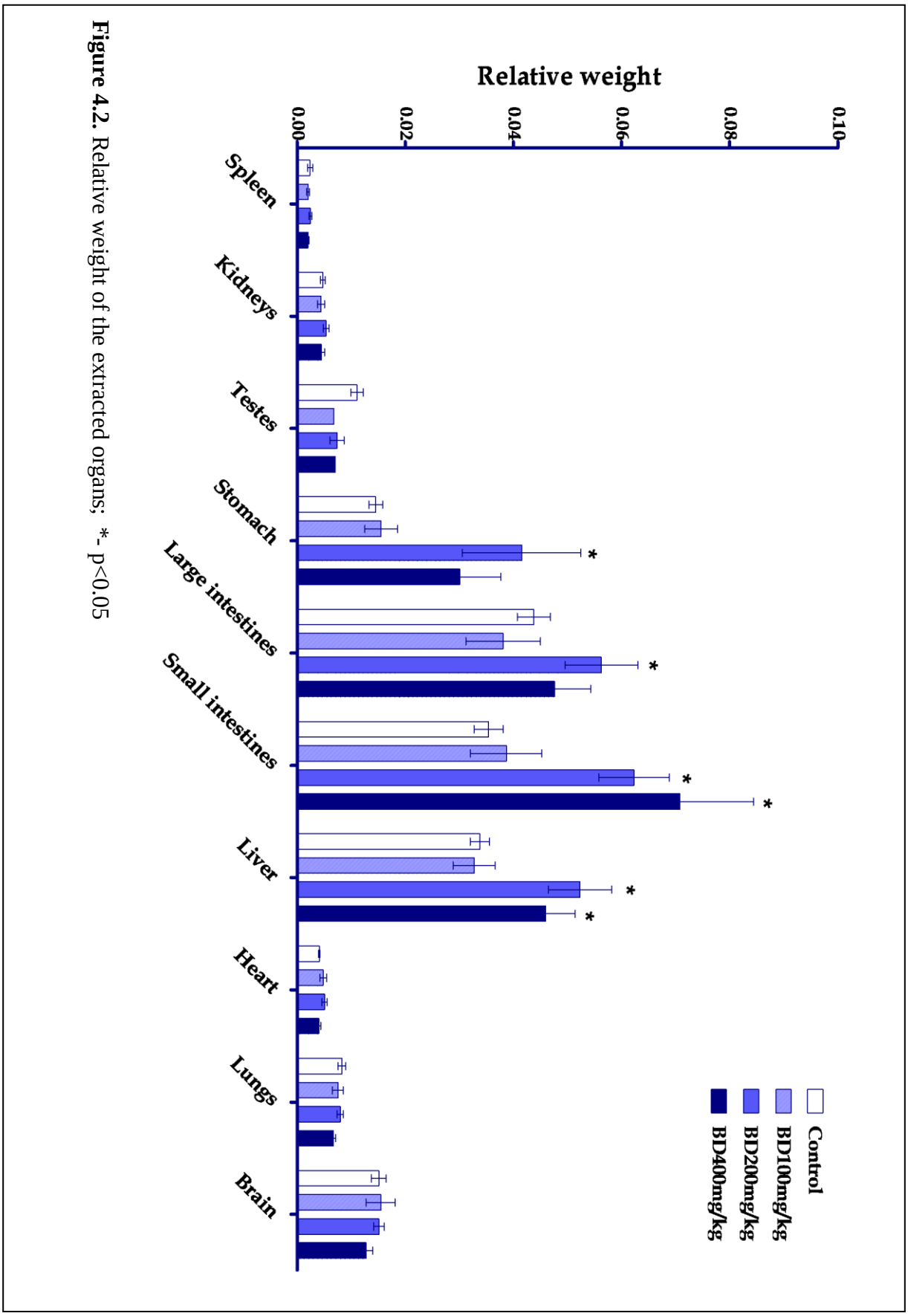


**Figure 4.2.** Relative weight of the extracted organs; *- $p<0.05$

### 4.2.3. Neurobehavioural Assessment with Functional Observational Battery

#### *4.2.3.1.* Activity Observations

##### *4.2.3.1.1.Posture*

Posture abnormality in FOB followed a dose dependent trend (Figure 4.3). Significant differences were noted between control and all experimental groups. The control group showed had a consistently low posture score throughout the study (Figure 4.3). On the other hand, 100mg/kg group had an average score of 1 on the first day which increased significantly on day 14 ($p<0.05$). However, for the 100 mg/kg dose, from day 14 to 28 there was no significant increase in the posture score compared to the control. For the 200 and 400 mg/kg the posture score was significant high throughout the study period.

##### *4.2.3.1.2.Arousal*

On day 1, the 100 mg/kg group had the highest score for arousal, with 60% (3) of the rats being slightly excited. However, this trend decreased with repeated dosing to days 14 and 28 (Figure 4.3). Rats in the control group were alert throughout the study period. Rats in the 400mg/kg group had the least score for arousal throughout [day 1, 3 (slightly sluggish), days 14 and 28, 1.5 (stupor)] the study period, with p values less than 0.005 for all test days. By day 14 levels of unprovoked activity for the 200 mg/kg and 100 mg/kg groups had dropped to below that of the control.

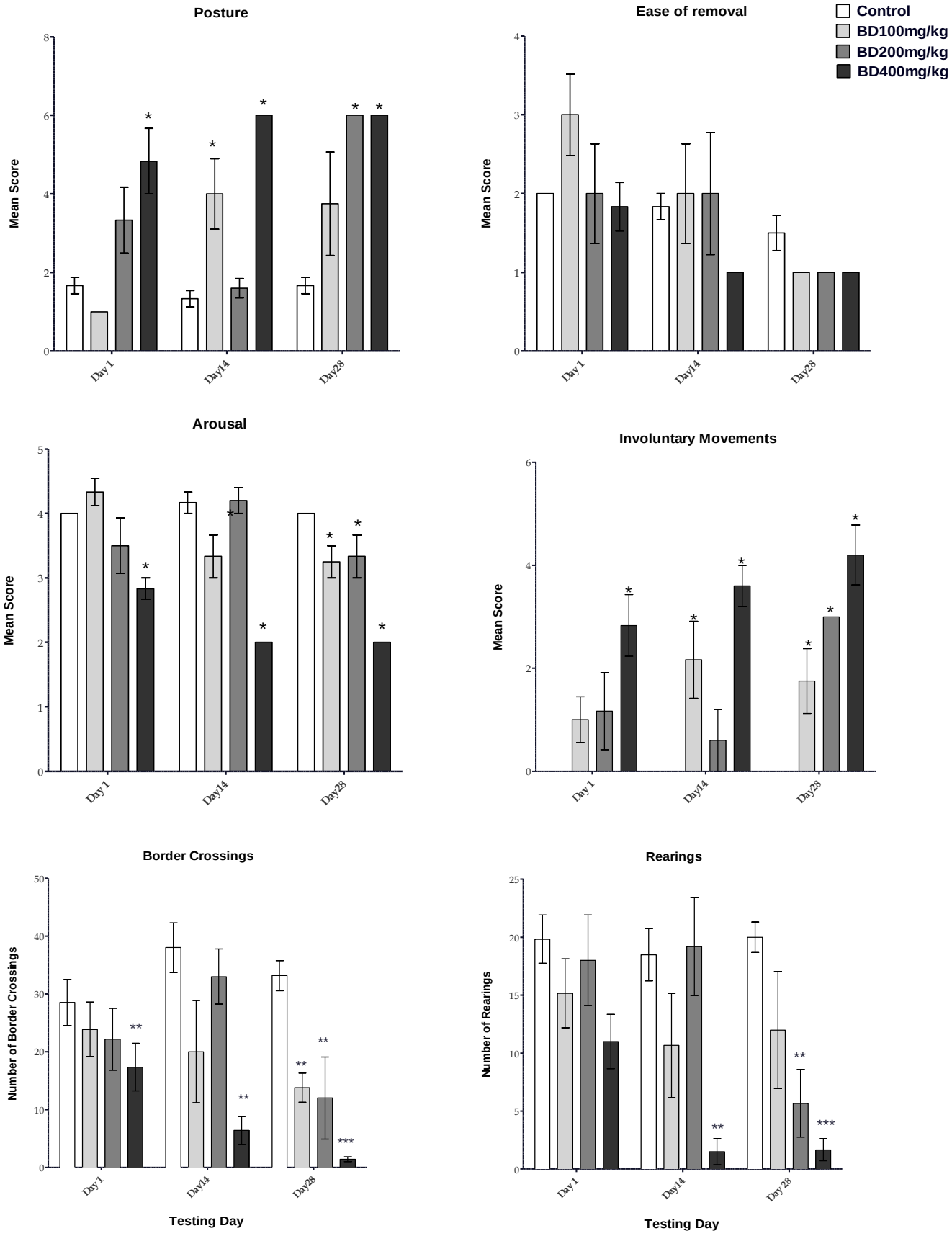


**Figure 4.3.**Activity observations in the FOB

*4.2.3.1.3.Involuntary Motor Movements*

The control group had no involuntary movements through out the study period. The 100 mg/kg had slight tremors on day 1 and these slightly worsened up to day 28 (Figure 4.3). A similar trend was also observed with both the 200 mg/kg and the 400 mg/kg. The effect was dose-dependent and the 400mg/kg dose had the most severe involuntary movements which also worsened with time. Explosive convulsions of the whole body were observed in the 400 mg/kg group. The forelimbs were mainly affected and were observed to have varying degrees of paralysis.

*4.2.3.1.4.Ease of removal from Home Cage*

Generally, the rats became easier to remove from their home cage with repeated dosing. On day one and day 14 the 100 mg/kg group was the most difficult to remove from their home cages. Rats in the 200 mg/kg group showed a statistically significant difference ($p<0.05$) in ease of handling between days 14 and 28. All experimental groups were almost stuporous on day 28, showing no resistance to being handled.

*4.2.3.1.5.Border Crossings*

Rats in the control group showed the highest activity with consistently high number of border crossings, throughout the study. For the experimental groups there was a sigificant dose dependent decrease in border crossings which was also time dependent (Figure 4.3). On day 14 the 400 mg/kg group had average crossings of below 7, which was significantlly lower than that of all other test groups ($p<0.05$). The 400 mg/kg group showed the least

number of crossings for all three days. Number of crossings decreased with repeated dosing for all dose groups.

*4.2.3.1.6.Rearing*

Control group had a consistently high average number of rears (20) throughout. On day one 200mg/kg dose group had highest number of rears, with the 100mg/kg and 400mg/kg groups having the lowest (average of 13). However, there was a dose-dependent decrease in rearing on day 14 (Figure 4.3). Only the 400 mg/kg group had statistically significant differences with the rest of the test groups ($p<0.005$). A similar trend was also observed on day 28. The 400 mg/kg had statistically significant differences with all groups ($p<0.005$). The 400 mg/kg dose group had the least number of rears throughout. Rearing showed a general decrease with repeated dosing for all the groups.

### *4.2.3.2. Autonomic Observations*

*4.2.3.2.1.Palpebral Closure*

On day one, the 100 mg/kg group had the highest palpebral closure score, which was significantly higher than the control. Generally all the experimental groups had a high palpebral closure score on all test days and the score increased significantly with time compared to the control group (Figure 4.4).

*4.2.3.2.2.Piloerection*

On day 1 all the animals in the Control group had no piloerection however some of the control animals showed piloerection on day 14 and day 28. On day one, only the 200

mg/kg group had a significantly high ($p<0.05$) piloerection score compared to the control. From day 14 all experimental groups had piloerection, which was significantly high compared to the control (Figure 4.4).

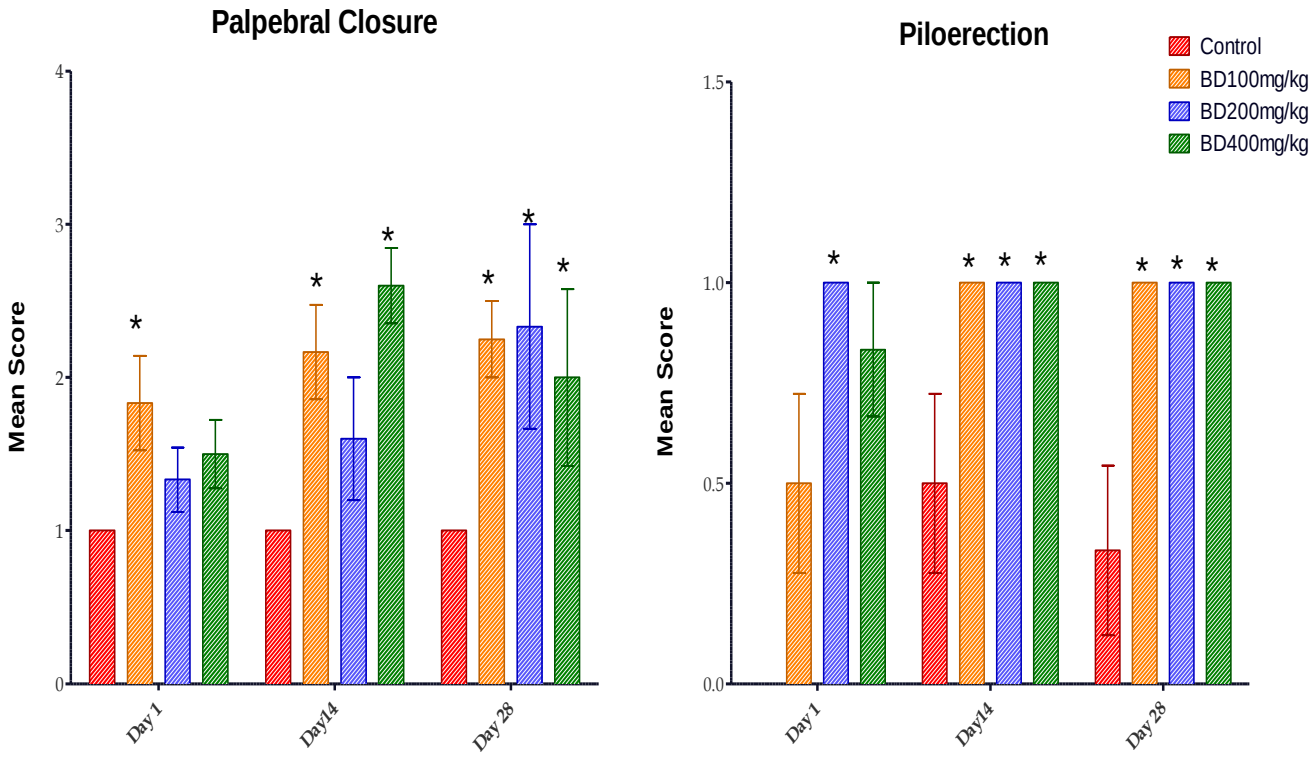


**Fig 4.4.** Effects of repeated dosing on Palpebral closure and piloerection

### *4.2.3.3. Neuromuscular Observations*

#### *4.2.3.3.1. Gait Analysis*

The Control group showed normal gait throughout the study. On day one, all the experimental groups had gait abnormality, which was significantly higher than the Control (Figure 4.5). On days 14 and 28 all the experimental groups remained significantly impaired. The 400mg/kg group had the most severe impairment even compared to the other dosage groups (Figure 4.5).

*4.2.3.3.2.Mobility*

Mobility followed an almost similar trend to gait analysis. Initially all experimental groups showed slight impairment which was not significant compared to the control. However after repeated dosing (day 14 and day 28) there was significant increase ($p<0.05$) in the Mobility score which was most severe with the 400 mg/kg dose. This trend was dose dependent with the exception of the 200 mg/kg dose on day 14 (Figure 4.5).

*4.2.3.3.3.Righting Reflex*

This parameter was ranked 0 (absence of reflex) to 1 (presence of reflex). On day 1 all animals from all the groups had the righting reflex and there was no significant difference, however, on day14 some rats in the 400mg/kg group had lost the reflex as well as on day 28. The rest of the rats in other dosing groups maintained the righting reflex throughout the test period (Figure 4.5).

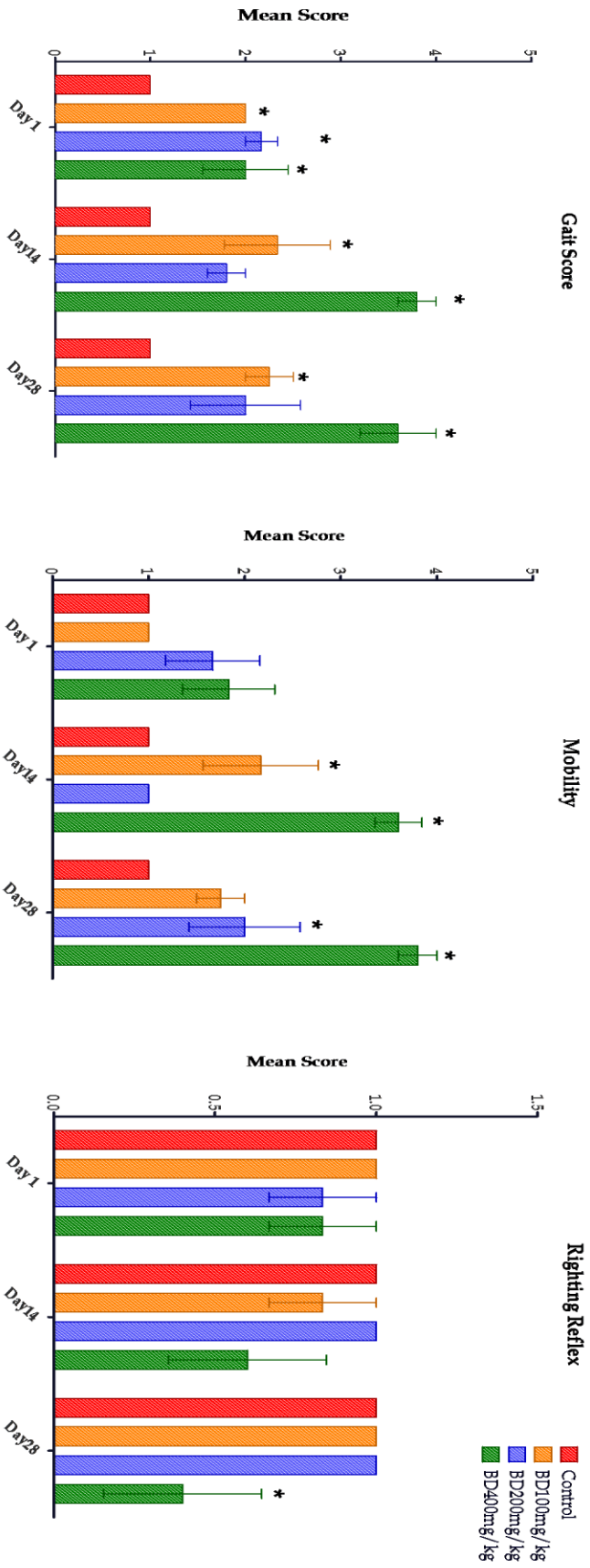


**Fig 4.5.** Effects of repeated dosing on neuromuscular observations in Open field

#### *4.2.3.4. Sensorimotor Observations*

Generally all sensorimotor observations did not show much deviation from the expected normal behaviour. And where differences occurred they were not consistent throughout the study duration. The 100 mg/kg dose showed significant decrease in the approach and click response son the first day and on day 14 and the other groups had no significant changes (Figure 4.6).

Touch response decreased among the experimental groups with repeated dosing and showed significantly difference with the control with 200 mg/kg (day1), 100 mg/kg (day 28) and 400 mg/kg (day 28).

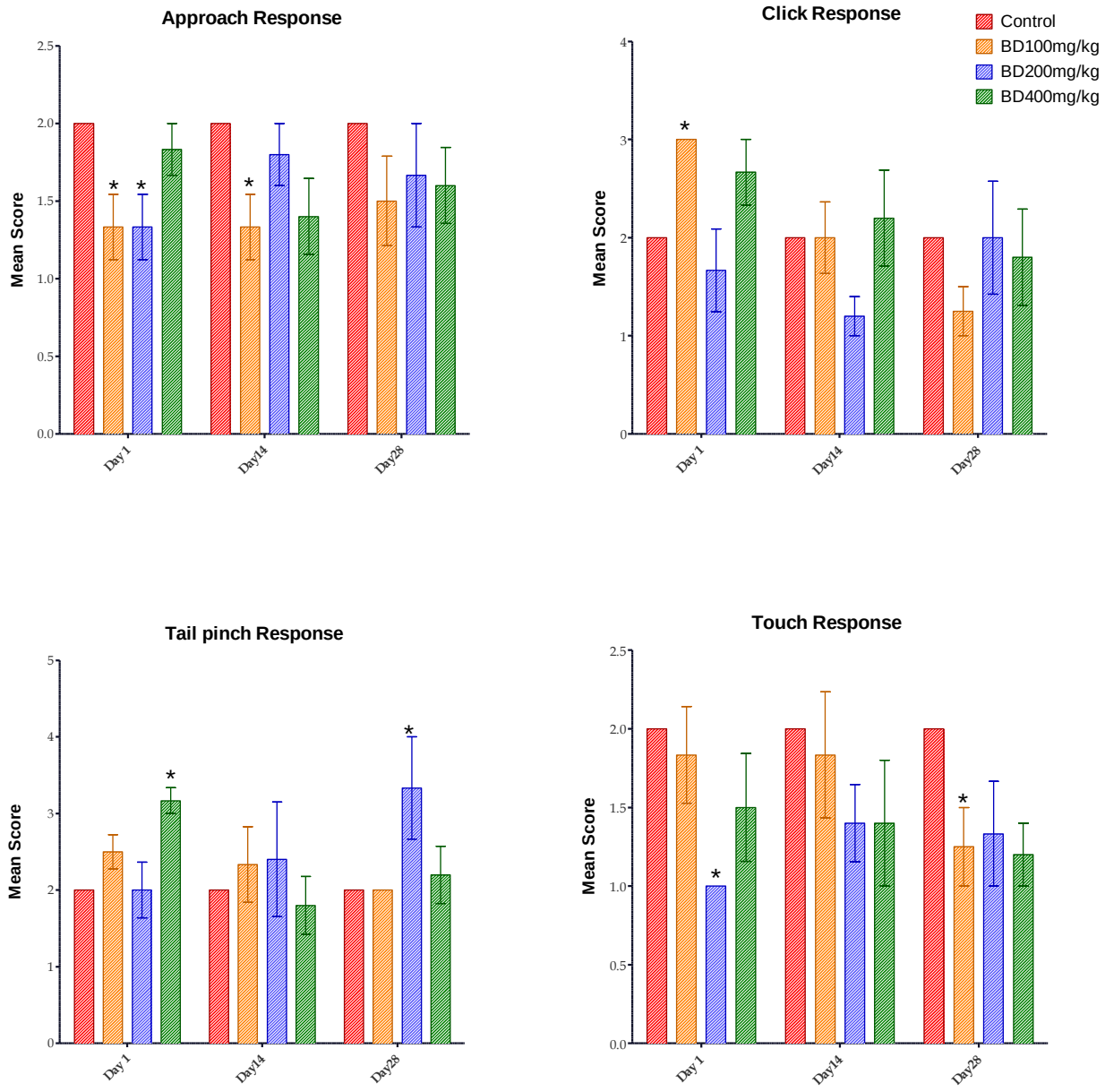


**Fig 4.6.** Effects of repeated dosing on Sensorimotor Observations in Open field

## 4.3. Genotoxicity effects of a hydroethanolic extract of *Boophone Disticha*

### 4.3.1. Effects of *Boophone disticha* HE on TA98 strains of *S. typhimurium*

A concentration dependent increase in the number of revertants produced was noticed for both tests (i.e. -S9 and +S9; Table 4.3) from the lowest concentrations of 500 μg/plate with the maximum number of revertants being found at 2000 μg/plate concentrations. For both sets, a further increase in concentration to 2500 μg/plate resulted in significantly lowering the revertant number and the appearance of a brownish colouration on the background lawn of the petri dishes. In the set that lacked metabolic activation (-S9), all the means from different concentrations were significantly ($p<0.05$) different while with +S9, the 500 μg/plate (9.3) and 2500 μg/plate (5.0) concentrations were not significantly different from the negative control (Table 4.3) Histidine revertants were found to be significantly higher in experiments that lacked metabolic activation than in those in which it was employed. However the two experimental sets followed a similar dose dependent trend in histidine revertant output with the respective highest quantities being attained at concentrations 2000μg/plate for each set (Table 4.3.)

**Table 4.3.** The effects of *Boophone disticha* HE on TA98 strain of *S. typhimurium.*

| | ***No. Revertant colonies per plate*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Conc.*** | **AC** | | **BD** | | **NF** | | **BD:NF** | |
| ***(μg/plate)*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** |
| 500 | 1.0±1.00 | 1.0±0.58 | 19.6±1.20$^{\#}$ | 9.3±0.88$^{\#}$ | 63.0±1.76* | 41.0±1.15* | 0.31 | 0.23 |
| 1000 | 2.7±1.33 | 0.3±0.33 | 29.3±0.88*$^{\#}$ | 20.3±1.20*$^{\#}$ | 89.3±4.20* | 83.3±4.41* | 0.33 | 0.24 |
| 1500 | 1.3± 0.88 | 2.7±0.33 | 162.0±3.46*$^{\#}$ | 85.0±3.61*$^{\#}$ | 303.7±4.20* | 174.0±4.16* | **0.53** | 0.49 |
| 2000 | 0.7±0.67 | 1.7±0.33 | 181.0±2.08*$^{\#}$ | 136.0 ± 3.46*$^{\#}$ | 323.0±6.24* | 187.7±4.33* | **0.56** | **0.72** |
| 2500 | 0.3±0.33 | 4.3±0.33 | 7.33±1.86*$^{\#}$ | 5.0±1.15*$^{\#}$ | 41.7±2.19* | 30.7±1.20* | 0.18 | 0.16 |

Data expressed as mean± S.E.M (n= 3 plates); Significant difference: $^{*}p<0.05$ versus AC (negative control), $^{\#}p<0.05$ versus NF (positive control)

#### *4.3.1.1. Effects on TA98 strains of S. typhimurium -S9 metabolic activation.*

The *Boophone disticha* extract (BD) and 2-nitrofluorene (NF) followed a similar dose dependent histidine revertant output with highest respective quantities being attained at 2000 μg/plate (Table 4.3). At all levels, the output in Boophone disticha extract was statistically lower than those in 2-nitrofluorene tests. As expected acetic acid (AC) produced insignificant histidine revertants at all concentrations (Table 4.3).

#### *4.3.1.2. Effects on TA98 strains of S. typhimurium +S9 metabolic activation.*

The *Boophone disticha* extract (BD+S9) and 2-nitrofluorene (2-NF+S9) followed a similar concentration dependent histidine revertant output with highest respective levels being attained at 2000 μg/plate (Table 4.3. At all levels, the outputs in *Boophone disticha* extract tests were significantly lower than those in the 2-nitrofluorene tests. However as with –S9 and also TA100 the 500 μg/plate and 2500 μg/plate were not significantly different from the negative control (acetic acid).

#### *4.3.1.3. Ratios of the effect of Boophone disticha HE to that of 2-nitrofluorene on TA98*

A concentration dependent increase in the ratios was noted from low from 500 μg/plate up until 2000 μg/plate. Further increases in the concentration to 2500 μg/plate decreased the ratio for both sets (i.e. –S9 and +S9). For both sets only the 1500μg/plate and 2000 μg/plate had a ratio (BD: NF) greater than 0.5 (Table 4.3) Metabolic activation tended to lower slightly lower the ratio at all concentrations.

### 4.3.2. Effects of *Boophone disticha* HE extract on TA100 strains of *S. typhimurium*

A concentration dependent increase in the number of revertants produced was noticed for both tests (BD and BD+S9; Table 4.4), from the lowest concentrations of 500 μg/plate with the maximum number of revertants being found at 2000 μg/plate concentrations. Further increase in concentration to 2500 μg/plate resulted in significantly lowering the revertant number and the appearance of a brownish colouration on the background lawn of the petri dishes. Histidine revertants were found to be significantly higher in experiments that lacked metabolic activation than in those in which it was employed. The two experimental sets followed a similar dose dependent trend in histidine revertant output with the respective highest quantities being attained at concentrations 2000 μg/plate (Table 4.4)

#### *4.3.2.1. Effects on TA100 strains of S. typhimurium - S9 metabolic activation.*

The *Boophone disticha* extract (BD) and sodium azide (NA) followed a similar dose dependent histidine revertant output with highest respective quantities being produced at 2000 μg/plate (Table 4.4). At all concentrations, the outputs in *Boophone disticha* extract tests proved to be significantly lower than those in the sodium azide tests. Acetic acid (AC) tests followed a different pattern as the histidine revertants from all concentrations used proved not to be statistically different with very insignificant quantitative outputs at every concentration. The *Boophone disticha* extract and acetic acid 500 μg/plate tests concentrations also proved not to be statistically different.

#### *4.3.2.2. Effects on TA100 strains of S. typhimurium +S9 metabolic activation.*

Generally metabolic activation reduced the number of revertants all the substances and at all the concentrations. The *Boophone disticha* extract (BD+S9) and sodium azide (NA+S9) followed a similar dose dependent histidine revertant output with highest respective quantities being attained at 2000 µg/plate for *Boophone distich*a extract and 1500 µg/plate for sodium azide (Table 4.4). At all concentrations, the output in *Boophone disticha* extract tests was significantly lower than with sodium azide tests. The 500 µg/plate concentration of *Boophone disticha* was not significantly different ($p>0.05$) from that of acetic acid (negative control).

**Table 4.4.** Effects of *Boophone disticha* HE on TA100 strain of *S. typhimurium.*

| | ***No. Revertant colonies per plate*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Conc.*** | **AC** | | **BD** | | **NA** | | **BD:NA** | |
| ***(µg/plate)*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** | ***-S9*** | ***+S9*** |
| 500 | 2.0±0.58 | 1.3±1.33 | 8.0±0.58* | 8.0±1.53$^{\#}$ | 35.7±2.40* | 24.7±1.86* | 0.22 | 0.32 |
| 1000 | 2.7±0.88 | 2.0±1.15 | 73.7±3.53*$^{\#}$ | 32.0±1.73*$^{\#}$ | 202.3±4.29* | 89.3±6.36* | 0.36 | 0.27 |
| 1500 | 1.7±0.88 | 2.6±0.33 | 178.0±4.36*$^{\#}$ | 114.3±4.70*$^{\#}$ | 313.3±7.26* | 203.3±4.41* | **0.57** | **0.56** |
| 2000 | 1.7±1.20 | 2.0±1.53 | 204.3±10.90*$^{\#}$ | 120.0±5.03*$^{\#}$ | 355.0±2.89* | 174.3±3.48* | **0.58** | **0.69** |
| 2500 | 2.3±0.88 | 2.7±1.76 | 21.7±1.20$^{\#}$ | 20.7±0.88*$^{\#}$ | 135.3±3.48* | 98.7±5.78* | 0.16 | 0.21 |

Data expressed as mean± S.E.M (n= 3 plates); Significant difference: *$p<0.05$ versus AC (negative control), $^{\#}p<0.05$ versus NA (positive control)

#### *4.3.2.3. Ratio of the quantitative effect of on TA100*

A concentration dependent increase in the ratios was observed from low concentrations of 500µg/plate with the highest respective values being found at 2000 µg/plate with or without metabolic activation (Table 4.4). Further increase in the concentration to 2500 µg/plate saw a decrease in the ratios. For both sets the ratios were significantly lower at 2500 µg/plate

where they fell about 0.2 with intermediate values being attained at concentrations 500 μg/plate and 1000 μg/plate. Only the 1500 μg/plate and 2000 μg/plate concentrations had ratios above 0.5 for both sets (Table4.4).

## 4.4. Anxiolytic effect of a hydroethanolic extract of *Boophone Disticha* in Balb/c mice

### 4.4.1. Time spent in open arms

Generally, there was an increase in the percentage time spent in the open arms of the elevated plus maze for all the *Boophone disticha* groups and the diazepam group when compared to the control group (Figure 4.7). The BD10 mg/kg group spent the most time on the open arms of the elevated plus maze which was significantly higher ($p<0.05$) than the negative control. However, there were no statistically significant difference between the other experimental groups and the control. Among the experimental groups there was a dose dependent decrease in the time spent in open arms.

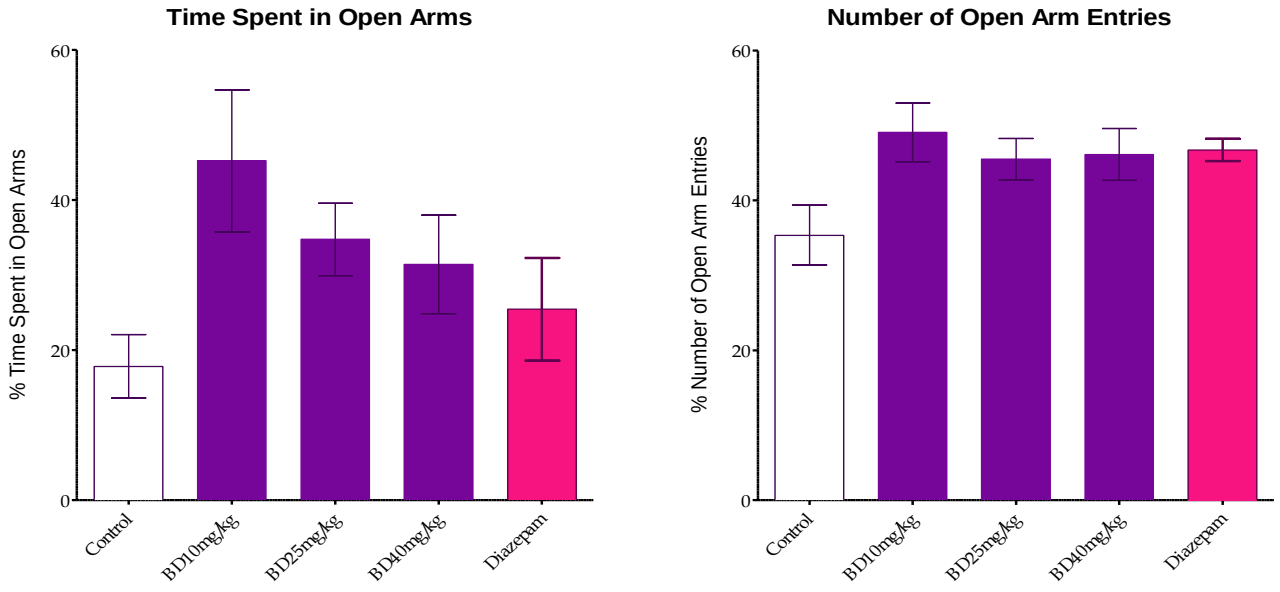


**Figure 4.7.** Effect of diazepam and *Boophone* extract on the time spent on the open arm of the EPM. Data is represented as mean ±SEM. *n*= 6-8. *p<0.05 vs. Control, Dunnett's test

### 4.4.2. Number of open arm entries

All *Boophone disticha* dosage groups increased the percentage number of open arm entries compared with the control group (Figure 4.7). However there was no statistically significant difference. As with time in open arms, the 10 mg group showed the highest percentage open arm entries (Figure 4.7). Again, the differences were not significantly higher ($p>0.05$) than the control for all the experimental groups including the positive control.

### 4.4.3. Unprotected head dips

There was generally a significant ($p<0.05$) increase in the percentage number of unprotected head dips for all the experimental groups and the diazepam group compared to the control (Figure 4.8.). The BD10 mg/kg group showed the highest percentage unprotected head dips, which were comparable to that of the positive control (diazepam group).

### 4.4.4. Unprotected stretched attend postures

All the *Boophone disticha* groups as well as the diazepam group showed an increase in the number of unprotected stretched attend postures when compared with the control. The BD10 mg/kg showed the highest percentage number of unprotected stretched attends postures, which was significantly higher than the control and comparable to the Diazepam group (Figure 4.8). A dose dependent decrease in the percentage unprotected stretch attend postures was observed among the experimental groups.

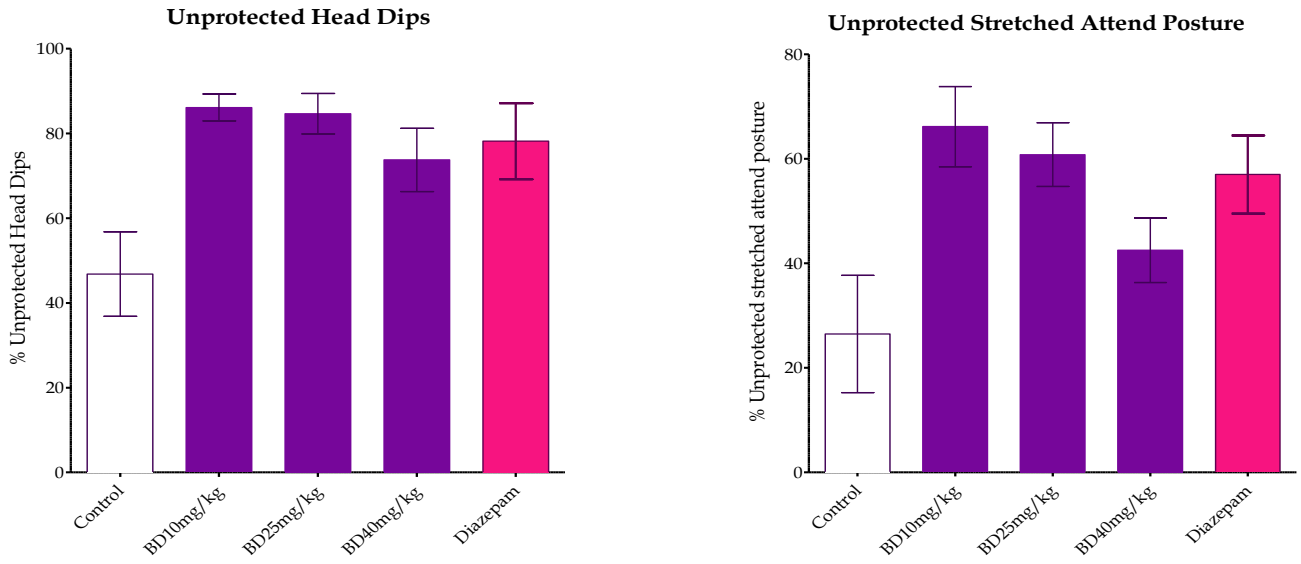


**Fig.4.8.** Effect of diazepam and *Boophone* extract on the number of unprotected stretched attend posture on the open arms of the EPM. Data are represented as mean SEM (n=6-8).*p<0.05 vs. Control, Dunnett's test.

### 4.4.5. Rearings

The *Boophone disticha* groups and the diazepam group showed a decrease in the number of closed arm rears when compared with the control group (Figure 4.9). The *Boophone disticha* 10 mg group had the least number of closed arm rears (Figure 4.9) but there was no significant difference compared to the control. Nevertheless, rearing among all the experimental groups was comparable to the diazepam group.

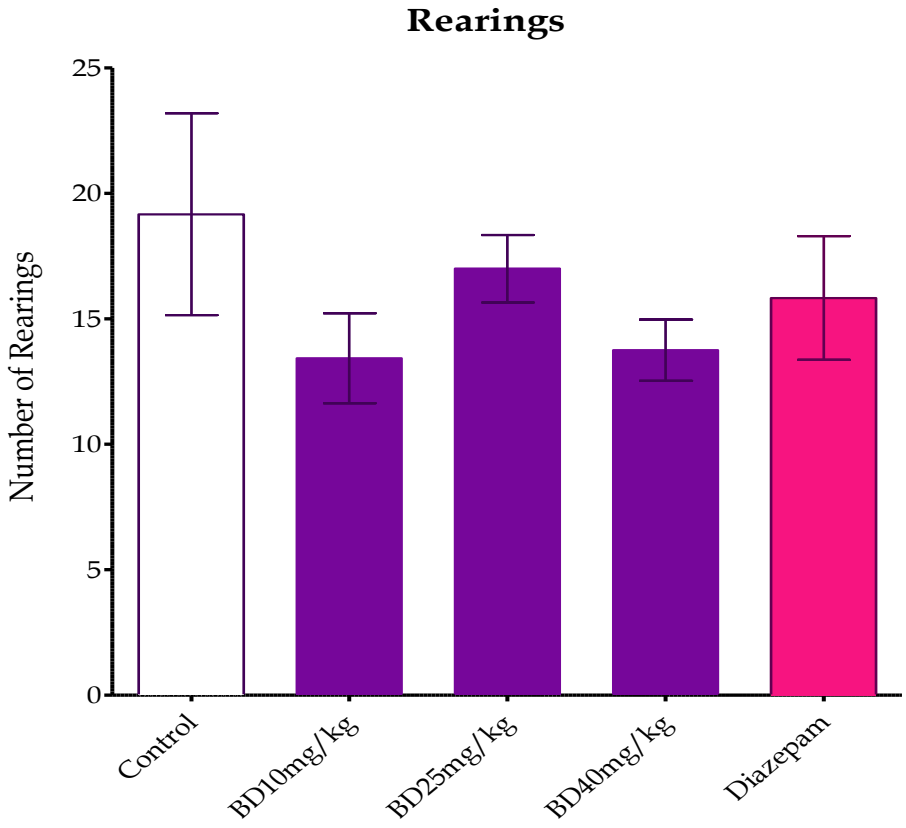


**Fig. 4.9.**Effect of *Boophone extract* and diazepam on the number of rears in the EPM. Data are presented as mean SEM (n= 6-8). *p<0.05 vs. Control, Dunnett's test.

## 4.5. Subacute Antidepressant-like effects of a hydroethanolic extract of *Boophone disticha* in Balb/c mice

### 4.5.1. Forced Swim Test: Day 1 (Acute Test)

#### *4.5.1.1. Total Swimming Time; Day 1*

There was no increase in swimming time in comparison to the control after single dosing (Figure 4.10.). Swimming time for all test groups and positive control was less than that for the negative control. Differences in swimming time had no statistical significance except for

the BD20 mg/kg group which had a significant difference with other test groups BD10 mg/kg and BD40 mg/kg as well as with the negative control group ($p<0.05$). No statistically significant difference was observed between this group and the positive control group although it was notably lower.

#### *4.5.1.2. Total Climbing Time; Day 1*

Generally total climbing time for all the groups was low compared to their swimming time, except for BD 20mg/kg group which showed a reverse trend (Figure 4.10). No notable differences were seen with the other experimental groups compared to the negative control and Fluoxetine group (positive control). Total climbing time for 20 mg/kg was notably higher than all other groups but not statistically significant.

#### *4.5.1.3. Total immobility time – Day 1*

Total immobility time for all the experimental groups was notably higher than the negative control (Figure 4.10.). However, only the total immobility time for BD20 mg/kg was significantly higher than that of the negative control. Total immobility time for Fluoxetine group was higher than all other groups except for BD20 mg/kg, but the difference was not statistically significant ($p>0.05$).

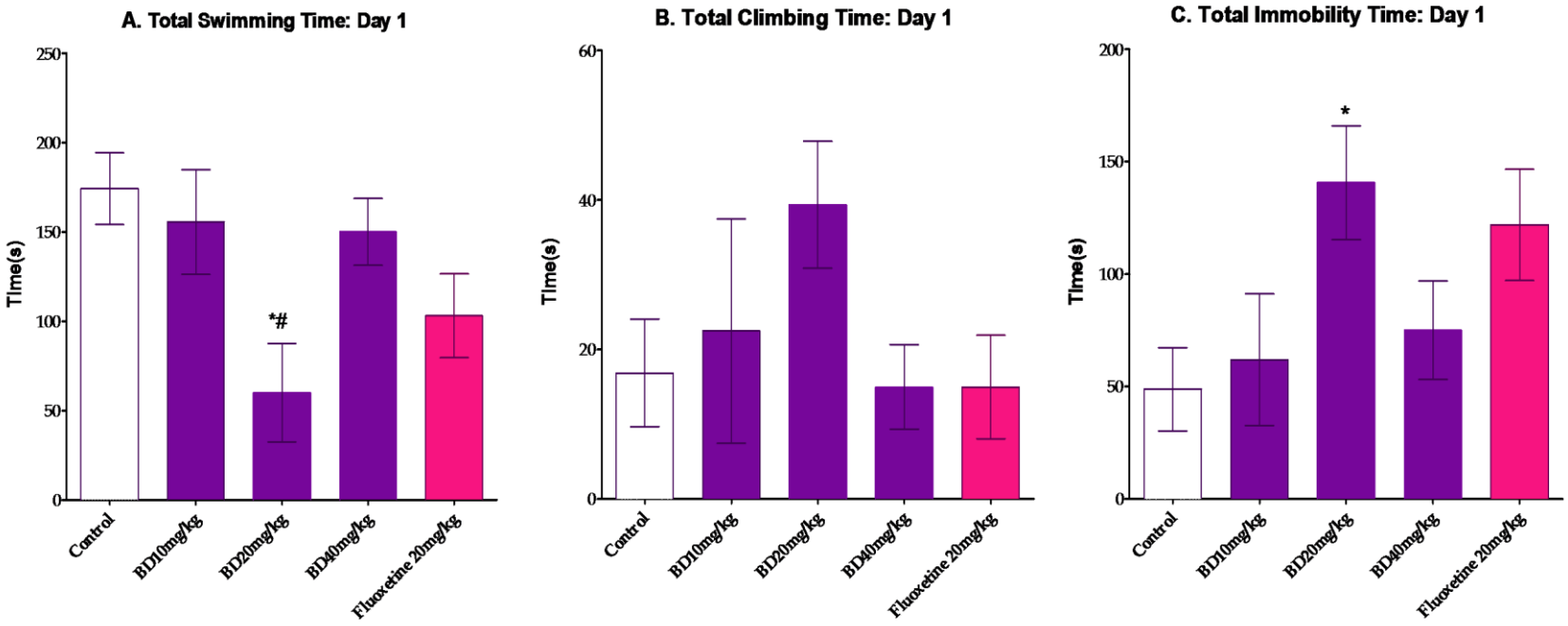


**Figure 4.10.** Effects of *Boophone* extract on latency to Immobility, Immobility time and Swimming time in the FST.*p<0.05 vs Control, Newman-Keuls test; #p<0.05 vs Fluoxetine (positive control), Newman-Keuls test

### 4.5.2. Forced Swim Test: Day 21 (Subchronic Test)

#### *4.5.2.1. Total Swimming Time; Day 21*

Total swimming times for BD10 mg/kg and BD40 mg/kg were significantly higher than those for fluoxetine group (positive control) and they were not significantly higher than the negative control (Figure 4.11). Total swimming time for fluoxetine group was significantly lower than all groups including negative control. Only the BD20 mg/kg was less than the negative control and it was also significantly lower than the BD10 mg/kg. Although total swimming time for BD20 mg/kg was also notably lower than BD40 mg/kg this difference was not statistically significant.

#### *4.5.2.2. Total Climbing Time; Day 21*

There was a significant difference between total climbing times of all other groups and that of fluoxetine. Very low climbing times were noted in comparison to swimming and immobility times for all groups except fluoxetine. The differences in climbing time of all groups compared to fluoxetine group were statistically significant ($p<0.05$). Climbing time for BD 40mg/kg was higher than other test goups and that for BD20 mg/kg was lowest although these differences were not statistically significant. Climbing time for BD10 mg/kg was more or less the same as that for negative control.

#### *4.5.2.3. Total immobility time: Day 21*

Total immobility time for BD20 mg/kg was significantly higher than that for BD40 mg/kg, Fluoxetine and BD10 mg/kg (Figure 4.11). Although total immobility time for negative control was lower than that for BD20 mg/kg this difference was not statistically significant

($p<0.05$). Total immobility time for the other two test groups was lower than that for negative control and fluoxetine although these differences were not statisticallly significant. Total immobility times for BD40 mg/kg and fluoxetine groups were more or less the same.

#### *4.5.2.4. Comparison of Swimming, Climbing and Immobility Times for Day 1 and Day 21*

A comparison of total swimming times for day one and day twenty one showed a general decrease with repeated dosing, but the change was not significant for all the groups. Only the BD10 mg/kg group showed a reverse trend, as the swimming time increased on day twenty one. No change was observed in the BD20 mg/kg. A notable decrease was observed in the Fluoxetine group although but it was not statistically significant ($p<0.05$).

Changes in climbing time with repeated dosing were also not significant with the experimental groups and did not follow any consistent trend (Figure 4.14). However a significant increase in total climbing time was seen with the Fluoxetine group. Interestingly the change in total climbing time for the BD40 mg/kg group followed the same trend as positive control (Fluoxetine) although the increase was not statistical significance ($p<0.05$).

Generally the change in the immobility time did not follow any consistent trend among the experimental groups and the changes observed were not statistical significant ($p<0.05$). Immobility was notably increased in the BD20 mg/kg and negative control groups although these differences were not significant (Figure 4.12). A decrease was seen in the BD10 mg/kg and BD40 mg/kg groups and the Fluoxetine group though these differences were also not significant.

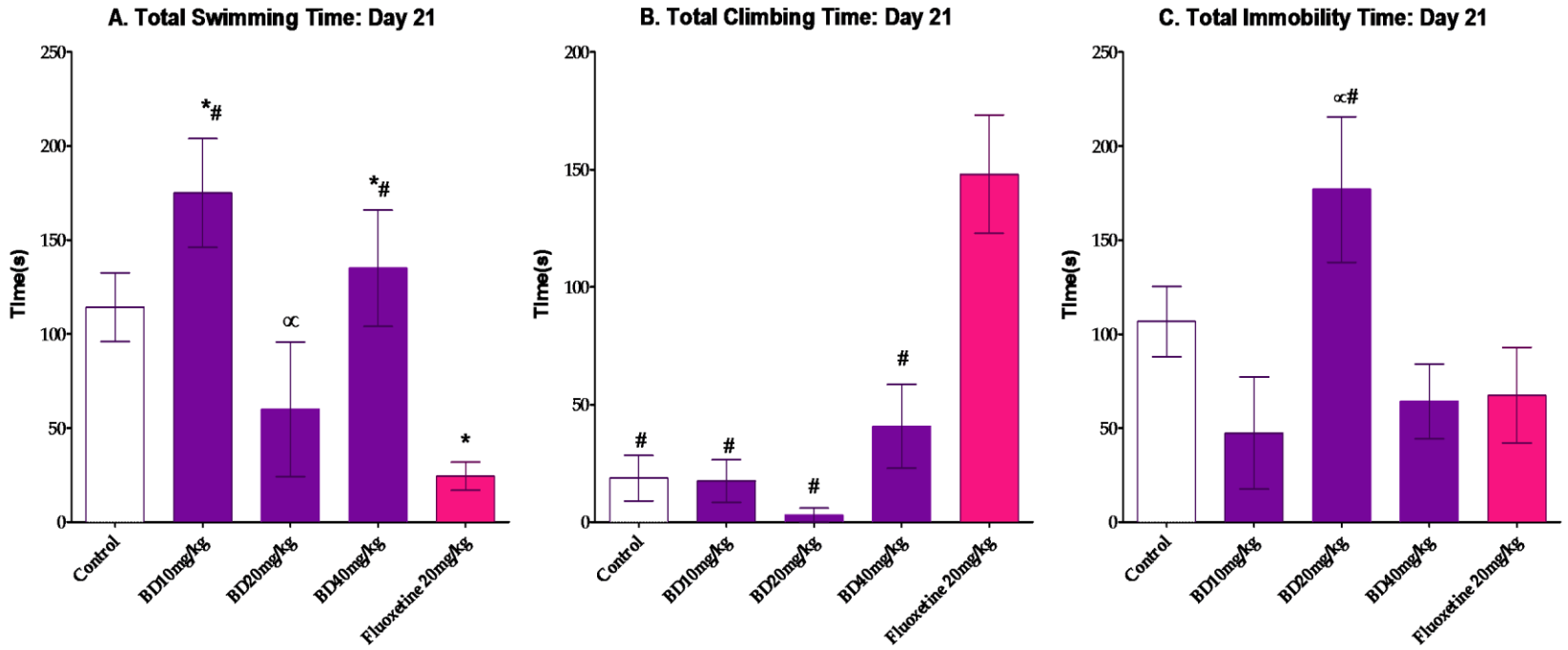


**Figure 4.11.** Effects of *Boophone* extract on in the FST after 21 day repeated dosing.* $p<0.05$ vs. Control, Newman-Keuls test; #$p<0.05$ vs. Fluoxetine (positive control), Newman-Keuls test; $p< 0.05$ vs. BD 10mg/kg, Newman- Keuls test

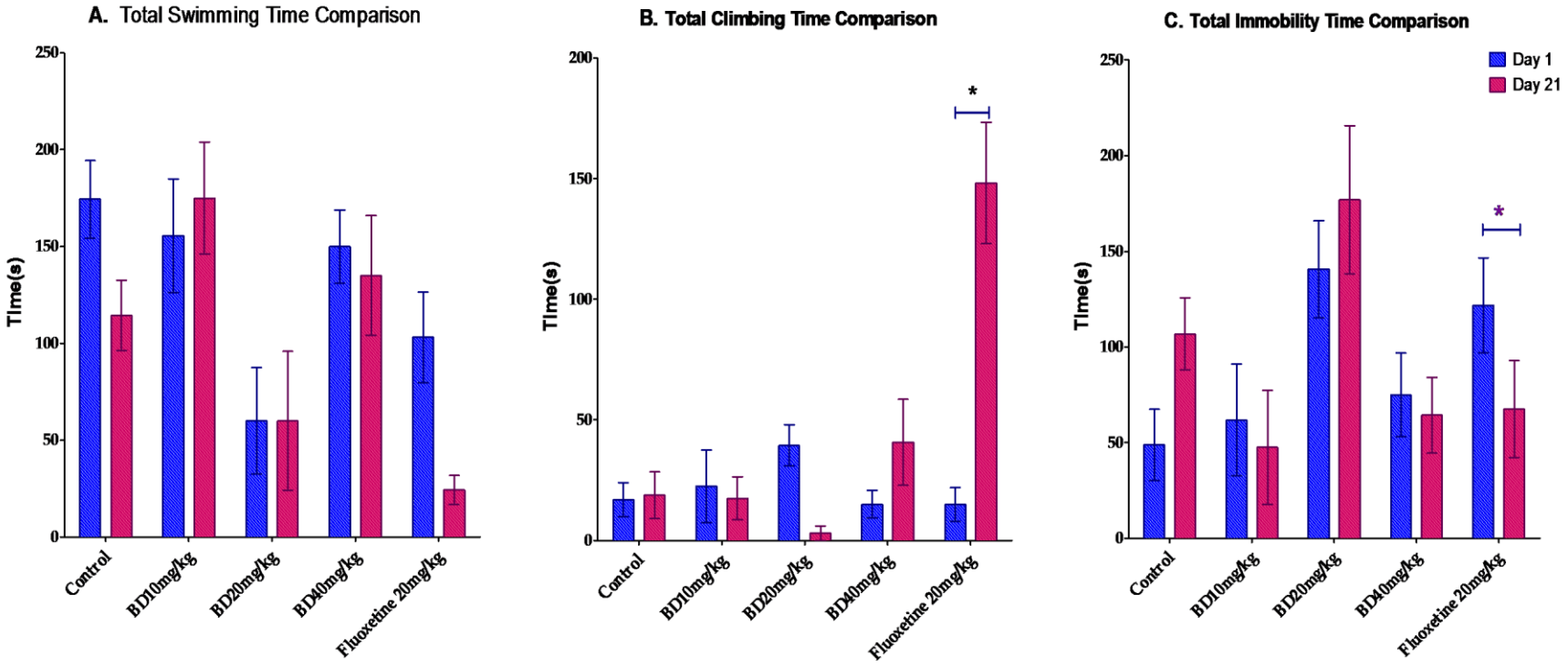


**Figure 4.12.** Comparison of the effect of the treatments on Forced swim test endpoints for Day 1 and Day 21.*- $p<0.05$ Day 1vs Day 21, Bonferroni test

# CHAPTER 5

# 5. GENERAL DISCUSSION

## 5.1. SUMMARY

There is a scarcity of literature on the experimental toxicity and efficacy of traditional medicines used in Zimbabwe and other African countries. This lack of knowledge and the wide spread reliance on traditional medicines among these population is a source of unwanted poisoning morbidity and mortality in these resource limited countries (Shale *et al.*, 1999; Louw & Korsten, 2002; McGaw *et al.*, 2008; Srivastava *et al.,* 1996; Stafford *et al.*, 2008). Furthermore the lack of toxicity information presents a challenge to the clinical toxicologist and/ or emergency physician in poisoning instances. However the importance of traditional medicines as potential lead candidate for drug development cannot be undermined.

*Boophone disticha* is one such plant that has been used extensively in ethnomedicinal practices and despite its known toxicity from case reports, is a very important candidate for future drug development. The present study was targeted at investigating the symptomology and toxicological effects of the bulb and potential efficacy of the bulb for selected indications based on traditional uses. This section presents a thorough discussion of present findings and correlates those with information hitherto reported human experience and experimental findings.

## 5.2. ACUTE ORAL TOXICITY AND NEUROBEHAVIOURAL EFFECTS IN RATS

In this study the $LD_{50}$ of the crude aqueous ethanolic extract was estimated to be between 120 and 240 mg/kg in rats, which is higher when compared to a previously published lethal dose of one *Boophone* alkaloid, buphanidrine, of 8.9 mg/kg s.c. and 10 mg/kg i.v. in mice (van Wyk *et al.*, 2002). The difference could be attributed to the difference in extraction method and to the purity of the extracted sample. Furthermore the crude extract is a complex mixture of different alkaloids with different properties. Since we estimated $LD_{50}$ to be above 120 mg/kg and in our protocol the 50 mg/kg dose showed little toxicity, we propose that doses with potential therapeutic use and for repeated dose toxicity studies should be below 50 mg/kg.

The results of the study indicate that the crude extract of *Boophone disticha* has acute CNS depressive effects. The early onset of intoxication symptoms, even at the lower doses, could point to a rapid gastrointestinal absorption of toxic principles in the crude extract. This is also supported by the quick onset of symptoms in reported acute poisoning cases (Laing, 1979; du Plooy *et al.,* 2001; Gelfand *et al.,* 1985).The moderately long duration of toxicity of the extract particularly with high doses for those animals that survived may be attributed to sustained effects on the nervous system due to high lipid solubility and high concentration of the alkaloids in the central nervous system or maybe related to the mechanism of toxicity.

The pattern of behaviours observed in the FOB is also suggestive of CNS depressant effect particularly at low doses. Higher doses are sometimes associated to a CNS stimulant and hallucinogenic effects. This is supported by the fact that weak decoctions have been used

traditional effectively as a sedative to relieve "hysteria" and insomnia (van Wyk *et al.*, 2002). High doses have been known to induce hallucinations, when used for divination and some of the reported cases of intoxication (van Wyk *et al.,* 2002; Laing, 1979; du Plooy *et al.,* 2001; Gelfand *et al.,* 1985). Lycorine is an anticholinergic alkaloid that has been extracted from *Boophone disticha* and many other Amaryllidaceae herbs (Watt & Breyer-Brandwijk, 1962). The mydriasis, palpebral closure, piloerection, tachypnoea and spastic hind limb paralysis observed at lower doses of *Boophone disticha* could be due to the anticholinergic effects of lycorine. An earlier *in vitro* study further supports these anticholinergic effects of the *Boophone* extract (Nyazema & Ndiweni, 1986). However, these effects cannot be all attributed to lycorine only since the pharmacological effects of the other *Boophone* alkaloids are not fully known, and further investigations need to be done.

The most notable effects with higher doses of the extract were convulsions, laboured breathing and flaccid forelimb paralysis. This suggests respiratory depression as a possible cause of death and CNS depression as an important toxic effect at these doses. A stereotypical behaviour, retropulsion was observed at higher doses ( 240 mg/kg). This is indicative of and further supports the reported hallucinogenic effects of the plant extracts (van Wyk et *al.,* 1997, 2002; du Plooy *et al.,* 2001; Gelfand *et al.,* 1985). The observed flaccid paralysis overrules spastic extensions of forelimbs as a cause of the retropulsion. This would point to the involvement of serotonin or dopamine or both and would agree with recent findings from *in vitro* studies on isolated alkaloids of *Boophone disticha,* which have shown selective binding to a serotonin transporter in the rat brain (Sandager *et al.*, 2005).

Locomotor activity is considered to be an index of alertness and a decrease in locomotion can indicate sedation (Thakur & Mengi, 2005). Our results show that locomotor activity was significantly reduced as evidenced by reduced rearing and grooming behaviour. However, the hypoactivity noted might be multifactorial, possibly involving impaired neuromuscular activity and other mechanisms. A finding that seems paradoxical is that all sensorimotor indicators were not significantly affected in all the dosage groups. This might indicate a restricted or selective neuropharmacological activity of the *Boophone* extract and warrant further investigation.

In conclusion the *Boophone* HE extract produced signs of acute reversible CNS depression, which probably explain its traditional use for anxiety disorders. However, it is highly toxic therefore its recreational use should be discouraged. The neurotoxicological effects of the *Boophone* Hydro-ethanolic extract have been described and range from mild tremors to limb paralysis and death at high doses. The observed toxicity and neuropharmacological effects are probably linked to several neurotransmitters like serotonin and acetylcholine. Although preliminary *in vitro* studies on several of the *Boophone* extract alkaloids have been performed, our ongoing research aims to investigate *in vivo* the anxiolytic and antidepressant potential of the *Boophone* extracts and the purified alkaloids.

## 5.3. Subacute Toxicity and Neurobehavioural Effects in Rats

Although *Boophone disticha* has been used traditionally to treat long term conditions like depression and anxiety (van Wyk *et al.,* 1997, 2002; van Wyk & Gericke, 2000), there are no documented reports about its long term adverse effects after both acute intake or repeated

exposure. Since some adverse effects can be delayed, subacute and chronic assessments are pertinent to determine its chronic toxicity.

The subacute toxicity results showed that the experimental groups generally had a higher increase in body weights as compared to the control (Figure 4.1). This also correlated with that of organ weights were the stomach, large intestines and small intestines had larger weights for the experimental groups (Figure 4.2). This could infer a possible enhancement of gastric absorption of alkaloids and by the extract and this is supported by the decrease in latency to onset of toxic responses with both repeated dosing and increasing the doses notwithstanding the likelihood of elevated serum levels as a result of the aforementioned repeated dosing. The increased gain in body weight may also be attributed to a possible modification of metabolism. Previous work by Sandager *et al.,* showed that crude *Boophone disticha* extract has selective serotonin reuptake inhibitory activity and this is known to increase food consumption as well as confer antidepressant properties (Purves, *et al.,* 2004). However, in the present study, daily food consumption was not measured to give a more concrete conclusion. Again, the increase in organ weights may also be attributed to lesions due to potential cytotoxicity or genotoxic effects of the *Boophone* extract. This is supported by GIT histopathological findings in the acute toxicity study. However, there is need for further histopathology analysis after repeated dosing to confirm or refute this theory.

Liver weight for the BD200 mg/kg and BD400 mg/kg groups were significantly high compared to the control (Fig 4.2). This when correlated with the increasingly rapid recovery of the rats from toxic responses may point to an increase in hepatic enzyme production to

metabolise break down the actives in the extract. This is supported by a study, which reported that hypertrophy was found to be an adaptive response of the liver to an orally administered toxicant (Dominic *et al.*, 1993). Nevertheless detailed hepatic assessments are necessary for the exact mechanism of this effect to be determined.

*Boophone disticha* was also found to cause death by respiratory failure of central origin in acute case reports, this is consistent with the experimental finding of a possible effect on the respiratory system**.** The smaller lungs of the rats in the test groups as compared to the control group inversely correlated with larger hearts of the rats in test groups and smaller hearts for the control (Fig 4.2). This like in the acute toxicity assessment might be indicative of an effect on the respiratory system, leading to long term compensatory adaptations to maintain adequate oxygenation of the extremities (Walker, 2006). In addition, some alkaloids are known to induce occlusive changes in pulmonary arterioles developing into pulmonary hypertension and right ventricular hypertrophy (Reddy &Hayes, 2001). The notable dose dependent decrease in testes weight, although not significant, may point to a possible effect on spermatogenesis. However, in this case, further investigations such as hormonal levels, sperm count and mating success are necessary to give conclusive findings.

*Boophone disticha* bulb has been exploited widely for its CNS effects (Acuda & Eide, 1994; Gelfand *et al.,* 1985; van Wyk *et al.*, 1997, 2002). As with the acute toxicity and case reports of intoxication, the neurotoxicological effects of *Boophone*, the chronic intake seems to affect the same neurological systems though the effects tend to be milder. The severity in abnormality of posture and head orientation with dose and with repeated dosing may be

attributed to a possible effect of the extract or its metabolites the vestibular system which is responsible for head orientation and posture during movement. This is also supported by the findings on gait that had a similar trend. (Figure 4.2)

The observed dose dependent increase in involuntary motor movements, might point to a possible effect on motor co-ordination due to an action on the basal ganglia which is responsible for gating the proper initiation of movements that is, suppressing unwanted movements and priming upper motor neuron circuits for initiation of movement (Purves *et al.,* 2004). However, more neuromuscular investigations are required to affirm this postulation.

*Boophone disticha* has been used traditionally for its sedative effects (Gelfand *et al.,* 1985). Eyelids were drooping for all the experimental groups, indicative of its CNS depressive effects (Purves *et al.*, 2004). This result is complemented by homecage observations that showed that rats became easier to remove from home cages and to manipulate after repeated dosing and administration of higher doses (Figure 4.3). This may also be attributed to effects on central transmission since it is documented that serotonin, as a central transmitter, is partly responsible for wakefulness (Purves *et al.*, 2004; Katzung *et al.*, 2009) and evidence from Sandager *et al.* (2005) suggests that *Boophone disticha* has Selective Serotonin Reuptake Inhibitory activity, thus the postulated drowsiness. Drowsiness may also have possibly resulted from effects on the parasympathetic transmission (Katzung *et al.*, 2009). Presence of piloerection in dosing groups points to a possible effect of *Boophone*

*disticha* on the sympathetic transmission, thus on peripheral adrenergic transmission. However, the lack of lacrimation and salivation points to possible lack of cholinergic effect.

Arousal, which showed the level of unprovoked activity, was higher on the first few days for the lower dose groups with bursts of movement as compared to the control (Fig 4.3). This may suggest that at lower doses *Boophone disticha* has central stimulatory effects, however, the rats became less active with repeated dosing hence it may have sedative or intoxicating properties at higher doses or blood levels. After repeated dosing retropulsion was observed and this may suggest presence of hallucination as a result of the extract, a finding that would corroborate with the use of *Boophone disticha* by traditional healers to get into a trance (Gelfand *et al.*, 1985).

The decrease in border crossings and rearing (Figure 4.3.) with repeated dosing points to reduced exploratory activity may be due to decreased levels of consciousness, or poor motor coordination which may be a result of toxicity on the neural structures (local spinal cord and brain stem circuits, descending modulatory pathways, or basal ganglia) involved in control of movement. This may also result from interference with central dopaminergic transmission (Purves *et al.,* 2004). Some symptoms (tremor of effort) were comparable to those for Parkinsonism, hence the inference to the dopaminergic system, and central cholinergic transmission. Thus, either the alkaloids themselves or their primary metabolites can cross the blood brain barrier and retain pharmacologic activity. Rearing is an activity that requires significant motor coordination again pointing to a possible effect on the sensory-motor coordination pathway and/ or the vestibular pathway.

Gait analysis showed a tendency (day 1 and day 14) to move on tiptoes with a scurrying motion and with the backs in a hunched posture. This observation infers a possible effect on muscle contractility and excitability, either directly or as a result of interference with transmission to the muscles as was postulated above. Beyond day 14 the rats were moving with forelimbs dragging, in crouched positions or with the body dragging against the surface. This again shows a possible effect on the motor complex, especially the lower motor neuron section as damage to this region is usually associated with paralysis, muscle atrophy (as observed by the decreasing body weight of the experimental groups), decreased superficial reflexes and reduced tone (Purves *et al.*, 2004). On the ranked gait abnormality there was a worsening of this parameter with repeated dosing as well as with increasing dosage. These increases in abnormality may infer a build-up of alkaloids in the system possibly as a result of the detoxification processes being overcome by the frequency of dosing or amounts administered respectively.

Righting reflex was lost in the 400 mg/kg group by day 14, while all other groups retained this reflex. These rats were stuporous and thus were disqualified from this test. However, even severely intoxicated rats from other groups retained the reflex suggesting that *Boophone disticha* may have no effect on reflex transmission that is the spinal cord and brain stem circuitry.

It can be concluded that *Boophone disticha* has constituents that are either highly toxic or have their primary metabolites being toxic especially at higher doses. These compounds are

also capable of crossing the blood brain barrier. At low single doses, however, it appears that *Boophone disticha* has stimulant properties or may cause anxiety.

In summary the main subacute toxic effects of *Boophone disticha* were like the acute effects and seem to be mediated via interference with the neuronal pathways, especially the central dopaminergic and motor neurons. Death due to *Boophone* poisoning, as with acute intoxication, can occur as early as 10 minutes post dosing or can take up to 12 hours. Target organs as observed by changes in organ weight appear to be liver, small and large intestines, stomach, central nervous system and peripheral nervous system.

## 5.4. Genotoxicity effects of a hydroethanolic extract of *Boophone Disticha*

Plants commonly used in traditional medicine are deemed to be safe due to their long and extensive usage. However, recent investigation of plants used in ethnomedicinal practices and even as food showed that the majority are potentially toxic and genotoxic (Fennell *et al.,* 2004). *Boophone disticha* due to its high toxicity and widespread availability and usage potentially might be genotoxic thus it is imperative that at preliminary genotoxic effects be carried out to determine its genotoxicity potential. In this context the following genotoxicity of *Boophone disticha* was done using the Ames test to provide preliminary characterization of genotoxic potential.

Nitrogen-containing organic compounds are electrophilic and thus can induce DNA structure destabilisation causing electron redistribution, which can lead to alteration in structure of DNA. Thus, these are highly cytotoxic, genotoxic, mutagenic and carcinogenic

compounds. *Boophone disticha* alkaloids are also nitrogen-containing compounds which in this study the extract has shown some genotoxicity potential. The genotoxic effects were concentration dependent as the mutagenic effects increased with increasing test concentration to a maximum at 2000 µg/plate. A marked decrease from the trend in colony forming units at 2500 µg/plate can be attributed to cytotoxicity that may have affected other revertant cells thus causing a reduction in the number of revertants observed. A brownish colouration observed at this concentration on the background lawn may also entail this cytotoxicity. The dominant effect, on the colony forming unit output, of the test set that lacked metabolic activation system (S9) at all concentrations over the set that employed S9 may explain the presence of the enzymatic system metabolised the genotoxicants into less genotoxic forms.

The lack of statistically significant difference for concentrations 1500 and 2000 µg/plate with metabolic activation might indicate that at these concentrations *Boophone disticha* produce the same genotoxic effects in a *Salmonella typhimurium* bacterial model. It also explains that an increase in concentration from 1500 to 2000 µg/plate may not produce a change in the genotoxic effects.

The similar trend followed by *Boophone disticha* extract and the positive control in the tests with TA100 *Salmonella Typhimurium* (sodium azide) all in the absence and presence (Table 4.4) of S9 shows that the extract has some genotoxic effects. Although they are less pronounced than those for the standard positive control and this is supported by the statistically differing means at all concentrations between the two chemicals.

A lack of statistical difference at 500 µg/plate shows that at this concentration and lower concentration *Boophone disticha* may have no genotoxic effects irrespective of the metabolic status. General revertant colony unit output of the positive control (sodium azide) was higher in the set that lacked metabolic activation than with S9 (Table 4.4). This is also supported by the fact that metabolic activation lowers the genotoxicity of sodium azide and is consistent with the observed effect with *Boophone disticha* extract.

The ratio of the effect of a test substance with respect to that of its positive control on the colony output is a strong marker of genotoxicity of a test chemical, both in the absence and in the presence of metabolic activation. The higher the ratio, the greater the genotoxic potential of that test chemical and for a chemical to be labelled genotoxic, this ratio must be greater or equal to 0.5 (OECD, 1997).

The similar trend in the sets of ratios of effects of *Boophone disticha* extract (Table 4.4) with respect to those of sodium azide in the absence and presence of metabolic activation entails that S9 has no effect on the dose dependent genotoxicity pattern of *Boophone disticha* hydroethanolic extract. The ratios also show that lower concentrations (e.g. 500 µg/plate) have low genotoxic potential which increases with increasing concentration to the highest at 2000 µg/plate for both sets of tests. This shows that 2000 µg/plate is the optimal concentration in the Ames tests employing *Boophone disticha* that can produce the highest genotoxic effect in TA100 *Salmonella typhimurium* from the observable genotoxicity markers. Low ratio in 2500µg/plate may mean that most potential genotoxicant cells might have beenlysed and thus could not show growth in the presence of *Boophone disticha*.

The genotoxic potential of *Boophone disticha* in TA98 *Salmonella Typhimurium* was concentration dependent as the mutagenic effects increased with increasing test concentration to a maximum at 2000 µg/plate. A marked decrease from the trend in colony forming units at 2500 µg/plate can be attributed to cytotoxicity which killed some revertant cells thus causing a reduction in the number of revertants observed. As with TA100 the brownish colouration observed at this concentration on the background lawn may also confirm its cytotoxicity.

The results with TA98 species of *Salmonella typhimurium* are confirmatory of the observed trends with TA100 (Tables 4.3 & 4.4). Concentrations greater than 500 µg/plate but less than 2500 µg/plate were associated with high incidences of revertant units and this trend was not affected by metabolic activation with S9. Again this result was confirmed by BD: NF ratios, which were higher than for 0.5 for the 1500 µg/plate and 2000 µg/plate concentration.

However, further tests using a different battery are necessary to appropriate characterize the genotoxicity of *Boophone disticha*. Information referring to the genotoxic potential of *Boophone disticha* published in peer-reviewed scientific literature seems, to the best of the available knowledge, limited to the work conducted by Taylor *et al.* (2003) in which Salmonella/microsome assays were used with the use of human white blood cells.

The genotoxicity of *Boophone disticha* may be attributed to the nitrogen-containing alkaloids it contains.

In summary the 500 µg/plate concentrations of the *Boophone* extract were not associated with any genotoxicity with both TA100 and TA98 strains of *Salmonella typhimurium*. Concentrations greater than 500 µg/plate but less than 2500 µg/plate showed some genotoxic effects by increasing the revertant units. The 2500 µg/plate concentration is potential cytotoxic which could have masked the genotoxic effects if this concentration.

## 5.5. Anxiolytic effect of a hydroethanolic extract of *Boophone Disticha* in mice

*Boophone disticha* is used in traditional medical practices to treat anxiety disorders (Botha *et al.,* 2005). However, little or nothing is known about its efficacy and its mechanism for the purported anxiolytic effects.

The elevated plus maze test is a widely used and extensively validated etiological animal model of anxiety because it makes use of natural stimuli such as a fear of a relatively narrow raised platform (Dawson & Tricklebank, 1995). According to Pellow *et al.* (1985), anxiolytic agents increase the frequency of entries and the time in open arms of the EPM. Furthermore, the inclusion of ethological measures (head dipping (HD) and stretched attend posture (SAP)) results in a more sensitive methodology to characterize drug effects than if only classical (spatial) measures are used (Ohl, 2003). In the present work, classical measurements i.e. open arm entries and time spent in the open arm as well as the ethological measures were recorded.

In this study the *Boophone distich* dosed groups showed an increase in the percentage time spent on the open arms of the EPM compared to the control group. This anxiolytic-like behaviour in mice might indicate a homologous effect in human, which might support its use in traditional medicine. It is also noteworthy that the BD10mg/kg group had the highest percentage time spent in open arms compared to the experimental groups. A possible reason for this effect might be an adaptive mechanism, which results in a change in confirmation of the receptor after exposure to higher doses of a certain compound. Another possibility might be the involvement of a totally unrelated and yet not specified target receptor, which alters the pharmacological response. A combined agonistic/antagonistic effect occurring` at different sites might account for the reduced time spent in the open arms as the dose is increased (Grundmann *et al.*, 2007). The diazepam group also showed an increase in the time spent on open arms versus the control although this was not statistically significant. The lack of statistical significance could mean that the conditions employed in our study somehow led to a reduction in the sensitivity of the model. However, this does not nullify the positive results obtained with *Boophone disticha,* which showed a statistically significant increase in percentage time spent on open arms.

The *Boophone disticha* groups showed an increase in the number of open arm entries which was comparable to Diazepam (positive control) and significantly higher than the control. Anxiolytic agents are expected to increase the frequency of entries in the open arms of the elevated plus maze (Pellow *et al.*, 1985). Again, this effect was more prominent with the BD10mg/kg dose. This behavioural effect further reinforces the claim that *Boophone disticha* could be useful as an Anxiolytic. It is speculated that *Boophone disticha* might exhibit its

anxiolytic effects through its action on the serotonin transporter (Neergaard *et al.*, 2009; Sandager *et al.*, 2005) and it has been shown that drugs that act on the serotonin system do not show significant difference in the number of open arm entries in the EPM (Dawson and Tricklebank, 1995; Moser 1989). Therefore this could explain the number of open arms that were not significantly increased (Figure 4.7) and also further supports the theory that *Boophone disticha* could work as an anxiolytic through the serotonin system.

Postural elements are indicative of risk assessment such as head dipping and stretched-attend posture, have been shown to be sensitive to drug effect in the EPM (Navarro *et al.*, 2006). In this study, the probable anxiolytic-like properties of *Boophone disticha* were also fortified by a notable increase of unprotected head dips. However in this instance there was not much of a difference among the *Boophone disticha* groups even though the *Boophone disticha* 10 mg/kg had a slightly higher number of unprotected head dips. However, there was statistically significant difference between the control group and the *Boophone disticha* groups and also diazepam group (Figure 4.8). This confirms that the inclusion of behavioural items increases the sensitivity/reliability of the test and also helps in drawing conclusions regarding the behavioural specificity of treatment effects (Rodgers & Dalvi, 1997).

An increase in the number of unprotected stretched attend postures further supports the anxiolytic-like activity of the *Boophone disticha*. The dose-response effect observed in the time spent on open arm was again observed in this case in which the 10 mg/kg dose had the highest number of unprotected SAP and the high dose (40 mg/kg) had the least. This

reinforces the possible theories already mentioned on the effect of different drug doses on response (Grundmann *et al.*, 2007).

Anxiolytic compounds are expected to decrease the number of protected rearing in the EPM. In the experimental groups, there was a decrease in the number of rearings, however the change, though comparable to Diazepam, was not statistically significant. This parameter does not really show anxiolytic-like effect of a drug because even if an animal spends more time on the open arms of the EPM, if the drug is anxiolytic, the animal will be more active and want to explore more hence more rears when in the closed arm. Nevertheless, the slight decrease in the number of rears in the closed arm is indicative of the anxiolytic-like effects of *Boophone disticha*.

The majority of mechanistic studies on anxiety have centred on the monoamines 5-hydroxytryptamine (5-HT) and Noradrenaline, the Gamma amino butyric acid (GABA)-benzodiazepine receptor complex and a number of unrelated compounds that are known to provoke anxiety and/or panic in humans (Katzung, 2009). However from the findings of thesis present work it cannot be empirically determined which mechanism *Boophone disticha* could be using as an anxiolytic. However, *in vitro* studies showed that *in vivo Boophone disticha* binds onto the serotonin reuptake transporter (Neergaard *et al.*, 2009). Hence, it can be speculated that anxiolytic-like activity of *Boophone* extract might be due to this effect on the serotonergic system.

To summarise the *Boophone* extract showed Anxiolytic-like activity in the elevated plus maze test by significantly changing the percentage time spent in open arms, number of open

arm entries, rearings, unprotected head dips and stretch attend postures. The 10 m/kg dose had the most prominent effects compared to the higher dose which were comparable to the positive control.

## 5.6. Subacute Antidepressant-like effects of a hydroethanolic extract of *Boophone disticha*

Rodents under inescapable stress exhibit behavioural despair that is become immobile after an initial period of struggling (Porsolt *et al.,* 1977a). Evaluation of antidepressant-like activity was performed with the Forced Swim Test. Primary measures in the Forced Swim Test are immobility, swimming and climbing times. Antidepressant-like activity of a drug in the test is shown by a decrease in the duration of immobility and hence an increase in the duration of active periods with escape attempts i.e. an increase in swimming and/or climbing duration (Cryan *et al.*, 2002).

Single dose administration of extract of *Boophone disticha* gave immobility times that were higher than that for the control (Fig 4.10). This is indicative of depression-related behaviours with acute administration of *Boophone disticha.* This contradictory behaviour could suggest multiple effects of the *Boophone* extract components on CNS neurochemical systems. Among the experimental group, the 10 mg/kg/day dose had the lowest immobility time after single dose administration and also had the lowest immobility time compared to all groups including the Control group after repeated dose administration (Fig 4.11), however the difference was not statistically significant ($p<0.05$). This result is in agreement with previous acute studies (Dube, 2009; Pedersen *et al.,* 2008) which showed that lower doses had the most Anxiolytic-like activity. In one of these studies, the parameters measured included

latency to immobility, which is the time taken by the mouse to show the first signs of immobility (Dube, 2009). A high latency to immobility which was comparable to that of the positive control (imipramine) was observed at this dose. Thus both studies demonstrate a possible antidepressant like activity at this dose.

Pederson *et al.,* (2008) observed that extract of *Boophone disticha* exhibited anti-depressant like effects at the lowest concentration, higher concentrations showed a loss of effect. The 10 mg/kg/day dose showed a greater increase in activity times and greater reduction in immobility times than the higher doses. It is therefore possible that the *Boophone disticha* 10 mg/kg/day dose has some antidepressant-like activity more than the higher doses. However there was no specific dose-response relationship due to the anomaly seen with the 20 mg/kg/day dose.

The *Boophone disticha* 20 mg/kg dose showed the highest immobility time after both acute and repeated dose administration. This anomaly could be attributed to the presence in the extract of alkaloids exerting an opposing effect. Several alkaloids have been isolated from *Boophone disticha* of which only two, buphanidrine and buphanamine, have been shown to have affinity to the serotonin transporter and hence a potential antidepressant-like activity (Neergaard *et al.*, 2009). Other alkaloids present could have an opposite effect i.e., increase reuptake of serotonin. A noteworthy point is that during the experiment, mice in this particular group exhibited high aggressive behaviour and cage fights were rife amongst mice in this group. *Boophone disticha* is known to cause hallucinations (Sobiecki, 2002) and these neurological effects could have aggravated this behaviour in the cage which was one

of the smaller ones that were used. Total immobility time for the 10mg/kg group was notably lower than that for the positive control after both single and repeated dosing but no statistical significance was noted, suggesting comparable antidepressant-like effects of the plant and positive control at this dose.

Swimming and climbing in the Forced swim test are escape-oriented activity behaviours. It has been demonstrated that swimming is sensitive to serotonergic compounds such as the selective serotonin reuptake inhibitors and that climbing is sensitive to tricyclic antidepressants and drugs with selective effects on noradrenergic transmission (Cryan *et al.*, 2002).

Total swimming time after single dose administration for all the experimental groups was notably but not significantly lower than that for the negative control, with the *Boophone disticha* 20mg/kg group showing the lowest total swimming time and subsequently higher climbing time (Fig 4.10.). This could suggest that while this dose might not have increased swimming time satisfactorily, an increase in total activity behaviour was still seen, as there was an increase in climbing time. This is indicative of selective effects on noradrenergic rather than serotonergic transmission (Cryan *et al.*, 2002). Again the *Boophone disticha* 10 mg/kg/day and *Boophone disticha* 40 mg/kg/day dose had total swimming and climbing time comparable to that of Fluoxetine although both activity times for 4 mg/kg were not significantly lower than those for 10 mg/kg.

Total swimming time after repeated dose administration was highest for *Boophone disticha* 10 mg/kg/day followed by *Boophone disticha* 40 mg/kg/day. At these doses the plant showed some antidepressant-like activity. The present findings showed that Fluoxetine group had the lowest swimming time after sub-chronic treatment with a subsequent increase in climbing time, and it had significantly higher climbing time compared to all other groups. This however is inconsistent with previous studies done on Fluoxetine using the same test (Cryan and Lucki, 2000; Cryan, *et al.,* 2002). However in a recent study (Dulawa *et al.,* 2004), a dose of 18 mg/kg/day of Fluoxetine in the same strain of mice increased swimming after subchronic treatment. Again, in the same study a dose of 25 mg/kg/day was also administered and a trend for an increase in climbing was seen. The authors attributed this increase in climbing time to a possible loss of specificity for the serotonergic system of 'this high dose'. In the present study a dose of 20 mg/kg/day was used and similar results were observed as with the 25 mg/kg/day dose in their study.

No significant differences were noted for swimming time between day one and day twenty one (Figure 4.12). Climbing time increased significantly for positive control on day twenty one. Immobility also decreased significantly for the same group. Notable increases in swimming time and subsequent decrease in immobility were observed for 10 mg/kg/day group. Other groups demonstrated less active behaviours in the second test. This passive behaviour could be considered as unwillingness to maintain effort in this inescapable situation, it could be that the increased immobility instead demonstrates a learning within the animal and a positive behavioural adaptation, i.e. the animal has learnt it cannot escape

and is conserving energy until it is removed by the experimenter (Petit-Demouliere, *et al.,* 2005).

To summarise *Boophone disticha* extract appear to exhibit antidepressant-like activity in the Forced swim test by increasing the total activity times that is swimming and climbing. The effect was not dose dependent but more prominent with the lower dose 10mg/kg. Repeated dosing enhanced the antidepressant-like activity of the *Boophone* extract.

# CHAPTER 6

## 6. CONCLUSIONS

This thesis was focused on revealing the toxicological and pharmacological effects of the bulb *Boophone disticha*. The aim was to test a crude extract of the bulb using mainly rodent models to bring to light its effects both harmful and beneficial.

The major findings of this work and recommendations are:

*Boophone* HE extract produced signs of acute reversible CNS depression, which probably explain its traditional use for anxiety disorders. However it is highly toxic therefore its recreational use should be discouraged. The neurotoxicological effects of the *Boophone* Hydro-ethanolic extract have been described and range from mild tremors to limb paralysis and death at high doses. The observed toxicity and neuropharmacological effects are probably linked to several neurotransmitters like serotonin and acetylcholine. Although preliminary *in vitro* studies on several of the *Boophone* extract alkaloids have been performed, our ongoing research aims to investigate *in vivo* the anxiolytic and antidepressant potential of the *Boophone* extracts and the purified alkaloids.

*Boophone disticha* has constituents that are either highly toxic or have their primary metabolites being toxic especially at higher doses. These compounds are also capable to cross the blood brain barrier. At low single doses, however, it appears that *Boophone disticha* has stimulant properties or may cause anxiety. The main subacute toxic effects of

*Boophone disticha* like the acute effects seem to be mediated via interference with the neuronal pathways especially the central dopaminergic and motor neurons. Death due to *Boophone* poisoning as with acute intoxication can occur as early as 10 minutes post dosing or can take up to 12 hours. Target organs as observed by changes in organ weight appear to be liver, small and large intestines, stomach, central nervous system and peripheral nervous system.

*Boophone disticha* extract is genotoxic from concentrations 1000 µg/plate and above with both TA98 and TA100 *S.typhimurium* species irrespective of the metabolic status of the system. The 500 µg/plate concentrations of the *Boophone* extract were not associated with any genotoxicity with both TA98 and TA100. The 2500 µg/plate concentration is potential cytotoxic which could have masked the genotoxic effects if this concentration. Further studies using a large battery of tests, metabolic approaches, and eukaryotic systems are required before the safety of *Boophone disticha* can be ascertained even at concentrations, which were found to be safe in this study.

*Boophone disticha* extract showed Anxiolytic-like activity in the elevated plus maze test by significantly changing the percentage time spent in open arms, number of open arm entries, rearings, unprotected head dips and stretch attend postures. The 10 m/kg dose had the most prominent effects compared to the higher doses that were comparable to the positive control. Further work should be done using individual alkaloids to determine the most active principle and to determine the exact mechanism of its effects.

*Boophone disticha* extract appears to exhibit antidepressant-like activity in the Forced swim test by increasing the total activity times that is swimming and climbing. The effect was not dose dependent but more prominent with the lower dose 10 mg/kg. Repeated dosing enhanced the antidepressant-like activity of the *Boophone* extract. Further investigations are necessary with extracted alkaloids to give a more conclusive evidence of its potential antidepressive effects.

In conclusion *Boophone disticha* exhibits high toxicity that affects mainly the CNS, However at low dose *Boophone* or its constituents have potential therapeutic benefits and maybe important lead candidates for drug development. Despite the few pharmacological effects that were assessed there are a number of other purported therapeutic benefits that warrant further investigation.

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# APPENDICES

## APPENDIX 1: FOB PROCEDURES AND SCORING CRITERIA

# FOB PROCEDURES AND SCORING CRITERIA

*Adapted from: McDaniel K.L. & Moser V.C. (1993). Utility of a Neurobehavioral screening Battery for differentiating the effects of two Pyrethroids, Permenthrin and Cypermethrin. Neurotoxicology and Teratology 15:71-83*

❖ Home-cage measurements

*I. Posture (Descriptive)*

1. Sitting or standing
2. Rearing
3. Asleep, lying on side or curled up
4. Flattened, limbs may be spread out
5. lying on side, limbs in air
6. Crouched over
7. Head bobbing

*II. Involuntary Motor Movements (Descriptive)*

*a. Clonic*

1. Repetitive movements of mouths and jaws
2. Quivers of limbs, ears, head, or skin (sometimes seen in untreated rats)
3. Mild tremors
4. Severe or whole body tremors
5. Myoclonic jerks
6. Clonic convulsions
7. Wet dog shakes

*b. Tonic*

1. Contraction of extensors such that limbs are rigid and extended
2. Opisthotonus: head and body rigidly arched backward
3. Emprosthotonus: head and body rigidly extended forward
4. Explosive jumps into the air with all feet leaving the surface
5. Severe clonic and/or tonic convulsions resulting in dyspnea, postictal depression, or death

*III. Vocalisations (Quantal):*

Spontaneous, not in reaction to being handled. Also includes spontaneous vocalisations in the open field.

*IV. Palpebral Closure (Ranked)*

1. Eyelids wide open
2. Eyelids slightly drooping
3. Eyelids drooping approximately half-way
4. Eyelids completely shut

❖ **Manipulative**

The rat was removed from its Home Cage and its ease of removal and reactivity to being handled was scored according to the ranking criteria below

I. *Ease of Removing Rat from cage*

1. Very easy(rat sits quietly, allows investigator to pick it up)
2. Easy (vocalisations, without much resistance to being picked up)
3. Moderately difficult (rat rears, often following investigator's hand)
4. Rat flinches (with or without vocalisations)
5. Difficult (runs around cage, or is hard to grab, with or without vocalisations)
6. Very difficult (tail and throat rattles, with or without vocalisations)

II. *Reactivity to Being Handled*

1. Low (no resistance, rat is easy to handle)
2. Moderately low (slight resistance to being handled, with or without vocalisations)
3. Moderately high (rat may freeze, or be tense, or rigid in hand, with or without vocalisations)
4. High (squirming, or twisting. Or attempting to bite, with or without vocalisations)

❖ **Measurements made while handling rat**

Whilst the rat is held the rat was observed for lacrimation, salivation, Palpebral closure, piloerection, increased or decreased body tone, bite marks, soiled fur appearance, missing toe nails. Lacrimation, salivation and Palpebral closure were scored according to the ranks below.

I. *Lacrimation*

1. None
2. Slight
3. Severe

II. *Palpebral Closure*

1. Eyelids wide open
2. Eyelids slightly drooping
3. Eyelids drooping approximately half-way
4. Eyelids completely shut

III. *Salivation*

1. None
2. Slight
3. Severe

IV. *Piloerection*

"+" indicates presence of piloerection (i.e., coat does not lie down after stroking).

❖ Open-field measurements

The rat was placed in the centre of a flat surface (60 × 90 cm with a 10 cm rim): with a perimeter barrier covered with clean absorbent paper for exactly 5 minutes. Whilst in the open the rat was observed for rearing, mobility, gait, arousal, stereotypical behaviour and other observations and this were recorded.

I. *Rearing*

Rearing was defined as each time the front legs of the rat come completely off the surface, without the rat necessarily have to raise itself up. This also included times when the rat used the side or lip of a cart top as support.

II. *Involuntary Motor Movement*

*a. Clonic*

1. Repetitive movements of mouths and jaws
2. Quivers of limbs, ears, head, or skin (sometimes seen in untreated rats)
3. Mild tremors
4. Severe or whole body tremors
5. Myoclonic jerks
6. Clonic convulsions
7. Wet dog shakes

*b. Tonic*

1. Contraction of extensors such that limbs are rigid and extended
2. Opisthotonus: head and body rigidly arched backward
3. Emprosthotonus: head and body rigidly extended forward
4. Explosive jumps into the air with all feet leaving the surface
5. Severe clonic and/or tonic convulsions resulting in dyspnea, postictal depression, or death

III. *Vocalisations*

Spontaneous, not in reaction to being handled. Also includes spontaneous vocalisations in the open field.

IV. *Gait*

*a. Descriptive*

Note; if rat did not move during the 5 min. observation period, it was gently prodded (after the 5 min is over) in order to observe the gait.

1. Ataxia, excessive sway, rocks, or lurches
2. Hind limbs show exaggerated or overcompensated movements, drag, or are splayed
3. Feet markedly point outward from body
4. Forelimbs drag, are extended, or unable to support weight
5. Walk on tiptoes
6. Hunched or crouched body position
7. Body drags or is flattened against surface

*b. Ranked*

Ranking of gait abnormalities

1. No abnormal gait
2. Slightly abnormal
3. Moderately abnormal
4. Severely abnormal

V. *Mobility Score*

The ability of rat to move around despite gait abnormalities was ranked according to the scale below

1. No impairment
2. Slightly impaired
3. Somewhat impaired
4. Severely impaired

VI. *Arousal*

The rat's level of unprovoked activity and alertness in the open field was ranked according to the scale below

1. Very low (stupor, coma)
2. Low (somewhat sluggish, some head or body movement)
3. Somewhat low (slightly sluggish, some exploratory movements with periods of immobility)
4. Alert, exploratory movements
5. Somewhat high (slight excitement, tense, excited, sudden darting or freezing)
6. Very high (hyper alert, excited, sudden bouts of running or body movements)

VII. *Stereotypical Behaviour*

Any behaviours that are excessive or repetitive such as circling, stereotypic grooming, pacing, repetitive sniffing, or head weaving were noted for each individual rat in the Open Field. Bizarre Behaviour such as self-mutilation, retropulsion, writhing, flopping were also noted.

VIII. *Excretion*

At the end of the 5 minute observation, the number of faecal boluses and pools of urine were recorded.

NB. "D" was recorded if diarrhoea is present and "X" if there is polyuria, or overlapping pools, were present...

❖ **Stimulus reactivity**

I. *Approach Response*

The rat was approached head-on with the end of a glass rod, held approximately 3 cm from face for 4s.

1. No reaction
2. Rat slowly approaches and sniffs or turns away
3. Rat flinches, actual muscle contractions

4. More energetic response than 2) or 3)
5. Exaggerated reaction: jumps, bites, or attacks

II. *Touch Response*

The rat's rump was gently from the side with a glass rod and its response was recorded according to the rank below.

1. No reaction
2. Rat may slowly turn or walk away, or vocalisations with little or no movement
3. Rat flinches, actual muscle contractions
4. More energetic response than 2 or 3
5. Exaggerated reaction: jumps, bites or attacks

III. *Click Response*

The rat's response to the snapping of the fingers placed approximately 5cm above the rat, was score and recorded according to the ranking below.

1. No reaction
2. Slight reaction, some evidence that noise was heard
3. Rat flinches, actual muscle contractions
4. More energetic response than 2 or 3
5. Exaggerated reaction: jumps, bites or attacks

IV. *Tail Pinch Response*

The tail of the rat (approximately 2-3 cm from the tip) was pinched with metal tweezers. The rat's reaction to the pinch was scored and recorded.

1. No reaction
2. Rat may turn or walk forward, or vocalisations with little or no movement
3. Rat flinches, actual muscle contractions
4. More energetic response than 2 or 3
5. Exaggerated reaction: jumps, bites or attacks

V. *Righting Reflex*

The rat was held supine, and then dropped from a height of about 30 cm. The rat's ease of landing noted and scored according to the scale below.

NB: Paralysed or severely affected, were not tested for the righting reflex.

1. Slightly uncoordinated
2. Lands on side
3. Lands on back

## FOB Procedures for Acute Toxicity Testing Form

*Adapted from: McDaniel K.L. & Moser V.C. (1993). Neurotoxicology and Teratology 15:71-83*

Date:…… Animal Code:…….. Weight:…… Body Temp. $^{0}$C……..

| Tests Parameter | Score | Comments |
|---|---|---|
| **a) Home-cage & Manipulative Measurements** | | |
| I. *Posture* | | |
| II. *Involuntary Motor Movements* | | |
| III. *Vocalisations* | | |
| IV. *Palpebral Closure* | | |
| **b) Manipulative Measurements** | | |
| V. *Ease of Removing Rat* | | |
| VI. *Reactivity to Being Handled* | | |
| VII. *Lacrimation* | | |
| VIII. *Palpebral Closure* | | |
| IX. *Salivation* | | |
| X. *Piloerection* | | |
| **c) Open-Field Measurements** | | |
| XI. *Rearing* | | |
| XII. *Involuntary Motor Movement* | | |
| XIII. *Gait* | | |
| *a. Descriptive* | | |
| *b. Score* | | |
| XIV. *Mobility Score* | | |
| XV. *Arousal* | | |
| XVI. *Stereotypical Behaviour* | | |
| XVII. *Excretion* | | |
| *a. Defecation* | | |
| *b. Urination* | | |
| **D. Stimulus Reactivity** | | |
| XVIII. *Approach Response* | | |
| XIX. *Touch Response* | | |
| XX. *Click Response* | | |
| XXI. *Tail Pinch Response* | | |
| XXII. *Righting Reflex* | | |
| XXIII. *Other Observations* | | |

APPENDIX 2: STANDARD OPERATING PROCEDURES FOR THE FORCED SWIM TEST

# STANDARD OPERATING PROCEDURES FOR THE FORCED SWIM TEST

## 1. Purpose

The forced swim test is used to assess depression and anti-depressant like behaviour in rats and mice

## 2. Scope of the S.O.P

Procedures described below are to be performed accurately and precisely by experimenters who are well versed with the test.

## 3. Safety requirements

Good laboratory practices should be followed at all times and the investigator must be careful to ensure the safety of self and the test animals throughout the study. There will be no eating or drinking in the work area, neither will cosmetics be worn. Protective clothing must be worn at all times (lab coats and gloves and/or goggles) unless the protocol states otherwise.

## 4. Responsibilities

Good animal husbandry should be practiced at all times and the following will be the responsibilities of the experimenter:

i. Ensuring that the animals have sufficient food and water throughout the duration of the study (food and water to be available *ad libitum*).
ii. Ensuring that the water supplied to the test animals is changed at least once every 2 days.
iii. Ensuring that the bedding in the animal cages is changed at least three times a week.
iv. A Habituation or familiarisation period of at least 7 days prior to the experiments will be done by daily handling of all the animals. This will reduce variability and improve validity of the results.

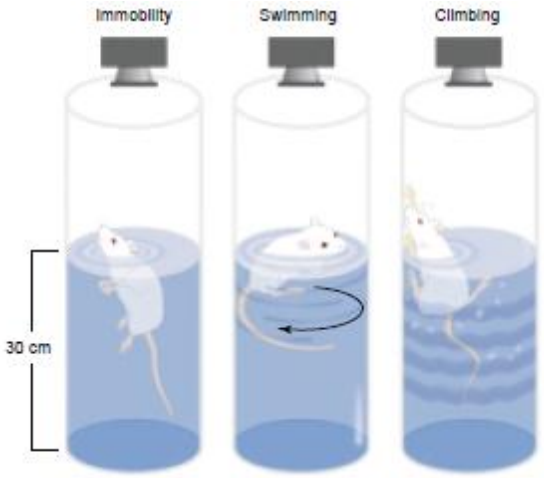
v. The following environmental factors should be at appropriate and constant levels in the animal holding room and the test rooms;
   a. A 12:12 hr light/dark cycle is maintained with darkness from 0700 to 1900 daily
   b. Humidity of 70%.
   c. Room temperature of 20-23$^0$C
   d. Minimal noise

vi. Tests should be carried out at the same time of day. Physiological and biochemical changes occur throughout the day

vii. The animals to be tested should be of the about the same age, strain and kept under identical environmental conditions.

## 5. Materials Required

- ✓ Stopwatch
- ✓ 10% v/v ethanol
- ✓ Paper towels
- ✓ Digital camera
- ✓ Permanent marker
- ✓ Drying tissue
- ✓ Thermometer
- ✓ Clean water 20-25degrees Celsius
- ✓ 4 cylinders



## 6. Preparation of Test Sample and Test Solutions

### 6.1. Preparation of the stock solution of *Boophone disticha* extract

a) Carefully weigh 5000mg of the dried hydroethanolic extract of *Boophone disticha* into a clean 50ml volumetric flask and make up to volume with normal saline.
b) Shake carefully to evenly distribute the *Boophone disticha* extract throughout the suspension.
c) Pipette 5ml of the resulting suspension into a 500ml volumetric flask and make up to volume with normal saline. This produces a *Boophone disticha* extract suspension with a concentration of 1mg/ml.

### 6.2. Preparation of the fluoxetine test solution

a) Randomly select and weigh twenty (20) fluoxetine 20mg capsules
b) Empty the contents of the capsules into a mortar and pestle and grind to a fine powder
c) Suspend the powder in 50ml of normal saline (this creates a suspension with a concentration of 8mg of fluoxetine/ml of suspension)

## 7. Animal Groups and Dosage Administration

### 7.1. Dosage Groups

Animals will be divided into 5 groups and each group will receive the following:

i. Group A (negative control) will receive 0.5ml of normal saline by oral gavage for 3 weeks
ii. Group B ( positive control) will receive 20mg/kg/day of Fluoxetine by oral gavage for 3weeks
iii. Group C will receive 10mg/kg/day *Boophone disticha* extract by oral gavage for 3 weeks
iv. Group D will receive 20mg/kg/day *Boophone disticha* extract by oral gavage for 3 weeks
v. Group E will receive 20mg/kg/day *Boophone disticha* extract by oral gavage for 3 weeks

### 7.2. Background to conduct of the tests

a) Testing will conducted between 0900 and 1300hrs
b) Each group of test animals will be made up of five (5) male and five (5) female Balb/c mice. Mark mice on their tails to enable identification of the animals.
c) Animals will be moved from the animal holding room to the testing laboratory and left to acclimate for an hour in their home cages
d) The water in the test cylinders should be kept at 22-23$^0$C throughout all the experiments

## 8. Forced Swim Test

### 8.1. Protocol

1) The cylinders are placed on flat surface and dark boards are placed between the cylinders so that each mouse is unable to see what is happening in the next cylinder. For improved camera viewing, dark boards are also placed behind the cylinder.
2) Place camera horizontally and at the same level as the cylinders to capture a horizontal view of the cylinders.
3) Take four mice from the same group one by one and gently place them in each cylinder, noting the time of insertion for each mouse.
4) Investigators must retreat approximately 2m so as to avoid distracting the animal
5) Observe the animals in the cylinder for six minutes.
6) Observe the following behavioural parameters for the test period and record them in the FST form; Time spent swimming, climbing and immobile, and the latency to immobility.
7) After the test period remove the mice from cylinders and dry them using drying paper towels/ tissue paper and return them to the homecage
8) The water is then removed and the apparatus is cleaned with 10% ethanol solution and fresh water put.
9) The test process is them repeated with the rest of the mice
10) The whole test including acclimatisation period would require approximately three and a half hours

### 8.2. Parameters measured

Predominant behaviour in 5s intervals is recorded. First two minutes in the water are for acclimatisation.

- ✓ **Swimming time**: defined as movement throughout the swim chamber, which included crossing into another quadrant
- ✓ **Climbing time**: defined as upward-directed movements of the forepaws along the side of the swim chamber or breaking the water surface
- ✓ **Immobility time**: when the mouse makes no further attempts to escape remained upright, except for the movements necessary to keep its head above the water.

APPENDIX 3: STANDARD OPERATING PROCEDURES FOR THE FORCED SWIM TEST

# STANDARD OPERATING PROCEDURES FOR THE ELEVATED PLUS MAZE TEST

## 1. Purpose

The elevated plus maze is used to assess anxiety responses of rodents

## 2. Scope

Individuals who have a full and clear understanding of this test are to perform the procedures described herein accurately and precisely

## 3. Safety Requirements

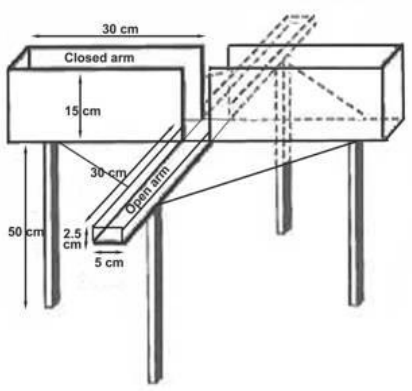
Common laboratory practices are to be followed at all times which include: no food and drink in the laboratory and on the work area. Laboratory coats must be worn at all times in the work area unless otherwise stated in the protocol.

## 4. Notes

i. To reduce variability and improve validity of the results obtained in this test, the experimenter must be aware of good animal husbandry and practice it. This includes, feeding the mice, changing bedding three times a week, interacting with the mice to improve familiarity.
ii. It is important to have mice of the same sex, age and strain since they contribute significantly to results in behavioural studies
iii. Environmental factors, i.e. humidity, temperature, light and noise are to be kept at appropriate and constant levels for mice
iv. All test are to be conducted at approximately the same time (0900-1300hrs)of the day since physiological and biochemical change throughout the day
v. There is no pre-training in this test

## 5. Equipment

a) *Elevated Plus Maze*




b) Camera connected to computer

## 6. Supplies

- ✓ Stopwatch
- ✓ 10% alcohol solution
- ✓ Tissue papers

## 7. Procedure

### 7.1. Preparation of stock solution of *Boophane disticha* extract

i. Accurately weigh 1 gram of the *Boophane disticha* hydroethanolic extract into a conical flask and make a 10 ml suspension with normal saline

ii. Pipette 1ml into a conical flask and make a 100ml suspension which will give 1mg/ml suspension

### 7.2. Test protocol

i. Day before the test, the mice are weighed

ii. On the day of the test the mice are randomly assigned to five different groups of eight mice each and each mouse of each group is given a distinguishing mark on the tail which is common to that group : Group A is the negative control which is given 0.5ml normal saline by oral gavage, Group B is the positive control which is given 10mg/70kg diazepam suspension by oral gavage, Group C is given 10mg/kg

*Boophane disticha* extract by oral gavage, Group D is given 25mg/kg *Boophane disticha* extract by oral gavage and group E is given 40mg/kg *Boophane disticha* extract by oral gavage

iii. One hour before the test begin the mice a dosed with their respective suspensions and taken to the testing room and left to acclimatize.

iv. Lighting in test room is provided by a 60 watt bulb directly above the Elevated Plus Maze

### 7.3. Test phase

i. Take mouse from cage and place in the middle of elevated plus maze facing an open arm whilst simultaneously switching on camera

ii. Investigator must move about 2 meters from apparatus, taking care not to make sounds that might distract the mouse

iii. Observe mouse actions for 5 minutes taking note of time spent in open and closed arms, number of entries into open and closed arms

iv. Stop camera, remove mouse and gently return to cage. Wipe maze arms with 10% ethanol solution and dry. Bring the next mouse.

v. Repeat process for all mice

vi. The whole test for all mice, excluding the acclimatizing phase will require approximately 3 hours

## 8. Parameters and Measures

- ✓ Number of open arm entries ( 4 paws)
- ✓ Number of closed arm entries
- ✓ Time spent in open arm
- ✓ Time spent in closed arm
- ✓ Stretched attend posture (protected and unprotected)
- ✓ Rearing (protected and unprotected)
- ✓ Head dips (protected and unprotected)

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# Acute oral toxicity and neurobehavioural toxicological effects of hydroethanolic extract of *Boophone disticha* in rats

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## Abstract

*Boophone disticha* (*B. disticha*) has been used systemically in traditional medical practice in Zimbabwe and neighbouring countries for the management of various central nervous system conditions including hysteria. Abuse of the plant by teenagers in Zimbabwe for its claimed hallucinogenic effects has also been reported, with the advent of serious toxicity in some cases. In the present work, we describe the acute toxicity and neurotoxicological effects of a freeze dried hydro-ethanolic plant extract of the bulb of *B. disticha*. Thirty-three adult (6–12 weeks old), non-pregnant female Sprague Dawley rats were used for the oral $LD_{50}$ estimation. Animals were given doses of 50, 120, 240, 360, 500 and 700 mg/kg and were observed using a modified Functional Observation Battery (FOB) for behavioural toxicity. The estimated oral $LD_{50}$ of the plant extract was between 120 and 240 mg/kg. For doses of 240 mg/kg and less, signs of toxicity began approximately 10 minutes after gavage, and the most prominent initial signs were head tremors (at 50 mg/kg) and body tremors, severe body tremors(>360 mg/kg) followed by convulsions. Generally, symptoms of toxicity lasted approximately 2 hours for doses of 240 mg/kg and less; and 3 hours for doses over 240 mg/kg for animals that survived. These results point to a rapid gastrointestinal absorption of the active principles in the plant extract. The most prominent neurotoxicological effects were increased flaccid limb paralysis and spastic hind-limb paralysis. Tachypnoea was noted at low doses and higher doses produced laboured breathing. The retropulsion observed with higher doses could indicate the reported hallucinogenic effects of the plant extract.

## Keywords

behavioural toxicology, natural toxins/toxinology, neurotoxicology, toxicity testing

## Introduction

*Boophone disticha* (L) (family/Amaryllidaceae; tumbleweed/sore-eye flower) is a highly poisonous indigenous psychoactive bulb that is widely used in Southern Africa. *Boophone disticha* belongs to the Amaryllidaceae family, *Boophone* species, which can be found throughout Southern and Tropical Africa.[1,2] The name of Herbert's Amaryllid genera, Boophone, has been spelled in four different ways (Boophane, Boophone, Buphane and Buphone); however, the current taxonomic nomenclature uses Boophone.[3] *Boophone disticha* was also known under the names of *Buphane toxicaria*, *Haemanthus toxicarius*, *Amaryllis disticha*, *Brunsvigia toxicaria* and *Boophone toxicarius*[3] and popularly as *munzepeti* in the Shona language; *ingcotho* in Isindebele. Despite its toxicity,

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the plant is commonly used in traditional medicine in Zimbabwe and other countries in the region for the treatment of a variety of ailments including boils, burns and 'hysteria.'[4] Moistened scales are applied to boils, septic wounds and abscesses to alleviate pain and to draw out pus.[5] In addition, weak decoctions of the bulb are administered by mouth or as enema for various complaints such as headaches, abdominal pains, weakness and various eye conditions or to 'drive out spirits.'[2,6] Traditional healers use the bulb for its psychoactive properties during the initiation of possession in divination rituals.[1,7]

An increase in the abuse rate of *Boophone disticha* has been reported in Zimbabwe and neighbouring countries.[1,8,9] Several fatal cases reported in the literature are linked to recreational use of *Boophone disticha*.[4,9-11] The characteristic clinical presentation of non-fatal poisoning with *Boophone disticha* in humans includes rapid development of ataxia, hallucinations, impaired vision, depression, stupor and coma.[9,10]

Given the extensive illicit and medicinal use of the plant as well as its potential as a source of psychoactive pharmacological therapies,[6,12-15] it is important to accurately describe its acute toxicity profile. Thus, in the present work, we present results of acute toxicity studies conducted on a hydroethanolic extract of *B. disticha* in a rat model with emphasis on the neurotoxicological profile of the extract as well as on target organ toxicity. This work is part of a project by our research group to investigate the claimed neurotherapeutic effects of *B. disticha* as we work toward drug development.

## Materials and methods

### Plant materials

*Boophone Disticha* was collected in December 2005 in Mashonaland West province about 60 km from Harare. The plant sample was authenticated by a taxonomist from the Botanical Gardens and National Herbarium and a voucher specimen was refrigerated in the department of Clinical Pharmacology, College of Health Sciences (University of Zimbabwe).

### Preparation of the crude extract

The fresh bulbs scales (5 bulbs were used) were peeled and then sun dried until all the scales were papery dry. The dried bulb scales were ground in a small mill until a coarse powder (1.173 g) was obtained. The powder was then mixed (1:5 w/v) with aqueous ethanol (70%v/v; 4 L) and then refluxed for 60 minutes at 100°C. After (≈24 hours) the extract was then filtered using a mutton cloth to remove the coarse material. The filtrate obtained was further vacuum filtered to remove the finer particulates. The aqueous-ethanolic extract was volume reduced by rotary evaporation with a Heidolph 4000 Rotavapor (Heidolph, Germany) to a thick paste (100 mL). The extract was then freeze dried from −40 to −20°C for 3 days. The extract was then ground to a fine powder which was kept in tightly sealed container in a cool dark place.

### Acute oral toxicity study

*Animals and animal husbandry.* Animal husbandry and the toxicity tests were conducted according to published OECD guidelines for assessing acute oral toxicity.[16] The laboratory was licensed by the Veterinary Services Unit of the Ministry of agriculture in Zimbabwe, and all the experimental protocols were approved by the Ethical Committee University of Zimbabwe. Thirty-three adult, non-pregnant female Sprague Dawley rats (6−12 weeks old; 180−280 g) purchased from the Animal House Faculty of Veterinary Sciences, University of Zimbabwe, were used. The animals were acclimatised to the laboratory conditions for at least 5 days prior to the experiments. The rats were housed in groups of up to five per cage, with wood shavings or shred paper bedding. They were allowed standard rodent food and tap water ad libitum. The animal facility was maintained at 19−21°C and had a 12-hour dark-light cycle with light on at approximately 0630 to 0700. The relative humidity was less than 70%.

### Administration of the extracts

The animals were randomly selected and marked to permit individual identification prior to dosing. The rats were fasted overnight and then weighed prior to dosing to avoid dose discrepancies. The test extract was administered in a single dose by oral gavage using an intubation cannula at volume of 1 mL/100 g body weight. After the sample administration and behavioural tests, food but not water was withheld for a further 3−4 hours.

### Acute toxicity: sighting study

A sighting study was carried out to allow selection of an appropriate starting dose for the main study.

The test substance was administered to single animals in a sequential manner, with doses increasing by a log cycle increase, starting at a dose of 5 mg/kg. If the animal showed any evidence of toxicity that dose was used in the main study, and dosing was stopped when a dose was reached which showed significant evidence toxicity. A period of at least 24 hours was allowed between the dosing of each animal. All animals were observed for at least 2 days. Single rats were given doses of 5, 50 and 500 mg/kg. The 50 mg/kg dose produced observable pharmacological effects and the 500 mg/kg dose caused death. Therefore, doses for the main study were selected within the range 50 to 1000 mg/kg. Doses for $LD_{50}$ estimation were increased by approximately a quarter log cycles starting at 50 mg/kg (50, 120, 240, 360, 500 and 700 mg/kg).

### *Acute toxicity: estimation of the $LD_{50}$ of the crude extract*

The rats were divided into five treatment groups of five female rats and one group with three female rats. Six concentrations of the test extract were prepared by serial dilution of the crude extract. The concentrations were spaced appropriately to permit an acceptable determination of the $LD_{50}$. The extract was administered by oral gavage using an intubation cannula at a volume of 1 mL/100 g body weight. Soon after extract administration, the animals were observed according to a modified FOB described below (adapted from McDaniel and Moser, 1993).[17] The animals that did not die during the observation period were monitored for 48 hours. Animals that died during the test were necropsied, and some of those which survived that appeared moribund were humanely sacrificed chloroform asphyxiation and necropsied. All humanely sacrificed animals were considered as having died as a result of the plant extract in the data interpretation.

### *Functional observational battery*

The functional observational battery (FOB) was used to evaluate neurobehavioural and physiological changes resulting from toxicity of the plant extract. The experimental protocol for the FOB was based on procedural details and scoring criteria for FOB previously described by McDaniel and Moser.[17] Some of the parameters were scored (Table 1), and a few others were descriptive.[18]

On test days, rats were transported to an observation room and allowed at least 1 hour to acclimate before testing began. Soon after dosing the rats were returned to their home-cages and observed for a period of 10 minutes. Home-cage observations included posture, rearing, vocalizations or any involuntary movements and the procedures are outlined in the FOB protocol as described by McDaniel and Moser.[17] The observer then removed the rat, held it, and scored lacrimation, salivation, miosis, piloerection and handling reactivity, according to defined criteria. The rat was then placed on an open field (a laboratory cart 60 × 90 cm surrounded with a 10 cm perimeter barrier). During 5 minutes of exploration, the observer counted the number of rears and evaluated and scored any gait abnormalities, ataxia, arousal, activity level, involuntary motor movements, stereotypical behaviour and excretion level (urination, defecation). Aerial righting reflex was also ranked. After the open field observations, sensorimotor responses were assessed according to responses to a variety of stimuli.[17]

### *Histopathological evaluations for acute toxicity study*

All animals that died or were sacrificed were given a complete post-mortem examination. The abdominal, thoracic and cranial cavities were observed for any abnormalities, and all the organs were removed and examined for grossly visible lesions, and the following organs weighed; brain, heart, liver, spleen, kidneys, stomach, large and small intestines. After weighing the organ samples were placed in 10% neutral buffered formalin. Tissues were then imbedded in paraffin, sectioned to a thickness of 4–6 µm, transferred to slides, and stained with haematoxylin and eosin (H&E) for light microscopic examination.

### *Statistical analysis*

Data collected with the FOB was analysed by STATVIEW 5.1. statistical package. The statistical analyses were performed by non-parametric Kruskal-Wallis to evaluate significant differences between the groups. Differences were considered significant at $p < 0.05$.

## Results

### *Acute oral toxicity study and $LD_{50}$ estimation*

The toxicological effects of acute administration of the crude extract of *B. disticha*, both signs and

**Table 1.** Functional observational battery for neurotoxicity assessment

| Tests parameter | Score values | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Home-cage and manipulative | | | | | | | | |
| Posture (descriptive) | | Sitting or standing | Rearing | Asleep | Flattened | Lying on side | Crouched over | Head bobbing |
| Involuntary motor movements | Normal | Repetitive movements of mouths and jaws | Non-rhythmic Quivers | Mild tremors | Severe or whole body tremors | Myoclonic jerk | Clonic convulsions | Wet dog shakes |
| Palpebral closure | | Eyelids wide open | Eyelids slightly drooping | Drooping approxi-mately half-way | Completely shut | Ptosis | | |
| Ease of remov-ing rat from cage | | Very easy | Easy | Rat flinches | Moderately difficult | Difficult | Very difficult | |
| Reactivity to being handled | | Low | Moderately low | Moderately high | High | | | |
| Lacrimation | | None | Slight | Severe | | | | |
| Salivation | | None | Slight | Severe | | | | |
| Piloerection (+/−) | No | Yes | | | | | | |
| Open-field | | | | | | | | |
| Involuntary motor movement | Normal | Repetitive movements of mouths and jaws | Non-rhythmic quivers | Mild tremors | Severe or whole body tremors | Myoclonic jerks | Clonic convulsions | Wet dog shakes |
| Gait (descriptive) | | Ataxia | Exaggerated or overcompensated hind limb movements | Feet markedly point outward from body | Forelimbs drag, are extended | Walk on tiptoes | Hunched or crouched body | Body is flattened against surface |
| Gait score | | Normal | Slightly abnormal | Moderately abnormal | Severely abnormal | | | |
| Body tone | Normal | Hypertonia | Rigidity | Fasciculation | | | | |
| Mobility score | | No impairment | Slightly impaired | Somewhat impaired | Severely impaired | | | |
| Arousal | | Very low (stupor, coma) | Low (sporadic) | Somewhat low (reduced) | Alert, normal exploratory movements | Somewhat high (enhanced) | Very high (hyper alert, excited) | |
| Stereotypical/ bizarre behaviour | None | Head weaving | Body weaving | Grooming, self-mutilation | Circling, abnormal movements | Others | | |

*(continued)*

**Table I (continued)**

| Tests parameter | Score values | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Stimulus reactivity | | | | | | | | |
| Approach response | | No reaction | Normal | Slow reaction | Energetic reaction | Exaggerated reaction | | |
| Touch response | | No reaction | Normal | Slow reaction | Energetic reaction | Exaggerated reaction | | |
| Click response | | No reaction | Normal | Slow reaction | Energetic reaction | Exaggerated reaction | | |
| Tail pinch response | | No reaction | Normal | Slow reaction | Energetic reaction | Exaggerated reaction | | |
| Righting reflex | | Normal/rat lands on feet | Slightly uncoordinated | Lands on side | Lands on back | | | |
| Pupil size | Normal | Mydriasis | | | | | | |
| Pupil response | No reaction | Normal | | | | | | |

symptoms and duration of symptoms are summarized in Table 2. No lethal effects were observed during the 14-day observation period for the 50 and 120 mg/kg dosage groups. However, deaths were observed with doses equal to or higher than 240 mg/kg. In all cases, the animals died between 30 minutes and 3 hours after dose administration. In these cases, the most prominent symptoms preceding death were generalized convulsions, respiratory distress, tachypnoea and flaccid paralysis. The estimated oral $LD_{50}$ of the crude plant extract was determined to be between 120 and 240 mg/kg.

### *Neurotoxicological assessment with FOB*

The data obtained from neurotoxicological evaluations are presented in Tables 2 and 3. Generally, home-cage and open-field observations, autonomic and activity endpoints were indicative of CNS depression with severity increasing with increasing dose. For doses of 240 mg/kg and less, signs of toxicity began approximately 10 minutes after dosing, with the most prominent initial signs according to home cage observations being head tremors (at 50 mg/kg) and body tremors. Generally symptoms of toxicity lasted approximately 2 hours for doses of 240 mg/kg and less and 3 hours for doses over 240 mg/kg for animals that survived. Of the parameters assessed in the home-cage, only handling reactivity was significantly decreased in experimental groups compared to control.

The most prominent neurotoxicological effects with doses 240 mg/kg and higher were increased flaccid limb paralysis, retropulsion (backward movement) and hypoactivity. This is evidenced by significantly rearing, reduced mobility and gait scores (Table 3). However, sensorimotor evaluations showed no significant difference between all experimental groups and the control group.

### *Histopathological analysis*

Gross examination and microscopic histopathological analysis of the extracted organs did not show any extract-related abnormalities. However, microscopic minimal-to-mild gastrointestinal sequelae was noted in a few animals including controls.

## Discussion

There is a paucity of literature on the experimental toxicity of traditional medicines used in Zimbabwe and other African countries,[19] including *Boophone disticha* crude extract and the alkaloids that have been extracted from it. In this study, the $LD_{50}$ of the crude aqueous ethanolic extract was estimated to be between 120 and 240 mg/kg, which is higher when compared to a previously published lethal dose of one *Boophone* alkaloid, buphanidrine, of 8.9 mg/kg s.c. and 10 mg/kg i.v. in mice.[2] The difference can be attributed to the difference in extraction method and to the purity of the extracted sample. Furthermore, the crude extract is a complex mixture of different alkaloids with different properties. Since we estimated $LD_{50}$ to be above 120 mg/kg and in our protocol, the 50 mg/kg dose showed little toxicity, we propose that doses with potential therapeutic use and for repeated dose toxicity studies should be below 50 mg/kg.

The results of our study indicate that the crude extract of *B. disticha* has acute CNS depressive effects. The early onset of intoxication symptoms, even at the lower doses, could point to a rapid gastrointestinal absorption of toxic principles in the crude extract. This is also supported by the quick onset of symptoms in reported acute poisoning cases.[4,9,11] The moderately long duration of toxicity of the extract particularly with high doses for those animals that survived may be attributed to sustained effects on the nervous system due to high lipid solubility and high concentration of the alkaloids in the central nervous system or maybe related to the mechanism of toxicity.

The pattern of behaviours observed in the FOB is also suggestive of CNS depressant effect particularly at low doses. Higher doses are sometimes associated to a CNS stimulant and hallucinogenic effects. This is supported by the fact that weak decoctions have been used traditional effectively as a sedative to relieve 'hysteria' and insomnia.[2] High doses have been known to induce hallucinations, when used for divination and some of the reported cases of intoxication.[2,4,9,11] Lycorine is an anticholinergic alkaloid that has been extracted from *B. disticha* and many other amaryllidaceae herbs.[4,5] The mydriasis, palpebral closure, piloerection, tachypnoea and spastic hind limb paralysis observed at lower doses of *B. disticha* could be due to the anticholinergic effects of lycorine. An earlier in vitro study further supports these anticholinergic effects of the *Boophone* extract.[20] However, these effects cannot be all attributed to lycorine only since the pharmacological effects of the other Boophone alkaloids are not fully known, and further investigations need to be done.

Table 2. Toxicity after single-dose administration by oral gavage of the hydroethanolic extract of *Boophone disticha*

| Dose (mg/kg) | T/M | Latency | Signs of toxicity observed |
|---|---|---|---|
| Control | 5/0 | – | – |
| 50 | 5/0 | >10 minutes, <2 hours | Piloerection, mydriasis, head tremors, hypoactivity, increased respiratory rate, lethargy |
| 120 | 5/0 | >10 minutes, <2 hours | Piloerection, mydriasis, body tremors, hypoactivity, increased respiratory rate, lethargy |
| 240 | 5/3 | >10 minutes, <2 hours | Piloerection, mydriasis, body tremors, convulsions, hypoactivity, hind paralysis, ataxia, laboured breathing, back arching, retropulsion |
| 360 | 5/4 | >5 minutes, <3 hours | Piloerection, mydriasis, body tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion, excessive sniffing |
| 500 | 5/4 | >5 minutes, <3 hours | Piloerection, mydriasis, tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion |
| 700 | 3/3 | >5 minutes | Piloerection, mydriasis, tremors, convulsions, hypoactivity, forelimb paralysis, ataxia, laboured breathing, retropulsion |

Abbreviation: T/M: total initial number of rats in group/number of rats that died due to the treatment.

**Table 3.** Acute effects of the hydroethanolic extract of *Boophone disticha* on behavioural endpoints of the FOB

| FOB endpoint | Overall significance $\chi^2$ ($p$ value) | Dose (mg/kg p.o.) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | 50 | 120 | 240 | 360 | 500 | 700 |
| Activity/reactivity | | | | | | | | |
| Posture | Not significant | 1.00 | 1.00 | 1.00 | 2.00 | 1.00 | 2.00 | 6.00 |
| Removal reactivity | Not significant | 3.00 | 2.80 | 2.00 | 2.00 | 2.00 | 1.80 | 2.00 |
| Handling reactivity | $\chi^2 = 15.916$ (0.0071) | 3.00 | 3.20 | 2.00 | 1.80 | 2.00 | 1.80 | 2.00 |
| Arousal | Not significant | 3.00 | 3.60 | 3.40 | 3.20 | 3.00 | 2.40 | 2.00 |
| Open-field rears | $\chi^2 = 10.038$ (0.0742) | 28 | 14.4 | 9.00 | 3.40 | 4.80 | 4.20 | 0.67 |
| Involuntary movements | $\chi^2 = 12.563$ (0.0278) | 3.00 | 3.4 | 3.40 | 4.40 | 4.00 | 4.20 | 4.33 |
| Autonomic | | | | | | | | |
| Lacrimation | $\chi^2 = 16.221$ (0.0062) | 1.00 | 1.00 | 1.00 | 1.00 | 1.60 | 2.00 | 2.00 |
| Salivation | $\chi^2 = 17.308$ (0.0040) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.67 |
| Palpebral closure | $\chi^2 = 14.775$ (0.0140) | 1.00 | 1.80 | 1.40 | 2.00 | 2.00 | 2.20 | 3.00 |
| Defecation/urination | Not significant | 1.00 | 0.80 | 0.80 | 0.40 | 0.80 | 1.00 | 1.67 |
| Neuromuscular | | | | | | | | |
| Gait score | $\chi^2 = 12.880$ (0.0245) | 1.00 | 1.20 | 1.80 | 2.20 | 3.00 | 3.60[a] | 4.33[a] |
| Mobility score | $\chi^2 = 11.084$ (0.0497) | 1.00 | 1.20 | 1.80 | 2.20[a] | 3.00[a] | 3.60[a] | 4.00[a] |
| Righting reflex | Not significant | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Sensorimotor | | | | | | | | |
| Approach response | Not significant | 1.00 | 1.00 | 1.60 | 1.60 | 1.80 | 1.60 | 1.00 |
| Tail pinch response | Not significant | 3.00 | 3.00 | 2.60 | 2.80 | 3.00 | 2.80 | 2.67 |
| Touch response | Not significant | 2.00 | 1.60 | 1.60 | 2.00 | 2.00 | 1.60 | 1.00[a] |
| Click response | Not significant | 2.00 | 2.80 | 2.60 | 2.60 | 2.60 | 2.80 | 2.33 |

Abbreviation: FOB: functional observation battery.
[a] Significant difference versus control group.

The most notable effects with higher doses of the extract were convulsions, laboured breathing and flaccid forelimb paralysis. This suggests respiratory depression as a possible cause of death and CNS depression as an important toxic effect at these doses.

A stereotypical behaviour, retropulsion, was observed at higher doses (≥240 mg/kg). This is indicative and further supports the reported hallucinogenic effects of the plant extracts.[1,2,4,9] The observed flaccid paralysis overrules spastic extensions of forelimbs as a cause of the retropulsion. This would point to the involvement of serotonin and/or dopamine and would agree with recent findings from in vitro studies on isolated alkaloids of *B. disticha*, which have shown selective binding to a serotonin transporter in the rat brain.[15]

Locomotor activity is considered to be an index of alertness and a decrease in locomotion can indicate sedation.[21] Our results show that locomotor activity was significantly reduced as evidenced by reduced rearing and grooming behaviour. However, the hypoactivity noted might be multifactorial, possibly involving impaired neuromuscular activity and other mechanisms. A finding that seems paradoxical is that all sensorimotor indicators were not significantly affected in all the dosage groups. This might indicate a restricted or selective neuropharmacological activity of the *Boophone* extract and warrant further investigation.

In conclusion, the *Boophone* HE extract produced signs of acute reversible CNS depression, which probably explain its traditional use for anxiety disorders. However, it is highly toxic, therefore, its recreational use should be discouraged. The neurotoxicological effects of the *Boophone* hydro-ethanolic extract have been described and range from mild tremors to limb paralysis and death at high doses. The observed toxicity and neuropharmacological effects are probably linked to several neurotransmitters like serotonin and acetylcholine. Although preliminary in vitro studies on several of the *Boophone* extract alkaloids have been performed, our ongoing research aims to investigate in vivo the anxiolytic and antidepressant potential of the *Boophone* extracts and the purified alkaloids.

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